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In vitro selection of aptamers and their applications

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Abstract

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The introduction of the in-vitro evolution method known as SELEX (Systematic Evolution of Ligands by Exponential enrichment) more than 30 years ago led to the conception of versatile synthetic receptors known as aptamers. Offering many benefits such as low cost, high stability and flexibility, aptamers have sparked innovation in molecular diagnostics, enabled advances in synthetic biology and have facilitated new therapeutic approaches. The SELEX method itself is inherently adaptable and offers near limitless possibilities in yielding functional nucleic acid ligands. This Primer serves to provide guidance on experimental design and highlight new growth areas for this impactful technology.

Introduction

The concept of single-stranded RNA or DNA oligonucleotides serving as molecular recognition agent akin to antibodies was unprecedented until the late 1980s. Then, the pioneering concept of Systematic Evolution of Ligands by EXponential enrichment (SELEX) led to the discovery of oligonucleotide-based molecular affinity agents, now known as aptamers, [1, 2] SELEX enabled isolation of high affinity-binding oligonucleotides from a large population of random sequences, expanding the known functions of DNA and RNA into unexpected roles. Aptamers offer specificity and affinity normally expected of antibodies but in small, chemically synthesized molecules free from cell-culture-derived contaminants. Additionally, aptamers can be easily synthesized and chemically modified, can be reversibly denatured and have tuneable affinity and kinetic properties. The in vitro nature of SELEX offers opportunities to control the conditions of the binding event to best suit a goal. The process follows the rules of combinatorial chemistry and directed evolution [3,4]. Chemically, SELEX uses a heterogeneous library **[G]** of nucleic acids that folds into conformations unique to their sequence identity. SELEX starts with the premise that within this multitude of three-dimensional nanostructures, an oligonucleotide with exclusive affinity to nearly any target molecule exists and can be sequestered. The affinity between an aptamer and its target is often described using the equilibrium dissociation constant (K_D), where lower K_D values signify tighter binding:

$$K_D = \frac{[A][T]}{[AT]}$$

where A is the aptamer, T is the target and AT is the aptamer-target complex. K_D can also be described using the ratio of the on-rate and off-rate,

$$K_D = \frac{k_{off}}{k_{on}}$$

where k_{on} is the rate of association and k_{off} is the rate of disassociation.

Determination of the dissociation constant by either method reveals the optimal working conditions of an aptamer for detecting its cognate target. Most commonly, aptamers are reported with an apparent K_D when working with concentration-based measurements, owing to the fact that a true K_D is not affected by varying concentrations of the limiting reagent[5].

The aptamer concentration should be much less than the K_D[6]. However, in aptamer systems it is not always possible to control experimental parameters to sufficiently eliminate this variable[6].

A typical SELEX process is shown in Figure 1. The main steps include incubation, partitioning **[G]** and amplification. The idea of molecular evolution stems from using a polymerase reaction to generate a screened library with potential winners, for example aptamers with strong binding affinity, to be utilized in the next round of SELEX. Multiple SELEX cycles generate a competition among active sequences, ideally enriching the highest affinity sequences in an evolved SELEX library. Success is measured by selection of aptamers with high affinity to their cognate target, high specificity, as well as other features, for example tolerance to nuclease digestion.

The elegance of SELEX is in its simplicity – powerful molecular recognition agents can be generated at low cost, using basic biochemistry/molecular biology equipment (for example, thermocycler, gel electrophoresis unit) with techniques taught in most undergraduate science curricula, such as polymerase chain reaction (PCA) and sequencing. The accessible nature of SELEX has democratized molecular affinity and led to a surge of start-up companies and academic labs working on aptamer development and applications, after the initial patents around the process expired. Oligonucleotides with these unique properties are the exception, not the rule. While SELEX is extremely proficient at finding these exceedingly rare molecules, careful consideration of the conditions and design of the experiment could help avoid potential pitfalls and greatly improve the likelihood of success. This provides guidance on experimental design and data analysis, while providing a snapshot of nascent opportunities for aptamer applications. While the recommendations described here could equally be applied to the generation of other functional nucleic acids, for example DNAzymes and riboswitches, this Primer focuses on nucleic acid aptamer selection, characterization, and applications. Considerations to improve reproducibility in experiments, limitations and workarounds, as well as the future outlook of the field are also discussed.

Experimentation

Proper preparation greatly improves the likelihood of success in a SELEX experiment. Ultimately, a comprehensive SELEX plan will require considerations involving nine aspects of the selection experiment: library design; incubation conditions; partitioning method **[G]**; stringency **[G]**; amplification; generation of single stranded nucleic acids; monitoring enrichment **[G]**; sequencing and bioinformatics; characterization. The flow chart in Figure 2 offers a blueprint to guide the planning process for a successful selection, including examples of queries and how they may influence the SELEX experiment. Considering the nature of the target, the desired application for the aptamer and the medium within which the aptamer will encounter the target, balanced by an understanding of the resources available for the experiment, will help guide decisions about all aspects of the SELEX experiment. For example, the end-application of the aptamer should be carefully considered prior to starting a SELEX experiment. Before starting an experiment, the ideal affinity of the aptamer to be successful in a particular application should be considered. The desired affinity could, for example, stem from the required limit of detection of the aptamer-

based assay, or the biological concentration of a key target of the aptamer's therapeutic intervention. Understanding this value can help inform what partitioning method should be employed and what the stringency of the selections must be to achieve that desired affinity. Furthermore, it impacts choices on characterization methods that are used to assess the selected hits. For example, techniques to estimate binding constants are limited in the range of K_D values they can assess by their own sensitivity,[7] thus the choice of method used should be guided by the range of K_D values expected and required. The following sections give more detail about the critical parameters in a typical SELEX process and give some examples of the typical data generated for both aptamer selection and characterization experiments.

Library Design

SELEX starts with naïve nucleic acid library, from which the aptamer hits will be revealed. The SELEX library is typically designed with a central randomized region of a variable number of nucleotides flanked by two fixed primer regions at both ends. The fixed regions facilitate the amplification of the library using PCR. The base composition, as well as the length of the randomized region of a SELEX library governs the extent of diversity of the initial library. A typical SELEX library consists of A, G, T/U, C. Considering an 'n' nt randomized region, the number of unique sequences that could be generated is 4^n. For example, a 40 nt randomized region can generate 4^{40} unique sequences. Notably, the sequence space **[G]** that can be sampled in a SELEX experiment is governed by the practical limitations of DNA synthesis; any library with a random region of more than 25 random nucleotides would not be expected to contain every possible random sequence (see box 1). Nevertheless, strategies for increasing sequence space coverage exists, such as using error-prone PCR conditions[8] to introduce point mutations. Additionally, a growing number of available modified bases have been utilized in SELEX libraries to generate aptamers with high nuclease stability and affinity. For example, to increase nuclease stability, efforts have been primarily focused on RNA SELEX, substituting pyrimidines with Xeno Nucleic Acid (XNA) bases[9,10]. Several XNA bases are currently available, including, 2'-F[11], 2'-O-Me[12], 2'-NH_2[13], LNA[14], TNA[15], FANA[10], and HNA[16] monomers. Additionally, while this approach is less common, the nuclease stability of a SELEX library can be enhanced by backbone modifications by using nucleic acids with phosphorothioate backbones[17]. Strategies to enhance diversity of a DNA-SELEX library and to produce protein-like binding have been accomplished by incorporating Unnatural Base Pairs (UBPs). These bases are decorated with hydrophobic or hydrophilic residues to enhance the chemical space of a SELEX library. For example, Ds: Px[18], AEGIS bases[19,20], and dnAM: d5SICS, dPTMO and dMTMO bases[21], have been used to select high-affinity aptamers. Chemical diversity can also be expanded by synthesizing uridine analogs with substituted groups at the 5th position on the purine ring[22]. This strategy led to the generation of SOMAmers with high specificity and high affinity against a large array of soluble biomarker proteins[22]. Furthermore, libraries can be designed with reactive functional groups, such as an alkyne, followed by the introduction of functional arms decorated with azide groups added via bioorthogonal reactions, such as click chemistry, to select high-affinity aptamers with novel functional groups[23,24]. The synthesis of a typical, unbiased SELEX library should yield an equal ratio of A:G:T:C in the random region; to achieve an equal nucleotide distribution, the molar ratio of A:C:G:T phosphoramidites on the DNA synthesizer should be optimized

to account for the different reactivity of these building blocks. For example, ratios of A:C:G:T have been varied from 1.30:1.25:1.45:1.00,[25] or 1.50:1.25:1.15:1.00[26]. These ratios can be achieved using a hand-mix in which an appropriate number of moles of AGTC phosphoramidites are premixed and added to a single position in the DNA synthesizer or machine-mix by programing the synthesizer to deliver an appropriate volume of each base to the column during synthesis. Generating RNA or XNA libraries is done by synthesizing a DNA library and then transcribing it into RNA/XNA libraries.[10] Since most UBPs are based on DNA bases, UBP-containing libraries are directly synthesized using a standard DNA synthesizer.

Design of fixed regions

Regardless of the composition of the random region, the fixed region typically contains natural RNA or DNA bases because of their compatibility with polymerases utilized to amplify SELEX libraries. The primer region normally contains 18–20 nts with a nucleic acid base composition optimized to achieve high PCR efficiency. Factors in primer design include optimal melting points, no primer-dimer formation and avoidance of secondary structure formation within the primer sequence. Several commercial sources are available for primer design, such as the Oligoanalyzer tool in IDTDNA, Bio-RAD, and Thermo Fisher. When the SELEX library is composed of RNA/XNA molecules, most XNA libraries are generated by primer-extension, though rarely an additional promoter sequence is included in the 5’-end for the T7 RNA polymerase reaction. A note of caution when designing multiple SELEX experiments that may be running concurrently: always design the libraries to have either different primer pairs or unique barcodes to avoid cross-contamination during parallel experiments. When multiple selections are on-going in the same space, it is very easy to cross contaminate selections, through shared instrumentation and reagents. Though good lab practices for handling nucleic acids and consistent decontamination (of pipettes, shared spaces, shared equipment) with ethanol and bleach can usually eliminate these issues, using unique libraries is an effective strategy to eliminate this problem entirely.

In addition to the fixed primer regions, the library may also contain fixed regions for other purposes. For example, a capture sequence may be included to facilitate binding of the library to a solid support such as capture SELEX, *vide infra*. A SELEX library can also be predesigned to select the desired aptamers with unique structural and functional properties. For example, structural components, such as stem-loop,[27] and three-way junctions[28], can be added within the randomized region to select aptamers containing predefined structures. A sequence can also be modified to facilitate the selection of aptamers that can bind to recalcitrant targets, for example, a library with predefined hairpin structures incorporating clustered glycans,[24] was used to select aptamers for carbohydrate-binding proteins. Predesigned SELEX libraries can also be centered on parent natural nucleic acid ligands by partially randomizing or by adding a doping mixture of nucleic acid bases to a set position.[29] This unconventional approach has been successful in generating second-generation aptamers with higher affinities and functionalities. Furthermore, a SELEX library can be designed for post-SELEX selection to add functional arms to already selected aptamers to enhance their affinity.[29,30]

Incubation conditions

The incubation of a SELEX library and the target can be achieved with both free in solution or by immobilizing either the target or the SELEX library on a solid support. This choice is usually based on the nature of the target and end-use of the aptamer. The conditions (pH, salinity, temperature) of this step should match those of the application as best as possible to ensure optimal fit for purpose.

During selection, it is necessary to present a target with consistent and uniform structure. Therefore, target preparation must be predominantly aimed at optimizing the conditions to facilitate generating a conformation like one in its natural environment. Several types of targets have been utilized in SELEX, and preparation steps are unique to the target.

Small molecules—Generally, when the target of interest is a small molecule, SELEX is more challenging.[31] However, the target molecule can be immobilized on a solid support, such as a bead, using standard bioconjugation procedures.[32] Here, it is essential to ensure that the structure of the target is not perturbed by immobilization and that the target functional groups used for immobilization are not critical for ensuring the specific binding of the aptamer. If the target molecules cannot be immobilized on a solid support, the SELEX library can be immobilized on a solid support.[33] This strategy is known as capture SELEX, and it has proven to be effective in generating structure-switching aptamers against various targets. Here, the SELEX library is hybridized to a bead-bound short complementary strand, and upon binding, the sequences undergo a conformational switch allowing the sequences to bind to the target, followed by spontaneous dissociation of the complex from the beads that leads to elution.[34] If the target or the SELEX library cannot be immobilized, then the binding can be done in a free solution, but here, the separation of bound sequences to the target is needed.

Proteins—In contrast, when the target is a protein, the SELEX experiment will have a higher expectation of success. However, the buffer conditions need to be optimized to ensure that protein folding is consistent and stable, enabling unperturbed presentation of the target's binding moieties to the SELEX library.[35] The protein target can be presented free in solution, allowing unrestricted binding of potential aptamers followed by partitioning. Proteins can also be immobilized on a solid support using several immobilization methods, such as Ni columns[36], biotin-streptavidin[37], magnetic beads[38] or direct chemical attachment[39,40]. In general, care must be taken to avoid selecting aptamers toward a protein's conformation that may not exist in its natural environment. If the protein is a membrane protein, a practical method is to use whole cells with endogenous expression levels of the target or whole cells engineered to express the protein of interest.[40,41]

Cells and tissues—A particular advantage of SELEX is that the target need not be known to carry out the selection. This feature is commonly exploited in cell-SELEX. The challenges and opportunities of whole-cell SELEX for bacteria, parasites and animal cells has been reviewed recently[42]. Generally, for cell selections, the selection matrix needs to be carefully considered to accommodate for potential challenges, for example nucleases or highly abundant non-targets that may deplete the library. As a specific example, when whole

cells are used, the culture conditions need to be optimized to avoid generating aptamers with a high bias towards cell-surface proteins only expressed on cultured cells. One way to avoid this bias is to use primary cells known to express the desired target.[43] Another consideration is the number of dead cells that could remove potential binders from the SELEX pool.[44] Use of fluorescence-activated cell sorting (FACS) to remove dead cells from the population helps increase SELEX efficiency. In addition to whole cells, SELEX can be used to select aptamers against whole tissues. When the target is whole tissue, the selection can be performed either *in vitro* or *in vivo* using animal models.[45,46] When using animal models, the SELEX library is injected systemically into the animal and allowed to circulate, subsequently removing a tissue of interest to extract bound sequences.[45]

Partitioning

Partitioning of winning sequences with high affinity is an essential part of the SELEX process. Partition efficiency[47] can be defined as $\mathrm{PE} = \frac{k_B}{k_N}$, where k_B is the transmittance of binders and k_N is the transmittance of non-binders after a partitioning step. Better partitioning efficiency leads to fewer SELEX cycles generating aptamers with desired affinity with low background sequences. Partition efficiencies vary widely by selection method, ranging from 10–100 for traditional methods[33] such as magnetic bead separation, to 10^5-10^6 for more advanced methods such as microfluidic-SELEX or capillary electrophoresis SELEX.[39,48,49]

If the target is immobilized on a solid support,[50] nucleic acids can be separated by simple washing, while bound aptamers can be eluted by applying heat[51], chaotropic agents or using enzymatic approaches.[52] Capture-SELEX employs a unique way of partitioning that involves a conformational switch facilitating the elution of an aptamer/small-molecule complex from the beads.[33] The most common method of partitioning a protein/aptamer complex free in solution is the use of nitrocellulose filters.[53,54] Free nucleic acids show minimal non-specific binding to nitrocellulose, while proteins are often tightly held. Thus, only oligonucleotides in a tight complex with the target protein will be expected to bind to the nitrocellulose, and weakly bound sequences are washed away. The retained nucleic acid sequences with target can be extracted using standard nucleic acid extraction methods. In addition, based on the changes of electrophoretic mobility, protein/nucleic acid complexes can be separated by gel electrophoresis or capillary electrophoresis.[55,56] When whole cells are the target, the nucleic acid sequences bound to cells can be separated by simply heating or using a higher affinity competitor, such as a monoclonal antibody.[57,58] In the case of tissue-SELEX, of the bound nucleic acid sequences can be eluted using standard nucleic acid extraction methods, such as homogenization and organic solvent extraction, or commercially available kits.[59,60]

Stringency

Stringency of the selection can be judiciously controlled in the planning phase. To complement the conditions of the positive selection **[G]** with the chosen target, counter and negative selections are frequently included. Counter-selections **[G]** involve incubating the selection library with a similar molecule to remove aptamers with cross reactivity,

whereas negative selections **[G]** are done to remove sequences with affinity to the selection matrix (for example, magnetic beads). The target concentration is also a key parameter of a selection experiment and the optimal target concentration will depend on a variety of factors.[61] In theory, the higher the target concentration, the greater the possibility of isolating aptamers with low to moderate affinity. In practice, however, low frequency but high affinity binders could be easily lost in early rounds if target concentration is too low. Gradually reducing the target concentration throughout the selection can be one approach to increasing stringency to ensure enrichment of true binders. Several studies have suggested that the optimal library to target ratio for SELEX experiments should range from 100:1–1000:1[62,63] Setting the final target concentration to be in the range of the desired K_D, and tracking your optimal target concentrations with the changes in the bulk K_D of the enriched pool, is a sound strategy.[22,64] To minimize enriching aptamers against the matrix, such as the solid support or the nitrocellulose filters, the performance of a negative selection step, using the matrix, is necessary.[65] When whole cells are utilized, a similar cell line that lacks the target receptor of interest to remove binders common to both cell types should be used for a counter-selection step.[66] When the whole cell is engineered to express a high level of target protein, a native cell line that does not express the same target is used to remove amplicons in the library.[67] Implementing a negative selection step has been proven to remove a significant number of nonspecific sequences from the pool. Other counter selection steps against potential cross-reacting species can also be very effective. When a specific application is desired, it is advisable to introduce conditions related to that application (pH, salinity, temperature, interferences) in the later rounds of SELEX to ensure the enrichment of robust, versatile aptamers.[43]

Amplification

Once the winning sequences are partitioned using an appropriate method, sequences are ready for PCR amplification. Since only a handful of truly functional aptamers to the target is present at any given early round of a SELEX pool, careful optimization of the amplification step is crucial for the successful selection of aptamers against the desired target.[68,69] At the beginning of SELEX, the nucleic acid library is heterogeneous and may contain sequences that are difficult to amplify, with high GC content, tending toward the formation of exotic secondary structures.[70] Therefore, at the beginning of SELEX (approximately rounds one to five), the probability of forming primer-dimers and overamplifying sequences with high PCR bias, also known as PCR parasites, is high. This could lead to a potential loss of aptamer sequences present in the pool. Therefore, it is essential to optimize PCR conditions to account for the concentration of primers and the number of cycles to minimize the overamplification of PCR parasites. Strategies to minimize PCR bias include limiting the amplification cycles, monitoring the amount of template, decreasing primer concentrations, and utilizing emulsion PCR.[71] In particular, quantitative (q)PCR is particularly useful for quantifying target-bound ssDNA after each round and revealing the optimal (minimal) number of PCR cycles for amplifying the target-bound fractions for the next round of SELEX.[72] Because PCR depends on the efficiency of recovering winning sequences at the partitioning step, no standard number of PCR cycles can be recommended. Therefore, when the copies of winning sequences are low during the initial rounds of selection, performing a two-step PCR reaction is advisable to avoid

the potential loss of aptamer sequences.[73] When UBP's are used in the SELEX library, the PCR procedure needs to be optimized to ensure that the modified SELEX library is uniformly amplified.[74,75,76,77] When an RNA library is used first, reverse transcription (RT)-PCR converts the RNA library into a DNA library. RT-PCR is followed by PCR to generate amplified double-stranded DNA pools subsequently converted into the evolved RNA library using transcription. When modified bases are used in a SELEX library, engineered polymerases are available mostly as plasmids from respective academic labs, which requires expression and the purification prior to beginning of SELEX.[78] PCR product can be detected using either a polyacrylamide or agarose gel electrophoresis. Typically, the number of PCR cycles required to obtain a reasonable amount of an evolved SELEX pool is relatively high during the first rounds of SELEX, but then decreases and eventually plateaus. When using modified bases or UBPs, at special polymerases are sometimes required so experiments should be planned accordingly. [79]

Generation of single-stranded DNA—When an RNA library is used, transcription of the amplified DNA library typically generates single-stranded RNA. The product of the amplified DNA library is double-stranded, which then needs to be converted to single-stranded aptamer sequences. One common method of generating a single-stranded SELEX pool is using a biotinylated reverse primer captured by streptavidin-coated magnetic or agarose beads, followed by alkaline denaturation.[80] The released sense strand is collected, desalted, and quantified. There are some challenges with this method, including carry over contamination of the selection library with streptavidin, which can interfere with the selection (an example that illustrates the importance of counter-selections). Only a few other approaches, such as asymmetric PCR,[81] Lambda exonuclease digestion[23], and denaturing PAGE separation[82,83], are commonly used, usually based on the availability of resources. Each of these methods have their own advantages and drawbacks.[80] Asymmetric PCR can generate a much higher concentration of single-stranded DNA than other methods, but PCR or proofreading issues can introduce bias. Exonuclease digestion is efficient in the sense that it produces a relatively high yield compared to magnetic beads, but it is a multiple step process. Finally, denaturing PAGE methods are useful in physically separating DNA strands of different sizes, but they are also laborious to carry out.

Monitoring enrichment

When selecting aptamers from large libraries, it is critical to remember that you get what you select for. As such, it is essential to evaluate whether the selection is working to maximize the chance of identifying aptamer candidates exhibiting the desired functional properties following the months-long selection experiment. Enrichment can be monitored by measuring the amount of target-bound nucleic acid, by measuring the affinity of the oligonucleotide pool to the target or by assessing the decrease in the diversity of the selected library, after each round. This term is also used for individual aptamer sequences or clusters from high throughput sequencing data by comparing cycle-to-cycle abundance. Using the following equation[84], relative sequence enrichment over each round can be compared and calculated:

$$\% \text{ Sequence Enrichment} = 1 - \frac{Unique\ Reads}{Total\ Reads} \times 100$$

First, for researchers employing typical, primer-based SELEX, confirmation of PCR amplification product by gel electrophoresis remains the method of choice. The PCR amplification product from the selection round should show a band matching the size as the original library (positive control), without any by-product formation and no band in the negative control (non-template control) lane. By-products or undesired amplicons may result in parasitic elongated or ladder-like products.[68] Figure 3A shows an example of an ideal PCR amplification analyzed by gel electrophoresis.

Next, as the selection proceeds, it is critical to monitor the round-to-round selection progress by assessing both library diversity and relative affinity. The progress of SELEX should be assessed to understand the enrichment of winning aptamer sequences, to decide when to introduce more stringent conditions and to avoid amplification of nonspecific sequences in the SELEX pool. Several methods are available to assess SELEX progress.[85–87] With the use of high-throughput sequencing (HTS – *vide infra*), the ability to deconvolute a SELEX library has become feasible. qPCR-based remelting curve analysis has also been used to track changes in sequence diversity as a surrogate for enrichment.[71,88] However, because enriched sequences may not represent putative aptamers,[89] it is ideal to also monitor and measure the relative affinity of the library to the target-of-interest.[90] The method of assessment of affinity depends on the target and end-use of the aptamer. One simple indirect method to do this involves quantifying the amount of library that is recovered or eluted from the target each round compared to the amount of library recovered from a control experiment – such as a counter target or a negative control.[91] As an alternative, in the later rounds, the entire library can be subjected to a head-to-head binding affinity experiment in bulk to determine the relative affinity of the enriched library to the target compared to the original library or a random sequence. For example, in a protein-SELEX experiment, affinity can be assessed by Surface Plasmon Resonance (SPR) analysis,[92,93] biolayer interferometry[94],[95]qPCR,[96] enzyme-linked oligonucleotide assay,[97] capillary electrophoresis,[98] or flow cytometry[43,99] and high-throughput sequencing[43]. The SELEX library can also be labeled with a fluorophore or ^{32}P, while nitrocellulose filter binding[100] or gel mobility shift assays[101] can be utilized to assess the affinity of an evolved SELEX pool. When the target is a whole cell, the most common method of affinity analysis is flow cytometry[73] While there is no definitive fold change or clear cut-off value to strive for (an enrichment value of 80% has been proposed as optimal in stopping SELEX[43]). Monitoring both the diversity and affinity throughout the entire selection process can provide important insight into the enrichment success, suggesting whether the stringency is suitable, provide encouraging support that high affinity and selective aptamer candidates are emerging in the library and help determine which sequences should be ultimately moved forward to the aptamer characterization phase. (Figure 3B shows one method of monitoring the enrichment process by comparing the quantity of library-target versus library-control interactions). Notably, high affinity aptamers that bind to small molecules have emerged from enriched libraries demonstrating as low as 3-fold increased bulk binding to the target compared to the negative control[91], while a 10-fold or greater interaction is expected for

aptamer selection against protein and cell targets compared to a negative control.[22,96] On the other hand, sequencing results processed via the bioinformatics platforms may reveal as little as two[102] or hundreds of potential aptamer families with fold enrichments varying between 1 (not at all) to 10^5 compared to the previous round (see Figure 3C for an example of library landscape comparisons used to show sequence enrichment throughout rounds of SELEX). As such, the more putative candidates that can be individually screened, the higher the probability of identifying a high-affinity aptamer.

Results

Sequencing and Bioinformatics

HTS is the most common method to sequence a SELEX library, allowing comprehensive coverage of all sequences in a SELEX library. Sequencing of selection libraries is typically achieved using HTS. The 454 Roche,[103] was the first commercially available HTS platform, although it is no longer commonly used. More recent platforms include the Illumina platform,[89] the SOLiD sequencer[104] and the Ion Torrent sequencing platform[105]. Details of HTS chemistry are reviewed elsewhere.[106,104] All sequencing platforms require converting the SELEX library to a double-stranded SELEX library with an adaptor sequence to facilitate sequencing. Such aptamers can be added to a SELEX library using commercial kits provided by vendors or by simply using the adapter sequences tethered to the primers utilized in SELEX. The Illumina sequencing platform is widely known for HTS in SELEX among other sequencing platforms.[89] SELEX-derived libraries can be prepared for Illumina HTS by introducing a two-step PCR approach. First, PCR is performed to introduce overhang adapter sequences to an enriched library to amplify. This step is known as amplicon PCR. The amplicon PCR is purified to remove primers and primer-dimers, ready for the second PCR called index PCR. Indexing allows the pooling of several samples, providing a cost advantage when analyzing multiple samples. Upon completion of index PCR, the product is purified and then quantified or characterized by gel electrophoresis to verify the size. Quality control can be performed prior to sequencing. More recently, nanopore sensing of DNA has been described. Rather than rely on some variation of sequencing by synthesisDNA amplification, each individual DNA molecule is unraveled and passed through the nanopore, during which time a characteristic disruption in the current across the nanopore is converted into an electrical readout of the DNA sequence.[107]Though this method has a lot of potential it has not yet been widely incorporated into SELEX experiments...

Processing sequencing data requires bioinformatics approaches to analyze the information obtained from HTS. The processing of data depends on the sequencing method employed. However, in general, initial preprocessing is required for all sequencing data, including removing sequencing artifacts, removing constant regions, such as primer and adapter sequences, and performing filtration of sequences for expected length. Once the HTS data is preprocessed, readily available bioinformatics software platforms can be used to analyze sequencing data. Features of available bioinformatics platforms are outlined in Table 1.[86,87,108–115] The most common approach of selecting aptamers involves looking at the fold enrichment of individual sequences as a function of the SELEX round. Levenshtein

distance **[G]** is one of the most common strategies of clustering sequences, while the use of shared motifs, consensus sequences, and secondary structure can also be utilized to cluster and identify potential aptamer families. When modified libraries with UBPs are used, the SELEX libraries are first converted to natural libraries, followed by conversion of selected aptamers back to modified sequences using customized algorithms.[116]

Characterization methods

Once the lead aptamer sequences are deciphered from the sequencing data, there are a wide variety of methods that can be used to characterize structure, affinity and other key features of aptamers. Thorough validation of individual aptamer sequences and characterization of their metrics are essential for implementing functional aptamers into diverse applications.[117] The most important aptamer property is its affinity for the target. Several other properties, such as specificity, structure, and stability may also be assessed, depending on the application.

Binding affinity—A plethora of assays are available to assess aptamer binding affinity, but no single technique can be considered generally applicable to all aptamer cases;[31,117,118] therefore, multiple methods must be employed to fully validate aptamer binding affinity. The dissociation constant K_D is used to describe the affinity, and represents the concentration in which 50% of the aptamer-target interaction dissociates to its separate constituents.[119] Bioinformatics analysis suggests that both RNA and DNA aptamers produce high affinity binding. In contrast, the range of possible K_Ds is dependent on the target type.[35] As an example, a successful small molecule binding aptamer will display K_Ds in the micromolar to high nanomolar range[35], whereas aptamers to large targets such as proteins and cell surfaces can exhibit K_Ds in the low nanomolar and picomolar range. Additional binding characteristics that are more rarely reported but can be very important for downstream applications include binding kinetics (on rate and off rate) and thermodynamics (change in enthalpy (ΔH) and entropy (ΔS) of binding), which can be measured using specialized methods such as surface plasmon resonance[120] and isothermal calorimetry[121], respectively.

Specificity/selectivity—Aptamer specificity is the second most important property of aptamers and thus must be carefully assessed. All the binding methods described to-date can be used to measure the K_D or relative binding of the aptamers to a suite of targets that are similar in structure or function to the target-of-interest. See Figure 4A and 4B for examples of methods used to characterize aptamer binding and specificity.

By comparing the binding affinity, it is common for aptamers to display up to 10,000 fold improved binding to the target of interest compared to similar compounds.[122] Competition assays are also employed to demonstrate high affinity binding of the target-of-interest despite the presence of other interfering targets.[22] In general, achieving 100% specificity **[G]** is unlikely, and thus a more reasonable goal is to achieving sufficient selectivity **[G]** needed for the particular application of interest. There are several selection strategies, post-selection methods, and application designs that can be implemented to help achieve maximal selectivity.[7]

Aptamer structure—Of the thousands of aptamers reported to-date, the length and sequence composition varies immensely.[35] However, the functional aptamers described to date typically fold into only a few common structural motifs, with G-quadruplexes[123] and stem-loops representing the majority. In fact, more complex aptamer structures, such as 5-way junctions, are very rare and not well-sampled in a typical SELEX library.[124] Identifying the structural motifs or overall structure of aptamers is extremely useful in aptamer development. For example, knowledge of the structure can be used to create aptamer switches, to rationally modify the aptamer with chemical tags or other biomolecules, or to minimize the functional sequence to allow scalable synthesis at higher yields.

Several methods are available to probe aptamer structure in varying levels of depth and detail. As a starting point, almost all researchers will employ a variety of secondary structure prediction programs such as MFold and RNAstructure. However, most programs do not capture the entire global 3-D structure or interactions with the target molecules, and therefore require follow-up laboratory experimentation. The most common means for obtaining some structural information is by synthesizing a variety of truncated[125] or mutated[126] versions of a given aptamer sequence (Figure 4C) and performing a structural activity relationship study using a quick binding assay or by measuring the K_D for each variant (see [127]for example). Figure 4B shows a surface plasmon resonance binding assay set-up and the subsequent kinetic analysis from which a K_D can be determined. Using this strategy, the initial aptamers emerging from a SELEX experiment, that are between 40 and 100 nucleotides in length, can be reduced to the minimal stable stem-loop or G-quadruplex scaffold representing as little as 15 nucleotides.[35] Another frequent method involves circular dichroism (CD), which has long been employed to determine the signature of a given nucleic secondary structure or monitoring structural changes resulting from changes to the environment.[128] As such, some aptamer structural insight can be gained using CD. In particular, CD is very powerful in identifying different G-quadruplex aptamers, where certain spectral features have been associated with different with quadruplex topologies.[129] Finally, the gold standards for structural determination are NMR spectroscopy and X-ray crystallography and have been extensively reviewed.[130] However these methods are labour intensive, and require specialized equipment, explaining why there are relatively few in vitro selected aptamer structures available via PDB.

Stability—A final parameter that is frequently characterized for therapeutic or in vivo applications is the stability of the aptamer. These studies are typically performed on chemically-modified aptamers given that RNA is highly unstable[131] and even DNA aptamers are quickly degraded in vivo.[132] It is relatively straight forward to assess aptamer stability: each candidate is incubated with different serum compositions or with specific nucleases, or in different environmental conditions with varying temperatures and pH for hours or days at a time. Then, the integrity of the full length sequence is examined via denaturing gel electrophoresis[91] or by performing an affinity experiment to assess whether binding has been impacted.[133] Figure 4D shows an example of aptamer stability characterization through denaturing gel electrophoresis analysis.

Applications

Aptamers can be antibody substitutes in most routine of applications in research and development [134]. However, their distinct differences from antibodies also mean that they can be used in unique applications as well. An important advantage that aptamers have over antibodies is they are made of DNA, a predictable and programmable molecule. This can be exploited as aptamers themselves can be amplified, and they are compatible with multiple nanotechnologies that can amplify a molecular recognition event.[135] Specifically, amplification strategies such as PCR, rolling circle amplification, strand-displacement amplification, and other methods can be used to directly amplify molecular recognition. Alternatively, aptamers can also be easily chemically modified, with an enzyme or nanoparticle for example, that can indirectly amplify molecular recognition. This section will delve into some of these examples in more detail, focusing mostly on cancer and drug delivery. It is not possible to review the entire breadth of aptamer applications herein. Generally, aptamers have found wide applications ranging from environmental monitoring[136] to food safety[137,138] to medicine,[139,140] and many interesting reviews have been published on these topics.

Diagnostics

Aptamers have long been considered as replacements in applications typically the mainstay of monoclonal and polyclonal antibodies such as immunohistochemistry, immunophenotyping by flow cytometry and enzyme-linked immunosorbent assays (ELISA) [141]. With the ease of adding functional reporter molecules on to the end of aptamers, such as fluorophores and biotin, aptamers have seen use in these applications [142–144].

There are numerous instances where complex staining protocols are required to determine the phenotype of specific cells, whether they are cells of the immune system or cancer cells. This can often be challenging with antibodies, due to the need for antibodies to be raised in different species or have a different isotype. The larger size of the antibody can also impede binding to cell surface receptors. Complex protocols have been generated that involve sequential antibody staining then stripping to accomplish this, even for fluorescent microscopy to ensure complete phenotyping [145]. Three key unique properties of aptamers make this process less complex. Aptamers can be easily chemically synthesized, labelled with fluorophores during synthesis with limited or no loss of functionality (Figure 5A and 5B) and they are much smaller than antibodies, meaning more aptamers can attach to different receptors on the cell surface. This latter property could also lead to an increase in sensitivity [146]. Aptamers can be easily adapted for functional cell sorting via magnetic beads or FACS. The benefits of using aptamers are that the aptamer can be easily removed from the cell surface, either through nuclease treatment or the use of an antidote sequence that is complementary to the aptamer, allowing normal receptor functionality for downstream applications, including cell transplants that are contraindicated in antibody-based separation strategies [147].

One area where aptamers could surpass expectations and overtake antibodies is in single molecule array assays (Simoa).[148] These are adaptions of ELISA that offer increased sensitivity. The basis of the technique is that the molecule is captured by a paramagnetic

bead labelled with a ligand that recognises the small molecule. An excess of beads is included to ensure that only one or no molecule binds per bead. A second biotinylated ligand that recognises the molecule is then added to the mix and they are then incubated with a fluorogenic substrate solution and the mixture is loaded into an array of microwells that can only hold one bead each. The enzymatic reaction proceeds, which is then detected with a CCD camera [149]. Biotinylated slow off-rate aptamers (SOMAmers) to six cytokine targets [150] were recently tested using this approach (Figure 5C) and showed an ultralow detection limit and comparable sensitivity to the antibody pairs, while also minimising the issues associated with antibodies of cross-reactivity [151]. It is important to note that it is rare to be able to mutate antibodies to increase both affinity and specificity, and that this can be a time consuming process [152]. While mutating aptamers is not without these same problems, it can be a much easier and quicker process [153,154].

Another single molecule application studies the interaction in cellular events that are initiated through ligand-receptor interactions at the plasma membrane. For successful localisation to occur, the fluorophore must be as close to their targets as possible, which is achievable as aptamers are typically far smaller than antibodies. A study in 2012 compared aptamers with antibodies for this purpose and demonstrated superior performance of the aptamers. In addition, as aptamer size can be modified through truncation or coupling additional nucleotides, the authors found that a size of 15 kDa or lower led to superior image quality [155]. This technique has also been used to visualise individual insulin receptors in the plasma membrane and study real-time dynamics [156], as well as imaging and tracking of individual epidermal growth factor receptor (EGFR) molecules in their native state in live cells, as shown in Figure 5D [157]. The mutation of an aptamer for lower binding affinity and faster dissociation rate from EGFR to allow for no perturbation of endogenous activity and faster tracking without the need for photobleaching were also observed [158].

On the topic of small molecules, it is generally considered easier to develop aptamers to these rather than antibodies, owing to the inherent low immunogenicity of small molecules, resulting in an upsurge in reports on biosensors that utilise aptamers, (aptasensors). The challenge with small molecule detection is that their size often means that they only have one binding region, which has made it challenging to develop highly sensitive sensors. One development in the field of aptamers has been the split aptamer, pioneered in 2000 [159]. In this application, a complete aptamer is split into two or more aptamer fragments and denoted as Apt1 and Apt2. When the target is then incubated with them, Apt1 and Apt2 are drawn close to each other, thus activating a reporter molecule for optical aptasensors, or the refractory index for surface plasmon resonance or electrochemiluminescence aptasensors [160]. The additional benefit of these aptasensors is the stability of the aptamers, meaning they can be used for in-field testing without the requirement for cold-chain.

An interesting development in the selection of aptamers was the ability to generate aptamers to fluorophores, leading to several new aptamers named after vegetables and fruits (See Figure 6). These RNA aptamers exhibit fluorescence when bound to small molecules and have allowed the imaging and tracking of RNA in living cells. The first of these to be developed was named Spinach and was selected to the green fluorescent protein derivative, 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) [161]. Owing to some instabilities

of the original aptamer, mutagenesis led to the development of Spinach 2 [162] and truncation generated Baby Spinach [163]. The Broccoli aptamer was generated to DFHBI, with a superior fluorescence when compared to Spinach 2 [164]. Other aptamers have also been developed that produce yellow, red, and orange fluorescence and a full list is provided in Table 2.

Advancements in technology have also opened up an attractive and novel area of wearable biosensors [165] that are non-invasive and collect health information rapidly. While this is a relatively new concept, it would revolutionise our understanding of health. Issues with real time monitoring include, but are not limited to, drift and the need to perform several calibrations a day, no sample preparation, stability with high signal to noise ratios and biofouling. One of the first devices demonstrated that aptamers could be readily used in the microfluidic electrochemical detector for in vivo continuous monitoring (MEDIC) for the real-time monitoring of doxorubicin in human whole blood, as well as live animals drawing blood into the chip using an indwelling catheter [166]. Further updates to the technology using a dual-frequency approach allowed for the continuous real-time monitoring of doxorubicin and cocaine for 8 hours [167]. Further work has been performed to identify sensors capable of real-time monitoring of cytokines [168,169]

Therapeutics

Aptamers have shown great potential as therapeutic agents in preclinical trials, with some aptamers entering clinical trials, either as the therapy itself, as a diagnostic, or as part of biomarker discovery. To date, however, only one aptamer has been approved for use by the US FDA. Macugen was approved for use for age-related macular degeneration in 2004 following successful clinical trials. According to the U.S. National Library of Medicine's online database, there are a total of 54 registered studies involving aptamers. Consistent with Macugen, the studies that have progressed the furthest, into phase 3 or phase 4, are mostly for conditions relating to the eye (macular degeneration, macular edema, and diabetic retinopathy). Certainly, the next few years may see a boom in oligonucleotide therapeutics with the approval of the mRNA vaccines and the success of antisense oligonucleotides, however there are still several associated challenges to explore collectively for oligonucleotide therapeutics and individually for each technology.

There are certain advantages that make aptamers particularly suited for therapeutics. Typically, aptamers are suggested to be a much safer version of monoclonal antibodies owing to the lack of or very low immunogenicity. However, as more has become known about immune cell triggers, it has been discovered that aptamers can also be immunogenic if the right sequence is chosen, suggesting they can also be used for immunotherapy [170]. Another major advantage is that aptamers are much smaller than antibodies, which means better penetrance away from blood vessels. This is especially important for the targeted delivery of chemotherapeutic agents for solid tumors [171]. Finally, the tunable binding affinity of aptamers also offers advantages for receptors that are over-expressed on the target population but are expressed at a lower level on normal healthy cells. Table 3 lists some of the uses of aptamers as therapeutic agents with key references provided.

Perhaps one of the most striking advantages of aptamers in therapeutics is the availability of a ready-made antidote (complementary sequence) [172] for the aptamer. Aptamer/

antidote pairs have mostly been developed for the use in haematology as anti-coagulant agents[173]. The first aptamer/antidote pair to enter clinical trials was pegnivacogin (aptamer)/ anivamersen (antidote). Success was seen in terms of efficacy and safety in Phase 1 and 2a trials. This was also observed in a Phase 2b clinical trial, where it was compared to unfractionated heparin. Unfortunately, a large Phase 3 clinical trial was halted in 2014 owing to a small number of allergic reactions, though these were likely caused by the polyethylene glycol modification rather than the aptamer itself [174]. In fact, a similar allergic reaction was observed upon administration of COVID-19 vaccines containing polyethylene glycol.[175] Regardless of the challenges associatied with the delivery vehicle, these studies indicate the uniqueness of aptamers and how easy the generation of antidotes can be.

Theranostics

The combination of diagnostic and therapeutic agents is another area where aptamers are demonstrating their utility.[176] Molecular imaging is required to diagnose various diseases and can be used to assess treatment efficacy. This field has seen several technological developments where imaging has progressed from radioisotopes to magnetic particles to fluorescence-based assessment. While some of these modalities are currently restricted to animal models for classical imaging,[177–179] they are being pioneered for intraoperative uses [180] Traditional radiolabelling of aptamers is typically a simpler process than for antibodies, given the temperature stability [181]. Conjugation to nanoparticles can use the same chemical reactions for attachment or through click-chemistry, with companies such as Baseclick™ licensing their technology. Depending on the attachment to the aptamer, these can be used for molecular imaging of the tumour and treatment at the same time, which offers benefit to the patient. These attachments can include lutetium, a radioisotope that emits both beta and gamma particles or specific types of nanoparticles that have both diagnostic and therapeutic capabilities. There is also potential for aptamer chimeras; bivalent molecules that contain a therapeutic aptamer and a delivery aptamer in one, or therapeutic OR delivery aptamer conjugated to another type of functional nucleic acid.[182,183]Some of the aptamers and their mechanism of action are listed in Table 3, with a full review provided in this reference [184].

Not strictly fitting in the theranostics field but providing a combination of diagnostic and therapeutic effects is the use of a companion diagnostic test, a principle first drafted by the FDA in 2016. In terms of targeted therapeutics, it is important to demonstrate the presence of the receptor on the target cell population. Typically, this would involve a biopsy being taken from the patient for confirmation of the presence, using basic diagnostic techniques. In the case of antibodies, it is possible that one antibody may be used for diagnostics but a different one used for therapy as it may not be possible to functionalize the first antibody, or it may not show favorable kinetics for in vivo use. As the antibodies may bind to different epitopes of the receptor, a false positive result may be observed, resulting in treatment failure. As aptamers can be easily functionalized, the same one can be used for both applications [185,186].

Reproducibility and data deposition

Reproducibility

The interest in aptamer selections has grown, producing many synthetic ligands with antibody-like properties. However, scrutiny has been directed on certain aptamers owing to lack of reproducibility and with only one aptamer achieving clinical success.[187,188] Several reports offer minimal publication guidelines[189] or specific guidelines to assess aptamers selected against cell-surface proteins,[190–192] There are two primary contributing factors in aptamer's binding properties. Importantly, an aptamer's structure is highly variable, and its binding and function depend on several factors.[193] It is essential that the correct buffer systems are used to ensure a uniform functional fold of aptamers. Variations to buffer composition, such as the concentration of divalent metals, which is known to stabilize the secondary structures, can impact generating the uniform functional fold of the aptamer. Furthermore, the three-dimensional structure of the target governs the aptamer binding; maintenance of the structure of the target presented during SELEX is essential in reproducing the aptamers' similar binding and functional properties. Variations of the structural stability of the target are often disregarded in many SELEX studies, even though the structures of targets can also change based on salt and pH.[194,195] One way of minimizing these variabilities is to use multiple controls and conditions when assessing the aptamer binding followed by adequately reporting these conditions. To assess the background and propensity for non-specific nucleic acid binding to the target, use of a scrambled sequence of the aptamer is advisable. Moreover, binding should be assessed using multiple related samples such as isoforms of the protein used[196] structurally similar small molecules[117] or multiple cells lines that express the same protein to a different degree; cell lines that do not express the same protein must be used to ensure that the aptamer's binding is specific.[197] Finally, the specificity of an aptamer can be assessed by investigating the aptamer's biochemical function.[198] Competition assays using a secondary ligand against the same molecule or cross-competition experiments between aptamers from the same family can provide further clarity.[57,199] Additionally, interlaboratory testing[117] can be a powerful approach to confirming reproducibility. The field would benefit greatly from a database that is like the protein data bank. In this database, each aptamer could be given a unique identifier, like an accession number, that would allow all experimental data to be linked to the sequence. Further, the requirement of this sort of entry before publication would go a long way in improving the reproducibility of aptamer data.

Data deposition

Several aptamer data deposition platforms have been made available for data related to aptamer sequences and other parameters. These databases, demonstrated precursors to an aptamer data bank, have attempted to collect the data from multiple selections, and offer researchers varying degrees of access to the selection information. The aptamer database initiated by the Ellington lab consisted of comprehensive sequence information on aptamers and ribozymes identified using in vitro selection methods.[200] PPAI, is a web-based server allowing users to predict aptamer and protein-aptamers interactions.[201] RiboaptDB, includes comprehensive sequence information on aptamers and ribozymes generated using in vitro selection experiments.[202] Biclusters allows users to identify similarities with aptamer

sequences that have been reported in other studies.[203] Aptamer base was developed to allow investigators to report and access detailed information on experimental conditions and SELEX outcomes.[204] With the incorporation of HTS, currently, it is possible to deposit all sequences in SELEX libraries and the identified aptamer sequences in the Sequence Read Archive of the National library of medicine as reported in ref [43]. Two significant challenges exist with the maintenance of these databases, the first being that there is no recognized centralized database, so information curation is up to a subset of researchers. The other significant challenge of many of the aptamer databases has been keeping them current, as several have gone dormant over time.

Limitations and optimizations

Since the establishment of the SELEX method, thousands of aptamers have been developed to a wide range of different targets, demonstrating the remarkable power of this approach. However, in most cases the SELEX process remains relatively blind to the researcher, yields low affinity aptamers to certain targets (such as small molecules), is low throughput and sometimes does not produce application-ready aptamers. Although some challenges are due to lack of resources, most limitations can be optimized by more judicious experimental planning.[205] The past three decades have seen numerous modifications to the SELEX method with the goal of addressing some key limitations.

First, the SELEX process consists of so many different parameters and oftentimes limited predictive understanding of which parameters should be tuned for a given target or application of interest.[35] As such, an entire selection experiment, using published protocols may not see enrichment nor yield desired aptamers.[102] The way forward is to select aptamers with an end application in mind, and to combine methods that monitor the evolving affinity, diversity and enrichment of the library throughout the entire SELEX experiment.[206,207] Additionally this information should be used to tune the stringency of selection, PCR amplification, number of rounds and counter selections. Largely this becomes a challenge of resources. If researchers could easily (and inexpensively) sequence every round, and monitor enrichment by real-time PCR, the selection could be adapted in real time to improve stringency and increase the probability of success. With next generation sequencing technologies becoming more accessible, quick and cheap sequencing may eliminate several barriers to successful selections.

Second, SELEX is low throughput. Most new aptamers described represent several years of effort, involving iterative trial-and-error experiments. In an effort to overcome the bottleneck of low-throughput experimentation, there has been remarkable progress involving the use of particle display,[208,209] microfluidics,[210,211] and automation[212,213] that can be leveraged for multiplexed aptamer selection. For example, a new selection procedure called de novo rapid in vitro evolution of RNA biosensors (DRIVER) uses aptamer-coupled ribozyme libraries to discover aptamer sequences in high throughput. [214] Additionally, the success and efficiency of SELEX experiments is often limited by availability of bioinformatic and computational tools. Interdisciplinary research is bridging the gap between bench science and big data. An example in aptamer selection experiments was the switch from cloning and sanger sequencing, where a small subset of sequences (typically <100) from the initial

and final rounds were examined, to high throughput sequencing methods where millions of sequences from each round were computationally processed for trends in enrichment, secondary structure and common motifs.

A third critical limitation of SELEX is that aptamers are often not immediately ready for their application of interest. In particular, the SELEX process usually involves identifying candidates in an ideal buffering environment and using highly purified targets. However, many applications require function at a variety of temperatures and pH, in the presence of organic solvents and in highly complex environments with molecular crowding and mechanical forces at play. Several aptamers function in complex matrices such as waste water, river water, or soil,[91, 136, 215, 216], but most aptamers must be heavily adapted, truncated, and/or modified to function in these differing environments. A more recent trend is to perform the selection experiment using conditions that closely mimic the environment of the intended application. This approach is especially important for health related applications that are not biosensing in nature. If aptamers are going to work in a live cell or animal they should be selected in conditions that mimic these environments as closely as possible, and very likely will require post-selection modifications for increased biostability. Most aptamer applications fall into biosensing, likely because biosensing conditions more closely resemble a simple buffer system. A future goal could be to continue to innovate SELEX approaches to minimize post-SELEX modifications and adaptations to yield a functional end-product from environmental monitoring to oligonucleotide therapeutics.

The final challenge is that academic researchers are often concerned with demonstrating the innovation of aptamer technology leading to novel assay designs that are overly complex and pay little attention to real world applicability, thus negating the inherent benefits of aptamer technology. Aptamers can take advantage of their nature as oligonucleotides to conceive of new uses and assays designs that would not be possible with antibody counterparts. Moreover, aptamers will always be well-suited for applications that benefit from their low cost of production and their extended shelf-lives, for example to develop economical screening technology for testing that require extensive sampling. Aptamers have been compared to antibodies since their earliest days, yet conventional antibody-based applications are well-established and their supremacy in certain applications is uncontested. Finding ways to capitalize on the distinct advantages of aptamers and to find unique niches for their application are keys to their future success.

Outlook

The last thirty years have seen enormous progress in aptamer research. The priorities for aptamer selections and applications for the next 5–10 years should be to address the select limitations of aptamers, to focus on areas of strength and to push for adoption and commercialization of aptamer technology. Despite some limitations, there is enormous potential for aptamer selections and applications. Only recently have new aptamer publications reported in depth analysis of selection experiments, in part due to the quantity of data afforded by next generation sequencing. However, with numerous bioinformatics tools available, such as in silico molecular docking (for aptamer design)[84] the application of machine learning to aptamers (to improve library design and sequencing

data analysis),[217,218,219] and other computational methods[220–222] it is expected that understanding of the selection process will improve, further improving reproducibility and enabling the strengths of aptamer technology. Moreover, like all realms of life, machine learning, artificial intelligence, and other computational methods likely lie at the intersection of challenge and opportunity. Predictably, the time will come that machine learning and AI may be able to predict aptamers for specific targets, but the success of such endeavors will most likely be dependent on careful and thorough curation of aptamer and selection data in a centralized data bank.

While translation of the strengths of aptamer technology from the lab into the real world has been somewhat elusive to date, the future looks bright. The expiration of the original patents in the early 2010s sparked an increase in academic and commercial interest in aptamer applications and the last ten to fifteen years have been marked with exciting advances in aptamer-based technology. Aptamer-based commercial products have emerged including New England Biolab's anti-Taq aptamers for Hot-Start PCR, and SOMAlogic's Slow-Off rate Modified Aptamer (SOMAmer) technology for proteomics platforms, culminating with the recent listing of SOMAlogic on the NASDAQ in 2021. As illustrated by the extensive demonstration of aptamers in biosensing for environmental monitoring and food safety, the best practice is to keep things simple. A renewed emphasis on simple, portable and inexpensive screening assays could allow aptamers to profit from their strengths. Gleaning potential from the COVID-19 pandemic, there is clear need for rapid and robust solution and paper-based assays that can be shipped and stored at room temperature for extended periods of time. To reiterate an advantage aptamers have over antibodies to this end is that aptamers are much more chemically stable and compatible with devices that meet these criteria.[223] Realizing the full promise of aptamer technology will hinge upon the rational design of effective SELEX experiments, a continued focus on aptamer reproducibility and the design of applications that take full advantage of all the assets that aptamers can offer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

Heterogeneous library

The pool of oligonucleotide sequences from which aptamers are selected, typically consisting of a random nucleotide core of between 20–60 nt, flanked by fixed regions used for library amplification. In a typical SELEX experiment, the library contains 10^{14}-10^{15} random sequences (1–10 nmol)

Partitioning

The process by which target binding oligonucleotide sequences are separated from non-binding sequences.

Stringency
Selection pressure used during the SELEX experiment to increase the competition between strong and weak binders and ultimately increase aptamer suitability (affinity and specificity). Stringency can depend on several factors, including the partitioning method, target concentration, buffer composition, counter selection rounds

Positive-selection
the incubation of the selection library with the desired

Counter- selection
Incubation of the library with a similar molecule(s) allows for increased stringency as sequences that have cross reactivity to the similar molecule(s) is removed from the selection. Typically the counter-selection would occur after a negative selection and before a positive selection

Negative-selection
It is important to remove sequences from the library that may bind to the selection matrix (such as magnetic beads, agarose beads, nitrocellulose paper). This is achieved by incubating the library with only the selection matrix prior to the positive selection

Enrichment
A measure of the progress of a selection, library enrichment describes the degree of convergence of the library on sequences with the desired properties

Selectivity
the ability of an aptamer to differentiate between components in a mixture

Sequence Space
A library synthesis yield of 1 nmol of DNA would be composed of approximately 6×10^{14} sequences. A library with a random region of 24 nt long would have 424 different possibilities = 2.8×10^{14} sequences. If the random region is increased by 1 nt, the number of possibilities (425) = 1.1×10^{15}. Thus, any SELEX library designed with a random region longer than about 25 nt will likely not contain every possible random sequence. Nevertheless, there may be reasons to consider a longer library length. There is some evidence to suggest that longer pools could be better for smaller targets.[28] Furthermore, libraries built from longer oligos will have a greater diversity of possible 3D structures.

Specificity
the ultimate selectivity, the ability of an aptamer to interact with only a single component in a mixture

Levenshtein distance
an algorithm that measures the difference between two sequences. In sequence comparison, the number of substitutions, insertions or deletions will affect the value. A common example

of where this is used is for the BLAST: Basic Local Alignment Search Tool maintained by the NIH

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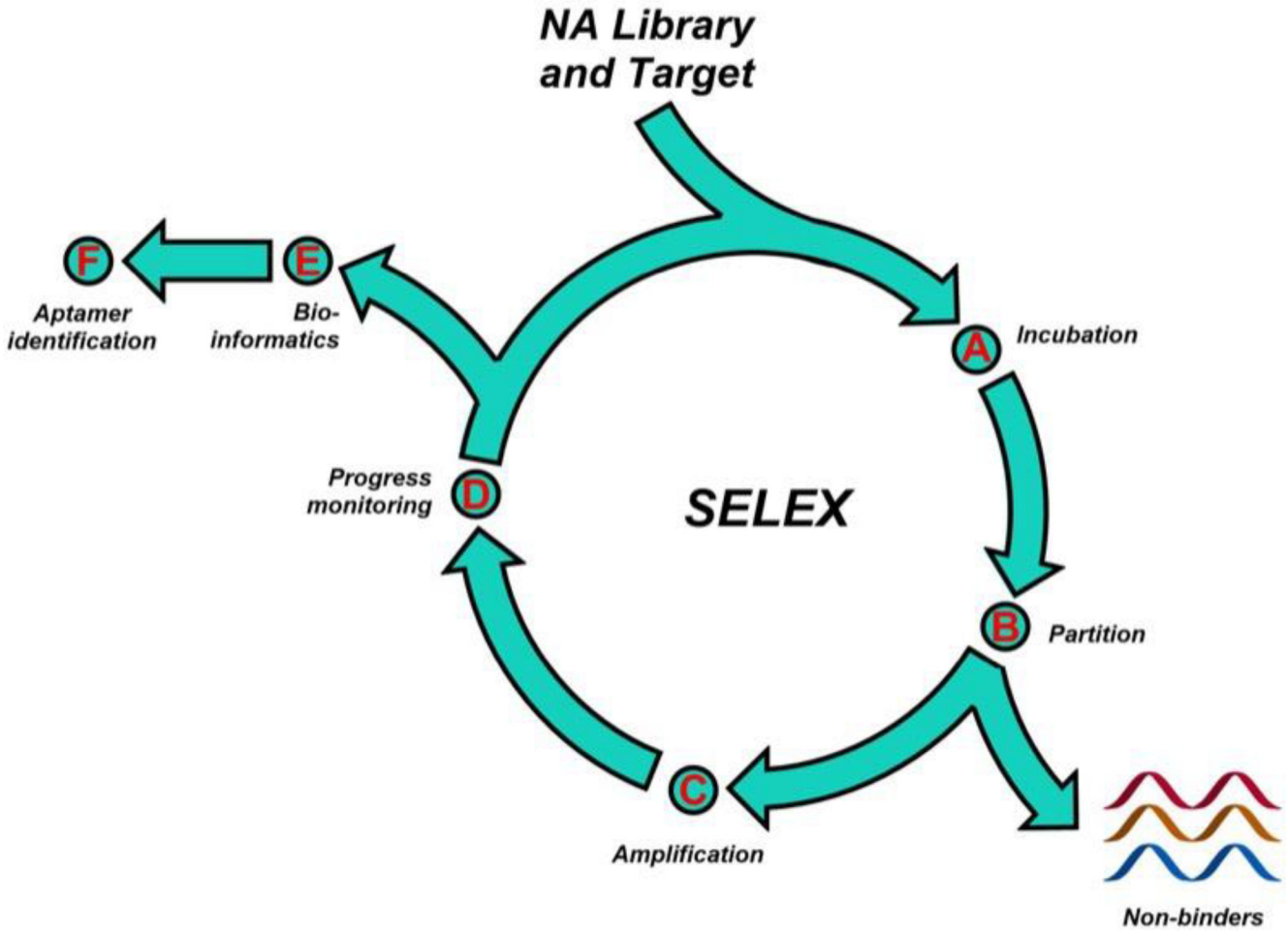
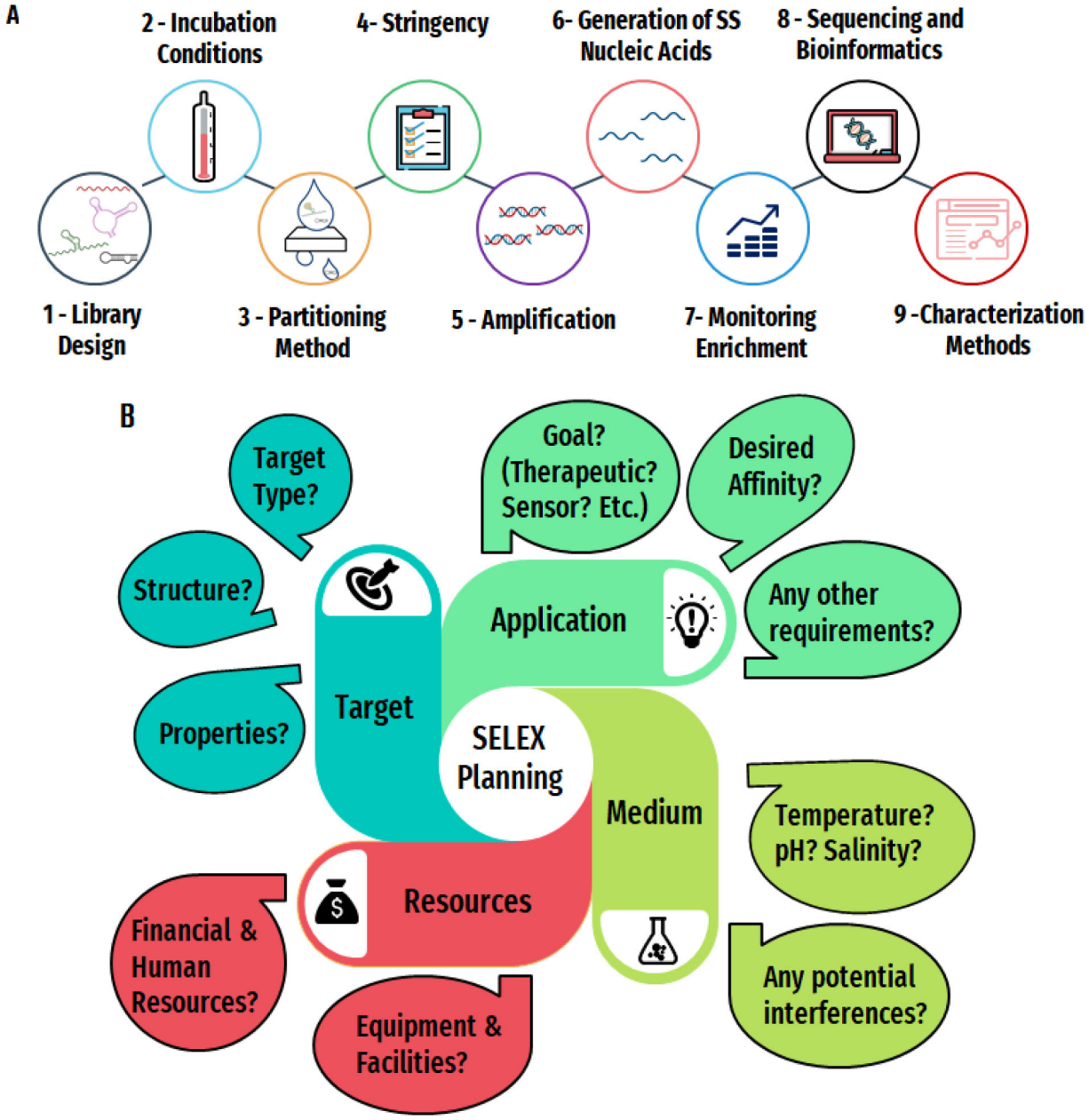


Figure 1. General outline of the SELEX process and aptamer identification.

A SELEX library is chemically synthesized using a DNA synthesizer, and PCR optimization prior to SELEX ensures efficient amplification. The SELEX library is incubated with the target, and binders are separated from non-binders using an appropriate partitioning method. The SELEX library with potential binders is then amplified and utilized in the next round of SELEX. The evolved libraries are monitored for enrichment and sequenced to identify hit aptamer candidates.



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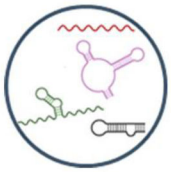
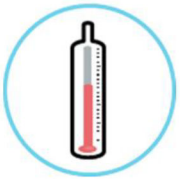


A hydrophobic target might require a modified library

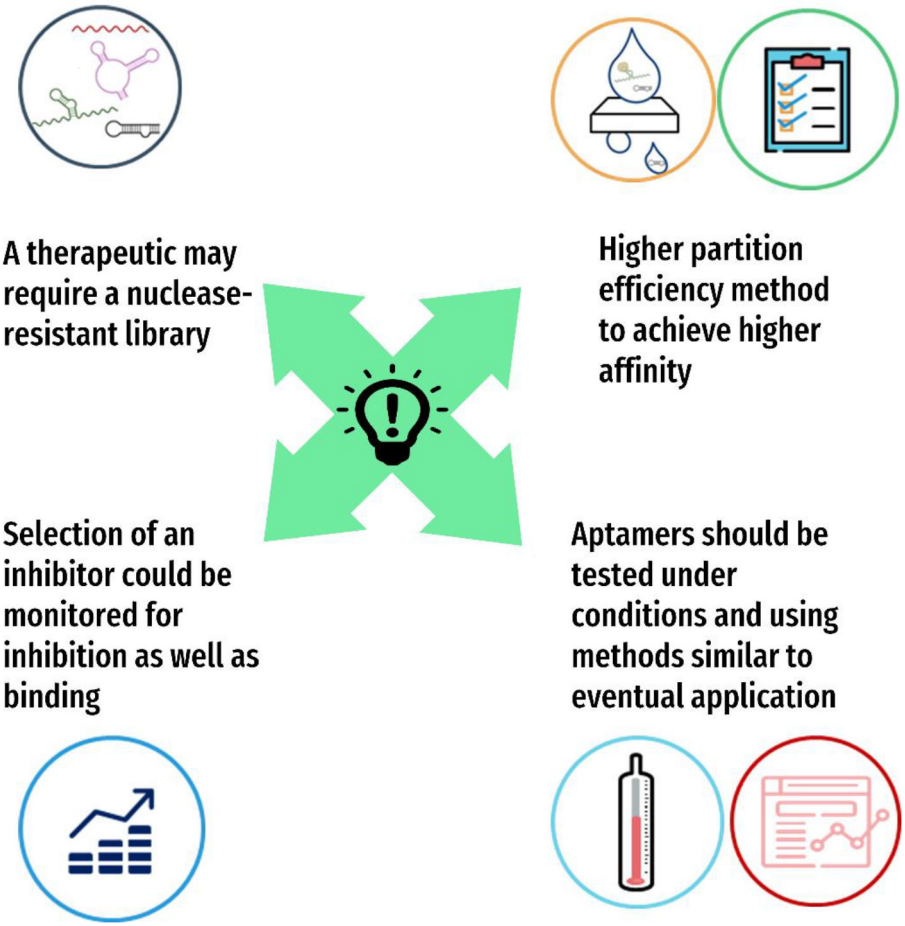


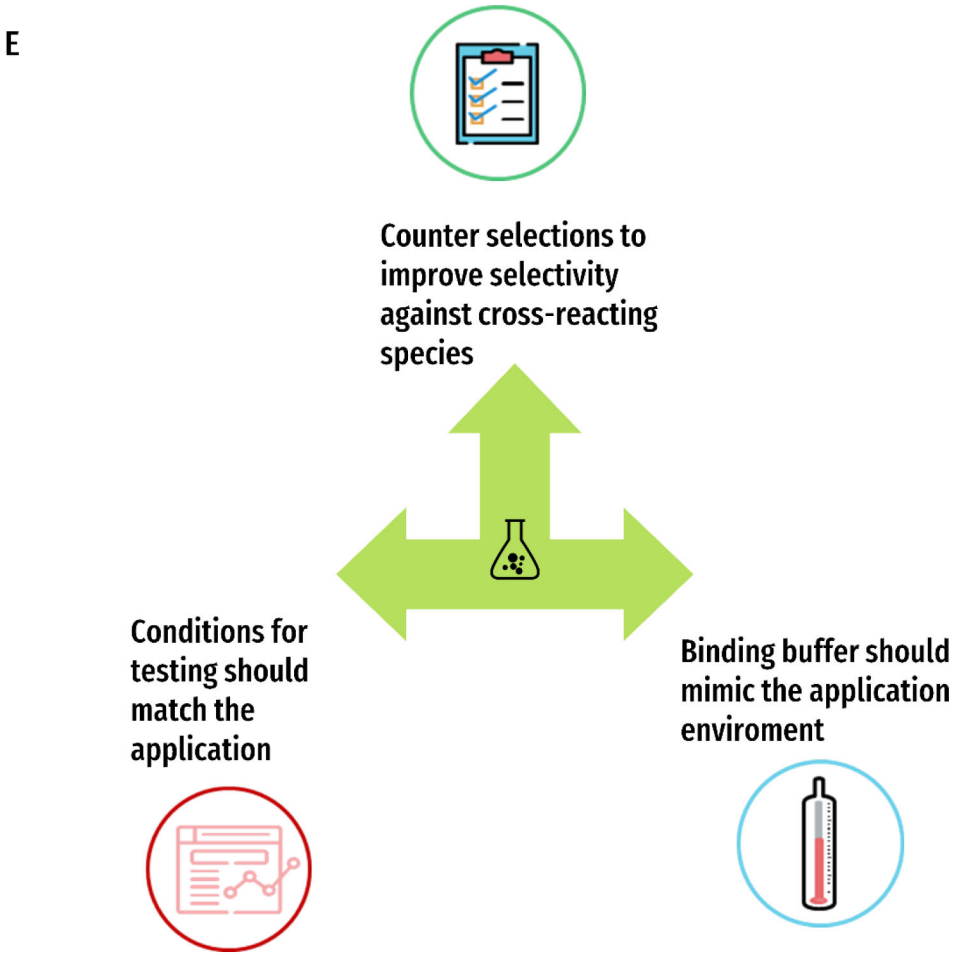
A flexible target might require conditions that ensure a stable 3D conformation

Targets of similar structure may be needed for counter selection

Limited functional groups could preclude target attachment to a solid phase

D





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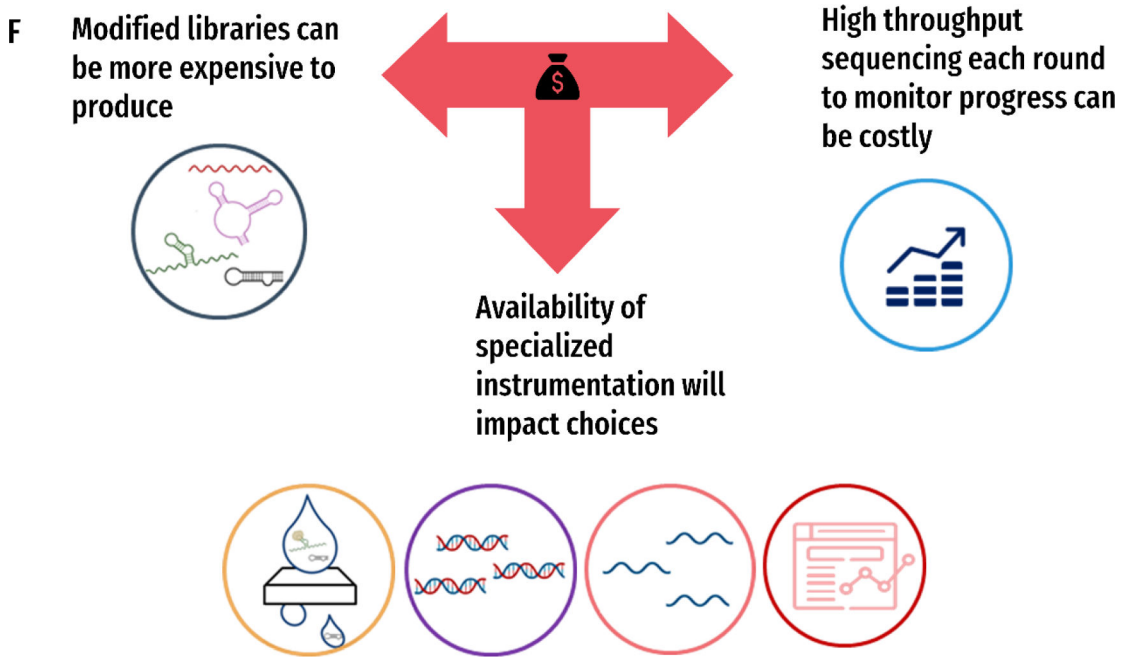

Figure 2. Decision-making for SELEX design.

A) The nine main aspects of a selection experiment: 1) Library Design 2) Incubation Conditions 3) Partitioning Method 4) Stringency 5) Amplification 6) Generation of Single Stranded Nucleic acids 7) Monitoring Enrichment 8) Sequencing and Bioinformatics and 9) Characterization Methods. B) The four key factors that will inform those decisions: 1) the target of interest 2) the eventual application of the aptamer 3) the medium and 4) the resources available. C)-F) Examples of ways these queries may influence the different aspects of the SELEX experiment for each of the four key factors C) Target D) Application E) Medium and F) Resources

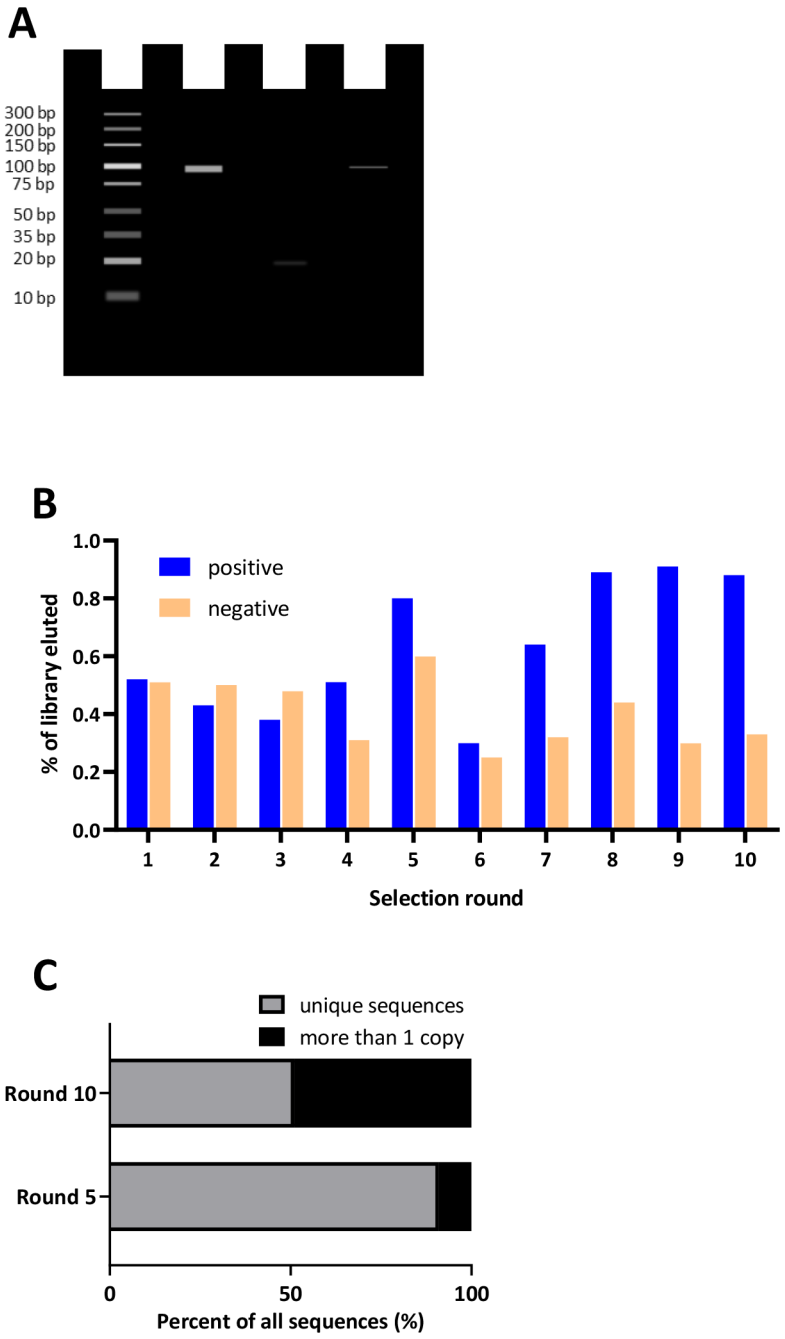

Figure 3. Representative analysis of the in vitro selection process.

A. Ideal Polymerase Chain Reaction (PCR) amplification analyzed by gel electrophoresis. Lane 1 ladder, lane 2 positive control, lane 3 negative control, lane 4 amplification of the library following a round of selection. B. One method for monitoring the in vitro selection process is by comparing the quantity of library that interacts with the target compared to controls. Blue bars represent percent of library from each positive selection round (the target of interest); orange bars represent the library recovered from a parallel negative control selection round (absence of target). C. Landscape of sequence enrichment throughout rounds ofin vitro selection. In early rounds, the library is not enriched and therefore sequencing

yields a diverse sequence pool with very few copies of the same sequence and are therefore mostly unique sequences. In later rounds, after enrichment has occurred, more sequences will emerge from the data that have numerous copies, thus resulting in fewer unique sequences.

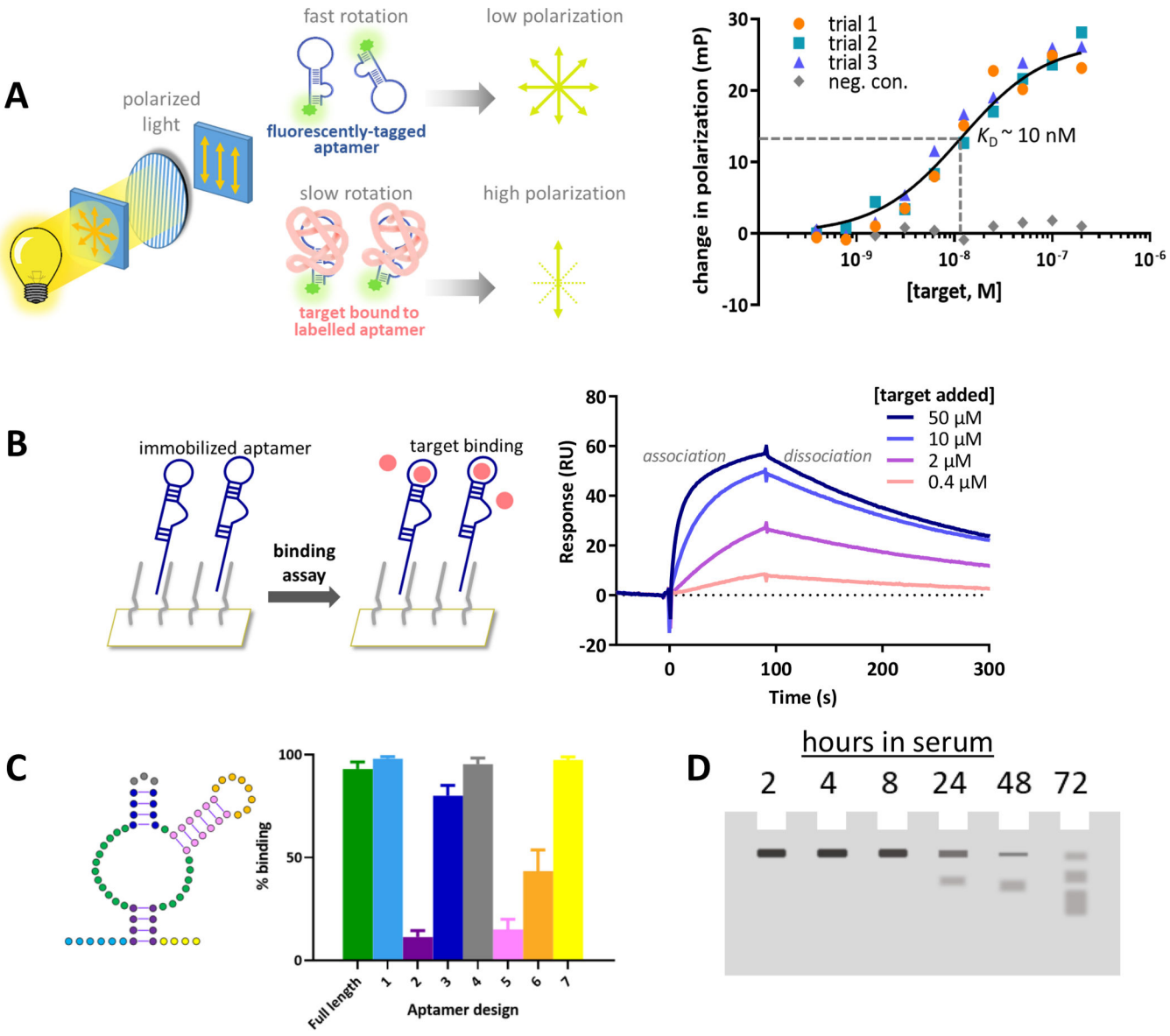

Figure 4. Representative characterization techniques of aptamer candidates

A) Aptamer binding measured using fluorescence polarization. The aptamer is modified with a fluorophore and is excited by polarized light. In solution, the aptamer rotates thus resulting in a depolarized emission of light. As increasing concentrations of the target are added and bind to the aptamer, the larger aptamer-target complex moves slower thus the emitted fluorescence remains more polarized. The change in polarization with increasing concentrations of target binding to the aptamer compared to a negative control sequence can be plotted as a binding isotherm to solve for the dissociation constant. B) Aptamer binding kinetics measured using a real-time surface plasmon resonance assay. The aptamer is immobilized to a surface and the target is flowed over a surface plasmon biosensor chip. The interaction at the surface is detected in real time resulting in on and off binding curves that can be fit to obtain kinetic data. C). Secondary structure prediction of an aptamer. By synthesizing truncated sequences, a simple binding assay can determine the importance of

different regions of the full-length aptamer necessary for binding. Here, aptamer designs are synthesized with each colored region removed and then tested for relative binding compared to the full-length sequence. D). Aptamer stability and half-life measured using denaturing gel electrophoresis. A labelled aptamer can be incubated with serum or nucleases and loaded into a gel at various time points to determine the time required to fully degrade the aptamer of interest.

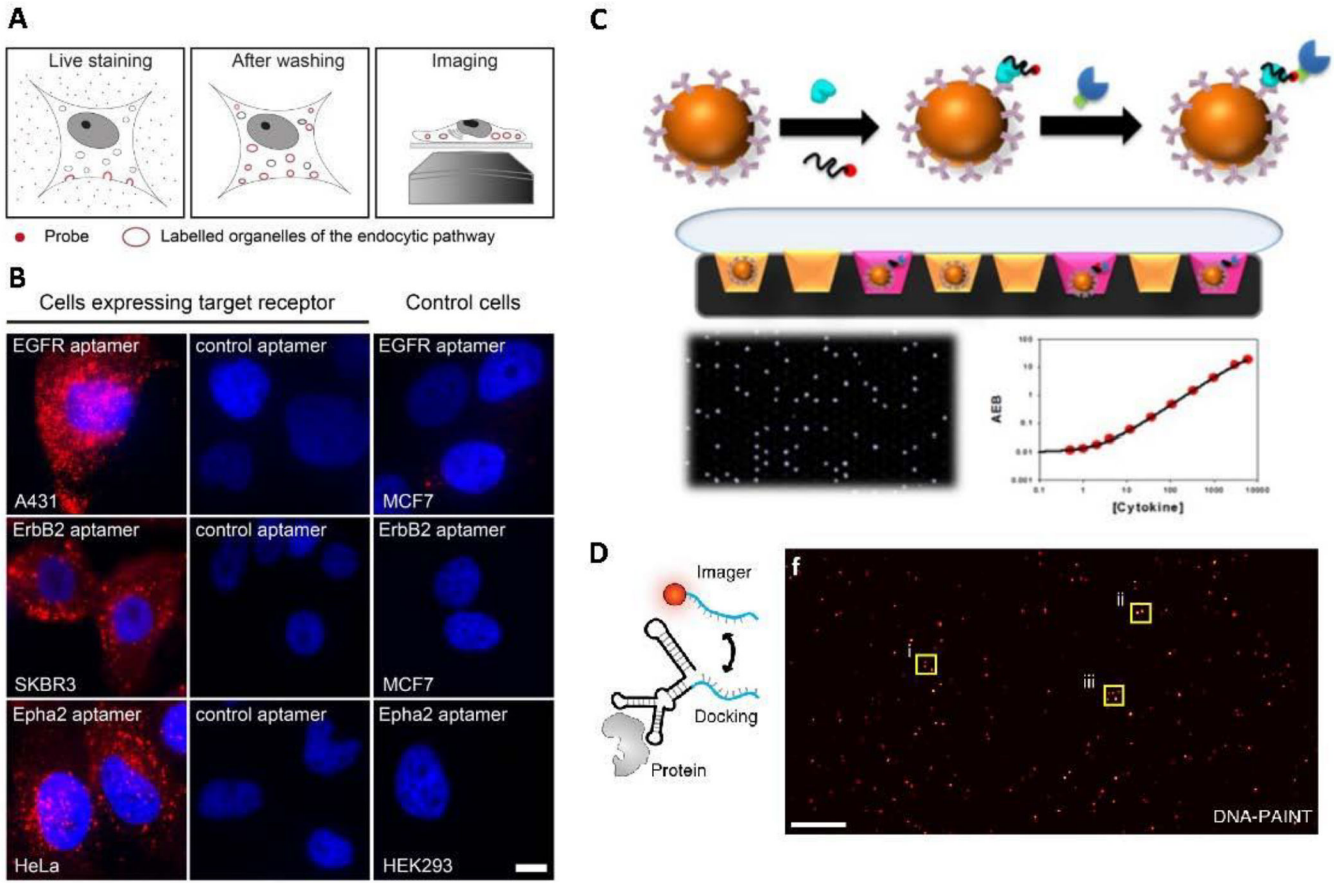

Figure 5:

Aptamers are powerful tools for molecular imaging. A) Schematic of staining live cells with fluorescently labeled aptamer. B) Aptamers that bind to epidermal growth factor receptor (EGFR), and two other proteins were compared to control cells to demonstrate the ability of aptamers for molecular imaging.. C) Schematic illustration of the Simoa assay,. Briefly, capture antibodies are immobilized on microbeads, which are then incubated with the target protein and biotinylated SOMAmer. Finally a streptavidin modified beta–galactosidase enzyme is added. The beads are distributed in microwells, and the presence of the target is revealed in the presence ofresorufin β-D-galactopyranoside substrate. Digital detection (black panel with lit up wells) is then translated into a concentration based reading (calibration curve shown). D) Schematic illustration of EGFR labelling by SOMAmer. The SOMAmer binds to the target protein, and a fluorescently labelled imager strand binds to a docking region of the SOMAmer. The SOMAmer DNA-PAINT based method allows for sensitive fluorescent detection of target protein expressed in cells. Panel A and B were reproduced under Creative Commons Attribution License from Gomes de Castro et al., 2017[146]. Part C reproduced with permission from Wu et al., 2016[150]. Part D Reproduced with permission from Strauss et al., 2018.[157]

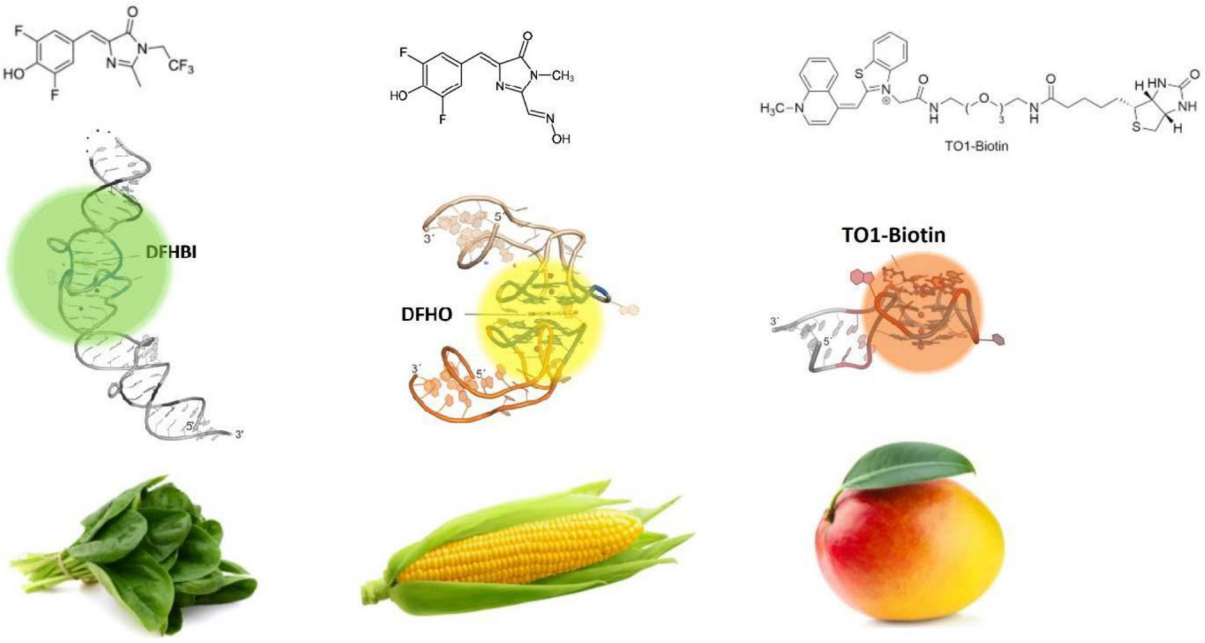

Figure 6:

Aptamers that have been selected to bind to light up fluorophores When bound to a fluorogen (DFHBI, DFHO, and TO1-biotin), aptamers can substantially enhance their emitted fluorescence. The spinach (a), corn (b), and mango (c) aptamers are shown Adapted from Neubacher and Hennig (2018). [237]

Table 1:

Bioinformatic platforms utilized in analyzing SELEX libraries

Software	Input file format	Operating System	Data Analysis	Clustering Method
Galaxy[86,87]	FASTQ	Web Linux Mac OS Windows	Preprocessing, enrichment ratio, read count, filtering and sorting data	Sequence-based Structure-based
FASTAptamer[108]	FASTQ	Linux Mac OS Windows	Preprocessing, enrichment ratio, read count, sequence clustering based on Levenshtein edit distance	Sequence-based
APTANI[109]	FASTQ	Linux Mac OS	Count enrichment, identifies binding motifs and target-specific aptamers	Structure-based
MEMESuite/ GLAM[110]	FASTA	Web Linux Mac OS Windows	Identifies sequence motifs	Sequence-based
MPBind[111]	Plain text	Linux Mac OS	Identifies sequence motifs and ranks potential binding of aptamers	Sequence-based
AptCompare[112]	FASTQ	Linux Mac OS Windows	Identifies sequence motifs and count enrichment	Sequence-based
MEMERIS[113]	FASTA	Linux Mac OS	Identifies sequence motifs and secondary structure analysis	Structure-based
RaptRanker[114]	FASTQ FASTA	Linux Mac OS	Preprocessing, count, enrichment, identifies motifs based on CapR algorithms	Sequence-based/ Structure-based
AptaSUITE[115] (AptaPLEX, AptaCluster, AptaTRACE, AptaMUT)	FASTQ FASTA	Linux Mac OS Windows	Preprocessing, count enrichment, identifies binding and structural motifs	Sequence-based Structure-based

Table 2.

Aptamers that have been selected to bind to “light up” fluorophores, the resulting fluorescence color, and their targets

Colour	Name	Target	Reference
Green	Broccoli	3,5-difluoro-4-hydroxybenzylidene imidazolinone	164
Green	Spinach	3,5-difluoro-4-hydroxybenzylidene imidazolinone	161
Green	Spinach 2	3,5-difluoro-4-hydroxybenzylidene imidazolinone	224
Green	Baby Spinach	3,5-difluoro-4-hydroxybenzylidene imidazolinone	163
Green	Bunch of baby spinach	3,5-difluoro-4-hydroxybenzylidene imidazolinone	163
Yellow	Corn	3,5-difluoro-4-hydroxybenzylidene-imidazolinone-2-oxime	225
Red and Orange	Red Broccoli	3,5-difluoro-4-hydroxybenzylidene-imidazolinone-2-oxime	225
Red and Orange	Orange Broccoli	3,5-difluoro-4-hydroxybenzylidene-imidazolinone-2-oxime	225
Red and Orange	Mango	Thiazole orange	226

Table 3.

Therapeutic applications of aptamers. Further information can be found in these review articles[184,227–229]

Modality	Example	Target (Aptamer)	Reference
Aptamer	Antagonist	Nucleolin (AS1411)	230
Aptamer	Blocking receptor for viral entry into cell	Hemagglutinin (HA12-16)	231
Aptamer-drug conjugates	Targeted delivery of chemotherapeutic agent (Doxorubicin)	Prostate specific membrane antigen (A10)	232
Aptamer-radioligand conjugates	Targeted delivery of radiotherapeutic agents for imaging	Tenascin-C (TTA1-^{99m}Tc)	233
Aptamer-RNAi	Targeted delivery of short interfering RNA	Prostate specific membrane antigen for knockdown of Lamin A (A9)	234
Aptamer-RNAi	Targeted delivery of microRNA	Tyrosine kinase Axl for knock-in of tumor suppressor gene (GL21.T-let7g)	235
Aptamer functionalised nanoparticles	Targeted delivery of liposomes	CEM-CCRF cells (Sgc8)	183
Aptamer functionalised nanoparticles	Targeted delivery of gold nanoparticles	Prostate specific membrane antigen (A9)	236