SELEX: Critical factors and optimization strategies for successful aptamer selection

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Abstract
Within the last decade, the application range of aptamers in biochemistry and medicine has expanded rapidly. More than just a replacement for antibodies, these intrinsically structured RNA- or DNA-oligonucleotides show great potential for utilization in diagnostics, specific drug delivery, and treatment of certain medical conditions. However, what is analyzed less frequently is the process of aptamer identification known as systematic evolution of ligands by exponential enrichment (SELEX) and the functional mechanisms that lie at its core. SELEX involves numerous singular processes, each of which contributes to the success or failure of aptamer generation. In this review, critical steps during aptamer selection are discussed in-depth, and specific problems are presented along with potential solutions. The discussed aspects include the size and molecule type of the selected target, the nature and stringency of the selection process, the amplification step with its possible PCR bias, the efficient regeneration of RNA or single-stranded DNA, and the different sequencing procedures and screening assays currently available. Finally, useful quality control steps and their role within SELEX are presented. By understanding the mechanisms through which aptamer selection is influenced, the design of more efficient SELEX procedures leading to a higher success rate in aptamer identification is enabled.

KEYWORDS
aptamer, oligonucleotide library, PCR, SELEX, sequencing

1 INTRODUCTION

The RNA- or DNA-oligonucleotides that are known as aptamers may have been discovered by as many as four different working groups within a relatively short time span.1–4 Since their inception 30 years ago, aptamers and the process by which they are identified have undergone fundamental improvements. Today, the Systematic Evolution of Ligands by EXponential enrichment (SELEX) can be conducted in a myriad of ways in vitro or even...
Aptamers are no longer just laboratory tools limited to the detection of different molecules. They can be used for biomarker discovery and specific diagnostics, as bioimaging agents, for drug delivery and even as pharmaceuticals. In the field of biochemistry, aptamers are a popular replacement for antibodies, which they surpass in terms of cost, quality, and shelf-life. However, in comparison to in vivo antibody generation, the success rate of SELEX is generally lower. A possible reason for this may be that aptamers are not only much smaller but also less structurally complex than antibodies, as they lack the conformational diversity offered by 20 different amino acids. Fortunately, these deficits can be addressed by the use of specialized SELEX techniques, optimized oligonucleotide libraries, and the introduction of quality control procedures. In this way, the generally estimated SELEX success chance of below 30% can be increased significantly.

In order to choose the proper methods for the identification of aptamers that bind to a desired target molecule, it is necessary to first gain a general understanding of the process of SELEX and its various aspects. While many existing reviews focus exhaustively on selected aspects of SELEX, this review shall provide a comprehensive discussion of the process as a whole. As such, the most critical factors of SELEX are described and summarized here, potential problems are highlighted, and possible solutions are presented (Table 1).

2 | TARGET TYPES AND SELEX VARIANTS

2.1 | Aptamer–target interaction mechanisms

The interaction between an aptamer and its target lies at the very core of the SELEX process and any downstream applications that follow afterward. The nature of this interaction will depend on which type of molecule the target is as well as its size. Whenever aptamers are larger than their target, they tend to integrate it into their structure. This is usually the case with small molecules and can be facilitated by stacking interactions (especially with flat, aromatic ligands, and ions), electrostatic complementarity (with oligosaccharides and charged amino acids), and/or the formation of hydrogen bonds. Surprisingly high specificity can result from steric hindrance, as seen with the popular example of the
| Factor of SELEX | Influence on the selection process |
|----------------|-----------------------------------|
| **Target-types and SELEX variants** | |
| Molecule type | The type of the target molecule can limit which SELEX variants are available. Some molecules may require a selection process in solution, while for other molecules an immobilization matrix may be preferable. |
| Immobilization matrix | If the target molecule is immobilized, the coupling process can influence its conformation. Furthermore, the immobilization matrix can function as a repellent for aptamers or allow them to bind via unspecific interactions. |
| Physicochemical properties | Molecules that are strongly hydrophobic, or which have strong charges on their surface, may have limited possibility for interaction with aptamers, potentially reducing the success chance of SELEX. |
| **Oligonucleotide library** | |
| Sequence diversity | The sequence diversity should be significantly higher than the total amount of sequences used for the first selection step. This ensures sufficient structural diversity, which can improve the success chance of selection. |
| Chemical modifications | Using modified nucleic acids may increase the success chance of selection, depending on the target. If modifications are necessary to prolong in vivo half-life of aptamers, these modifications should arguably be present during selection, as adding them after selection may disrupt the structure and function of identified aptamers. |
| Constant domains and primers | The constant domains should be optimized to have minimal influence on the aptamer structure. Primers should not be able to form dimers, which can otherwise lead to the formation of PCR by-products. |
| **Amplification and sequencing** | |
| PCR bias | Avoiding PCR bias can greatly improve the quality of the aptamer pool during SELEX. Strategies to minimize PCR bias include limiting the amplification cycles, monitoring the amount of template, increasing primer concentrations, skipping the elongation step, and/or utilizing emulsion PCR. |
| ssDNA regeneration | The ssDNA regeneration step provides the possibility of removing unwanted by-products if a gel-based method is used. Furthermore, asymmetric PCR can help to reduce PCR bias. The ssDNA regeneration step should be optimized to avoid excessive loss of DNA. |
| Sequencing | New generation sequencing provides a high-resolution analysis of the aptamer pool. The sequence data can be used to identify clusters and possibly even to estimate the influence of singular nucleotides on binding behavior. In contrast, Sanger sequencing is laborious and provides only little insight into the composition of the aptamer pool. |
| **Stringency and quality control** | |
| Selection stringency | The chosen selection method and its specific metrics determine how stringent the selection is, that is, how well good binders are separated from bad binders. Stringency should be low in early SELEX cycles to avoid loss of rare sequences but must be increased with subsequent cycles, or the progress of selection will stop and by-products will accumulate. Furthermore, the selection process should be designed to closely mimic the downstream assays that the aptamers are generated for. |
| Counterselection | Adding a counterselection step, also called negative selection, can help to remove aptamers that bind to the immobilization matrix or a target of choice. This can increase the specificity of the aptamer pool. |
| Quality control | Introducing quality control steps can help assess the necessary selection stringency, the relative amount of by-products and/or weak binders that are present, and the progress of SELEX in general. |

Theophylline aptamer, which can differentiate between two molecules that differ by only one methyl group. If the target is a large protein, the situation is generally reversed and the aptamer is integrated into the structure of its target, or it attaches to the surface. As proteins tend to exhibit high structural complexity, the mechanisms by which they can interact with aptamers are more variegated than those seen with small molecules. Hydrogen bonds still play a role, but often only in combination with polar interactions and structural complementarity. RNA- or DNA-binding motifs that exhibit such structural complementarity are frequently found in the natural world as well and include leucine zippers, homeodomains, helix motifs, and beta-sheet motifs, among others. The nature of the interaction will also depend on the structural complexity of the aptamer itself. Depending on the sequence, the aptamer may take on a multimeric state via the formation of G-quadruplexes or i-motifs,
both of which can lead to an interaction between multiple oligonucleotides.22–24 A visual size comparison between small molecules, aptamers, and proteins is provided in Figure 2.25,26 Interestingly, the formation of aptamer–target complexes can involve conformational changes in either the target, the aptamer, or both according to the “induced fit” principle.20,27,28 Such an induced fit results in better shape complementarity, which in turn facilitates short-range interactions, such as hydrogen bonds and van der Waals contacts.18,19,29 Conformational changes can also lead to catalytic activity, in which case the aptamers are called “aptazymes.”30 Negative charges on the surface of a target molecule can weaken or even prevent aptamer binding, as they can interact unfavorably with the electronegative phosphate groups contained in DNA and RNA.31,32 Positive charges, on the other hand, can increase the strength of interactions but may also exacerbate unspecific binding.11,33 Similarly, largely hydrophobic molecules have proven to be suboptimal targets, as aptamers are generally hydrophilic and have limited options to interact with such molecules unless modified nucleotides are introduced into their sequence.34,35

2.2 | SELEX variants with immobilized targets

Traditional SELEX variants involve the immobilization of the target molecule on a carrier matrix and the addition of aptamers in solution. Probably the most straightforward matrix used for target immobilization is a nitrocellulose filter, which has the advantage of being simple and affordable, but it is at the same time limited to large molecules like proteins or cells, according to its pore size.36 Bead-based SELEX is more versatile in the choice of target, as it adds the possibility of coupling small molecules to the matrix.37 Furthermore, beads are commercially available in a variety of different materials and sizes, and sometimes even have specialized coatings which allow specific orientation of the target molecule.38 The beads can be handled via centrifugation, filtering, or by using a magnet, depending on the material. They can also be used to pack a microcolumn, allowing for a greater degree of process automation, even though larger amounts of the target molecule may be necessary.39 This issue has been solved by microfluidic SELEX, where automation can be conducted even with picomolar amounts of the target molecule. Here, aptamers are incubated with target-coupled beads on a microchip and are sorted according to their binding behavior.40 Special microchips can even be coated with the target molecule directly, and enable close monitoring of the aptamer-binding behavior during selection.41 A large disadvantage of target-immobilization techniques is the fact that aptamers are identified for the complex between target and matrix. Depending on the molecule, the coupling itself can lead to profound changes in the structural conformation of the target, leaving it in a nonnative state.42 Furthermore, the surface of the immobilization matrix may lead to unspecific adsorption of aptamers, though this effect can be reduced by using a passivating agent like Tween20 or polyethylene glycol after target coupling.43 The matrix may also cause steric hindrance for the aptamers, which could weaken or completely prevent successful binding, especially with small molecules.44,45

2.3 | SELEX variants with immobilized aptamers

Problems associated with the immobilization of the target molecule can be avoided by using methods that immobilize the aptamer library instead while keeping the target
in solution. In microarray-based SELEX, aptamers are coupled to a microwell plate through chemical linkage, and the fluorescently labeled target molecule is added in solution. This process allows for a very high selection stringency, but the diversity of the library is limited by the number of available wells.\textsuperscript{46} Aptamer immobilization is also the most prominent feature of capture SELEX, which was designed particularly for small molecule targets. The principle of this process is the ability of some aptamers to change their conformation in the binding interaction. Predesigned, short oligonucleotides on a plate interact with complementary constant sequences added to the library aptamers and immobilize them. The native target is added in solution, and if aptamers bind and change their conformation, they are released from the plate.\textsuperscript{47} This method is especially effective for the identification of structure-switching aptamers.\textsuperscript{48} Another possibility is to immobilize aptamers to a plate and then “pick” them using a superfine needle that is precoated with the target molecule. Within this process called atomic force microscopy SELEX, high-affinity aptamers can be identified in as little as three rounds, but the disadvantages include a limited library diversity and of course the necessity to immobilize both the target and the aptamers. Furthermore, high-tech equipment is required.\textsuperscript{49} Another rapid-selection method that involves aptamer immobilization, but offers a slightly higher library diversity is known as particle display SELEX. Within this method, magnetic beads are enriched with numerous copies of only a single aptamer sequence and are then incubated with fluorescently labeled target molecules. They can then be sorted by a fluorescence-activated cell sorter (FACS) according to signal strength, excluding all but the strongest binders. While this method offers the possibility to generate high-affinity aptamers for small or large molecules in very few rounds, expensive equipment is needed.\textsuperscript{50,51}

2.4 | SELEX variants with target and aptamers in solution

It is possible to keep both the target molecule and the oligonucleotide library in solution by using techniques like capillary electrophoresis (CE) SELEX. This process utilizes electrophoretic mobility patterns to single out target–aptamer complexes and requires only very few rounds to generate high-affinity aptamers. In fact, aptamers can be identified after only a single selection cycle within the CE-based, highly specialized workflow known as non-SELEX.\textsuperscript{52,53} The necessity of CE equipment, paired with limited library diversity due to finite capillary load capacity are notable disadvantages.\textsuperscript{54} Theoretically, low-tech electrophoresis techniques are also available by using polyacrylamide or agarose gels to separate bound from unbound aptamers. However, these are limited to protein targets and generally offer the very little possibility of controlling the selection conditions.\textsuperscript{42} Another effective, but still somewhat high-tech method would be sol-gel SELEX, in which target molecules are trapped in a porous material on a microchip. The aptamers are then added in solution and pass through the porous material with a constant flow rate, binding to native target molecules in the process.\textsuperscript{55} Surprisingly, this technique even works with low molecular weight targets.\textsuperscript{56} To avoid the use of expensive equipment but still retain the ability of selection in free solution, a comparably simple technique called graphene oxide (GO) SELEX is also available. Here, aptamers and the target molecule are incubated in solution, and GO sheets are used to wash away unbound aptamers by selective adsorption. However, this process may only work for small molecules that have a strong positive charge. Alternatively, the GO sheets can be used as an immobilization matrix to select for structure-switching aptamers, within a setup similar to capture SELEX.\textsuperscript{57}

2.5 | SELEX variants involving cells or tissues

With the application range of aptamers expanding into the medical field, complex targets become more attractive. In order to represent the structural complexity exhibited by cells or even living organisms, the methodology of SELEX must be adapted accordingly.\textsuperscript{58} And indeed, aptamers that recognize particular types or conformations of cells can be generated using a process called cell-SELEX, in which folded aptamers are incubated with whole cells as a target. The selection can be conducted in a variety of ways, some as complicated as using fluorescently labeled aptamers and FACS, some as simple as utilizing a centrifuge to immobilize the aptamer-bound cells.\textsuperscript{59–61} Irrespective of the chosen method, a carefully optimized counterselection and the strict removal of dead cells are required to avoid unspecific binding.\textsuperscript{62} If an effective counterselection is employed, the generated aptamers may be able to differentiate between cancerous and noncancerous cells, identify pathogenic bacteria at the species level, and even deliver therapeutics to target cells.\textsuperscript{63–65} Surprisingly, cell-SELEX can also be used to generate aptamers for individual proteins within a process called target expressed on the cell surface SELEX. Here, eukaryotic cells are modified to express a preselected protein on their surface, which is useful if a complex protein cannot be obtained in sufficient quality using heterologous expression systems. After adding the necessary counterselection process, aptamers can be generated for the target protein in its
native configuration. In case the target protein is deeply embedded in the cell membrane, as is the case with many channels or receptors, virus-like particles can be used as expression vehicles instead. One step further toward mimicking in vivo conditions is 3D cell-SELEX, where aptamers are generated for cells that are oriented in a three-dimensional grid. The spatial separation causes the cells to be in more active contact with the medium and each other as if they were part of a tissue, which may lead to the availability of new target epitopes. Finally, it is even possible to generate aptamers via in vivo SELEX, where a library is injected into the bloodstream of a laboratory animal, and then the organ of interest is harvested together with the bound aptamers. Oligonucleotides specific for human cancer cells can be generated in this way by using xenografts in laboratory animals.

3 | Oligonucleotide Library

3.1 | Aptamer library diversity

What separates an aptamer from other oligonucleotides is not necessarily the ability to take on a structural conformation. Most oligonucleotides can exhibit a structure, if only through the formation of base pairs. Rather, an aptamer is defined by its capacity to take on a very specific shape that allows it to interact with an intended target. As such, the definition is very functional in nature. Whether a theoretical aptamer can form the desired structure will depend on its nucleotide sequence as well as the buffer environment. SELEX libraries contain large amounts of oligonucleotides with different sequences, most of which exhibit uniquely different structures. The more different structural elements are included in an oligonucleotide library, the more likely it is to contain an aptamer that can interact with the desired target. As a result, the structural diversity of a library is a key factor in the success or failure of SELEX. Understanding the advantages and disadvantages of available library types will make it possible to choose the one most suitable for the intended target and purpose.

3.2 | RNA or DNA oligonucleotides

Traditionally, RNA libraries were the most popular choice for aptamer generation, because it was believed that it was the special ability of RNA, but not DNA, to take on functional structures. And indeed, on average, RNA oligonucleotides may display a higher structural complexity than their DNA counterparts. Additionally, the natural occurrence of ribozymes clearly proves the viability of RNA aptamers. On the other hand, an RNA library necessitates adding a reverse transcription step to every SELEX cycle. This introduces the possibility of transcriptional errors, which become increasingly severe with longer RNA sequences around 100 nucleotides (nt). Furthermore, longer RNA sequences are also more likely to suffer from misfolding. These disadvantages, combined with the fact that the proof of principle for single-stranded DNA (ssDNA) aptamers, were published not long after the initial discovery of SELEX, increased the popularity of DNA libraries. Using DNA libraries not only allows avoiding the reverse transcription step but DNA oligonucleotides also offer higher chemical and biological stability and are easier to synthesize. Even in terms of enzymatic activity, RNA does not necessarily provide an advantage. Deoxyribozymes may not occur in nature but can be generated in vitro even from longer sequences, without the typical drawbacks of RNA. Eventually, DNA libraries took over RNA libraries in popularity, and today they are highly represented in the literature and the market. While DNA libraries may seem superior overall, it is important to note that both DNA and RNA libraries offer their own, uniquely different diversity of structural elements.

3.3 | Nucleotide balance

The structural diversity of an oligonucleotide library is further influenced by the amount of each nucleotide it contains. Because the different nucleotides are incorporated into the libraries at varying rates, the molar ratio needs to be optimized if an equal distribution between A, T/U, G, and C is desired. It has been shown that creating a predominance of G- and C-bases can increase the structural diversity of a library. This is because DNA libraries that contain more G and C bases rather than A and T bases tend to form more complex three-dimensional structures that also contain more stems. Furthermore, GC base pairs exhibit stronger hydrogen bonds than do AT pairs, which leads to the greater structural stability of hairpin structures. As a result, the structural diversity and stability of both RNA and DNA libraries can be increased by optimizing the ratio of the different nucleotides that are present during synthesis. This may be an important point to consider when buying aptamer libraries.

3.4 | G-quadruplex and i-motif

Another way a higher G and C content can increase the structural diversity of a library is by facilitating the formation of G-quadruplex and i-motif structures.
G-quadruplexes are planar structures, formed by the interaction of at least four guanine nucleotides with a stabilizing cation in the center, typically a potassium ion.\textsuperscript{79} Such structures allow hydrophobic interactions with the target and may possess side loops that are uniquely involved in target recognition. G-quadruplexes are found frequently in aptamers published in the literature, where they have been tied to cancer cell recognition.\textsuperscript{42} Interestingly, they can also be found in the human genome.\textsuperscript{80} Owing to their stackable nature, they have been observed to recognize small molecules exceptionally well.\textsuperscript{81} While it would seem that biasing the library toward one particular base would reduce the structural diversity of a library, this is not necessarily the case with guanine. This is because even small differences in the nonguanine bases can lead to changes in the G-quadruplex structures. As such, introducing fixed guanine blocks into the starting library may even increase structural diversity. There are, however, disadvantages to such a strategy. For example, G-quadruplexes may interact with targets based on unspecific hydrophobic interactions. They may also cause problems during amplification, where their high structural stability can hinder primer binding. However, even though G-quadruplexes may in some cases resist temperatures of up to 99°C, removing potassium from the PCR buffer can solve this problem by eliminating the major stabilizing factor for these structures.\textsuperscript{42} Interestingly, G-quadruplex structures do not necessarily depend on potassium ions but may also integrate magnesium ions, calcium ions, or other cations according to their atomic radius.\textsuperscript{79} This is why aptamers that form G-quadruplexes can in some cases be used to detect and even quantify the presence of certain positively charged ions.\textsuperscript{82} The presence of cytosine-repeat sequences, on the other hand, can lead to the formation of i-motifs. These tetrameric structures are composed of two oligonucleotide strand duplexes, which intercalate according to their C repeats. This interaction is only possible if the C bases are partially protonated. As a result, i-motifs generally require acidic, or at least slightly acidic conditions, which is why they can potentially be used as pH-sensitive detection agents.\textsuperscript{83}

3.5 | Patterned libraries

Adding particular nucleotide patterns can improve the efficiency of selection and thus increase the binding affinity of the generated aptamers. This was experimentally tested by adding alternating purine and pyrimidine bases to favor the formation of hairpin structures.\textsuperscript{81} The resulting aptamers bound more tightly to their targets and could be enriched earlier compared to the use of a standard library. Differences in the average folding energies of the libraries also suggested a greater amount of structural elements in the patterned library, which in theory increases the chance of a successful aptamer generation. Furthermore, such libraries were shown to be less dependent on the cations in the buffer, which may allow for a wider application range of the generated aptamers. On the other hand, these libraries showed a smaller possibility of G-quadruplex formation, which could in theory decrease their chance to bind to a target via the hydrophobic interaction and to recognize small molecules.\textsuperscript{81} Furthermore, caution needs to be taken not to predefine the sequences too much, as this may reduce diversity and ultimately reduce the chance of successful aptamer selection.\textsuperscript{84}

3.6 | Predefined sequences and motifs

Another possibility of manipulating the oligonucleotide library is the addition of well-known functional sequences.\textsuperscript{85} Upon analyzing multiple aptamers that are known to bind to the desired target and comparing their sequences, favorable motifs may be discovered. The addition of such motifs to the starting pool may then increase the chances of successfully generating a high-affinity aptamer or may alternatively be used to improve an already known aptamer.\textsuperscript{86} Similarly, adding regions that perform a known function can lead to the generation of specialized aptamers. Combining a ligand-binding region with a catalytic region, for example, can result in an aptamer with enzymatic activity.\textsuperscript{30} Adding a region that binds to a fluorescence-emitting molecule, and which is only formed after successful target binding, makes it possible to create self-reporting aptamers.\textsuperscript{87} Furthermore, by connecting two different known binding motifs with a random nucleotide linker sequence, it is possible to generate the so-called bivalent aptamers with exceptionally high affinities. In addition to their binding motifs, these aptamers rely on a balance between rigid and flexible elements, similar to certain peptides that can be found in vivo.\textsuperscript{88} Finally, the combined existing knowledge about aptamers and the structures they are able to form can be used in conjunction with sophisticated software, in a process called in silico SELEX. Briefly, this approach enables the optimization of starting libraries by adding favorable structural elements. It can also be used to improve existing aptamer sequences via computational calculations. Finally, it can even simulate the binding of theoretical aptamers with the desired target within a virtual selection, though the capabilities of this application are still limited.\textsuperscript{36}
3.7 | Chemical modifications and special nucleotides

Conventional RNA or DNA nucleotide libraries, no matter how much they may be optimized, still have two potential shortcomings. The first is the availability of only four different nucleotides, which limits the total structural diversity and the possible mechanisms of aptamer–target interaction. Depending on the target, conventional libraries may even be considered unsuitable for the generation of high-affinity aptamers. The second shortcoming is the relatively brief in vivo half-life of aptamers due to nuclease degradation and/or renal clearance. Conventional aptamers have a half-life of about 1 min in human serum, which makes a therapeutic use effectively impossible.

All of these issues can be addressed by using nucleotides that have modifications in one or multiple of their structural elements. The 5′-end of an aptamer, for example, can be coupled to a large molecule, such as polyethylene glycol or cholesterol. In this way, the total size of the aptamer is increased and it becomes resistant to filtration by the kidneys. The phosphodiester backbone, which is the direct target of nucleases, can be chemically modified to make recognition by these enzymes less likely. Unfortunately, such manipulations may also lead to the phosphate group losing its negative charge, which reduces the stability of the G-quadruplexes. Adding special chemical groups to the sugar molecule of nucleotides causes them to assume a nuclease-resistant conformation, increasing the aptamer half-life in vivo to multiple days.

In addition, some of these functional groups can facilitate hydrogen bonds and thus even enhance the ability of aptamers to bind to their target. Replacing the sugar moiety with a different sugar molecule altogether is also possible and yields so-called “xeno nucleic acids” or “XNA.” These can provide improved nuclease resistance, as is the case with threose nucleic acid, which is believed to be the historical predecessor of RNA. Locked nucleic acids, which have a cross-link added to the ribose, also exhibit higher half-life in vivo and, remarkably, allow the oligonucleotide to form unique triplex structures. Many other variations exist, and each has unique advantages and drawbacks. Nuclease-recognition can also be reduced by using aptamers with chirally reversed L-nucleotides. These so-called Spiegelmers have an L-ribose in their backbone, instead of the regular D-ribose. To identify such aptamers, the first step is to generate regular D-ribonucleotide aptamers for a chiral target in its non-natural orientation (e.g. the D-form for amino acids). Once a binding sequence has been identified, it is used to synthesize nuclease-resistant L-aptamers, which will then in a mirror-fashion recognize the target in its natural conformation. Finally, the manipulation of nucleobases may significantly increase the success chance of SELEX. For example, so-called slow off-rate modified aptamers, or SOMA-mers, have peptide-like side chains added to their nucleobase groups. Such modifications can increase structural diversity and can be highly hydrophobic by themselves, leading to recognition advantages with certain targets. The same can be achieved by using fully synthetic bases, which may offer improved structural stability along with excellent target recognition. Taking this to the extreme, so-called X-aptamers can be created from nucleotides modified with a theoretically unlimited number of random functional groups. This is a strategy similar to a traditional small molecule screening process, and it usually involves only a single SELEX cycle because of the impossibility of amplifying such sequences.

In general, the amplification step can pose a major hurdle to the handling of modified aptamers, because conventional polymerases may not be able to recognize the modified nucleotides. However, mutant polymerases have been developed and are available for many applications. Interestingly, even oligonucleotides with bases that cannot form hydrogen bonds can be amplified in this way, suggesting that shape complementary alone can be sufficient for replication. Furthermore, replication difficulties can be overcome by utilizing a process called click-SELEX, where chemical modifications are removed before, and reapplied after, amplification. In this way, even large functional groups can be added to the aptamers, potentially introducing antibody-like properties.

All in all, the use of modified nucleotides is a promising method that can increase the success chance of SELEX, improve stability and affinity of the generated aptamers, and add clinically relevant properties. The advantages and disadvantages of each individual modification need to be carefully considered, together with the necessitation of additional material and the possible obstacle of commercially held patents.

3.8 | Library length

The length of the random region of an oligonucleotide library can limit its potential structural diversity. As such, it can be a major determinant in the success or failure of SELEX and should be considered carefully in advance. Aptamers have been successfully generated from libraries with a random region as short as 22 nt or as long as 220 nt, while the average is around 30–80 nt. To elucidate a suitable length, it will be necessary to consider the amount of starting material that can be sampled in the experiment. If a library containing $10^{15}$ oligonucleotides are used, the random part should be at least 25 nt long. This is because,
assuming four different nucleotides, a 25-nt library offers just over $10^{15}$ possible different sequences at the maximum ($4^{25} \sim 1.1 \times 10^{15}$). In practice, such a library can still contain many identical sequences, and even more structurally similar aptamers, leaving the structural diversity at a suboptimal level. On the other hand, choosing significantly longer sequences will make it impossible to exhaustively sample the whole diversity of the library, and certain binding sequences or motifs may not be contained at all. For example, the total possible diversity for a library with 50 nt in the random part would be $10^{30}$ different sequences, which means that a library with $10^{15}$ oligonucleotides will only contain a fraction of all possible sequences, roughly the square root ($4^{50} \sim 1.3 \times 10^{30}$ and $\sqrt{10^{30}} = 10^{15}$). Another point to consider is the binding motifs that are desired in an aptamer. While small and single motifs are readily identified using short oligonucleotide libraries, they may not be found in libraries with longer sequences due to masking, misfolding, and/or incomplete sampling. In addition, longer sequences are outcompeted by shorter amplicons during amplification and are more prone to transcription errors. As such, libraries with short random sequences are likely more advantageous when small, low-complexity molecules are the target of SELEX. However, in case more complex molecules or even cells are targeted, the increased structural diversity offered by longer sequences can be an advantage. Especially complex binding motifs may even require a minimum oligonucleotide length to be formed, and the combination of multiple motifs in one sequence can further improve binding. On the other hand, a large meta-review on aptamer selection concludes that individual sequence and structure are more important for aptamer affinity than the length of the starting library. As such, when it comes to choosing the length of an oligonucleotide library, the most important factor to consider is the limited structural diversity found in very short libraries. In conclusion, a length of 50–70 nt in the random part can provide a suitable compromise between sequence diversity and ease of handling.

### 3.9 Constant regions

In SELEX, the random regions of oligonucleotides are commonly flanked by two constant regions. These constant regions serve as primer-binding sites, which are necessary for the amplification step. On average, they are 18–21 nt long, which means that they can make up more than half of the total nucleotide sequence of an aptamer. Their sequences should be chosen in such a way that the primers will not form primer dimers, in order to avoid unspecified PCR products which can reduce the success chance of the experiment. The influence of the constant regions on the structure and/or stability of an aptamer is hard to predict, but there are some general pointers. The aptamer structure is more likely to be affected by primer-binding sites when the length of the random region is 30 nt or less. In addition, if the constant regions form stable structures by themselves, the structural diversity of the library may be reduced. The longer the random region is, the smaller is the influence of the constant regions on the aptamer structure.

There are multiple possible explanations for this. First, the low complexity of the constant region limits structural diversity, making it more likely that binding motifs are found in longer, and thus more complex, random regions. Second, the constant regions are located at the edges of the oligonucleotide and are thus less accessible in long sequences. Third, aptamers that involve the constant regions in their structure impede primer binding, which may lead to disadvantages during amplification. As such, one strategy to avoid constant region interference is to choose a library with a long random part. For libraries with short random parts, engineering the primer sequences to form short hairpin structures with each other may keep their interference low. These hairpin structures should only be a few bases long, as they may predefine the possible aptamer conformations if they are too stable. Another possibility is the usage of a primer-free library, which involves the removal of primer-binding sites before the selection step via restriction enzymes. Alternatively, in “primer switching,” different constant sequences and primers are chosen in every SELEX-cycle and are kept during selection. Both of these methods introduce restriction and ligation steps, which are highly inefficient and may lead to the loss of binding sequences. Blocking the primer-binding sites with short, complementary sequences is another possible solution, which may, however, introduce PCR bias. All in all, considering that constant sequences can reduce the structural diversity of a library, optimization seems worthwhile, especially with short random regions.

### 4 Amplification and Sequencing

#### 4.1 PCR amplification

Over the course of SELEX, an initially highly diverse oligonucleotide library is reduced to comparably few, but very abundant aptamer sequences. This is achieved according to two general principles: the removal of non-binders during the selection step, and the enrichment of binders during the amplification step. In theory, aptamers
that bind to the target with high affinity are more likely to be retained during the selection step than weak or unspecific binders. As such, high-affinity aptamers will have more copies present in the amplification pool and will undergo greater enrichment than weak or unspecific binders. Therefore, it would seem suitable to conduct as many PCR cycles as possible to achieve the best enrichment of binding aptamers. In practice, however, such a strategy can actually reduce the success chance of SELEX. \(^\text{103}\)

### 4.2 PCR bias

Selective enrichment according to the copy number of aptamers in a template pool can only work if the PCR procedure does not favor individual sequences. However, whenever an oligonucleotide library with high heterogeneity is amplified via PCR, a certain bias is unavoidable. There are multiple different mechanisms by which bias is introduced into PCR. First, short sequences are generally favored by PCR and tend to be amplified at a higher rate than long sequences. \(^\text{84}\) Second, unspecific interactions can result in (PCR–)product–product, product–primer, and even primer–primer hybridizations, which may not only prevent primer annealing but can also lead to the generation of unwanted by-products. \(^\text{109}\) And finally, aptamers with high structural stability, especially those rich in G and C bases, are generally less accessible for primers and are outcompeted by aptamers with lower structural stability. \(^\text{110}\) In case an RNA library is used, additional bias may occur during transcription. Most amplification errors increase exponentially during every single PCR cycle. \(^\text{111}\) As a result, depending on the severity of the bias, some sequences may be lost from the selection entirely. In the worst case, the final aptamer pool may be conditioned for amplification frequency rather than target binding, leading to a suboptimal result or even failure of SELEX. \(^\text{110}\)

The failure of SELEX can also result when the aptamer pool is dominated by PCR by-products, which may outcompete and mask actual binders (Figure 3). Unspecific hybridization may lead to the formation of two particular types of by-products, one of which is ladder like, and other has a relatively constant length. \(^\text{111}\) The ladder-like product results when the 3′-constant region of one oligonucleotide strand hybridizes with the 5′-end of the random region of another strand. The DNA polymerase will bind to this partially hybridized DNA molecule and elongate the first strand from its 3′-end onward, adding a repeat of the primer-binding sequence without a gap. This additional primer-binding site can now be recognized by the primers, and the elongated oligonucleotide will be reproduced. If an elongated sequence repeats this process, another primer-binding site will be added. Finally, these elongated oligonucleotides are more likely to interact with each other according to the gap-less primer-binding site repeats, and even more, elongation will occur. The result is a DNA ladder that can be seen in an agarose gel of the PCR product. The by-products of constant size, on the other hand, result if a primer-binding site is added to an oligonucleotide with a gap. This happens when one strand hybridizes with the center or the 3′-end of the random region of another strand. As a result, the elongated section will contain parts of the random sequence, followed by a primer-binding site repeat. The gap that is created by the random region disfavors further elongation because it acts as a hindrance for hybridization according to multiple primer-binding site repeats. \(^\text{112}\)

### 4.3 Strategies to minimize PCR bias

It is possible to avoid or at least reduce PCR bias by optimizing several different aspects of the PCR procedure. Ideally, the number of PCR cycles should be kept at the necessary minimum. Furthermore, the template amount should be adapted after every selection step, because higher concentrations of starting DNA may lead to by-products in earlier PCR cycles. Under optimal conditions, as few as 10 cycles of PCR, rather than 20 or even 30, can be enough for suitable enrichment of binding species. \(^\text{109}\) Furthermore, a higher concentration of primers in comparison to the template DNA can reduce unspecific product–product hybridization and increase the yield of PCR. \(^\text{111,113}\) Problems with ladder-type by-products may be solved by skipping the elongation step of PCR. \(^\text{112}\) Finally, choosing special polymerases and amplification buffers can reduce PCR bias for structurally stable aptamers. \(^\text{114}\) In case an RNA library is used, it is possible to further reduce the cycles of PCR because the RNA transcription step will also amplify the aptamer pool. During transcription, the template strands are not reproduced, which lead to less unspecific hybridization, and therefore less bias. Furthermore, by using a heat-resistant RNA polymerase at higher temperatures, bias due to stable aptamer structures can also be minimized. \(^\text{111}\)

In order to limit PCR bias to the absolute minimum, a method called emulsion PCR (ePCR) can be employed. In ePCR, the amplification is not conducted in full solution but instead takes place in multiple small oil droplets simultaneously. Each of these droplets contains only a small fraction of the totality of aptamer sequences in the pool. This method confers two large advantages: first, it reduces the likelihood for partially complementary aptamers to be contained in the same reaction; second, it reduces the chance that PCR-disfavored aptamers are suppressed.
by easily amplified sequences. As such, ePCR not only reduces bias and by-products due to unspecific hybridization but it also preserves rare binding sequences that could have been lost in regular PCR. Preserving rare sequences is especially important when the diversity of the starting library is limited, as is the case with some variants of SELEX. The downsides of this method are the high degree of optimization that may be needed, and the requirement of greater amounts of DNA polymerase. Furthermore, rare sequences are less likely to be lost, but they are still disadvantaged during amplification. As a result, ePCR products should be sequenced after every cycle of SELEX. If only the final aptamer pool is analyzed, easily amplified sequences will be overrepresented, and the best binders might not be identified after all.

### 4.4 Regeneration of ssDNA or RNA

After successful amplification, the resulting PCR product will mainly consist of double-stranded DNA (dsDNA) in the form of aptamer strands hybridized with their complementary sequence. In order to continue to the next selection cycle, the ssDNA or RNA aptamers need to be regenerated from this pool. For RNA libraries, this can be done via RNA transcription, which is a relatively straightforward process. The known transcription bias against G- and C-rich sequences may be avoided by using higher temperatures along with a heat-resistant RNA polymerase. For the purpose of ssDNA regeneration, four basic methods exist among which bead-based applications are most frequently used.
One effective method of ssDNA regeneration is the size-dependent separation via denaturing gel electrophoresis. For this procedure, the amplification step is conducted with a 5′-elongated reverse primer that effectively increases the size of the antisense strands. Such primers usually have additional nucleotide sequences attached, either structure forming or with a PEG linker, to avoid errors during PCR. Using biotinylated primers is also possible, in which case streptavidin needs to be added before the gel separation. Alternatively, the antisense strand may be shortened instead by 3′-labeling the reverse primer with a ribonucleotide. Upon incubation in an alkaline solution, the primer will detach, leaving an oligonucleotide with reduced size. Using denaturing urea-PAGE, it is possible to suppress the effect of aptamer structure on migration speed, and the sense and antisense strands can be separated according to their size. Other components, such as by-products and mutated sequences, will also be visible on the gel. By extracting only the band of the desired size, unwanted products can be removed in every cycle of SELEX. Disadvantages of this method include the recovery of only around 30% of the total ssDNA and the generally long incubation times of up to 12 h. Both of these issues may be solved by utilizing an approach called electro-elution, which has only recently been applied for DNA aptamers.

Alternatively, regeneration of ssDNA can also be achieved by using streptavidin beads in conjunction with a 5′-biotinylated reverse primer. The dsDNA can be coupled to the beads according to the streptavidin–biotin interaction, and the sense strand will be released upon applying high temperatures or an alkaline pH. This method is comparably fast and straightforward, and ssDNA yield is around 30%, similar to the gel-based separation. There are, however, considerable disadvantages. First, streptavidin beads are comparably expensive. Second, biotinylated primers may take up space on the beads if they are not removed before ssDNA regeneration. Third, streptavidin may dissociate from the beads and interfere with selection in the following rounds, especially if heat is used to separate the dsDNA. And finally, PCR by-products may contain more than one biotin molecule, and thus bind more strongly to the beads than regular aptamers. As a result, it is very likely that by-products are carried over to the next cycle of SELEX.

Enzymatic digestion is another method to regenerate ssDNA. For this procedure, a 5′-phosphate-labeled reverse primer is used during PCR, leading to 5′-phosphorylation exclusively on antisense strands. Lambda-exonuclease is then added and will specifically degrade the antisense strands while leaving the sense strands intact. Some of the ssDNA material will be lost in the following phenol-chloroform extraction step, which is necessary for the removal of the enzyme. The entire process can be completed within about 2 h and will recover around 60% of the total ssDNA. Downsides are the high cost of the enzyme and the possibility of incomplete enzymatic reactions. Furthermore, PCR by-products will not necessarily contain the 5′-phosphorylated reverse primer and may thus resist digestion.

Finally, asymmetric PCR offers the possibility of directly producing ssDNA through the unequal distribution of primers. For this purpose, forward and reverse primers are added in vastly different amounts, for example at a ratio of 100:1 forward: reverse. As a result, the PCR reaction will be split into two phases. In the first phase, dsDNA is produced at an exponential rate, similar to regular PCR. After the reverse primer is used up, the second phase starts. Now, only the antisense strands serve as a template, and sense strands are amplified via the forward primer in a linear fashion. The final PCR product will contain overwhelming amounts of regenerated ssDNA and can in theory be used for the next selection step without removing the dsDNA. In practice, it is advisable to add an additional separation procedure to avoid interference of dsDNA in the selection. Traditionally, asymmetric PCR is followed by native gel electrophoresis. This provides the possibility of distinguishing the dsDNA band from the ssDNA band on the gel according to size. However, on a native gel, the ssDNA oligonucleotides retain their diverse structures, which can affect their migration behavior and may result in a smear on the gel. Alternatively, one of the three aforementioned ssDNA regeneration techniques can be used to remove the dsDNA. Denaturing PAGE with length-modified primers provides an excellent yield of ssDNA. Biotinylated primers and streptavidin beads are also suitable, and in this case the beads do not even have to be eluted. In this way, dsDNA by-products with multiple biotin molecules will also be removed from the selection. Finally, enzymatic degradation of the antisense strand is also a promising second step. Asymmetric PCR combined with any of these three methods will yield considerably higher amounts of purified ssDNA than could be achieved with symmetric PCR. However, a large downside of asymmetric PCR is the necessity of process optimization.

To ensure proper performance, the ratio of primers, amount of template, and the concentration of Mg²⁺ in the reaction buffer may have to be experimentally determined for every new cycle of SELEX.

### 4.5 Ethanol precipitation or filter purification

Between the individual steps of every SELEX cycle, the oligonucleotide pool will need to be concentrated,
buffer-exchanged, and/or purified multiple times. Generally, this is the case after selection-elution, PCR, and ssDNA regeneration. Two methods are commonly employed to this end: ethanol precipitation and spin-column purification. Ethanol precipitation is based on the principle that DNA becomes insoluble in the presence of 70% ethanol and sufficient amounts of positive ions. Different salts can be used to provide the positive ions, which are necessary to neutralize the negative charge of the phosphate backbone. While incubation at cold temperatures between +4°C and −80°C is often recommended, neither the incubation time nor the incubation temperature seems to influence the success of the procedure. If possible, the subsequent centrifugation step should be prolonged instead. Furthermore, coprecipitants like glycogen can improve the DNA yield and the ease of locating the pellet within the tube. Spin-column-based purification usually relies on the ability of DNA to bind to a carrier material, for example, silica, in the presence of a chaotropic salt. Alternatively, spin columns with filter membranes can also be used, ideally with a cutoff that is smaller than the respective size of the nucleotides. Because of the unusually small size of aptamers, using commercial kits may lead to the loss of a large part of the oligonucleotide pool. Therefore, such kits should be selected with care. Depending on the selection method, it may be possible to skip the postselection purification step by performing PCR with the target-bound aptamers directly. In the case of bead-based selection, this can be done by using the aptamer-coupled microbeads as the PCR template.

### 4.6 Sequencing

After multiple cycles of SELEX, target-binding aptamers should be highly abundant in the oligonucleotide pool. In order to identify the individual aptamers, the DNA pool needs to be sequenced, followed by an evaluation of the sequencing data. The traditional method of aptamer identification involves cloning and Sanger sequencing. Briefly, singular aptamers from the final pool are ligated into plasmid vectors on a random basis. The resulting plasmids are then transformed into *E. coli* bacteria, which are isolated and cultured. Ideally, each culture of *E. coli* contains many copies of a plasmid with the same aptamer sequence. The genetic material is then extracted from the bacteria and analyzed via Sanger sequencing, yielding one sequence per sample. Multiple different solutions exist for the individual steps of this process. The simplest method available for the ligation of aptamers into plasmids is TA cloning. For TA cloning, the final PCR amplification is conducted using Taq polymerase, which will preferentially add an adenine nucleoside to the 3′-end of sequences after synthesis. Thus, A-overhangs are created on both sides of the individual aptamers. These oligonucleotides can then easily be integrated into a linearized plasmid vector with T-overhangs. A variant of TA cloning that utilizes topoisomerase can also be used. However, this process called TOPO-TA cloning is known to cause cleavage errors, which may lead to sequencing issues down the line. Another available method is GC cloning, which is based on the same principle as TA cloning but uses different nucleotides. In both cases, overhangs can be added by polymerases during PCR but can also be attached to blunt-ended oligonucleotides in a separate reaction. The finished plasmids can then be transferred into any suitable electro- or chemically competent strain of *E. coli* bacteria. Individual bacterial cultures are usually selected according to antibiotic resistance. Blue/white screening can also be used, even though aptamers may be too short to disrupt the *lacZ* gene, meaning that blue colonies may also contain aptamers. Finally, the genetic material is purified from the bacterial colonies using a regular plasmid preparation kit, ideally yielding a high amount of plasmid DNA while avoiding contaminants that may disturb sequencing. The subsequent process of Sanger sequencing is conducted on a per-sample basis, with the amount of analyzed samples generally between 30 and 100. Depending on the samples, the sequencing reactions may need to be optimized to avoid the formation of stable structures, which can otherwise lead to sequencing failure. Advantages of the traditional Sanger sequencing based approach are its low-tech nature and easy accessibility. Disadvantages include the comparably small amount of sequences received, which strongly limits the depth of analysis of the final aptamer pool. This limitation may even lead to the loss of the most efficient binders, which may be outnumbered by other sequences as a result of PCR bias or unspecific binding.

The introduction of next-generation sequencing (NGS) techniques into SELEX has greatly expanded the toolkit for aptamer identification. Sometimes also called high throughput sequencing (HTS), this technology offers the generation of potentially more than a hundred million sequence reads from a single oligonucleotide pool. Only minimal sample preparation is required and usually consists of adding sequencing adapters to the oligonucleotides. This is routinely done by PCR amplification with modified primers. Interestingly, this amplification step is the largest source of sequencing bias, and it should be carefully optimized as a result. Alternatively, amplification-free protocols are also available but require a higher starting concentration of DNA. In case strand-specific RNA sequencing is needed, special sample preparation techniques will have to be applied before reverse transcription of the aptamer pool. The large amount of data gathered during NGS usually includes countless sequences of
different lengths and requires advanced bioinformatics software for the identification of individual aptamers.\textsuperscript{133} Overall, the NGS approach offers a range of advantages compared to traditional Sanger sequencing. Because of the high amount of sequence readouts, it is not only possible to identify unique aptamers but also entire sequence clusters and even binding motifs.\textsuperscript{49} Furthermore, according to the abundance of each aptamer in any given cluster, the effect of singular nucleotide changes on binding ability may be estimated. As such, it is possible to optimize aptamer sequences even before synthesis.\textsuperscript{110} Because NGS allows an in-depth analysis of an aptamer pool, less enrichment is necessary throughout SELEX than with traditional sequencing. Indeed, aptamers may already be identified between rounds two and four, which can drastically reduce the expenditure of material and time, and improve the success chance of selection by avoiding amplification bias.\textsuperscript{134–136} However, the number of SELEX cycles necessary for successful selection will depend on many factors, such as the amplification rate of individual aptamers and the number of potential binders in the library. Unspecific binding and PCR bias endanger the success of selection even with NGS. A possible solution is the sequencing of aptamer pools from several SELEX rounds. By analyzing the fold enrichment of unique sequences from cycle to cycle, rather than their total abundance, it is possible to evaluate their binding behavior.\textsuperscript{49}

Finally, the technique of nanopore sequencing has been implied as a promising future application. In this procedure, oligonucleotides are pulled through a nanopore via an electrical gradient and nucleotides are determined according to voltage changes in the nanopore as they pass through. In theory, this method of sequencing can be used to identify different types of modified RNA, DNA, and even proteins.\textsuperscript{137} As such, it offers the possibility of sequencing highly modified aptamer libraries with relative ease. Nanopore sequencing could also be applied within a diagnostic context, for example, by sequencing patient serum samples after the administration of pharmaceutical aptamers.\textsuperscript{138} However, while definitely promising, the technology has yet to be developed to the point where widespread use is possible.

5 | STRINGENCY AND QUALITY CONTROL

5.1 | Partition efficiency

The success of the SELEX procedure mainly depends on two factors: the enrichment of high-affinity binders and the elimination of low-affinity and unspecific binders. These two factors are interdependent. If unspecific binders are removed during the selection step, high-affinity aptamers will be subjected to less competition during PCR and can be enriched at a greater rate. Subsequently, a larger amount of high-affinity aptamers in the selection step will allow for more effective competition between strong and weak binders. This increases the possible degree of selection stringency that can be applied without endangering the success of selection. The selection stringency itself will depend on different factors, such as the method of selection, the concentration of the target molecule, and the buffers used for washing. Taken together, these factors can influence the number of SELEX cycles that are necessary, as well as the success chance of the procedure in general.\textsuperscript{42}

5.2 | Selection method

The method of aptamer partitioning during the selection step not only determines selection stringency but also the possible characteristics of the identified aptamers. SELEX variants that involve immobilization of the target, for example, allow for comparably high background binding. This decreases the partition efficiency and the success chance of selection in general because unspecific binders can outcompete high-affinity aptamers through sheer numbers in the amplification step.\textsuperscript{43} Furthermore, immobilizing the target molecule may change its conformation and may thus limit the ability of selected aptamers to bind to the target in its native form. In order to minimize the effect of background binding during selection, a negative-selection step can be added. By incubating the aptamer pool with the immobilizing matrix, but without the target molecule, aptamers that bind to the matrix can be removed. Of note, the negative selection requires no elution step, as the desired aptamers will be contained in the unbound fraction. This technique, however, is only effective for aptamers that bind to the matrix via structural features. If unspecific binding occurs due to electrostatic charge, for example, a negative-selection step will reduce the size of the entire aptamer pool and may eliminate high-affinity binders, especially in earlier rounds of SELEX.\textsuperscript{52} Adding a counterselection step, on the other hand, may also remove unspecific binders and increase the specificity of the aptamer pool. Here, the aptamer pool is conditioned for a molecule that is not the target of SELEX, in order to remove the oligonucleotides that cross-react with this molecule. This procedure is a necessary part of cell-SELEX but may also improve the results of other selection variants.\textsuperscript{63–65} The terms “negative selection” and “counterselection” are often used interchangeably but may mean different strategies depending on the
context. Another proven method of increasing the success of the selection is the combination of different SELEX variants. Such a strategy can lead to reduced background binding, higher specificity, and the requirement of fewer SELEX cycles. Downsides are the requirement of additional resources and expertise.\textsuperscript{103}

### 5.3 Target concentration

The amount of target molecule present during the selection step is an important determinant of selection stringency. According to mathematical studies, an optimal target concentration can be calculated but it will depend on multiple factors such as the concentration of the aptamer pool, the bulk dissociation constant of the pool, and even the employed selection method. Generally, lowering the target concentration increases the stringency of selection, because there is less opportunity for aptamer binding, which results in greater competition between binders. In theory, such an approach will lead to the enrichment of only the best binders, while weak or unspecific binders are removed. In practice, this is only the case with libraries that are dominated by strong binders to begin with. If a library is dominated by sequences with low-binding affinity, lowering the target concentration can actually increase the enrichment of such weak binders. At the same time, high-affinity binders with few copies in the pool are lost more easily.\textsuperscript{103} This is an effect of the generally high heterogeneity of an oligonucleotide library, which is composed of individual aptamers with different respective concentrations and dissociation constants. However, the bulk dissociation constant ($K_d$) of the aptamer pool as a whole can be used to calculate an optimal target concentration during the selection step.\textsuperscript{139}

Selection methods that involve high rates of background binding will limit the possible selection stringency due to the prevalence of weak binders. As a result, significantly greater target concentrations are needed to enrich high-affinity aptamers within a nitrocellulose-based selection (high background) than within a microfluidic system (low background). However, even if background binding is entirely unavoidable, an optimal target concentration can still be calculated when factoring in the bulk $K_d$ and the rate of unspecific binding.\textsuperscript{140} For SELEX variants that rely on target immobilization, one more aspect to consider is the density of target molecules on the matrix. If the density is particularly high, it is possible that individual aptamers interact with multiple target molecules at once by binding via nonspecific interactions. Optimal target-to-matrix concentrations can be calculated to avoid this particular error source.\textsuperscript{43}

### 5.4 Selection buffers and temperature

The structural conformation of an individual aptamer sequence can depend heavily on the buffer environment. The factors that exhibit the largest influence are the pH value, and the types of ions that are contained in the buffer, as well as their respective concentrations. Potassium ions, for example, may be necessary for the formation of stable G-quadruplexes.\textsuperscript{141} For this reason, potassium-free amplification buffers are generally recommended for use during PCR, to avoid bias due to stable structures.\textsuperscript{42} However, other positive ions like sodium, magnesium, and calcium are also known to stabilize G-quadruplexes.\textsuperscript{142,143} Magnesium is known to contribute to hairpin- and duplex structures, may stabilize the aptamer structure in general, and can facilitate aptamer–target interactions. The presence of sodium or potassium in the buffer can weaken electrostatic forces, which may lead to a decrease in binding due to electrostatic interaction. In this way, adding these elements to the buffer may reduce unspecific binding. However, in higher concentrations, sodium or potassium may even impair specific electrostatic interactions.\textsuperscript{29,104,141}

Calcium ions are known to contribute to the stability of aptamer structures, especially such parts of the sequence that form a corner or bend.\textsuperscript{19,144,145} Finally, the pH value of the buffer can also influence aptamer conformation, for example via the protonation of bases that are involved in structure formation.\textsuperscript{146} As a result, the buffer environment plays a key role in aptamer structure formation and binding behavior. This is especially relevant during the selection step, which can involve multiple different buffers for aptamer folding, incubation with the target, washing, and elution. These buffer systems can be adapted to increase selection stringency and condition the aptamer pool for a particular environment, for example, colorimetric assays or therapeutic environments. The final purpose of the aptamers should be kept in mind when choosing these buffers, as a later deviation from the original conditions of selection can lead to the failure of target binding.\textsuperscript{103} A similar principle applies to the folding procedure of aptamers. Before incubation with the target, aptamers are usually transferred to a specialized folding buffer, which contains ions that support structure formation. The sample is then heated in order to break up stable oligonucleotide structures that may have been formed as a result of different temperature or buffer environments. A subsequent cooling step rapidly decreases the sample temperature, causing the aptamers to take on a structural conformation that is suggested to be reproducible under the same circumstances. However, the folding step is probably of minor importance, as the structure of aptamers seems to depend mainly on their buffer environment. Folding may actually decrease
aptamer function if is done at temperatures of 95°C or more.\textsuperscript{147} Interestingly, the incubation temperature of the selection step seems to have a significant influence on the average $K_d$ of selected aptamers. A selection temperature of 37°C could be favorable even with aptamers intended for in vitro application, though this temperature may be incompatible with certain detection assays.\textsuperscript{104}

5.5 | Quality control procedures

Traditional SELEX methods offer very little insight into the progress of selection throughout the process. In many cases, the only known factors are the initial library and the sequencing results of the final aptamer pool. For this reason, various authors have described SELEX as a “black box” and have suggested the implementation of quality control procedures during or after the selection step. While such techniques require additional time and material, they offer valuable data that can help to avoid overselection. In this way, these processes may ultimately save resources and increase the chance for a successful selection.\textsuperscript{103,134,148}

One effective way to determine the selection progress is to measure the binding affinity of the aptamer pool to the target. During the course of a successful SELEX procedure, high-quality binders with a low $K_d$ are enriched and weak binders with a high $K_d$ are washed away. As a result, the bulk dissociation constant of the aptamer pool will decrease with every cycle of SELEX. If the $K_d$ of the oligonucleotide pool stops decreasing, individual sequences likely do not compete anymore and the selection should either be stopped, or the stringency should be increased.\textsuperscript{156} Techniques that can be employed for $K_d$ measurement are surface plasmon resonance and aptamer-binding assays such as the enzyme-linked aptamer sorbent assay (ELASA) and its variations.\textsuperscript{43,149} An additional advantage of this method is that the bulk $K_d$ can be used as a basis for determining the optimal target concentration in the next cycle of SELEX.\textsuperscript{139} Another way to measure the selection progress is by determining the quantity of RNA or DNA material that bound to the target during the selection step. In theory, the total amount of binding sequences should increase as long as selective enrichment is taking place. For this purpose, either qPCR or a fluorescently labeled aptamer library in tandem with a suitable detection method can be utilized.\textsuperscript{150,151} Finally, the selection state can also be determined by assessing the diversity of the aptamer pool. Starting with a highly diverse aptamer library, the conditioned aptamer pool will become less diverse with every cycle of SELEX. Once the diversity stops decreasing, the limits of selection are reached. Many methods for diversity assessment exist, and they can be based on restriction enzymes and low-tech gel electrophoresis (amplified polymorphism analyses) or high-tech applications (qPCR, HPLC, NMR).\textsuperscript{103,136} Of all these methods, qPCR probably offers the most advantages, as it can be used to determine selection progress via both the amplification curves and the melting curves. Furthermore, it offers the ability to accurately quantify the aptamer pool in between cycles. However, qPCR data may be limited by the applied fluorophore and should therefore be optimized carefully in advance.\textsuperscript{135} Even though most of the aforementioned applications correlate well in terms of data, a combination of multiple methods may lead to an even more accurate result.\textsuperscript{136} Finally, sequencing aptamer pools from multiple cycles via HTS will offer the most detailed insight into selection progress. HTS offers the additional benefit of aptamer identification in early rounds of SELEX, and the possibility of in silico post-SELEX optimization.\textsuperscript{103,134–136}

5.6 | Aptamer screening and post-SELEX optimization

After the identification of potentially binding aptamers through sequencing and data evaluation, these aptamers will need to be screened to determine their binding behavior. A simple possibility for prescreening the sequences in question is offered by online tools such as “mfold” and “QGRS-mapper.” These software tools can offer insight into the potential structures formed by aptamers as well as the possibility of G-quadruplex formation.\textsuperscript{152,153} This approach can be of use if certain structural elements are known beforehand and are desired for aptamer–target interaction.\textsuperscript{154} Other methods usually involve the synthesis of the aptamers in question and incubation with the target molecule within an analytical setup. Most popularly, this is achieved via ELASA- or dot blot-based binding assays. These assays often make use of the binding interaction between biotinylated aptamers and streptavidin-coupled detection enzymes. More modern versions exploit the interaction between aptamers and DNA polymerase, or aptamers and gold nanoparticles, in order to determine whether binding has occurred. Detection is conducted via colorimetric change or fluorescence signals.\textsuperscript{85} As a low-tech approach, such assays can be conducted by hand, whereas high-throughput microarray assays offer the screening of multiple thousand aptamers in a relatively short amount of time.\textsuperscript{155} The fluorescence resonance energy transfer (FRET) can also be used for the detection of aptamer binding. For this purpose, however, the aptamers need to bind to a fluorophore, or either the aptamer or target must be coupled to a fluorophore beforehand.\textsuperscript{85} Furthermore, aptamer–target complexes can also be detected on a polyacrylamide gel according to their migration behavior.\textsuperscript{156} An advantage of this method is the
easy detection of quadruplexes, which may show multiple bands due to their tendency for multimerization.\textsuperscript{157}

Post-SELEX optimization of the identified aptamer sequences is a popular approach to improve the binding affinity and saving material cost. Generally, the constant primer regions are unlikely to contribute to the aptamer structure or target binding. As a result, removing these parts of the sequence reduces the cost of aptamer synthesis and can even lead to improved binding affinity.\textsuperscript{158} However, in some cases the primer regions are necessary for proper aptamer folding, so careful monitoring, that is the secondary structure prediction is required before removing these domains.\textsuperscript{29,159} By utilizing specialized software, it may be possible to identify the consensus binding sequence of an aptamer and then truncate the sequence accordingly.\textsuperscript{160} Chemical modifications can be added to the finished aptamer in order to increase nuclease resistance or achieve other advantages, as discussed in the library section.\textsuperscript{161} High-affinity multivalent aptamers can be created by combining known aptamer sequences or binding motifs.\textsuperscript{162} And finally, in silico maturation offers a software-assisted approach to improve the affinity of an aptamer candidate.\textsuperscript{163}

6 | DISCUSSION

The success or failure of SELEX depends on a variety of different factors. Interestingly, many of these factors seem not to limit the overall possibility of aptamer selection. According to a large meta-review on successful SELEX experiments, aptamers could be identified irrespective of the chosen target type, oligonucleotide library, selection buffer, cycle number, and selection method. However, the meta-review shows that some target types are strongly linked to particular SELEX variants, which could suggest a limiting factor. Furthermore, the dataset does not include failed and thus likely unpublished SELEX experiments, which greatly limits the possibility of identifying aspects that influence the success chance of SELEX.\textsuperscript{104} These aspects, however, are discussed at length in the present review. Briefly, they include the structural diversity offered by the oligonucleotide library, the effective removal of nonbinders and weak binders during the selection step, and the selective enrichment of high-affinity binders during the amplification step, ideally without by-products. Furthermore, it is vital to choose a SELEX variant that fits the intended selection target, and detailed optimization is recommended for techniques that involve target immobilization. The selection step should be designed to mimic the desired future application of the aptamer as closely as possible in order to avoid downstream difficulties.

Integrating quality control steps will allow for excellent monitoring and optimization of the selection process on a cycle-to-cycle basis. And finally, choosing an appropriate sequencing strategy and aptamer screening process will ensure the best possible outcome of SELEX.

While the theoretical basics of SELEX have been covered extensively in the present review, the methods that are ultimately employed will depend on many factors. Of note, the material and laboratory equipment at hand will determine the availability of selection and quality control procedures. The goals of SELEX, as well as the nature of the target molecule, will determine the range of SELEX variants that can be employed. On top of that, a lively dynamic lies at the core of aptamer selection itself, as the relative importance of its many aspects may change not only from technique to technique but even from cycle to cycle. The structural diversity found in the aptamer library, for example, will be all the more important if the library size is limited, as is the case with some variants of SELEX. The degree of selection pressure may need to be low early on, in order to avoid loss of rare sequences, but will need to become increasingly stringent in later cycles, to avoid the accumulation of weak binders. But even then, the optimal stringency will be different for every single procedure, as it will also depend on the respective progress of enrichment. As such, a deep understanding of SELEX is necessary to make fully educated choices at every step of the way. The present review certainly provides an overview of the various facets of aptamer selection. To facilitate true expertise of the practical methods discussed herein, additional reading is encouraged. The publications referenced in this review, among others, will serve that purpose and are recommended for further study. Thus, the present discussion of SELEX, when combined with already existing works, will allow for a deeper understanding of aptamer selection, which in turn will lead to a broader application of aptamer technology in research and development.

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CONFLICT OF INTEREST

The authors do not report a conflict of interest in relation to this work.

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