Chapter

Aptamers for Targeted Delivery: Current Challenges and Future Opportunities

Chetan Chandola and Muniasamy Neerathilingam

Abstract

Aptamers are synthetic ssDNA/RNA molecules that are emerging as novel tools for the development of therapeutics, especially for targeted delivery. Aptamers are comparable to monoclonal antibodies, which are well-established therapeutic molecules, in terms of specificity and affinity to their target. The advantage of aptamers over antibodies includes their high stability, ease of synthesis, less batch-to-batch variation, easy chemical modifications that allow different conjugation chemistries, small size for better tissue penetration and low immunogenicity. These advantages make aptamers an important tool for use in therapeutics for targeted delivery. However, aptamers do have some limitations that have hindered their widespread clinical use as a therapeutic agent. Some of their common limitations include serum stability, renal filtration and endocytic escape. Other limitations that are more specific to aptamers include lack of diversity in the aptamer library, nuclease susceptibility and claims of aptamer specificity as well. This book chapter sheds light on these challenges, and using examples, it explains the scientific advancements that have been achieved in overcoming these limitations. We will end this chapter by discussing the use of high-throughput technology, which is the only way of truly industrializing the aptamer technology akin to the development of small molecule drugs.

Keywords: aptamers, SELEX, targeted delivery, serum stability, endosomal escape, renal clearance, high-throughput

1. Introduction

Aptamers are small single-stranded RNA or DNA oligonucleotides that specifically bind to their target due to their unique 3-dimensional structure. They were first independently developed by the groups of Gold and Szostak in 1990 [1, 2]. Aptamers are selected from a pool of random oligonucleotide library (>10^{15} random sequences) by iterative rounds of selection and amplification by a process called Systematic Evolution of Ligands by EXponential Enrichment (SELEX). Different intermolecular interactions facilitate the interaction of aptamers with their target including van der Waal's forces, electrostatic interactions between charged groups, three-dimensional shape, stacking and hydrogen bonds.

Functionally aptamers are same as antibodies; however, their advantages over antibodies include small size, less immunogenicity, ease of synthesis, easy chemical

modification that allows conjugation with a variety of molecules and stability at a higher temperature. Aptamer is comparably smaller (~3 nm) than an antibody (10–15 nm) [3], thus, allowing higher penetration in tissues and the ability to bind more dense cellular epitopes in living cells. Unlike antibodies, aptamers can be selected against non-immunogenic molecules, including proteins or peptides as well as toxins. Aptamers have been successfully developed against proteins, peptides, dyes, metal ions, viruses, bacteria, toxins, and whole cells. They have high specificity and affinity often comparable with antibodies. Unlike antibodies, they can be selected at non-physiological conditions such as extremely high or low temperature, or pH. Synthetic manufacturing of aptamers allows minimal batch-to-batch variation, which is a tedious task to maintain in antibody development. All these properties make aptamers as suitable candidates for therapeutic use, particularly targeted nano-delivery. Several aptamers have already been selected against cell surface targets for targeted delivery of therapeutic payload for different diseases, e.g., cancer and human immunodeficiency virus (HIV) infected cells [4]. **Table 1** lists the common differences between antibodies and aptamers.

In this book chapter, we discuss the challenges that hinder the clinical use of aptamers as targeted delivery molecules. For example, the small size of aptamers is like a double-edged sword. Though it helps in higher tissue penetration, but also results in high renal filtration that causes loss of its therapeutic efficiency. Using examples we show that how challenges in serum stability, renal filtration, selection

	Antibody (IgG)	**Aptamer**
Size	10–15 nm	~3 nm
Target	Only immunogenic targets. Can't be raised against toxic or non-immunogenic proteins	Produced against immunogenic and non-immunogenic (e.g., metal ions, dyes, small peptides, toxins etc.) targets
Specificity	High	High
Affinity	High	High
Penetration	Low tissue penetration due to large size	High tissue penetration due to small size
Stability (pH, Temperature)	Low	High
Shelf life	Few months (at low temperature; repeated freeze-thaw causes loss-of function)	Several months (at room temperature) to several years (frozen)
Nuclease susceptibility	Absent	Present (modified nucleotides minimize nuclease susceptibility)
Immune response	High (except humanized Ab)	Absent or low (rare cases)
Batch-to-batch variation	Present	Absent due to synthetic manufacture
Conjugation	Less possibilities	Easy conjugation due to chemical nature allows functionalization with a wide variety of molecules
Synthesis	Only in physiological conditions	Synthetic
Cost of synthesis	High. Requires animal house facility or reactors	Low. Synthesized using table-top instruments
Scale-of-synthesis	Low	Scalable

Table 1.
This table lists the common differences between an antibody (IgG) and an aptamer.

methodology and endocytic escape have been overcome. We explain the importance of proper controls and validation methods in SELEX methodology, which would otherwise result in the development of poor quality aptamers and, this might ultimately diminish the confidence in this technology. Lastly, we discuss about the development of automation in SELEX technology, an important step toward industrializing the aptamer selection process.

2. Serum stability

Currently, many aptamers are in different stages of clinical trials for various diseases [5]. Most of the drugs are administered systemically, i.e., intravenously. This method poses a challenge of overcoming serum stability, which involves overcoming nuclease activity and coagulation. Here in we discuss these two topics in detail:

2.1 Aggregation

Aptamers have been used to functionalize liposomes to increase their bioavailability through targeted delivery (**Figure 1**) [4]. Since aptamers are negatively charged it is easier to pack them with cationic liposomes. However, serum proteins being charged molecules bind to cationic lipids, thus, lowering their targeting efficiency due to change in structure, aggregation or dissociation of the aptamer-cationic lipid complex [6, 7–9]. Polycationic lipids have also shown cyto(toxic) effects [10]. The problem of aggregation incurred by the charge of cationic lipids can be overcome by using neutral helper lipids [11, 12]. But another challenge is that lipid conjugated aptamers may also bind to erythrocytes, and filtered out through liver and spleen. For example, 1,2-dioleoyl phosphatidylethanolamine (DOPE), a neutral helper lipid, binds to erythrocytes. As an alternative, another neutral helper lipid, cholesterol, may be used which shows significantly lower binding to erythrocytes as compared to DOPE [10, 13].

Cholesterol conjugated RNA aptamer against Hepatitis C virus (HCV) NS5B protein has been used to inhibit HCV replication in human liver cells without induction of cytotoxicity *in vitro*. Next, in this study, the same molecule was injected in a mouse model but found no induction of innate immunity. The half-life of the cholesterol-conjugated aptamer was longer than that of the

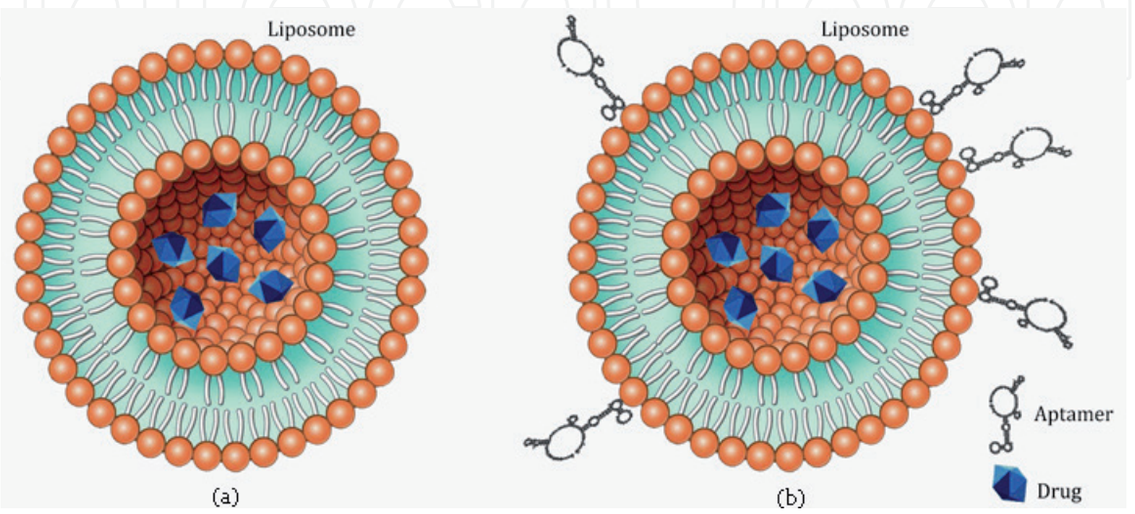

Figure 1.
Illustration showing (a) non-functionalized liposome and (b) aptamer-functionalized liposome carrying drug molecules.

non-cholesterol-conjugated aptamer, and in accordance, cholesterol conjugation reduced the clearance by 9-fold [14].

It is important to note that no one lipid composition induces cytotoxicity in all cell types, i.e., a conjugated lipid may be toxic to one cell type but not to another. Improvements of lipid formulations are constantly explored by the addition of different lipids, targeting molecules, or shielding moieties designed to prevent clearance *in vivo*. Use of neutral lipids for aptamer administration has been explored less due to the difficulty in loading hydrophilic aptamer molecules in the neutral lipids for formulation, as compared to (poly)cationic lipids. Liposomes are just one tool for enhancing the efficacy of aptamers for targeted delivery, and there are multitudes of vehicles that are being examined.

2.2 Nuclease activity

Nuclease activity of the serum is helpful in cleaving foreign DNA, thus, it is a way of prevention from pathogens. However, this activity is deleterious to the oligonucleotide-based therapeutics, including aptamers. Unmodified aptamers may have very short half-lives *in vivo* (<10 minutes) [15]. Two types of nucleases act on these aptamers, *viz.*, exonucleases and endonucleases. Hence, different strategies have been designed to prevent the cleavage activity of each of the nucleases.

In-SELEX and post-SELEX strategies can be utilized to develop nuclease resistant aptamers. Each of these two strategies has its own advantage and limitation. We discuss these two below:

2.2.1 In-SELEX

In this methodology aptamers with the desired modification are generated during the aptamer selection process using modified nucleotides, e.g., modified 2′ sugar position using 2′-amino pyrimidine nucleosides [16, 17], 2′-fluoropyrimidine nucleosides [18, 19], 2′-O-methyl purine [20], and 2′-O-methyl pyrimidine nucleosides [21, 22] and locked nucleic acids (LNAs) [23, 24] have been commonly used for this purpose, preventing resistance from endonucleases. Macugen, the only FDA approved therapeutic aptamer, was also selected using this approach [25]. The Advantage of this methodology is that no further modification is required in the selected aptamers, thus, ruling out the possibility of loss of function due to post-SELEX modification. However, use of unnatural nucleotides posed a challenge to early researchers since wild-type DNA/RNA polymerases could not amplify the unnatural aptamers during preparative PCR round of aptamer selection. However, using synthetic biology researchers developed new polymerases that could amplify oligos with unnatural nucleotides. Still, amplification using these mutant polymerases is not comparable with natural polymerases; hence, aptamer library amplification is a bit challenging.

SOMAmers are another class of aptamers that bear dU residues at the 5′-position. These are uniformly functionalized at the 5′-position with various moieties (e.g., benzyl, 2-naphthyl, or 3-indolyl-carboxamide). These moieties can interact with the target molecule and form novel secondary and tertiary structural motifs within the SOMAmer. Introduction of dU at the 5′-position offers nuclease resistance to these molecules [26].

2.2.2 Post-SELEX

Post-SELEX involves the introduction of desired modification in pre-selected aptamers during solid-phase chemical synthesis. This method may often lead

to reduced affinity or specificity of the aptamers due to the structural change in the aptamer that is brought upon by the modification. Hence, a combination of methods has to be tested on the aptamer, and one that renders no loss of function has to be selected. Modification of 5′ and 3′ nucleotides is most common and effective in this case. Sometimes unmodified aptamers may demonstrate high resistance to serum nuclease activity [27]. This feature might develop due to the unique 3-D conformation of the aptamer that protects the 5′- and 3′-termini from exonucleases.

Spiegelmers are excellent nuclease resistant *L*-form RNA or DNA aptamers that are chiral inversions of the natural D-forms. Since naturally occurring nucleases can act only on *D*-form of nucleotides, they are very less likely to degrade the *L*-form spiegelmers [28]. Also, due to their unnatural conformation, they are less likely to generate an immune response. The unnatural conformation of spiegelmers also renders it unfit for processing by polymerases for amplification during SELEX process. Hence, in the first step, an aptamer library of natural D-oligonucleotides is selected against a synthetic enantiomer of the *L*-protein target. After the aptamer selection is done, aptamers are converted from *D*-form to *L*-form. As per the rules of symmetry, the resulting *L*-aptamers (spiegelmers) would bind to the natural *D*-protein target with the same affinity as the *D*-aptamers bind to the mirror image selection target [29].

3. Diversity of aptamer library

As we know, aptamer binding to its target depends on its 3-dimensional conformation, which is ultimately governed by its sequence. Hence, an aptamer library with a high degree of sequence variability will have a high variety of structurally variant aptamers; therefore, such a library will have a higher degree of success in developing tight binding aptamers to its target. In contrast to proteins, which are made of 20 different amino acids due to a variety of functional groups, nucleic acids possess a very limited variety of functional groups, i.e., only four bases (adenine, guanine, cytosine and thymine/uracil). Thus, aptamers can be endowed with protein-like properties by addition of functional groups that mimic amino acid side-chains to expand their chemical diversity [26].

SomaLogic, a leading company in aptamer development, pioneered in increasing the repertoire of aptamer sequences in a library. They introduced new functional groups, i.e., hydrophobic side chains (e.g., naphthyl, benzyl, tryptamino and isobutyl) at 5′-position of dUTP nucleotide. Aptamers developed using these modified nucleotides were called Slow Off-rate Modified Aptamers (SOMAmers), and they showed higher success rate in aptamer selection against difficult proteins with success rate going from <30% for unmodified aptamers to >90% for SOMAmers [30].

Kimoto et al. used an extra base pair Ds:Px in the initial library to introduce variety, and developed aptamers with 100-fold higher affinity as compared to the unmodified analogs against VEGF-165 (vascular endothelial growth factor) [31]. Sefah et al. developed introduced non-natural nucleosides Z and P to develop aptamers specifically targeting liver cancer cells [32]. In another work, glycosylated aptamers were used in a method called SELection with Modified Aptamers (SELMA) to develop low-nanomolar affinity aptamers against HIV broadly neutralizing antibody 2G12 [33]. These antibodies protect against HIV by recognizing the unique carbohydrate epitope on HIV. Here, the glycosylated aptamers worked as a scaffold that presented carbohydrate in a manner that mimics their multivalent presentation on HIV, thus it could be used as a vaccine component.

4. Renal filtration

Renal filtration of small molecule drugs is a phenomenon that needs to be overcome for efficient therapeutic use. Owing to their small size, aptamers also undergo this challenge. An aptamer of 6–30 kDa mass has a size of <5 nm [34]. When an unmodified aptamer is administered intravenously, even using stabilizing backbone modifications, they are subjected to rapid excretion through renal filtration, hence, reduced circulation time. To overcome this challenge, aptamers are functionalized with bulky moieties *viz.*, polyethylene glycol (PEG), liposomes, proteins, cholesterol, organic or inorganic nanomaterials, or multimerized to reach a mass above the threshold of glomerulus cut-off (30–50 kDa) [34].

4.1 PEG attachment

PEGylation is one of the most common methods to prevent renal filtration of aptamers. PEG is a flexible, uncharged and a highly hydrophilic polymer that is widely conjugated with therapeutic drugs to reduce reticuloendothelial clearance, extend circulation time and improve drug efficiency [35]. Macugen, which is the only FDA approved aptamer drug in the market is also PEGylated. PEG decreases aggregation and increases the solubility of the conjugates. PEGylation of Macugen increased its half-life to 9.5 –12.5 hours after intravenous and subcutaneous injection, respectively, in the plasma, and up to 94 hours in vitreous humor [36, 37]. Similarly, PEGylation also increased *in vivo* half-life of M7, a DNA aptamer, from <1 to 24–48 hours [38]. This modification did not cause any change in the specificity of M7 aptamer toward its target PD-1 (Programmed death protein 1) and suppressed the growth of PD-L1 (Programmed death-ligand 1) positive colon cancer carcinoma *in vivo* [39].

4.2 Cholesterol

A 29 nucleotide-log 2′-F pyrimidine modified RNA aptamer was reported to inhibit Hepatitis C virus (HCV) replication *in vitro*. In the follow-up study, this aptamer was derivatized with cholesterol to form cholesterol aptamer conjugate (chol-aptamer). This conjugate entered the cell and inhibited HCV RNA replication in a cell-based system successfully. Systemic administration of chol-aptamer was well tolerated by mice and increased retention time in plasma [14].

4.3 Dialkyl lipids

Willis et al. conjugated diacylglycerol (DAG) to the 5′-end of a nuclease resistant VEGF aptamer [40]. This DAG-aptamer conjugate was then incorporated to a liposome bilayer, which resulted in aptamers with improved anti-VEGF activity *in vitro* and *in vivo*, both. Importantly, this DAG-aptamer-liposome complex had a considerably better residence time in plasma as compared to unmodified aptamer [40].

5. Toxicity

Toxicity, side effects, and immunogenicity are one of the most important criteria in drug evaluation, apart from shelf life and efficacy of the drug. Aptamers have a significant advantage over antibodies due to their minimal or non-immunogenic nature. Early preclinical studies using Pegaptanib sodium (Macugen), the aptamer drug for preventing wet age related macular degeneration (AMD), did not exhibit any intrinsic toxicity during preclinical studies [37, 41, 42]. The only side effect

reported arose almost exclusively due to the injection procedure instead of the drug itself [43]. Similarly, other works have also described aptamers as non-toxic therapeutic agents [44, 45].

Though aptamers may be inherently non-immunogenic, chemical modification or conjugation may render them immunogenic or cytotoxic. Hence, chemical modification of aptamers works as a double-edged sword. For example, LNA-modified nucleic acids have shown severe hepatotoxicity [46]. 2′-fluoro pyrimidine and 2′-orthomethyl pyrimidine modifications are commonly used in aptamers to introduce endonuclease resistance. 2′-fluoropyrimidine modified RNA aptamers lead to cytotoxicity and induction of interferon-beta expression in human cancer cells *in vitro*. Interestingly, 2′-orthomethyl pyrimidine completely abrogated the induction of cell death and cytokine expression in the same cells [47].

There is a limited toxicological data available on aptamers. Mainly because the field is still in its initial stage and the majority of aptamer-based drugs are still under clinical trials. In comparison, a significant amount of toxicological data is available for antisense oligonucleotides (ASOs). Some cues might be taken from toxicity studies on ASOs; however, we must remember that aptamers are structurally a different class of oligonucleotides; hence, they may not behave in exactly the same way as ASOs. Toxicological properties of ASOs have been described in earlier reviews [48–50]. Less information is available on other class of immunostimulatory oligonucleotides *viz.*, siRNA, gene therapy, and other partially or completely nucleic acid-based molecules. Three major oligonucleotide-based class effects that have been described are: polyanionic effect, the stimulation of innate immunity, and tissue accumulation of oligonucleotide material [51]. These class effects are briefly described below.

Polyanionic effects occur due to the non-specific off-target interaction with proteins that may affect protein function and it usually occurs at a high concentration of ASOs. The toxicological effects of polyanionic effect include activation of the alternative pathway of complement (C′) that results in pseudohypersensitivity responses and inhibition of the coagulation pathway, thus, resulting in delayed clotting. Complement activation has been studied predominantly in non-human primates, and to some extent in humans. Complement activation is a threshold-dependent phenomenon and will occur acutely once the threshold is crossed [52].

Stimulation of innate immunity has been reported for ASOs. This activation occurs due to the interaction of Toll-Like Receptor 9 (TLR 9) with the CpG motif within the single-stranded DNA. When CpG motifs are unmethylated, they act as immunostimulants [53]. Unmethylated CpG motifs are common in microbial genomes but occur quite less in vertebrate genome, hence they are called as pathogen-associated molecular patterns (PAMPs) [54]. CpG PAMP is recognized by pattern recognition receptor TLR9, which is expressed constitutively by B cells and plasmacytoid dendritic cells (pDCs) in humans and other higher primates [55].

Lastly, oligonucleotide accumulation is observed in certain tissues and cells. This accumulated material is termed basophilic granulation, and it is observed most commonly in renal proximal tubular epithelium and tissue macrophages. Accumulation of basophilic granules usually has no effect on associated cells; however, its accumulation at a very high level in cells, especially renal proximal tubular epithelium, may lead to cytotoxicity and eventually organ dysfunction [56].

Apart from the above mentioned three oligonucleotide class effects, there are other effects that should also be studied; however, these three are the most commonly observed and, hence, should be assessed carefully. In a very interesting work, Guo et al. demonstrated that RNA molecules of different shape, size or sequence can initiate immunogenicity at different levels *in vitro* and *in vivo*, both. Findings from this work suggest that properties of RNA nanoparticles may be tuned for safe

use as therapeutic nanocarriers [57]. Findings from this work may be extrapolated to RNA aptamers to some extent.

Conjugated moiety may also cause an adverse affect in aptamer drug. For example, penivacogin, a PEGylated aptamer drug, induced severe allergic reaction during first exposure to the drug during phase III clinical trials. The allergy occurred due to pre-existing anti-PEG antibodies [58]. PEG is widely used in several consumer products including topical and parenteral medications. Possibly, exposure to these wide varieties of products leads to the development of anti-PEG antibodies [35].

Although less clinical data is available to assess the cytotoxicity of aptamers, studies in animal models show low or no cardiotoxicity, hepatotoxicity or renal toxicity. Doxorubicin (DOX), a widely used anticancer drug causes cardiotoxicity as a side-effect due to non-specific uptake. Neither liposomal DOX nor PEGylated DOX has resolved this issue. However, aptamer-DOX conjugate is known to reduce this cardiotoxicity along with increase in efficacy toward tumor suppression in comparison to non-targeted delivery [59] *in vivo*.

The hepatic uptake of aptamer functionalized gold nanostars (Apt-Au NS) was studied by Dam et al. They found high accumulation of the complex in liver; however, there was no hepatic acute toxicity. This was probably due to the high accumulation of the complex in macrophages instead of the hepatocytes [60]. Similarly, other works also demonstrated lack of hepatic and renal toxicity *in vivo* for aptamer-mediated tumor suppression [45, 61].

6. Selection process

Aptamers are selected from a pool of random single-stranded DNA/RNA oligonucleotide library ($>10^{15}$ random sequences) by iterative rounds of selection and amplification by SELEX method as shown in **Figure 2** [4]. Therapeutic aptamers have been developed against cell surface targets [62] as well as for delivery of therapeutic conjugates into the cells, i.e., for internalization [3]. Though the selection method is fundamentally the same for developing aptamers for either use, the cell-internalizing aptamers require more points to be addressed to ensure a higher degree of success. Endosomal escape and effective delivery to the cytosol is an important aspect that should be considered for the effective biological activity of a cargo. Here we describe current selection methods commonly used for selection of cell-internalizing aptamers, and discuss the limitations of each method. For this purpose, here we compare the two commonly used strategies, i.e., SELEX and cell-SELEX.

6.1 SELEX

Aptamers have been historically selected against cell surface biomarkers using purified or recombinant proteins. Majority of aptamer therapeutics that are currently in clinical trials have been selected using this methodology. However, there are problems associated with this selection method since the protein against which selection is done might not be in its natural conformation. Proteins are present in physiological milieu in a unique 3D conformation due to either posttranslational modification or their association with other interacting proteins. Proteins that are produced *in vitro* usually don't possess these post-translational modifications, specifically, if they produced in a prokaryotic system, e.g., commonly used *E. coli*. Hence, it is advisable to use eukaryotic expression system *viz.*, mammalian, yeast or insect cells as they would possess post-translational modifications which may

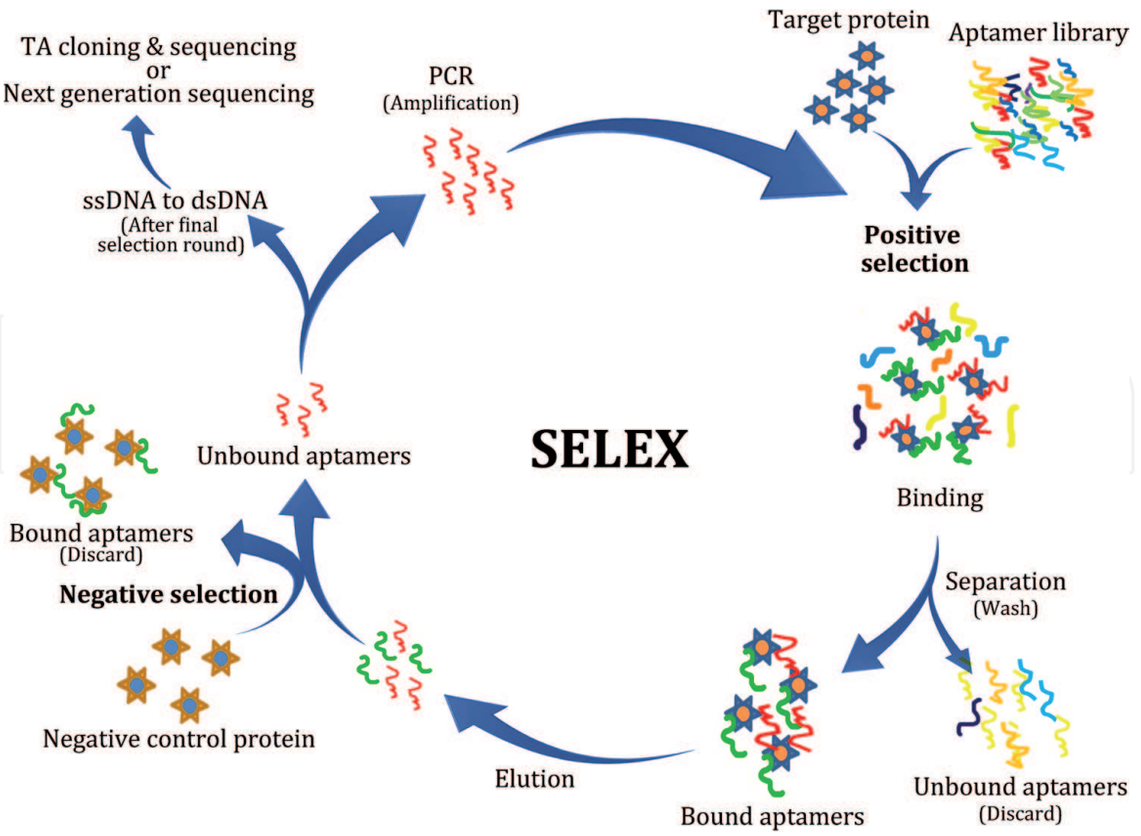

Figure 2.
Diagrammatic representation of the systematic evolution of ligands by EXponential enrichment (SELEX) method. A library of ~10^{15} different single-stranded DNA/RNA molecules is incubated with the target molecules followed by washing. Unbound sequences (non-specific aptamers) are discarded. Bound sequences are recovered and added to the negative control target. Here, the bound sequences (these are aptamers binding to common epitopes) are discarded, and the unbound sequences (target-specific aptamers) are recovered. These sequences are amplified and subjected to further rounds of in vitro selection followed by sequencing after the final selection round. The figure is adapted from Chandola et al. [4].

be crucial for the correct 3D conformation for selection of aptamers. However, low protein yield might be an issue if we chose a eukaryotic expression system over the prokaryotic one. There is also a possibility that recombinant cell surface proteins may be insoluble since they need interaction with other cell components (e.g., G-protein coupled receptor), or form multimeric and/or multivalent structures [63, 46]. Lastly, in the majority of cases, aptamer selection is done using only the extracellular domain of the target protein to avoid aptamer selection against the intracellular region of the protein; however, the absence of transmembrane domain may again alter the natural conformation of the protein. Therefore, there is a possibility that aptamers selected using protein-based SELEX approach might not be able to bind their target in a physiological milieu. Liu et al. selected RNA aptamers against histidine-tagged epidermal growth factor receptor variant III (EGFRvIII) ectodomain. This receptor is present in glioblastoma, but not in normal brain tissue. This protein was expressed in an *E. coli* expression system, hence, lacked a specific post-translational modification, i.e., glycosylation. Lack of glycosylation at this particular site resulted in significant alteration of EGFRvIII protein structure and, hence, the selected aptamer was unable to bind this receptor protein in target cells [64]. Using recombinant protein with a tag-normally used for affinity column based purification of a recombinant protein, e.g., His tag-may result in non-specific aptamer selection against the protein tag; hence, it is always recommended to cleave the tag before a purified recombinant protein is used for aptamer selection. Lastly, attachment of a protein to the matrix may result in non-specific aptamer selection against the matrix itself.

6.2 Cell SELEX

Above mentioned limitations, *viz.*, altered 3D conformation, undesired aptamer selection against the protein-tag or matrix can be overcome by using cell-SELEX methodology. In cell-SELEX aptamer selection is performed on live cells *in vitro*, hence, target surface proteins are present in their native conformation. Unlike protein-based SELEX, cell-SELEX does not need any prior information about the native conformation or biological function of the target protein. This method relies on the difference in the expression pattern of cell surface receptors between the diseased target cells (e.g., cancer cells) and the non-diseased cells (e.g., healthy cells). The aptamer selection is first performed on a negative control, i.e., healthy cells. From here, the unbound aptamers are collected and added to the test cell line, i.e., diseased cells. Only the unbound aptamers are collected from negative control cells because they are uncommon among the target and control cells, and it possibly has a set of aptamers that bind the upregulated cell surface receptors that are present only on target diseased cells. Using the above-mentioned methodology, cell-SELEX has been used to identify novel tumor biomarkers [65]. In our experience, major challenge of cell-SELEX is the optimization of aptamer enrichment by PCR as compared to protein-based SELEX. This is due to the inhibitors or other cellular contaminants (e.g., DNA or RNA) that come in the aptamer pool which inhibit the polymerase-based PCR reaction for enriching aptamer pool during each round of selection. Also, lack of prior knowledge about the identity and expression level of the biomarker may lead to aptamer selection against unrelated/unwanted off-target surface molecules that are co-expressed on target cells; hence, more rounds of counter selection with the control cell line is required to improve the selectivity of aptamers.

An ideal aptamer candidate for internalization purpose should bind to a cell surface receptor that is present in high quantity, and it should have a high rate of endocytosis upon binding of a ligand. However, aptamers selected by cell-SELEX may not fall in this category always. Hence, researchers have developed novel strategies to facilitate enrichment of cell internalizing aptamers by alteration of the traditional cell-SELEX protocol. For this purpose, Thiel et al. developed a methodology called cell-internalization SELEX. Here, target cells were incubated with an RNA library followed by high-salt wash [66]. This method eliminates the non-internalizing surface bound aptamers or those that internalize at a slower rate. Cell internalizing aptamer against Human epidermal growth factor receptor (HER2), a transmembrane protein overexpressed in breast cancer cells was selected using this method [66]. Later studies reported that a high-salt wash is insufficient to remove the non-internalizing surface bound aptamers. Hence, Levy et al. (2012) used a cocktail of RNAses to digest the cell surface bound non-internalizing aptamers [67, 68]. Using this strategy, this group selected cell internalizing 2′-fluoropyrimidine RNA aptamer called c2 against human transferrin receptor (hTfR).

Proteinase K is a broad-spectrum serine protease that cleaves peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha-amino groups. Proteinase K was used as an alternate approach to digest protein domains present on the cell surface after incubation with aptamer library. Thus, digestion of protein domains would render loss of cognate epitopes needed for aptamer binding. After proteinase K treatment and washing of the cells, the aptamers non-specifically bound to the cell surface proteins would be removed, and only those which have internalized the cells can be obtained, as desired. Proteinase K treatment can be used for DNA and RNA aptamers both, unlike RNase treatment used by Levy et al. [68] which is applicable only for RNA aptamers. Iaboni et al. used this method to select cell-internalizing aptamers against insulin receptors on human glioma cells [69].

7. Strategies for endosomal escape of cargoes

The therapeutic efficiency of a cell-internalizing platform depends on its ability to escape from endosomes and deliver the therapeutic cargo to the cytoplasm. The efficiency of endosomal escape, which is 0.01% for traditional delivery systems, should be increased significantly to achieve higher therapeutic efficacy [70]. Two types of strategies are commonly employed to facilitate endosomal escape. First method uses endosomal destabilizing agents that alter the permeability of endosomal membranes, e.g., chloroquine. However, the limitation of this method is that these endosomolytic agents may reach lysosomes, thus, lysing them and lead to cytotoxicity due to the release of lysosomal lytic enzymes. Second method, which is found to be non-toxic in several studies, uses engineered viral or bacterial cell penetrating peptides (CPPs) (e.g., truncated HIV-1 TAT peptide or Aurein1,2) [71–73]. However, CPPs can increase the escape of cargoes from endosomes to the cytoplasm by only 5- to 8-fold, which is not enough to generate an effective platform for clinical applications [70]. Below we will discuss the strategies that could be used with aptamers to enhance their endosomal escape properties or that of the attached cargo.

Any targeted delivery platform for internalization of a cargo to cells is endocytosed by receptor-mediated endocytosis (RME) to a large extent [74], and aptamers fall in this category. The endocytosed cargo first reaches early endosomes, from where it is either sent back to cell surface (recycling endosomes) or sent to late endosomes, and finally lysosomes. The cargo may be degraded by hydrolytic enzymes in lysosomes. Therefore, if a cargo enters an endosome that is destined for lysosomes, it has a short period of time within which it has to escape to the cytosol to prevent its degradation. Different strategies have been developed that can be used in conjugation with aptamers, which may help in their endosomal escape. Endosomal sorting complex required for transport-I (ESCRT-I) is a protein complex that plays an important role in maturation and trafficking of endosomal cargoes. Wagenaar et al. silenced two components of ESCRT-1 (i.e., TSG101 and VPS28) to induce endosomal escape of delivered anti-miRNA *in vitro* and *in vivo*, both, and resulted in tumor regression [75].

Nieman-Pick type C1 (NPC1) is a late endosomal/lysosomal membrane protein required for extracellular recycling of endosomal contents. A small molecule inhibitor of NPC1 protein, termed NP3.47, was earlier used to delay maturation of non-recycling endosomes [76]. The Study found that NP3.47 caused 3-fold higher accumulation of lipid nanoparticle (LNP) formulations of siRNA, i.e., LNP-siRNA as compared to the controls in late endosomes/lysosomes in a variety of cell lines causing 4-fold higher gene silencing. Authors proposed that trapping LNP-siRNA in late endosomes extended the window of time for a more efficient escape into the cytosol to facilitate siRNA induced gene silencing [76].

pH-dependent cargo release is also an effective way for endosomal escape. Anti-PSMA aptamer-siRNA chimera was conjugated to a pH-dependent polyhistidine domain *via* dsRNA binding domain (dsRBD). In the acidic compartment of a mature endosome (pH ≤ 6), Histidine becomes protonated and facilitates osmotic swelling that leads to cargo release into the cytosol, a mechanism known as the proton sponge effect [77]. By this mechanism, the polyhistidine tag facilitated the escape of anti-PSMA aptamer-siRNA chimera from late endosome to cytosol resulting in improved silencing of the target gene [78].

Another straightforward strategy for cytoplasmic delivery is to perform aptamer selection against a cell surface target protein that internalizes to other compartments by receptor-mediated endocytosis. RME facilitates more rapid internalization of the targeting moiety as compared to untargeted complexes. Depending on

the intracellular receptor-dependent or independent trafficking path, the fate of targeting moiety can be controlled, e.g., macromolecules internalized by clathrin-dependent RME are mostly destined for lysosomal degradation whereas clathrin-independent internalization leads to endosomal accumulation and sorting to a non-degradative path [74]. However, this method can be used against those targets that have previously been characterized, e.g., nucleolin. Nucleolin is a nuclear ribonucleoprotein that is overexpressed in cancer cells where it shuttles among the cell-surface, cytoplasm and the nucleus. AS1411 aptamer, a G-quadruplex forming oligonucleotide, specifically binds nucleolin and displays antiproliferative activity against cancer cells [79]. However, there are conflicting reports regarding nucleolin-mediated uptake of AS1411 since one study suggest that it is nucleolin dependent and the other suggests that it is nucleolin independent. Though the mechanism of escape of AS1411 from endosomes to cytoplasm is unclear, it is likely that its interaction with nucleolin, which shuttles between different cellular compartments, plays a role in it [80].

8. Quality of known aptamers

Aptamers have generated excitement in the scientific community in the last 2 decades due to their unique characteristics that helped them gain popularity for use in therapeutic purposes. They have been used as both agonists and antagonists targeting cell surface receptors [34]. The Ability of aptamers to specifically bind the cell surface targets catapulted them into the field of targeted delivery of therapeutics. They can be easily conjugated with small molecule drugs, liposomes, proteins, siRNAs, miRNAs, or other nanoparticles. However, when industry leaders in the field of siRNA/miRNA delivery tried to recapitulate the results of siRNA delivery, they failed [81]. Also, since the approval of Macugen, in 2004, by Food and Drug Administration (FDA), no other aptamer-based drug has made to the market. Failure of Fovista (anti-PDGF aptamer to prevent age-related macular degeneration) and Reg1 (an antifactor IXa aptamer paired with its complementary reversal agent, reverseran, to restore blood clotting) failed in phase III clinical trials due to no improvement over current standard care methods has also been disappointing [81]. However, Reg1 also resulted in serious allergies in patients, albeit it was due to conjugated PEG, and not due to the aptamer itself. Though failure of drugs in clinical trials is quite common as many more drugs fail during trials than pass them, but more disappointingly, there is a lack of aptamer-based drugs that are even advancing to the clinic [5]. Unlike antibodies and antibody-based drugs which are supported by big pharmaceutical companies, aptamer development has been largely relegated to academic labs that are often, comparatively, poorly funded.

Levy group observed lack of reproducibility in aptamer binding to its target or therapeutic effects in several cases [81]. This unreliability among novel molecules is a bad precedent that may generate a lack of confidence in an upcoming technology. The poor reproducibility, or robustness, of these aptamers might be due to poor downstream characterizations, which is, in fact, very important for validation of novel aptamers. There is also no standard way of assessing aptamer binding results, as different labs use a variety of methods to assess the binding function of their selected molecules. Hence, there is no straightforward way to compare or validate results between different labs. Other labs often cite the work from previous labs without revalidating the aptamer that they intend to use. Lack of funds, resources or time are often the reason nor not revalidating the aptamers for their own purposes; however, if the aptamer field has to grow, a bit more care and diligence will be needed from the labs that are developing aptamers as well as the end user.

Here, we briefly discuss the validations and controls that should be used to ensure a more reliable selection of aptamers for targeted delivery. Soluble proteins or peptides are often used as targets for aptamer selection, and their binding affinity or specificity is confirmed by binding assays. However, further validation using a negative control cell line or a knockout cell line is quite important for such aptamers since aptamers may be selected against an epitope that is buried in the plasma membrane or it might be located in the cytoplasmic region.

Varieties of assays are performed to study aptamer binding to target cells, e.g., radioactively or fluorescently labeled aptamers. However, limitation of these assays is that they are performed on bulk cells, hence, it is not known whether the observed signal is due to interaction with the entire cell population, or only a subset of cells. Confocal imaging is often used as a qualitative method to confirm internalization of aptamers, or localization in a particular organelle. Quantification of the signal and statistical methods are often not performed. Since a very small number of cells are typically interrogated, statistical analysis is not performed [81]. The low throughput of imaging, i.e., low cell counting in confocal imaging is usually due to strong lasers that may bleach the fluorescent labeled aptamers. However, with the advent of LED-based high content imaging systems, quantitative analysis in a high-throughput fashion can be easily performed. It is recommended that aptamers should be conjugated to high-quality fluorophores in these studies, i.e., Alexa Fluor dyes or quantum dots instead of traditionally used FITC or TRITC, as these are less resistant to photobleaching. Flow cytometry is also highly recommended to study aptamer internalization due to its high sensitivity, and its ability to interrogate each cell in a population individually.

It is advisable to limit the incubation time with aptamers during cell-internalization SELEX, typically up to ~1 hour. Incubation with cells for an extended period of time may lead to significant levels of non-specific uptake. It is also recommended to use blocking agents to prevent non-specific binding of aptamers to cell surface. Aptamers are polyanions, therefore, they can readily interact with cell surface moieties due to charge-charge interactions, especially at high concentrations (>1 μM). A Nonspecific polyanionic competitor like ssDNA or tRNA can be used successfully to significantly inhibit non-specific aptamer binding to cells [81]. As an alternative, dextran sulfate may also be used for the same purpose [5].

9. Automation of SELEX protocol

Aptamer selection is a repetitive, time-consuming and a laborious process. The immense number of PCR reactions during preparative PCR step for the enrichment of selected aptamers is also a monotonous process which might lead to manual errors. Various tasks in molecular biology such as DNA sequencing, plasmid preparation, and microarray construction have been automated [82, 83]. The automation of these processes has substantially increased the throughput of sample preparation or assays and significantly reduced the amount of time a researcher must spend to perform purely repetitive mechanical tasks.

Automation reduces variability during the selection process, leading to better reproducibility of results. Depending on the degree of automation, manpower, time and consumables can be reduced, whereas throughput can be increased by parallelization. The extent of lab automation depends on scope and timeline of the project, and the allocated budget [84]. In principle, there are two concepts of automation, *viz.*, full automation and unit-automation. A full automation pipeline is the one where all steps of the process or assay are performed without any human intervention [85]. In unit automation, human intervention is required in certain

stages and only individual stages in a pipeline are partially automated, independent of each other [86]. The advantage of unit-automation is that it keeps a highly open architecture of the pipeline allowing individual modules to be easily modified or exchanged; hence, the pipeline can be easily extended or modified [87].

Unlike the discovery of small molecule-based drugs in industry, where automation is a norm, development of novel aptamers has happened in individual labs in an academic research setting. For truly industrializing the aptamer discovery process, it is imperative to develop high-throughput strategies for aptamer development at different levels, *viz.*, aptamer selection process, sequencing, and characterization of aptamers involving affinity and specificity studies toward its target. Here we discuss about the high-throughput technologies which have been developed facilitating high-throughput selection of aptamers.

The first attempt to automate aptamer selection process was performed by the group of Ellington [82] followed by some other improvements [88–92]. It brought down the selection time from weeks or months to days. Automation of the SELEX process increased the throughput of selections, but shifted the bottleneck within a selection pipeline toward the identification and evaluation side.

In 2011 the group of T.M. Soh developed a Quantitative Parallel Aptamer Selection System (QPASS), which integrates microfluidic aptamer selection (M-SELEX) and next-generation sequencing (NGS) with in situ synthesized aptamer arrays, enabling simultaneous measurement of affinity and specificity of several 1000 candidate aptamers in parallel. Major advantage of this high-throughput platform is that it automated the characterization, i.e., finding the affinity and specificity of aptamers which is a major bottleneck in aptamer selection, since traditionally the affinity and specificity of aptamers is measured individually in a serial manner [93]. This group used angiopoietin-2 (Ang2) as a target and performed four rounds of M-SELEX followed by next-generation sequencing to obtain enriched aptamer sequences. Next, using aptamer arrays they simultaneously measured dissociation constant (K_d) of ~1000 aptamer candidates in parallel, identifying six high-affinity Ang2 aptamers with Kd <30 nM.

Development of NGS platform in the last decade has revolutionized the aptamer selection process. The Schroeder group in 2010 made the initial attempt in this direction as they combined genomic SELEX with high-throughput sequencing for the identification of genomic aptamers [94]. The same year, Soh group (2010) integrated microfluidic SELEX with high-throughput sequencing to obtain novel aptamers against platelet-derived growth factor BB (PDGF-BB) protein in just three rounds of selection, which traditionally takes 12–20 rounds [95]. Advantage of HTS is that it can track the copy number and enrichment fold of more than 10 million individual aptamer sequences through multiple rounds of selection. This enables the identification of high-affinity aptamers without the need of fully converging the aptamer pool to a small number of sequences, which is required for cloning-based selection method. Similarly, Hoon et al. obtained high-affinity aptamers after just one round of positive selection followed by HTS [96].

10. Conclusion

Since the inception of aptamer technology, this field has significantly evolved with its foray into a wide variety of applications, including targeted delivery. Aptamers caught the attention of researchers due to their unique advantages for use in therapeutics in nanomedicine due to their small size that gives higher penetration in tissues as compared to antibodies, ease of synthesis, high specificity and affinity that is comparable with antibodies and ease of chemical modifications that allowed

easy conjugation with a variety of molecules like siRNA/miRNAs, proteins, peptides, antibody, drugs and nanoparticles. However, there have also been challenges in the application of aptamers due to their unique characteristics, *viz.*, small size that lead to quick renal filtration, serum instability and lack of diversity in aptamer library. It is important to note that each of these issues has been addressed in different possible ways. Quick renal filtration of aptamers was tackled by conjugation with high molecular weight moieties, e.g., PEG. Similarly, serum instability was dealt with introduction of modifications to the nucleic acid backbone and modifications at 5′- or 3′-end of the oligonucleotide. The diversity of aptamer library was increased by introduction of modified bases, and this lead to the development of mutant polymerases which were capable of amplifying these unnatural aptamers. Endosomal escape of cell-internalizing aptamers is a very important issue that needs to be addressed for clinical translation. Conjugation of aptamer with endosome escaping peptides will facilitate this issue. But lysosomal localization of aptamers is not always a problem, since lysosomal delivery of proteins or drugs is an important aspect of treating lysosomal storage diseases.

Efforts to up the speed of aptamer selection for industrializing this technology were achieved by development of high-throughput SELEX, and CE-SELEX which brought down the duration of selection process from months or weeks to just a few days. Additionally, the development of next-generation sequencing platform has been a boon for aptamer selection since it allowed assay of millions of aptamer sequences at one go, and alleviated the need to perform several rounds of selection that was earlier a norm. It significantly reduced the amount of time and resources that went in the selection of aptamers. From the point of selectionologists, it is quite important to characterize aptamers in the best possible way, i.e., keep right controls and perform vigorous validation experiments to make sure that aptamers coming out of a lab are robust, and work in hands of different people. Development of working reliable aptamers will pave their way for targeted delivery of therapeutics.

Macugen was the first FDA approved aptamer drug that appeared in market for clinical use in 2005. No other aptamer drug has entered the market since then. Several aptamers underwent clinical trials in the last 2 decades as listed in earlier reviews [5, 97, 98]. However, many of them have failed [5, 98]. In many cases, the failure in clinical trials is not due to lack of aptamer specificity or affinity to the target, but because they were unable to provide better treatment over the existing state-of-the-art treatment regimens. However, it is a common phenomenon that more drugs fail during clinical trails than pass it. In the past 2 decades the advances described in this chapter have facilitated in the development of stabilized aptamers that have entered clinical trials. In future, application of aptamers in targeted delivery and therapeutics is only poised to increase due to the establishment of ancillary technologies that facilitate faster and more stable aptamer development. Due to this, a number of aptamers are entering clinical trials. In summary, aptamer-mediated targeted delivery remains a work in progress; however, with bright results at *in vivo* level and several aptamer drugs in clinical trials, the coming decade will determine their efficiency at the clinical level.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Chetan Chandola and Muniasamy Neerathilingam*
Department of Lipid Science, CSIR-Central Food Technological Research Institute, Mysore, India

*Address all correspondence to: mneerathilingam@gmail.com

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