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Strategies for the discovery of therapeutic Aptamers

Xianbin Yang[†], **Na Li**, and **David G. Gorenstein**[‡,*]

[†]AM Biotechnologies LLC, 12521 Gulf Freeway, Houston, TX 77034, USA

[‡]Centers for Proteomics and Systems Biology, The Brown Foundation Institute of Molecular Medicine, The University of Texas Health Science Center at Houston, 1825 Pressler St., Suite 205, Houston, TX 77030, USA

Abstract

Importance of the field—Therapeutic aptamers are synthetic, structured oligonucleotides that bind to a very broad range of targets with high affinity and specificity. They are an emerging class of targeting ligand that show great promise for treating a number of diseases. A series of aptamers currently in various stages of clinical development highlights the potential of aptamers for therapeutic applications.

Area covered in this review—This review will cover *in vitro* selection of oligonucleotide ligands, called aptamers, from a combinatorial library using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process as well as the other known strategies for finding aptamers against various targets.

What the reader will gain—Readers will gain an understanding of the highly useful strategies for successful aptamer discovery. They may also be able combine two or more of the presented strategies for their aptamer discovery projects.

Take home message—Although many processes are available for discovering aptamers, it is not trivial to discover an aptamer candidate that is ready to move toward pharmaceutical drug development. It is also apparent that there have been relatively few therapeutic advances and clinical trials undertaken due to the small number of companies that participate in aptamer development.

Keywords

aptamers; *in vitro* selection; oligonucleotide phosphorodithioate; thioaptamer; SELEX

1. Introduction

Historically, the pharmaceutical industry focused on discovering small organic molecules that modulate the function of target proteins such as enzymes, receptors, or ion channels. The structure and mode of action for these small molecules are usually very complicated and often not completely understood. Thus these traditional drugs were discovered either by identifying the active ingredient from traditional remedies, through large screening efforts, or by serendipitous discovery. The process of drug discovery involves the identification of candidates, synthesis, characterization, screening, and assays for therapeutic efficacy. On average, it is necessary to synthesize and screen about 10,000 new compounds in order to

[*]To whom correspondence should be addressed at: David Gorenstein, Ph.D., Deputy Director and James T. Willerson Distinguished Chair, Centers for Proteomics and Systems Biology, The Brown Foundation Institute of Molecular Medicine, The University of Texas, Health Science Center at Houston, 1825 Pressler St., Suite 205, Houston, TX 77030, Tel: 713-500-2233, Fax: 713-500-2420, david.g.gorenstein@uth.tmc.edu.

discovery one new active substance worth developing. Drug discovery is a lengthy, expensive, difficult, and inefficient process with a low rate of new therapeutic discovery [1]. It is estimated that the average cost of discovering and developing a successful new drug in the United States today is more than $800 million. The high cost of drug discovery and development combined with the increased regulatory hurdles and pressure from third-party payers and governments to keep prescription medicine costs down are putting tremendous pressure on the pharmaceutical industry. It is clear that the pharmaceutical industry must adopt changes in the manner in which it discovers and develops new drugs.

One way that the pharmaceutical and biotechnology industry may decrease costs and increase efficiency is to identify innovative technologies and apply them to discover and develop non-traditional drugs such as antibodies and nucleic acids. One innovative technology for producing potential drug candidates involves combinatorial chemistry to discover high-affinity ligands for target molecules. Nucleic acids are particularly well suited for combinatorial selection. Using them has generated high-affinity ligands for a variety of protein and small molecule targets. Nucleic acids can be easily synthesized automatically, they can fold into well-defined secondary, tertiary, and quaternary structures, they are easily amplified by the polymerase chain reaction (PCR) or *in vitro* transcription, and they can be easily identified by sequencing. Nucleic acid ligands that are isolated from combinatorial oligonucleotide libraries are termed aptamers, which can be single-stranded (ss) or double-stranded (ds) nucleic acids [2–5]. Aptamers have demonstrated remarkably tight binding affinity for their targets with dissociation constants typically in the nanomolar and even picomolar range [6–8], better than those of conventional small-molecule drugs and comparable to monoclonal antibodies. Due to their relatively larger size and more complex structure than small-molecule drugs, aptamers can be tuned to access non-druggable targets. It has been proven that aptamers are generally useful at inhibiting protein-protein interactions. For instance, some aptamers targeting cell surface receptors could block the interaction between ligands and their receptors, therefore inhibiting the receptor-mediated signal transduction for cancer therapy [9]. In addition to their discriminate recognition, aptamers offer advantages over antibodies as they can be engineered completely in a test tube, are readily produced by chemical synthesis, possess desirable storage properties, and elicit little or no immunogenicity in therapeutic applications [10,11]. The utility of aptamers is reflected in the growing number of applications [7,12], which encompass the development of diagnostic reagents and therapeutic applications [13]. The approval by the US Food and Drug Administration of an aptamer that binds to and inhibits vascular endothelial growth factor highlights the potential of aptamers for therapeutic application. Several other aptamers are now in clinical trials for human diseases including those targeting thrombin and transforming growth factor-β2 etc. [10]. It is worth noting the aptamer-control agents pairs developed by the Sullenger lab at Duke University [14]. This technology was transferred to Regardo Biosciences followed by the formation of the REG1 and REG2 anticoagulation systems. The systems contain two components: 1) RB006 is an anti-coagulant aptamer binding to blood coagulation Factor IXa and preventing blood clot formation. 2) RB007 is a control agent that could base-pair with RB006 and partially or completely reverse its activity. The REG1 anticoagulation system with the intravenous injection of RB006 and RB007 is currently in a phase 2b study in acute coronary syndrome. In REG2 anticoagulation system, phase 1 study for venous thromboembolism has been completed with subcutaneous injection of RB006 and intravenous injection of RB007.

The short timeframe from the initial discovery of synthetic aptamers in 1990s to the regulatory approval of the first aptamers therapeutic in 2004 is impressive, and upcoming clinical trials on additional aptameric compounds will provide important validation of their effectiveness and cost saving.

Since their discovery 20 years ago [2,3], aptamers have shown bright prospects in scientific research, clinical diagnosis, and therapy. The goal of this review is to summarize the most well-known technologies for identification of aptamers in drug discovery and development.

2. Strategies for identifying aptamers

2.1 SELEX or *In vitro* aptamer selection

Since its first description in 1990, the SELEX [2] or *in vitro* selection [3] technology outlined in Figure 1 is widely used to evolve oligonucleotide ligands, called aptamers, with novel functionalities [7]. This traditional method has been described in several detail review articles [6–8], so an outline will be given here of only the basic steps of a SELEX process, for comparison with the recent variants described below. The starting point of a typical SELEX process is a random DNA oligonucleotide library synthesized by an automated DNA synthesizer that is programmed to include equal amounts of all four monomer bases of the oligonucleotide during the coupling of residues in a randomized segment.. The random region of the library typically ranges from 20–200 nucleotides in length flanked by two constant regions are generally from 18 to 30 nucleotides long [15]. Because primers are fixed sequences and they may positively or negatively compromise identification of aptamers in the selection process, protocols to isolate primer-free aptamers using a random DNA library have also been developed and aptamers targeting S100B, a protein marker for melanoma have been isolated [16]. For a DNA-based aptamer selection, the library can be used directly, whereas a conversion into an RNA library has to be accomplished prior to starting an RNA SELEX process. In either case the randomized DNA or RNA pool is typically heated and cooled in an appropriate buffer to promote formation of stable structures (folding), and then incubated directly with a target. The binding complexes are subsequently partitioned from unbound and weakly bound oligonucleotides by means of a physical separation technique such as nitrocellulose filters, which is often used with protein targets that are retained on nitrocellulose. Thus, those oligonucleotide sequences that do not bind the target are washed away and discarded, whereas those which favorably interact with the target are selectively retained. Following affinity elution, the population of sequences bound to the target is isolated and amplified by PCR (DNA aptamer selection) or reverse transcription (RT)-PCR (RNA aptamer selection). The pool of the enriched library is then subjected to another selection/amplification process, often with increasing stringency conditions for selection. After a number of rounds, the initial random oligonucleotide pool is reduced to relatively few sequence motifs with the highest affinity and specificity for the target. If the selection returns ‘mediocre’ nanomolar aptamers, further selection can be performed with a partially randomized library to improve the aptamer’s binding affinity and specificity. The randomized library will be prepared based on the aptamer in which the original random sequence regions are 70% wild-type and 30% non-wild-type, i.e., if a given residue in the aptamer was G, then in the randomized pool it would be 70% G, 10% A, 10% T and 10% C [6].

The number of rounds depends on a variety of parameters, such as target features and concentration, the components of library, ratio of target molecules to nucleic acid pools, and the efficiency of the partitioning method. In addition, the affinity of the aptamers to their target can be influenced by the stringency of the selection conditions such as progressively reducing the target concentration in later rounds [17]. The last SELEX cycle is stopped after the amplification step and the PCR products are cloned to get individual aptamer clones from the selected pool. The individual aptamers are sequenced and analyzed. The representative aptamer clones are synthesized and used in binding assays to characterize their binding features including affinities and specificities. It should be mentioned that the SELEX process for the selection of target-specific aptamers is a universal process characterized by repetition of several of the above mentioned steps, however there is no

standardized selection protocol for any target. So far, most of the published aptamers were selected via this traditional strategy. There have been considerable efforts to develop automated platforms for aptamer selection, for example to meet the increased need for aptamers as reagents in rapid development of proteome analysis [18–22]. However, there is no practical “automated SELEX instrument” due to a very complex automation challenge to integrate and automate different molecular biology methods.

Nuclease sensitivity of natural DNA and RNA aptamers compromises their usefulness for *in vitro* and *in vivo* applications, thus requiring the development of nuclease-resistant molecules. The aptamers identified through the SELEX involve enzymatic amplification of enriched nucleic acid pools. In order to increase resistance to degradation from nucleases, practitioners of SELEX-related processes have needed to either chemically modify their aptamers post-selection, or include modified nucleic acids during SELEX selection. Rather than stabilize the aptamer from nucleases by post-selection monothiophosphate modifications, it is best to select the modification and base sequence during the *in vitro* combinatorial selection process. This avoids problems where modifications perturb either the structure of the aptamer or interfere with the binding to target molecules. Monothioaptamers, in which one of the non-bridging phosphoryl oxygens is replaced by sulfur, are attractive choices for aptamer development. In recent publications from our laboratory we described modifications to the normal *in vitro* selection technology by incorporating one or more dNTP(αS) or NTP(αS) analogs during the polymerization step [4,5,23–26]. This creates 5'-monothiophosphate substitutions in one or more of the nucleotides in random or high sequence-diversity libraries. We have demonstrated that ds and ss combinatorial monothiophosphate libraries can be screened successfully for binding to many different protein and nucleic acid targets, including NF-IL6 [5], NF-κB [4,26], HIV reverse transcriptase [25], Venezuelan Equine Encephalitis nucleocapsid [24], and cytokines [23]. We and others have also demonstrated that monothioaptamers generally exhibit enhanced binding affinities and nuclease resistance [27–29]. Thioaptamers offer advantages over traditional aptamers in their enhanced affinity and specificity and higher stability, largely due to the properties of the sulfur backbone-modifications. 2'-modified RNA libraries are also created for isolating aptamers with improved *in vivo* stability, facilitated by the use of mutant T7 RNA polymerases. 2'-fluoro-pyrimidine modified RNA library was transcribed by $T7^{Y639F}$ RNA polymerase and used to isolate a number of aptamers including those targeting Tenascin C [30], prostate specific membrane antigen [31], and human tyrosine kinase RET [32]. This polymerase was also used to generate 2'-*O*-methyl-containing libraries to discover 2'-*O*-methyl pyrimidine and full 2'-*O*-methyl aptamers [33]. 2'-*O*-methyl aptamers may be less toxic than other 2'-modified aptamers because 2'-*O*-methyl substituted nucleotides occur naturally at relatively high abundance in the context of ribosomal RNA [34].

In parallel with the traditional positive selection for a known target, a negative selection step against a non-target such as a support resin or the filter used for immobilization of any target is generally recommended. Briefly, two processes are involved: first, the nucleic acid library is loaded onto the support or matrix first, and to exclude the ones absorbed by the support or matrix, the flow through, unbound portion is used to incubate with the target protein or target attached on the same matrix such as a small molecule or protein. Compared with the strategy without negative SELEX, the specificity of the oligonucleotide aptamers obtained by involving negative SELEX is usually higher. For example, Haller and Sarnow have used this strategy to isolate RNA aptamers specifically targeting guanosine residue with 7-methyl group [35]. Additionally, negative SELEX is also often used for complex targets such as cancer cells [36]. Ohuchi et al. first incubated the 2'-fluoro-pyrimidine-modified RNA pool with Chinese hamster ovary (CHO) cells that lack growth factor-beta type III receptor (TbRIII) protein to eliminate RNAs bound to undesired molecules on the cell surface. Then,

cells containing TbRIII were incubated with the unbounded RNAs to do the positive selection. After 11 rounds, an RNA aptamer against cells containing TbRIII protein with high affinity and specificity was isolated [37]. Similarly, Blank et al. have incorporated this strategy to isolate ssDNA aptamers for transformed endothelial cells as a complex target [38]. It may be quite challenging to isolate a highly specific aptamer that binds to its target but not to an analog of the target or structurally similar target using only positive selection. Negative SELEX is used to first incubate the oligonucleotide pool with an analog of the target or structurally similar target. The unbound fraction is then used to incubate the target for normal selection. As a consequence, negative SELEX provides highly specific aptamers that can discriminate between closely related structures. It is worth noting that the negative selection can be included in every round, every other round or every several rounds. This strategy is employed for aptamer selection targeting small molecules, proteins, and cells as well [4,35,39,40]. For example, Jenison et al. have included a negative SELEX procedure and isolated RNA aptamers that bind with high affinity and specificity to the bronchodilator theophylline, but not to caffeine. One of the RNA aptamers binds to theophylline with a dissociation constant K_D of 0.1 μM. This binding affinity is 10,000-fold greater than the RNA aptamer's affinity for caffeine, which differs from theophylline by only a methyl group at nitrogen atom N-7. Analysis by nuclear magnetic resonance spectroscopy indicates that this RNA aptamer undergoes a significant change in its conformation or dynamics upon theophylline binding. Binding studies of compounds chemically related to theophylline have revealed structural features required for the observed binding specificity. These results demonstrate the ability of RNA aptamers to exhibit an extremely high degree of ligand recognition and discrimination [40]. Similarly, to enhance the specificity towards a single NF-κB protein dimer, Bassett et al. [4] have included the negative SELEX procedure in the generation of phosphorothioated aptamers (referred to as thioaptamers [41]) used to enhance the selectivity towards the RelA/RelA homodimer. Briefly, the initial phosphorothioated library (created by substituting dATP(αS) for normal dATP in the PCR amplification step) was incubated with the p50/p50 homodimer protein. The wash, representing a phosphorothioated library that doesn't bind tightly to the p50/p50 homodimer, was then used to select sequences that bind more tightly to the targeted RelA/RelA homodimer (Figure 2). This approach eliminated any p50/p50-binding thioaptamer sequences to enhance specificity for RelA/RelA.

2.2 Cell-SELEX and *in vivo* SELEX

As we are nearing the 20th anniversary for the first publication of these in vitro selection processes, the majority of aptamers have been selected via this traditional method using purified, soluble forms of recombinant proteins as targets. Using purified proteins as targets has the obvious advantage of convenient control to achieve optimal enrichment during the selection process, but relatively large amounts of high-quality protein must be available in hand as a prerequisite for SELEX. Due to the instability, post-translational modifications or the complexity of some proteins such as cell surface receptors and some other membrane proteins, preparation of these purified proteins has often proven to be difficult. Thus, in many cases it is hard or impossible to obtain the relevant active protein(s) in purified form with enough quantity for SELEX. For many interesting therapeutic targets or diagnostic markers expressed on the cell surface, the selected aptamers may show weak binding or no binding at all because the aptamer's binding domain may be shielded and inaccessible on a cell surface. Recently, several groups have reported the isolation of aptamers by using whole living cells as targets, using a process referred to as cell-SELEX [36]. In contrast to the traditional SELEX process using a purified protein target, the cell-SELEX process uses whole living cells as targets. Thus, the cell-surface proteins keep their native conformations which are critical for biological functions. Complex targets such as human red blood cell membrane preparations and membrane-bound acetylcholine receptors have been used to

extend the SELEX method [42,43]. The early examples of Cell-SELEX targeted live African and American trypanosomes [44,45]. Although the whole SELEX procedures were similar to those used for recombined protein targets, the number of selection rounds in this case was significantly greater. This Cell-SELEX was carried out over 25 rounds, but still yielded pools with high sequence complexity and convergence, comparable to ca. 15 rounds in general for selection against purified targets. Aptamers targeting these red blood cells were generated, and the binding affinities of these aptamers for the cells were comparable to those found in similar experiments against pure proteins. This study demonstrated first that nucleic acid libraries could be used to isolate aptamers against unspecified targets on cell surfaces. Thus it is possible to obtain therapeutic or histochemical staining aptamers that only bind to the specific type of disease cells, but not normal cells or other types of cells [22]. Since many drug targets are receptor proteins existing on the cell surfaces, an aptamer or a panel of aptamers can be selected to target these protein directly on the cells. The Cell-SELEX approach is also highly valuable in identifying new markers for different types of cancer and other diseases [46]. Several articles have also described cell-type SELEX targeting bacterial, viruses, and parasites. (reviewed in [47–49]).

SELEX has also been performed in the context of animals recently and aptamers capable of localizing to intrahepatic tumor deposits have been isolated by this in vivo selection process [50]. A 2'-fluro-pyrimidine modified random RNA library was intravenously injected into mice implanted hepatic tumor. Liver tumors were harvested and RNA molecules were extracted, reverse transcribed, and PCR-amplified to complete one round of selection. After 14 rounds of selection, an aptamer that identifies an intracellular target protein within the tumor compartment was obtained. Because the binding of nucleic acids is dependent on their target conformation, *in vivo* SELEX represents a strategy for the selection of molecules in their native environment, which in turn offers broad applications in creating reagents that may be useful to target and in vivo escort of tissues of interest.

2.3 Blended-SELEX

Blended SELEX [51] is the process of incorporating an extraneous molecule into a nucleic acid library and then selecting the most active of these composite assemblies. The extraneous small molecules extend the chemical power of the nucleic acid library. As an example, Smith et al. [51] first described this new approach via coupling a small molecule inhibitor of elastase to a randomized RNA library and then selected aptamer sequences to promote the covalent reaction of the inhibitor with the human neutrophil elastase (hNE) active site. Indeed, the aptamers selected from this RNA library show an approximately 20-fold increase in activity compared with the small molecule inhibitor. In addition, low doses of the inhibitor were found to prevent lung damage inflicted by human neutrophils in an isolated rat lung model of acute respiratory distress syndrome (ARDS) [51]. Similarly, Charlton et al. [52] incorporated a phosphonate irreversible inhibitor of neutrophil elastase into a randomized DNA library and selected the most potent elastase inhibitors, which are also significantly more potent than R-1 proteinase inhibitor in blocking degradation of elastin by activated neutrophils [52]. Not only can the small molecule inhibitor be employed in the randomized oligonucleotide library, biopolymers such as a peptide can also be incorporated into the randomized oligonucleotide library. For example, Radrizzani et al. [53] have developed reagents that behave as "synthetic antibodies via using synthetic peptides and a combinatorial library of 56-mer random oligonucleotides to select so called "oligobodies." These examples demonstrate that blending small molecules or peptides into combinatorial libraries is a feasible method of drug discovery.

2.4 Covalent SELEX or cross-linking SELEX

Covalent SELEX [54,55] is used to generate aptamers that bind a protein target with high affinity and specificity and can in addition, form a covalent link with the target. A nucleic acid molecule that could associate in such a manner with a target protein might be valuable for biochemical and structural studies of nucleic acid-protein interactions and possibly for therapeutic applications. This approach is based on the uracil analogs 5-iodouracil (5-IU) or 5-bromouracil (5-BrU), which can be incorporated into a randomized oligonucleotide pool for SELEX. Jensen et al. [54] demonstrated this method using a 5-IU-substituted RNA library to carry out a cross-linking SELEX experiment. They reported RNA aptamers that can bind HIV Rev protein with high affinity and can also be crosslinked to the target protein with high specificity and high efficiency by using UV laser light. Similarly, Brody et al. isolated DNA aptamers containing 5-bromodeoxyuridine residues for photo cross-linking to a protein [56,57]. Golden et al. [57] have shown that basic fibroblast growth factor (bFGF) aptamers crosslink low picomolar concentrations of target in the presence of serum, with very little non-specific crosslinking. Crosslinking activities for non-cognate, but related, proteins are substantially smaller than crosslinking for cognate proteins.

2.5 Mirror-image SELEX - Spiegelmer technology

A natural DNA or RNA molecule is composed of nucleotides containing D-2'-deoxyribose or D-ribose sugars. These sugars also exist in the mirror image versions (enantiomers) L-2'-deoxyribose or L-ribose, respectively. Aptamers composed of these L-form nucleotides are called Spiegelmers [40]. Spieglemers are attractive because they are not recognized by natural enzymes such as nucleases, and are thus extremely resistant to degradation in biological systems. However, the L-form nucleotides also prevent recognition by polymerases, which prevents the use of conventional SELEX to isolate Spiegelmers. To circumvent this problem, the first step of the mirror-image SELEX or Spiegelmer technology is to identify natural D-oligonucleotide aptamers against the enantiomeric form of the desired target. For example, if the desired target is a small polypeptide composed of natural L-amino acids, the enantiomeric version is synthesized using D-amino acids. This target is used in SELEX to select a conventional DNA or RNA aptamer. After trimming the aptamer to the minimal binding motif, the equivalent L form of the aptamer composed of L-2'-deoxyribose or L-ribose units, the Spiegelmer, then is synthesized. Because of the reciprocal chirality, this Spiegelmer binds with high affinity to the natural target just like the initial aptamer bound to the mirror-image selection target [58]. The basic concept of combining molecular evolution with chiral inversion stemmed from the identification of a D-peptide ligand for the SH3 domain of c-Src by using a phage display approach [59]. Mirror-image RNA aptamers to adenosine and arginine as well as an enantiomeric DNA specific for vasopressin were identified by mirror-image SELEX and have been published [59,60]. Since there are no nucleic acid degrading enzymes in vivo to degrade a molecule synthesized as the mirror image L-oligonucleotide, the Spiegelmer will not be degraded by any nucleases [61,62]. Spiegelmers have been demonstrated to be stable in human plasma for over 60 hours at 37 °C, while non-modified RNA aptamers are degraded in seconds. Finally, Spiegelmers appear to be non-immunogenic, even under the most inductive conditions for antibody formation in rabbits. This is of critical importance if Spiegelmer therapy is to be used repeatedly to treat chronic diseases [58]. However, this method is restricted to targets that are available as stereoisomers because most proteins are too large to be synthesized using all D-amino acids.

2.6 Tailored-SELEX

Aptamers are usually isolated using oligonucleotide libraries with 20–40 nt long randomized regions plus two fixed primer sites of ~15–25 nt on each side. Thus, the initially identified aptamer sequences typically comprise 60–90 nt. The aptamers sequences are then rationally

and experimentally truncated before they are further tested in biological systems [63]. Since the flanking fixed primer regions are usually not part of the motif structures and do not participate in forming the scaffold that surrounds the binding interface, to obtain aptamers largely free of the fixed sequences of the initial libraries, the *tailored* SELEX method was proposed [61]. For example, a library with very short fixed regions (4–6 nt) on both sides of the random region was used to identify a Spiegelmer that inhibits the action of the migraine-associated target calcitonin gene related peptide 1 (a-CGRP) with an IC_{50} of 3 nM at 37 °C in cell culture [61].

2.7 Toggle-SELEX

SELEX can produce oligonucleotides that bind to a particular target with high affinity and high specificity. However, being too specific may be problematic if species cross-reactivity is desired, as is usually the case during the preclinical evaluation of a molecule in animal models. Many potentially therapeutic candidates that demonstrate excellent efficacy *in vitro* fail to enter clinical trials due to lack of compelling efficacy *in vivo*. Therapeutic aptamers generated against human protein targets may show decreased efficacy or no *in vivo* efficacy at all in appropriate animal models. To overcome this potential problem, White et al. [64] proposed the toggle-SELEX strategy that generates specific therapeutic aptamers that are cross-reactive between different species such as human and mouse. As shown in Figure 3, in the first round of in vitro selection, the starting library is incubated with both target A (such as the human protein) and target B (such as the mouse protein). The first enriched library is assumed to bind to either target and then is recovered and amplified. In round 2 of selection, the enriched library is incubated with target A. In round 3, the human-focused library is incubated with target B. The toggle selection process is repeated using the alternate proteins for alternate rounds. Thus, White et al. demonstrated this strategy to isolate RNA aptamers that bind both human and porcine thrombin.

2.8 Capillary electrophoresis-SELEX

The traditional SELEX method is usually carried out using either filter binding or affinity chromatography to separate aptamer-target complexes from free nucleic acids. In addition, it usually takes 10–18 rounds of selection and PCR amplification to identify aptamers with acceptable binding affinity and specificity. To improve the traditional SELEX method, the Bowser and Krylov groups independently developed the capillary electrophoresis SELEX, termed CE-SELEX protocol [65–67]. For the CE-SELEX approach, an oligonucleotide library is mixed with a target molecule and the mixture is introduced into a free-solution CE system. Unbound oligonucleotides migrate at one rate, while the oligonucleotides that are bound to the target molecul migrate through the capillary at a different rate. Thus, this approach is similar to an electrophoretic mobility shift assay (EMSA). Similar to conventional SELEX selections, the collected bound fraction of oligonucleotides are then amplified by PCR and purified and prepared for further rounds of selection. It has been shown that aptamers can be identified by CE-SELEX in as few as 2–4 cycles [66]. Bowser and colleagues have used the CE-SELEX to isolated aptamers for IgE [68], HIV reverse transcriptase [69], and neuropeptide Y [65]. A very appealing advantage of the CE-SELEX is that the total number of rounds can be reduced significantly. For example, high affinity aptamers for IgE were obtained in as little as two rounds of selection. Tang et al.[70] were able to isolate aptamers with similar affinities for ricin using conventional SELEX and CE-SELEX selection protocols in nine and four rounds of selection, respectively. Remarkably, Berezovski et al. [71] were able to isolated aptamers with subnanomolar affinity for PFTase with only a single CE-SELEX. Minimizing the number of selection rounds greatly reduced the time required to isolate aptamers, and as a consequence, shortening the process from weeks to days. Recently, Krylov's group introduced the equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM) [72] selection method. The unique feature

of ECEEM is pivotal to its ability to select smart aptamers with predefined values of K_D. The ECEEM selected panels of aptamers with K_D value could be used in competitive screening of drug candidates. Very recently, Krylov's group also introduced the non-SELEX selection process for aptamers [73]. This non-SELEX strategy involves repeated partitioning steps without amplification. Importantly, the non-SELEX procedure can take as little as 1 h to complete to improve aptamer affinity by four orders of magnitude as demonstrated via a DNA library targeting the h-Ras protein.

2.9 Micromagnetic Selection (M-SELEX)

Although CE-SELEX has shown selection efficiencies for protein targets, the CE method is less effective for other classes of targets that do not induce sufficient electrophoretic shift of aptamer complexes (such as for small molecules). Magnetic beads have been used as a solid support or matrix for linking to the targets [74,75]. However, the efficiency of using magnetic beads has lagged behind what can be achieved by CE with multiple time-consuming selection rounds. Recently, Soh's group has developed a platform that integrates magnetic bead-assisted SELEX with microfluidics technology and demonstrated the capability to isolate high-affinity DNA aptamers against the light chain of recombinant *Botulinum* neurotoxin type A with only a single round of selection [76]. This chip-based microfluidic SELEX is referred to M-SELEX. The M-SELEX strategy takes advantage of a number of unique phenomena that occur at the microscale and implements a design to precisely manipulate small numbers of beads and rapidly isolate high-affinity aptamers. Very recently, Soh's group has upgraded M-SELEX [77], which employs a disposable microfluidic chip to rapidly generate aptamers with high affinity and specificity. The utility of upgraded M-SELEX has been demonstrated by generation of DNA aptamers against streptavidin in three rounds of selection. Importantly, the upgraded M-SELEX exhibits exceptional separation efficiency in removing weakly bound and unbound ssDNA to rapidly enrich target-specific aptamers. Since a wide spectrum of molecular targets can be rapidly conjugated to magnetic beads, the M-SELEX may provide a general platform for rapid generation of specific aptamers.

2.10 Atomic force microscopy (AFM)-SELEX

AFM is a method of choice to scan the atomic sample surface using a cantilever probe, detecting the weak force between sample surface and probe. AFM can also dynamically detect the adhesion or affinity force between a sample surface and cantilever. Taking advantage of these features, Miyachi et al. [78] reported AFM-SELEX to isolate aptamers against thrombin with three rounds of selection. Importantly, many of the resulted aptamers via the AFM-SELEX had a higher affinity to thrombin than the thrombin aptamers published from conventional SELEX method. Independently, Peng et al. [79] utilized a combined AFM and fluorescence microscopy to select aptamers from nucleic acid library in only a single cycle.

2.11 Surface Plasmon resonance (SPR)-SELEX

SPR is a very sensitive method to measure binding interactions of various biomolecules, including peptides, proteins, nucleic acids, carbohydrates, and phospholipids [80]. This technology has been widely used to determine the binding constants of many aptamers [81]. As an extension, Misono and Kumar [82] used the SPR-SELEX to actually isolate RNA aptamers binding to hemagglutinin (HA) of human influenza virus. Thus, they injected the nucleic acid pool into the flow cell of a CM4 chip coated with target for 2 min. Unbound nucleic acids were washed off the chip, and then the bound aptamers were eluted into the fraction tray. The collected fractions were ethanol-precipitated to recover the bound RNAs and amplified further for the next round of selection. SPR-SELEX has several advantages, such as the repeated use of an immobilized target and the identification of binding species

fractions. In addition, the great advantage of SPR is that it provides information about the target binding abilities of molecules as they are being selected.

2.12 On-chip selection

Various aptamer chips have been used for protein detection [83,84]. Current technology allows rapid synthesis of a large custom chip with tens of thousands of probes (Agilent Technologies, CombiMatrix). The application of high density microarrays to the aptamer selection process has the potential to speed up the generation of aptamers with high affinity and specificity. For example, Asai et al. [85] have isolated DNA aptamers targeting resorufin using on-chip selection in combination with a method for point mutations. One of the main problems in the application of chip technology for the selection of aptamers is the design of the initial pool of oligonucleotide molecules for screening. It is quite challenging to design one with only 10^4–10^5 nucleic acid sequences for the microarray chip from a set of 10^{13}–10^{14} possible sequences normally found in *in vitro* selection libraries. However, recent advances in *in silico* approaches have enabled a reduction in the size of the nucleic acid sequence space for experimental screening and selection of nucleic acid aptamers [86]. Katilius et al. [87] used DNA microarrays to explore the relationship between the aptamer sequences and binding properties of immunoglobulin E (IgE)-binding aptamers.

2.13 Bead-based selection

Since the nucleases degrade nucleic acids by nucleophilic attack at the oligonucleotide phosphorus, modifying the phosphate ester backbone of an aptamer is expected to enhance stability. A number of chemically-modified phosphate ester backbones of oligonucleotides have been used in nucleic acid based drug development. The substitution of both non-bridging phosphate oxygen atoms with sulfurs gives rise to a phosphorodithioate (PS2) internucleotide linkage which, like normal phosphorodiester linkages of RNA, is achiral at phosphorus. Like the normal phosphodiester, the PS2 modification is isosteric and isopolar and should have other biochemical and biophysical properties similar to natural RNA/DNA. Additionally, it has been shown to have high nuclease resistance and to increase binding affinity to proteins without sacrificing specificity [88]. However, it is not possible to select achiral PS2 containing aptamers using SELEX since nucleoside dNTP(αS2) are not substrates of polymerases [89]. Therefore, Yang et al. [41,90] demonstrated a bead-based combinatorial library selection methodology. There are three essential steps for this strategy. The first step is to create via split/pool synthesis a PS2-modified nucleic acid library attached to beads. A split-pool method using two synthesis columns can created 2^N different members of the library for N split-pool steps (see www.thioaptamer.com for automation of the bead-based library synthesis). Utilizing more columns (M) allows synthesis of M^N different beads with one unique nucleic acid sequence on each bead. This approach has been used to create hybrid backbone PS2-ODN libraries as well as libraries using other modified nucleotides such as 5-aminoallyl modified dU. The second step involves screening the beads for binding to a target. The bead library is exposed to a target that has been covalently labeled with a dye [90]. Alternatively, the bead library is incubated with unmodified target, followed by addition of a specific primary antibody to the target protein and then a second antibody conjugated with a fluorescent dye or with a fluorescently labeled antibody that binds to the complex [91]. For manual selection the beads are viewed under a fluorescence microscope, and the beads showing the most intense fluorescence can be picked with the aid of a micropipette coupled to a micromanipulator (Figure 4). These positive beads have aptamer sequences that demonstrate high affinity to the target. Alternatively, the beads can be sorted using high-throughput/multi-color flow cytometry [91] or magnetic bead separation using magnetic nanoparticles attached to the target. The final step in this aptamer selection process involves sequencing the aptamers on the selected beads. Yang et al. [90,92] used PCR followed by cloning to sequence the beads. For selection of aptamers

containing specific PS2 modifications (or other modified nucleotides), the sequence is used as a “barcode” to indicate the location of the thiophosphates or other modifications.

The bead-based process has several significant advantages. As noted above, the bead-based process can be used to select aptamers containing modified nucleotides that cannot be incorporated enzymatically. Thus, it can incorporate a significantly broader chemical diversity. The bead-based process also reverses the conventional SELEX approach to binding and separation. During SELEX, a mixed library of oligonucleotides in solution is selected for binding to an immobilized target (e.g. a protein bound to a filter or chromatography resin). In this situation, a large mass of weak-binding sequences are recovered along with the small number of tight binding aptamers. Thus, numerous rounds of selection are typically required to enrich for the tightest binding sequences. In contrast, the bead base process incubates the soluble target with an immobilized library. Thus, there is no direct binding competition between tight and weak binders. A single bead carrying a high-affinity aptamer will bind large amounts of target at low concentrations, while the majority of beads will show little or no binding. Since the high-affinity beads are isolated directly and individually, tight-binding aptamers can be isolated in a single step. This eliminates the time-consuming requirement for multiple selection rounds. A further benefit is that this eliminates the repeated PCR amplification steps required during SELEX. Several groups have noted that PCR can impose an “amplifiability bias” during aptamer selection, and may significantly limit or even prevent recovery of desirable aptamers that amplify with low efficiency [93,94].

3. Conclusion

Because of their high affinity and specificity, oligonucleotide aptamers have been finding numerous applications beyond therapeutics, such as in analytical fields [95], target validation [96], drug discovery processes [97], and as diagnostic agents [11,56]. In spite of many different strategies reported to select aptamers, currently, the conventional SELEX strategy still dominates the other strategies outlined here. Clearly, in order to develop as many aptamers as possible to meet the broad application potential, cost-effective automation of the selection procedures is desirable. Indeed, much progress has been made on this matter[18–21,98]. Additionally, while numerous strategies have evolved to select aptamers, most still rely upon PCR amplification of the binding sequences. These SELEX-based selection strategies are limited to natural nucleotides and to only a very small set of PCR-compatible modified nucleotides. Further work on selection strategies that expand the chemical diversity of aptamers would benefit the field.

4. Expert opinion

Combinatorial chemistry is an important technology for biotech and pharmaceutical research to discover new molecules with desirable properties such as new drug candidates and catalysts. Oligonucleotide aptamers obtained from SELEX and many other *in vitro* selection strategies have demonstrated high affinities and specificities for a considerably wide range of targets including metal ions, small organic molecules, biomolecules such as proteins, and cells. In reality, any kind of biological molecule can be targeted by aptamers. Aptamers with the K_D values in the low nanomolar to picomolar range are a highly competitive class of molecules that rival antibodies in diagnostics and therapeutics. Several groups are already starting to transfer antibody-based diagnostic platforms to aptamers. The enzyme-linked oligonucleotide assay based on ELISA technology is one example for using aptamers instead of antibodies [99]. Unlike antibodies which are generated from animals and cell lines, aptamers are selected by an *in vitro* process. Importantly, aptamers also display little to no immunogenicity, which is important for human therapeutic applications [100]. Another

important advantage of aptamers is that they can recognize a specific domain of a multidomain protein target. Thus, when the selected aptamer modulates the targeted domain of the protein, the untargeted domains often retain their functions. In addition, oligonucleotide aptamers can be easily functionalized with many function groups such as biotin and dyes, which make them excellent diagnostic or therapeutic tools. Like many oligonucleotides, aptamers can have a fast clearance rate *in vivo* making them more suitable than antibodies for in vivo imaging.

In spite of the number of aptamers currently in various phases of clinical trials, Macugen is the only aptamer to gain approval as a therapeutic agent. Clearly, one principal challenge that remains in achieving the application of aptamer therapeutics is the hurdle of intracellular delivery. Once that challenge is met, the development of aptamers therapeutics may fulfill its exciting potential as the next major class of drug molecules.

Article highlights

- Affinity selection using nucleic acid combinatorial technology enables rapid and comprehensive exploration of drug-binding nucleic acid molecules.
- Nucleic acid aptamers offer benefits to drug discovery from several aspects: a) leads from non-traditionally used medicines, b) novel structured compounds from combinatorial chemistry screening, c) better binding affinity than those of conventional small-molecule drugs, d) advantages over antibodies as produced by chemical synthesis and little or no immunogenicity in therapeutic applications.
- The structured oligonucleotides can provide bioactive compounds for a wide range of therapeutic targets from small molecule to complicated target such as cancer cell.
- Identification of targeting aptamers is available through the SELEX process or *in vitro* selection and many variants such as CE-SELEX, M-SELEX, and bead-based *in vitro* selection.

The box summaries key points contained in the article.

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Declaration of interest

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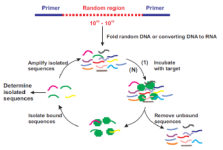

Figure 1. General scheme of the SELEX or in vitro selection procedure

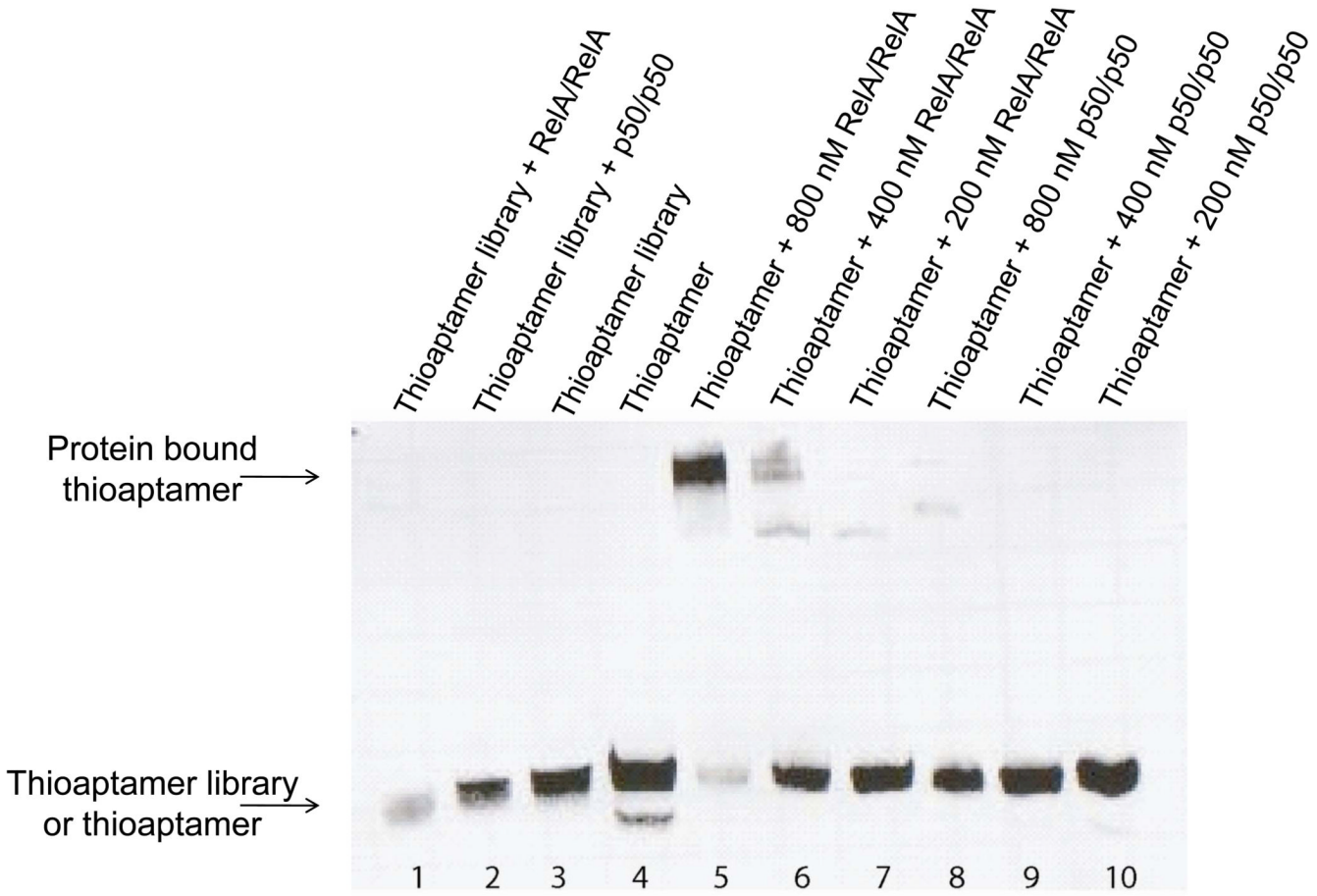

Figure 2. A negative-SELEX selected thioaptamer sequence binds more selectively to RelA/RelA than to p50/50 homodimers of NF-κB

Lanes 1–3 show the initial thioaptamer library, either incubated with RelA/RelA (Lane 1), incubated with p50/p50 (Lane 2), or with no added protein. Lane 4 is a selected thioaptamer clone isolated from the initial library after 10 rounds of selection for binding to RelA/RelA and negative-SELEX against p50/p50. Lanes 5, 6, and 7 represent the thioaptamer with 800, 400, and 200 nM RelA/RelA, respectively. Lanes 8, 9, and 10 represent the thioaptamer with 800, 400, and 200 nM p50/50, respectively. For a complex target such as a cell, the negative SELEX procedure is often included. In one example, a library of ssDNAs that contained a 52-mer random sequence was incubated with the T-cell acute lymphoblastic leukemia cell line CCRF-CEM to allow binding to take place [36]. The cells were then washed, and the DNA sequences bound to the cell surface were eluted. The collected sequences were then allowed to associate with excess negative control cells (B-cell line from human Burkitt's lymphoma, Romos), and only the DNA sequences remaining free in the supernatant were collected and amplified for the next-round selection. After multiple rounds of selection, this negative-SELEX process efficiently reduced the DNA sequences bound to the control cells, while those target-cell-specific aptamer candidates were enriched [36].

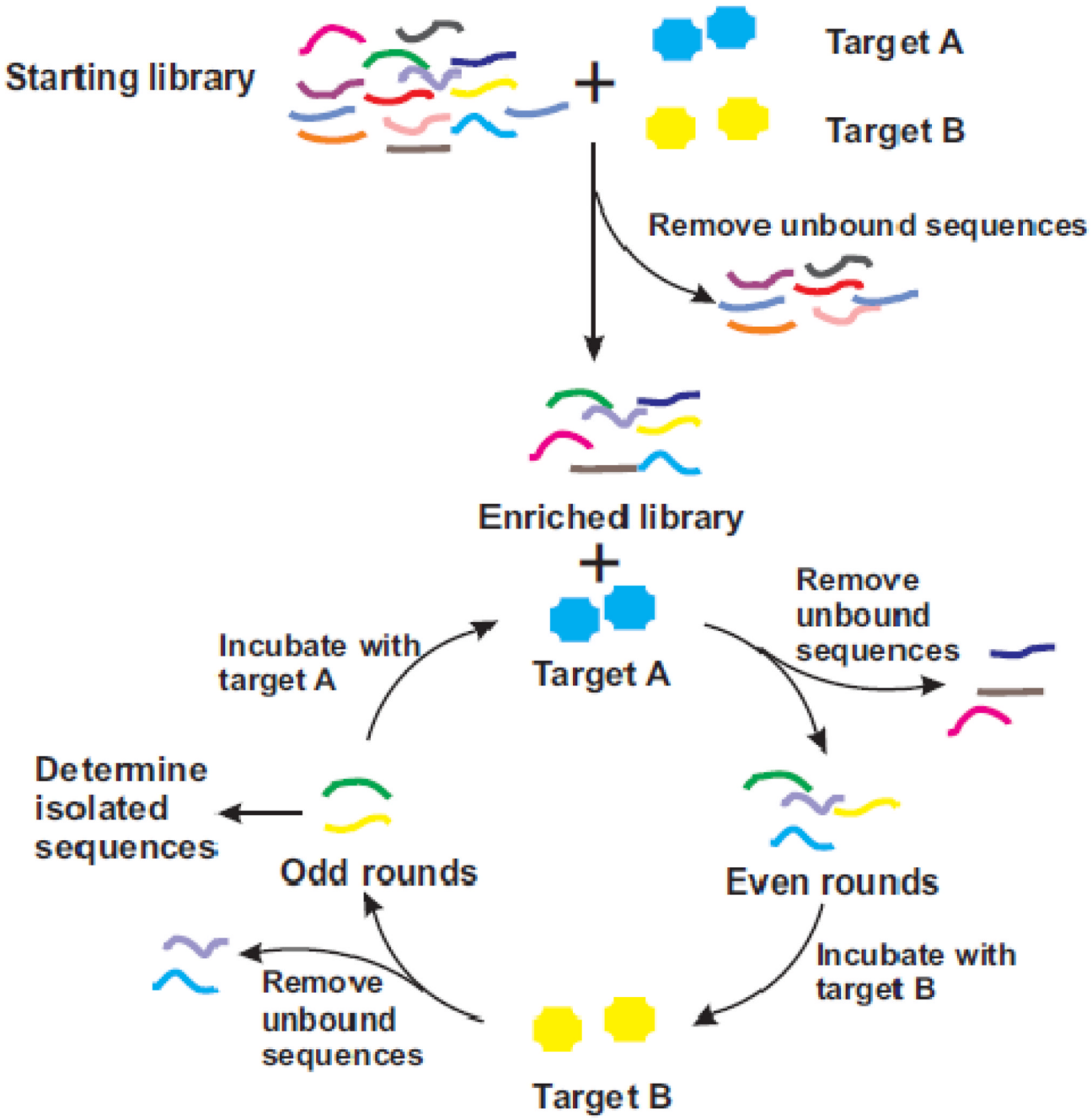

Figure 3. Toggle SELEX

Aptamers that bind both human and mouse proteins are selected by toggling the protein target between human and mouse during alternating rounds of selection.

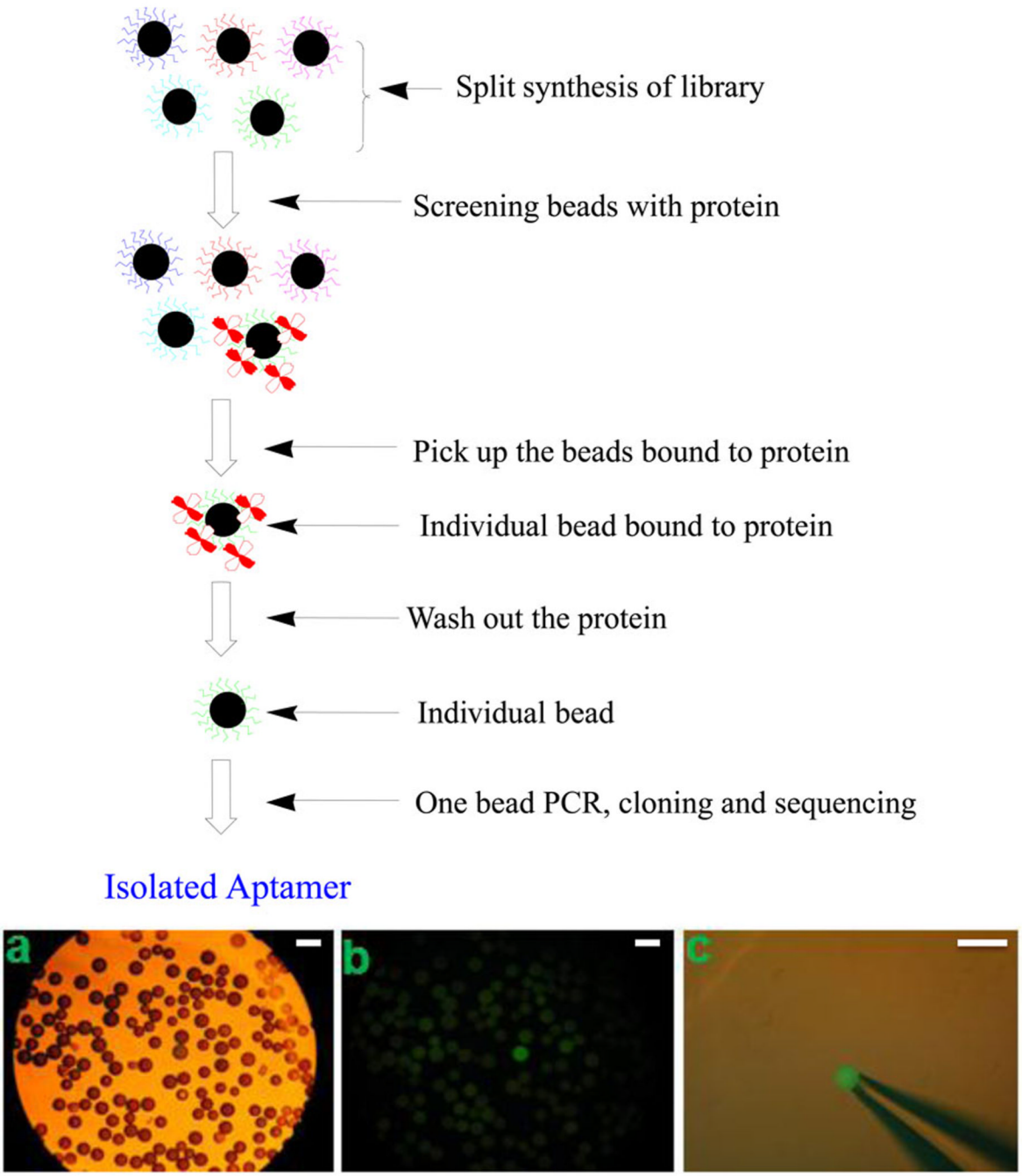

Figure 4. A bead-based aptamer library screen

(a) An aliquot of aptamer beads bound to target protein labeled with the Alexa Fluor 488 dye viewed under light microscopy. (b) The same beads viewed under fluorescence microscopy, in which a positive green bead stained with Alexa Fluor 488 dye can be easily identified in a background of many hundreds of nonreactive beads. (c) A single positive bead can easily be retrieved with a hand-held micropipette under a fluorescence microscope. Scale bar, 100 μm.