Journal of Genetic Engineering and Biotechnology 22 (2024) 100400

Contents lists available at ScienceDirect

Journal of Genetic Engineering and Biotechnology

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

Current developments of SELEX technologies and prospects in the aptamer selection with clinical applications

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ARTICLE INFO

Keywords:
Aptamer
SELEX
Biosensing techniques
Biotechnology
Nucleic acids

ABSTRACT

Aptamers are single-stranded oligonucleotide sequences capable of binding to specific ligands with high affinity. In this manner, they are like antibodies but have advantages such as lower manufacturing costs, lower immunogenicity, fewer batch-to-batch differences, a longer shelf life, high tolerance to different molecular milieus, and a greater number of potential targets. Due to their special features, they have been used in drug delivery, biosensor technology, therapy, and diagnostics. The methodology that allowed its production was the "Systematic Evolution of Ligands by Exponential enrichment" (SELEX). Unfortunately, the traditional protocol is time-consuming and laborious. Therefore, numerous variants with considerable optimization steps have been developed, nonetheless, there are still challenges to achieving real applications in the clinical field. Among them, are control of *in vivo* activities, fast renal filtration, degradation by nucleases and toxicity testing. This review focuses on current technologies based on SELEX, the critical factors for successful aptamer selection, and its upcoming biomedical and biotechnological applications.

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https://doi.org/10.1016/j.jgeb.2024.100400
Received 17 April 2024; Received in revised form 28 June 2024; Accepted 12 July 2024

1. Introduction

Aptamers are oligonucleotide single-stranded sequences, both DNA and RNA, that have a mean length between 25 and 80 base pairs that fold into stable three-dimensional structures and recognize specific targets with high affinity.[28,102] The first aptamers were selected from RNA molecules in 1990 by different research groups in an independent manner.[33,123,160] Firstly, Tuerk & Gold[160] isolated two RNA sequences from a pool of 65,536 species that interacted with T4 DNA polymerase. For his part, Ellington & Szostak,[33] generated subsets of RNA from a variety of 10^{14} random sequences that bound specifically to organic dyes. Similarly, Robertson & Joyce[123] developed a method for the *in vitro* selection of RNA sequences with catalytic properties on DNA. Overall, these studies laid the foundations for the *Systematic Evolution of Ligands by Exponential Enrichment* (SELEX), which represents a universal method for aptamer selection.

Over the last few decades, significant progress has been made in the medical field.[44,116] As a result of their thermal stability, post-SELEX modifications, specificity, low immunogenicity, and wide spectrum of targets *in vivo*, aptamers have become essential components of the development of more precise, accurate, and reproducible medical toolsNi et al.[103,166] Several innovative uses of aptamers should be highlighted, including recognition of pathogenic microorganisms, biomarker detection, targeted drug delivery systems, and therapeutics for diabetes, infectious diseases, and cancers.[35,67,68,79,153] However, there are still challenges to improving technologies in pathogen detection, pharmacology, and treatments for cancer and other diseases.[76]

Despite its effectiveness, conventional SELEX is a time- and resource-intensive method. As a result, over the past three decades, several alternatives and modifications have been made to the original protocol.[166] During this period, thousands of aptamers were produced for the recognition of small metallic ions, inorganic molecules, peptides, proteins, pathogens, whole cells, and numerous targets within animals.[182] This has enabled them to be used in various fields, such as drug delivery, *in vitro* diagnostics, therapy, biomarker discovery, and biosensor technology.[28,166] This review discusses the latest tools for the optimization of SELEX technology, as well as the critical factors of aptamer selection, the challenges, and some prospects for their application in therapy and diagnostics.

2. Conventional SELEX method

In the conventional SELEX method, a synthetic library is built with ssDNA or ssRNA molecules, each containing a random central region between 20 and 100 nucleotides. These oligonucleotides are flanked by defined sequences at their 5′ and 3′ ends, providing the target for primers used in PCR amplification throughout the selection rounds. Usually, the pool consists of approximately 1 or 2 nmol, meaning 10^{15} different molecules, giving a generous amount of diverse possible structures to select the aptamers with the highest affinity for a ligand (Fig. 1A). SELEX starts with a mixture of the library and target, generating aptamer-protein complexes (Fig. 1B). Afterward, non-specific sequences are removed, and candidate aptamers are eluted for PCR amplification (Fig. 1C). Then, double-stranded amplicons are separated to obtain a new library with increasingly specific candidates at the end of each cycle (Fig. 1D). After many rounds of selection, generally 2–15, the aptamers are subjected to cloning and sequencing for their modeling and characterization (Fig. 1E).

DNA and RNA aptamers are manufactured with some differences. In the former case, after the recovery step, conventional PCR is used, while in the latter, RT-PCR is required. RNA aptamers often exhibit more diverse three-dimensional structures and stronger intra-strand RNA-RNA interactions, increasing the affinity and specificity[139], while DNA aptamers are more stable, and their production cost is lower.[182]

3. Crucial points to optimization of the aptamers selection

SELEX is a combination of different steps repeated several times (Fig. 1). Each of these contributes to the success or failure of generating a specific and high-affinity oligonucleotide.

Variables in the selection environment that affect aptamer affinity and function include target-library ratio, the size and type of aptamer candidates (DNA or RNA), annealing regions, target molecules, PCR amplification bias, sequencing method, and tools for modeling aptamer structure and predicting interaction with targets, pH, buffer components, temperature, time, and binding force. Likewise, ligand features are considered to design the experimental conditions, including net charge, isoelectric point, and hydrophilicity.[182] Before starting the aptamer generation, it is necessary to obtain a wide and deep knowledge of each step, especially in novel SELEX variants, to design the most adequate protocol possible. Thus, some breakthroughs to solve these specific drawbacks are presented.

3.1. Library design

A typical SELEX synthetic library contains large amounts of different sequences with unique structures. The higher the number of oligonucleotides, the greater the chance of selecting aptamers with

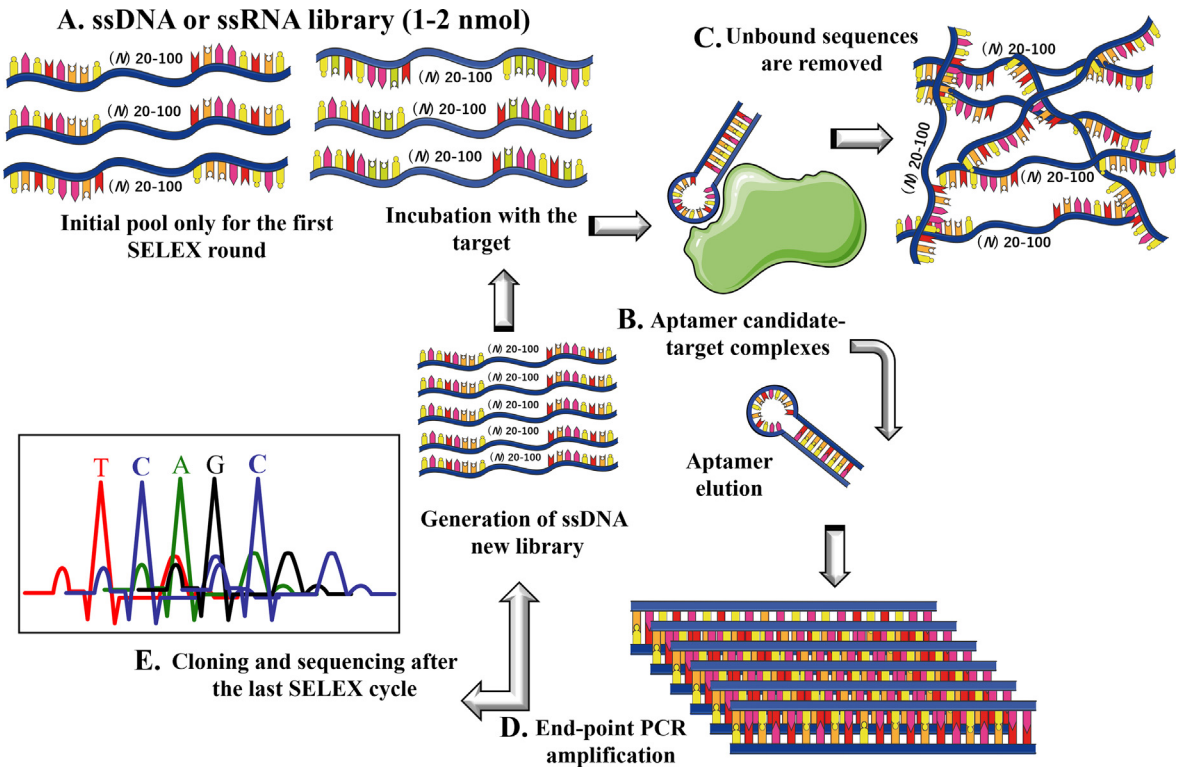

Fig. 1. Schematic representation of DNA aptamers and RNA aptamers selection through conventional SELEX methodology. A synthetic library of ssDNA or ssRNA molecules with random sequences is created (A). These molecules are mixed with the target to form aptamer-target complexes (B). Non-specific sequences are removed, and specific aptamers are isolated (C). The selected aptamers are amplified through PCR to enrich the library (D). This process is repeated in several cycles to enhance specificity (D). Finally, the aptamers are sequenced and characterized (E).

high affinity for the target. This is why libraries usually contain 10^{11}–10^{16} DNA or RNA molecules. However, as was mentioned above, *in silico* SELEX is a variant with the advantage of virtual screening of candidates before the *in vitro* synthesis, being cost-effective and eliminating time-consuming processes.

Early studies on aptamer generation used RNA libraries[33,123,160], and this preference continued for about ten years. One of the reasons was that RNA folds into intricate structures involving its 2′-hydroxyl group.[32] In the case of RNA SELEX, double-stranded DNA is transcribed to generate an initial RNA library, and reverse transcription is performed after PCR step for each SELEX cycle.[12,130] The obtained DNA is transcribed for the next round until cloning and sequencing. This process can introduce synthesis mistakes, affecting the desired specific sequence, which becomes severe with RNA sequences of around 100 nucleotides.[74] Besides, the protection of RNA from RNAases is required throughout the SELEX protocol.[181] These concerns increased the interest in DNA libraries. At the same time, strategies to improve RNA stability, such as sugar modifications, base modifications, and truncation, were also being explored.[12,42,130] As a result, various alternatives have been developed to enhance both RNA and DNA pools so that they are now widely used, achieving aptamers with similar levels of affinity and specificity.[137,150,157,177]

3.2. Aptamer-target interaction

Optimization of the SELEX process involves elucidating the mechanisms underlying the interaction between the aptamer and its target molecule. Understanding these mechanisms is crucial for enhancing the efficiency and specificity of aptamer selection. Several approaches can be employed to optimize SELEX based on these interactions:

3.2.1. Systematic variation of selection conditions

By systematically varying selection conditions such as buffer composition, pH, temperature, and incubation time, the binding affinity and specificity of aptamers can be optimized. This approach allows for the identification of optimal conditions that favor the selection of high-affinity aptamers while minimizing non-specific binding.

3.2.2. Structural characterization of aptamer-target complexes

Structural studies, such as X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, or molecular modeling, can provide insights into the binding interface and conformational changes associated with aptamer-target interactions. This structural information can guide the rational design of aptamers with improved binding properties.

3.2.3. Mutagenesis and binding site mapping

Site-directed mutagenesis coupled with binding assays can be used to identify critical residues involved in aptamer-target interactions. This information can be utilized to engineer aptamers with enhanced binding affinity or altered specificity through rational design or directed evolution approaches.

3.2.4. Kinetic analysis of binding interactions

Kinetic studies, such as surface plasmon resonance (SPR) or isothermal titration calorimetry (ITC), can provide quantitative information about the association and dissociation rates of aptamer-target complexes. Optimization of SELEX conditions based on kinetic parameters can lead to the selection of aptamers with optimal binding kinetics for specific applications.

By integrating these approaches, the optimization of SELEX can be tailored to the specific requirements of aptamer selection, leading to

the generation of high-affinity and specific aptamers for various biomedical and biotechnological applications.

4. Development of aptamers through different SELEX technologies

The conventional SELEX requires several weeks or months, and even so, the success ratio is low.[28] Consequently, alternative methods have been developed to enhance the efficiency of the selection and affinity of the aptamers obtained. Methodologies based in SELEX selection are *in silico* SELEX, Capillary electrophoresis-SELEX (CE-SELEX),[95] SELEX combined with fluorescent labels and magnetic beads (Flumag-SELEX),[145] microfluidic-based SELEX (M-SELEX),[89,119] SELEX assisted by graphene oxide (GO-SELEX),[109] real-time quantitative PCR coupled to SELEX (qPCR-SELEX),[25] and SELEX using complete living cells (Cell-SELEX).[6,39] Someones principles, advantages, and disadvantages of SELEX variants are discussed.

4.1. In silico SELEX

It has been well-documented that the classical SELEX and its variants can produce aptamers from a synthetic library of between 10^{11} and 10^{16} molecules through up to 15 cycles of selection,[28,33] Ni et al.[103] Nevertheless, computational tools have been extensively used to optimize the process, resulting in an increased affinity of the evolved aptamers via a library of enhanced candidates.[3,57,188]

The first *in silico* SELEX research was developed by Chushak & Stone,[22] who designing a complete strategy for the selection of RNA aptamers. The process implies the selection of RNA sequences based on their secondary structure; the generation of a library of 3D structures and the virtual screening of the candidates to select aptamers with binding affinity to desired targets. This methodology can reduce the *in vitro* SELEX by 4–5 orders of magnitude, accelerating the experimental steps and selection of final aptamers. Besides, *in silico* SELEX has a lower cost than *in vitro* SELEX due to no need to invest in chemicals, laboratory equipment, or technical personnel.[188] Rather than this, hardware and software, selection of suitable programs and validation of computational process are required, with times and economic costs affordable. Key features of the library design that are improved with these tools include the length of the internal random sequence, the values for randomization, chemical orientation, and affinity of the constant regions for primers annealing.[188]

Currently, many computer-based methods are used to model aptamer's 2D, 3D, and G4 quadruplex structures, as well as molecular docking for predicting optimal binding sites between the oligonucleotides and targets.[78] Both DNA and RNA aptamers can be studied using online platforms since their designing processes are similar. Among the most used *in silico* tools are including AptaSUITE,[56,57] AptaLoopEnc,[176] SMART-Aptamer,[142] AptCompare[136] and AptaNet.[34] The details of *in silico* design of both DNA and RNA aptamers through computational-based methods were studied by Lee et al.[78]

4.2. Capillary electrophoresis-based SELEX

The CE-SELEX technique was introduced by Mendonsa & Bowser in 2004, who selected an anti-IgE aptamer in only four days. In contrast, classical SELEX procedures take anywhere from 3 weeks to a month to complete. In CE-SELEX, the binding step occurs in a free solution, eliminating fixed substrate and linker biases. Using CE, sequences with target affinity are separated from those not bound by the difference in electrophoretic migration and collected as CE fractions. Thus, there is no need to carry out a washing step as in a traditional SELEX. The collected ssDNAs are enriched with PCR amplification for the next round of screening. Nevertheless, the effective resolution depends on the size of the target. For great molecules, such as proteins, the specific change in the electrophoretic migration after complex formation is easily reached. Nonetheless, the shift is not seen for small molecules, or with similar charges.[144]

In recent years, CE-SELEX has been used to develop aptamers with strong binding affinity and possible clinical applications. An example is the anti-thyroglobulin (Tg) aptamer created by Zhu et al.[184] in only two selection rounds of CE-SELEX. With further studies, this aptamer could be useful for the detection of Tg, an important thyroid cancer biomarker. Similarly, aptamers targeting proprotein convertase subtilisin/kexin 9 (PCSK9) were successfully selected after 3 rounds by Sattari et al.[131] and could be used in therapeutics for hypercholesterolemia and cardiovascular disease. In addition, several approaches of CE-SELEX have been proposed to increase the yield of this method, among others: nonequilibrium capillary electrophoresis of equilibrium mixtures, equilibrium capillary electrophoresis of equilibrium mixtures, non-SELEX, ideal-filter capillary electrophoresis, and capillary transient isotachophoresis.[45]

4.3. Fluorescence-Based SELEX

Fluorescence-based SELEX is a pivotal advancement in the field of aptamer selection, offering significant improvements in sensitivity and specificity compared to traditional SELEX methods. The use of fluorescence allows for real-time monitoring of the selection process, providing a highly sensitive means to detect and quantify aptamer-target interactions.[8] This sensitivity is particularly advantageous when dealing with low-abundance targets or when high precision is required.[146] The fluorescence-based approach enhances the ability to distinguish between specific and non-specific binding events, thereby improving the enrichment of high-affinity aptamers.[8,146] Furthermore, the integration of fluorescence facilitates high-throughput screening, enabling the rapid processing of large libraries and accelerating the overall selection process.

Despite these advantages, fluorescence-based SELEX also presents certain challenges such as the complexity and cost associated with the method.[101] The incorporation of fluorescent labels necessitates specialized equipment, such as fluorescence microscopes or flow cytometers, which can be expensive and require skilled operators. Additionally, the process of labeling targets or aptamers with fluorescent tags can introduce biases or alter the native binding properties of the molecules, potentially affecting the selection outcome. Moreover, the photobleaching of fluorescent dyes over time can lead to signal degradation, complicating the interpretation of long-term experiments.[31] Despite these challenges, the benefits of enhanced sensitivity and specificity make fluorescence-based SELEX a valuable tool in the selection of aptamers for diverse applications, ranging from diagnostics to therapeutic development.[18]

4.4. Magnetic beads-based SELEX

Another promising approach broadly used involves the combination of magnetic beads (MBs) into SELEX procedures. For MB-SELEX, the target is immobilized on the surface of a compatible MB, and the ssDNA or ssRNA library is incubated with magnetic nanoparticles ligand-coated. Then, magnetic separation (i.e., magnetic rack or bar magnet) is used to remove unbound sequences from the target-bound aptamer candidates. The desired oligonucleotides are eluted from the ligand for enrichment via PCR. As a result, a new double-stranded library is obtained and denatured by heating or alkali treatment for subsequent SELEX rounds. In contrast to agarose and sepharose beads, MBs require lower target amounts, which increases the sequence competition for the ligand, and generates high-affinity aptamers. Furthermore, magnetic separation principles are simple, low-cost, and easier to apply.[28]

4.5. SELEX combinated with fluorescent labels and magnetic beads

FluMag-SELEX is a sophisticated variant of the SELEX technique that integrates the use of fluorescent labels and magnetic beads to enhance the selection and enrichment of aptamers with high specificity and affinity for their target molecules.[145] This method leverages the advantages of fluorescence for detection and magnetic beads for efficient separation, making the SELEX process more effective and less time-consuming.[29] The process begins with the preparation of a synthetic oligonucleotide library containing a large diversity of sequences, each typically including a central random region flanked by constant regions for PCR amplification.[111] The library is then incubated with target molecules, which are often immobilized on the surface of magnetic beads such streptavidin for large protein.[91,111] Fluorescent labels can be attached either to the target or the aptamers to facilitate detection.

In the magnetic field, the beads with bound aptamers are separated from unbound sequences, ensuring that only aptamers with an affinity for the target are retained.[29] The magnetic beads are then washed to remove any non-specifically bound sequences, improving the specificity of the selected aptamers.[29] Subsequently, the bound aptamers are eluted from the target molecules on the magnetic beads, and these eluted aptamers are amplified using PCR.[91] The amplified pool serves as the input for subsequent rounds of selection.[94] Throughout the process, fluorescence detection can be used to monitor the progress of aptamer enrichment and to quantify binding efficiency.[94]

The primary advantages of FluMag-SELEX include enhanced sensitivity, as the use of fluorescent labels allows for highly sensitive detection of aptamer-target interactions, facilitating the identification of high-affinity sequences.[77] Additionally, magnetic beads enable rapid and efficient separation of bound from unbound sequences, reducing the time and labor required for the selection process.[94] The combination of multiple washing steps and fluorescence detection also helps to enhance the specificity of the selected aptamers.[147] However, the integration of fluorescent labeling and magnetic bead separation adds complexity to the SELEX process, requiring specialized equipment and expertise.[94] The use of fluorescent dyes and magnetic beads can also increase the overall cost of the SELEX procedure. Furthermore, the attachment of fluorescent labels or the use of magnetic beads might introduce biases that could affect the selection of aptamers.[94]

This methodology has been applied to the design of aptamers targeting drugs, organic pollutant, hazardous chemicals, bacteria and hormonal and pregnancy biomarkers.[64,72,90,92,148,173] These studies underscore the significant advancements that FluMag-SELEX represents in the field of aptamer selection, combining the strengths of fluorescent labeling and magnetic bead separation to achieve high sensitivity and specificity. Despite the challenges associated with this technique, its advantages make it a powerful tool in both research and clinical settings.

4.6. Cell-SELEX

In Cell-SELEX methodology, whole-live cells are used to develop aptamers. As native surface proteins are employed, is not require previous protein purification or a prior understanding of the target on the cell. Besides, aptamers obtained can be applied *in vivo* without complications or bias of using proteins produced *in vitro*, such as the lack of post-translational modifications.[187] In general, Cell-SELEX is used for selecting aptamers for identifying and discovering biomarkers on cells. Thus, this technology has mostly been used to identify cancer cell targets for biomedical purposes.[138] In that sense, it has allowed the evolution of aptamers against targets on many types of cancer, including colorectal,[81] breast,[23] gastric,[82] ovarian,[51] lung,[183] liver,[172] prostate,[124] and pancreatic cancer.[59]

Generally, Cell-SELEX protocol includes the basic steps of conventional SELEX, being binding, elution/recuperation, and enrichment. Differential steps are positive screening with cellular membrane components rather than only one target, then negative screening with non-target cells.[28] This additional step is necessary to remove DNA/RNA oligomers that bind to normal cells and enhance the specificity of candidate aptamers.[17]

Despite the great potential of Cell-SELEX, there are methodologic limitations, such as dead cells in the initial suspension leading to non-specific binding candidates, with a negative impact on selection rounds.[181] Additionally, the whole process is time-consuming and more expensive than other variants and requires a high level of technical expertise.[181] A technology termed “ligand-guided selection” (LIGS) was successfully coupled to a partially evolved Cell-SELEX library to develop aptamers against cell-surface proteins.[189] This approach is promising for producing aptamers with high affinity and therapeutic applications, as was shown by Zumrut et al.[189] who selected an aptamer that binds to the membrane of immunoglobulin M-expressing B-cell neoplasms using LIGS. Besides, Cell-SELEX linked to LIGS can be applied to evolve multiple aptamers for multiple targets expressed on a single cell.[169] Deep details of Cell-SELEX and LIGS technology have been recently reviewed.[170]

4.7. High-throughput sequencing-SELEX

While conventional SELEX needs PCR and cloning in the last round to sequence by the Sanger method, High-Throughput Sequencing (HTS) SELEX integrates massively parallel sequencing technologies to eliminate experimental biases and laborious processes. Therefore, HTS-SELEX gives the opportunity to analyze sequencing data obtained from the libraries produced during and after aptamers isolation providing a deep basis for precise aptamer identification, modeling its structure, and predicting binding sites.[75]

Frequently, the high-affinity aptamer may be missed due to errors generated during PCR. In consequence, the enriched sequences at the final amplification cycle could be different or not necessarily the best candidate aptamers.[28] Besides, the limitations of insufficient clone numbers and inefficient cloning cannot go unnoticed. Using HTS, also called next-generation sequencing (NGS), millions of sequences can be read in one experiment, reducing clone and PCR bias. Additionally, HTS-SELEX allows the detection of changes within the pool of sequences throughout the selection process. Thus, high-affinity aptamers are identified much earlier, in other words, in fewer rounds of SELEX.[28,187]

The first work of HTS-SELEX was reported by Cho et al.[21] using microfluidic selection and HTS to discover aptamers recognizing the platelet-derived growth factor-BB protein. After three rounds, they selected aptamers with approximately 3–8-fold higher affinity and 2–4 higher specificities compared to oligonucleotides developed through conventional SELEX.[21] In recent years, HTS-SELEX has been used to develop aptamers mainly targeting cancer biomarkers, proteins, bacteria, and ligands that induce apoptosis.[75,132,148,185]

4.8. Biolayer interferometry SELEX

Several studies have used Biolayer interferometry (BLI) in combination with SELEX to examine aptamer affinity and binding properties.[41,66,84,88] For this purpose, a Dip-and-Read approach is implemented, in which aptamer coated-biosensor are submerge into microplate wells.[88] BLI detection is based on an optical analytical technique sensitive to interferences between waves of light (interferometry) by using two surfaces or layers. The mechanism of detection is composed of one internal layer ubicated inside an optical biosensor and one layer that is biocompatible with aptamers at the interphase between the fiber tip and a surrounding liquid phase (outer membrane). Light is reflected towards both layers and each reflection generates interferences that oscillate in wavelength. Then, the changes are detected by means of a charge-coupled device (CCD) array.[88,113]

In a typical BLI-SELEX assay, the tip of the biosensor is dipped into a 96-well microplate that contains a target molecule, which binds to immobilized candidate aptamers on the biosensor surface depending on the affinity, and consequently, generating interferences patterns being detected in real-time.[88,113] Finally, the monitoring of these patterns provides information regarding kinetic data and molecular interactions of proteins and aptamers. This novel approach has been widely applied in aptasensors and has advantages over other SELEX variants such as label-free, simple principles of detection, rapid measurement, and real-time sensitive lectures.[66,113] Among targets used to develop aptamers through BLI-SELEX are found toxins, recombinant human proteins, disease biomarkers and environmental pollutants.[41,52,66,84,108,118]

4.9. Droplet digital PCR-SELEX

Endpoint PCR could hinder the SELEX process due to the formation of heteroduplexes hybridized in the common flanking sequences, PCR products improperly extended, and the accumulation of aberrant non-desired sequences in the library during the screening rounds.[5,107] In response to the need to enhance the PCR enrichment of candidate aptamers, digital PCR and droplet digital PCR (ddPCR) have been integrated into the SELEX protocols.[38,48] The library diversity is preserved, and the loss of highly structural aptamer sequences is avoided with the application of these methods. In this way, PCR bias and selection failure are eliminated from the process, increasing overall SELEX efficiency and candidate aptamer efficacy.[5,48]

4.9.1. High-fidelity aptamer selection

ddPCR-SELEX has led to the development of techniques such as the high-fidelity aptamer selection, Hi-Fi-SELEX, which introduces fixed-region blocking elements to improve the functional heterogeneity in the starting library.[5,107] Then, the partitioning used in ddPCR allows for ultra-high-fidelity amplification of a selected pool of sequences, ensuring that each amplicon exists as a fully complementary duplex.[5]

Furthermore, aptamers have speflf-life, no batch-to-bath variation, low or even no immunogenicity, reversible folding features, lower cost and shorter manufacturing time, *in vitro* synthesis, chemical stability, and the flexibility to incorporate modifications that improve their tolerance of nuclease degradation, serum half-life and binding target stability.[182,103,166,187]

4.10. CRISPR-SELEX

The CRISPR-Cas system, renowned for its precise gene-editing capabilities, has been adapted for aptamer development, enhancing the specificity, efficiency, and high-throughput potential of traditional SELEX techniques.[125] By leveraging the RNA-guided DNA targeting ability of Cas proteins, particularly Cas9, CRISPR-Cas allows for the programmable targeting of specific DNA sequences.[65,125] Guide RNAs (gRNAs) direct the Cas proteins to these sequences, facilitating the isolation and enrichment of aptamer sequences through iterative rounds of selection and amplification.[65]

A notable application of this integration is in the development of aptamers targeting pathogenic entities. The CRISPR-Cas system has been employed to identify aptamers that can bind and inhibit viral proteins, presenting a novel approach to antiviral therapy.[186] This method significantly enhances the efficiency and precision of aptamer development, reducing the time required by traditional SELEX procedures and increasing the likelihood of identifying high-affinity aptamers.[186]

Moreover, the CRISPR-Cas system has been incorporated into the creation of aptamer-based biosensors for pathogen detection.[126,186] These biosensors combine the high specificity of aptamers with the programmability of CRISPR-Cas, enabling the detection of a wide range of pathogens with remarkable sensitivity and specificity.[125] They are designed to recognize specific DNA or RNA sequences from pathogens, triggering a detectable signal upon binding. This innovation is particularly impactful for rapid and accurate pathogen detection in clinical diagnostics, food safety, and environmental monitoring.

5. Aptamers with medical applications

Several aptamers have been designed and evaluated for monitoring viral and bacterial infections, and cancer types.

5.1. Aptamers applied in the microorganism detection and pathogen inhibition

Similarly, a DNA aptamer was designed as a screening method for human immunoglobulin G (IgG) Fc fragments, besides the fact that several aptamers could be used for bacteria, parasite and virus detection, and thus as potential diagnostic tools (Table 1).[40,71,80,175] Although they are promising, there are still several crucial points that need to be addressed.

5.1.1. Aptamers targeting bacteria and parasites

Aptamers can detect bacteria and bacterial toxins.[35,121] In the case of bacterial species, early identification of important foodborne pathogens such as *Salmonella enterica* has been at the center of the latest research. An aptamer binding to SipA protein from *Salmonella* Enteritidis has shown inhibition of biofilms formation up to 26.81 %.[135] Likewise, a DNA aptamer that targets *Salmonella* Typhimurium was developed to detect 10^2 CFU/mL from agar plates and 10^3 CFU/mL for samples artificially contaminated.[104] Additionally, aptamers were used to detect and decrease the growth levels of both *Salmonella* Enteritidis and *Salmonella* Typhimurium *in vitro*.[47] Other recent studies have successfully detected *Salmonella* Typhimurium using Cell-SELEX[46] and aptamer-based bioluminescent approaches.[30,163]

In addition, different aptasensors were successfully used to detect methicillin-resistant *Staphylococcus aureus* and alpha-toxin from *S. aureus* in human samples, described as simple, rapid, cost-effective, and reliable.[114,167] Comparably, an important advance in bacteria inhibitors was achieved by means of DNA aptamer against intracellular protein DevR from *Mycobacterium tuberculosis*, showing disruption in the function of the cytosolic bacterial response regulator.[15] Besides, DNA and RNA aptamers were used to detect the LipL32 protein from *Leptospira*, which represents a possible marker for leptospirosis diagnosis.[137,177] It is necessary to highlight a recent work in which an aptamer-based enzyme-linked oligonucleotide assay was developed for sensitive and specific detection of the principal etiologic agents of bacterial meningitis.[115]

On the other hand, aptamer-coupled recognition methods have been developed with a high rate of success for important protozoan parasites, as well as *Trypanosoma cruzi*,[99] *Trichomonas vaginalis*,[36] *Leishmania infantum*,[40] *Cryptosporidium parvum*,[49] *Plasmodium falciparum*,[87] and *Toxoplasma gondii*.[24] A deeper review of the current advances in aptamers as a diagnostic tool for human parasites was recently published by Brosseau et al.[13]

5.1.2. Detection of mycotoxins and fungal cell components

Fungus-contaminated food can cause disease and death in both humans and animals. Therefore, the potential application of aptamers as recognition elements for fungal pathogens has been explored, focusing on their characteristic secondary metabolites produced as propagation mechanisms and cell wall components. The major mycotoxin in nature, aflatoxin B1, is the principal target studied in recent studies, including the development of label-free aptamer assays[7,62,174] and colorimetric and fluorometric aptasensors.[20,168]

Further, BLI-SELEX was performed to evolve an aptamer that targets a non-proteic toxin, the patulin produced by mold species, which

Table 1
Representative aptamers for detection of human pathogens and control of infectious diseases.

Pathogen	Target	Aptamer type	SELEX method	Binding Affinity (Kd)	Reference
Pan-coronavirus	N protein	DNA	Magnetic beads-based SELEX	1.31–135.36 nM	196
SARS-CoV-2	RBD	DNA	Magnetic beads-based SELEX	7 nM	86
Ebola Virus	GP protein and NP protein	DNA	FluMag-SELEX	4.1 nM and 41.3 nM	197
Zika Virus	Envelope protein	DNA	Magnetic beads-based SELEX	106.9 nM	198
Avian Influenza Virus Subtype H5N1	Whole Virus	DNA	Membrane Filtration Column-SELEX	70 nM	(Kim et al., 2020)
Dengue virus serotype 2	NS1 protein	RNA	ELISA plate-based SELEX	37.57 nM	(Thevendran et al., 2023)
Hepatitis B	E antigen	DNA	*In silico*-SELEX followed by qPCR and ELISA validation	0.4 nM	(Liu et al., 2020)
HIV	HIV-1 Reverse Transcriptase	DNA	Capillary electrophoresis-SELEX	75.10 nM	(Ratanabunyong et al., 2021)
E. coli O157:H7	Whole bacteria	DNA	Whole cell-SELEX	41 nM	(Renuka et al., 2018)
Salmonella Typhimurium	Outer membrane usher protein FimD	DNA	Counter-SELEX/Cell- SELEX	76 nM	104
Staphylococcus aureus	Alpha-Toxin	DNA	Counter-SELEX	93.7	(Hong et al., 2015); 114
Pathogenic *Leptospira* spp.	Leptospiral major outer membrane protein LipL32	DNA	Magnetic beads-based SELEX	36.3–43.6 nM	(Hsu et al., 2024)
Pathogenic *Leptospira* spp.	Leptospiral major outer membrane protein LipL32	RNA	Hybrid SELEX	54.45 nM	137
Leishmania infantum	LiH3 histone	DNA	SELEX/ELONA	0.52 nM	40
Toxplasma gondii	SAG1 Protein	DNA	Magnetic beads-based SELEX	41.57 nM	24
Plasmodium falciparum	Histidine-rich protein II	DNA	Magnetic beads-based SELEX/Counter SELEX	51.84 nM	87

resulted in aptamers with high affinity and potential uses in the screening of food samples.[98] Moreover, aptamers and *in situ* fluorescence imaging have been shown to be highly sensitive, specific, and promising tools for detecting (1,3)-β-d-glucan from fungal cell walls.[190]

5.1.3. Isolation of aptamers for virus recognition and inhibition

Smith et al. (2021) discussed the application of FluMag-SELEX for the rapid detection of viral particles, demonstrating enhanced sensitivity and specificity compared to traditional methods.

SARS-CoV-2 pandemic, rapid, specific, and reliable tests have been required for the epidemiological tracing of COVID-19. Numerous studies have performed SELEX obtaining aptamers to detect domains of the spike protein from the virus, providing DNA and RNA sequences that could be used as diagnostic agents of COVID-19 infection.[10,60,79,143] In the same way, aptamers that bind with high affinity to the receptor binding domain (RBD) protein and inhibit its interaction with the angiotensin-converting enzyme 2 (ACE2) receptors on human cells could prevent the host infection (Fig. 2A).[86,140,149] Another example involving the selection of DNA aptamers against SARS-CoV-2 is the use of biosensors, which can be used both to detect and differentiate virus variants.[16,110]

Other studies achieved the detection of human immuno-deficiency virus-1 (HIV-1),[120,122,134] hepatitis B and C virus genotypes 1–4[10,157,191,192,134], influenza A H1N1 virus,[11] avian influenza H5N2,[70] ebola virus,[155] dengue virus,[69] and zika virus (Fig. 2B). Morais et al.,[97] showing potential diagnostic and even therapeutic applications.

5.2. Aptamers for cancer cell targets

Cancer is the second-leading global cause of death.[158] Detection of biomarkers early and targeted therapy are crucial for treating the disease and increasing survival rates. In this scenario, the specificity and affinity in target recognition provided by aptamers can improve the efficiency of the diagnostic tools in clinical oncology (Table 2).

In silico SELEX has been used to cancer biomarkers.[9,116,162] Additionally, Garcia et al. (2019) reviewed the use of FluMag-SELEX in identifying novel biomarkers for cancer diagnosis, emphasizing its potential for clinical applications. Hi-Fi SELEX was applied recently to detect cancer biomarkers and could be used in the future in diagnostics and the development of biosensors.[48] Similarly, HTS and ddPCR was coupled to SELEX for RNA oligonucleotide sequences selection against cell surface targets.[152] In this respect, several aptamers have been generated to detect different types of cancer cells, including lung, breast, prostate, gastric, ovarian, pancreatic cancer, and so on.[23,51,59,82,124,183]

In lung cancer, aptamers application has been focused on treatment and therapy for the disease. An aptamer that binds to the histone acetyltransferase 1 (HAT1) enzyme may reduce cell viability, induce apoptosis, and cause cell cycle arrest in lung cancer cells.[73] Similarly, the aptamer apMNKQ2 has shown the capacity to arrest colony formation, migration, and epithelial-mesenchymal transition processes in non-small cell lung cancer cells.[14] In addition, an RNA aptamer to TGF-B1 have been used together with anti-cancer drugs enhancing the antitumor effect and to suppressing repopulation of lung cancer cell lines.[151] Likewise, Cell-Selex was used to develop a DNA aptamer that can recognize annexin A2 from the cancer stem cells, which could be a therapeutic tool for lung cancer.[171]

For breast cancer, a SELEX strategy called Exo-SELEX was presented by.[37] They used exosomes as targets for aptamer selection; these extracellular components are early biomarkers and regulators of many cancers. The study shows an RNA molecule for specific exosome detection from breast cancer cells and patient serum samples.

The aptamer PDGC21-T recognizes poorly differentiated cancer cells and tumor tissues of triple-negative breast cancer.[19] In the same work, aptamer-engineered NK cells were tested *in vitro*, inducing apoptosis in targeted cancer cells.[19] Different biosensors based on aptamers also have been developed to detect biomarkers of breast cancer.[67,85,133] Interestingly, the aptamer apMNK2F used in therapy for lung cancer, was used previously as anti-tumor drug which produces inhibition of proliferation, migration, and colony formation of breast cancer.[112] According to these studies, the same aptamer sequence could be used to target a variety of cancers in the future.

Regarding ovarian cancer, Hi-Fi SELEX was performed recently to evolve two aptamers that recognize the ovarian cancer biomarker human epididymis protein 4 in urine samples, which is overexpressed in this type of cancer.[48] Thus, aptamers AHE1 and AHE3 could be useful in diagnosing ovarian cancer by means of urine tests and biosensors. In a similar way, Cell-SELEX allowed the generation of four specific aptamers for ovarian tumors, and molecular docking was

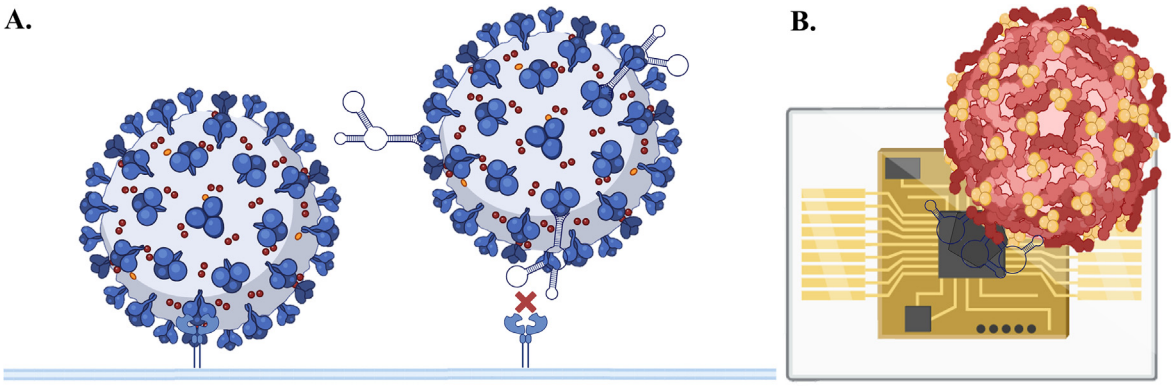

Fig. 2. Biomedical applications of aptamers in virus inhibition and detection. **A**. Inhibition of SARS-CoV-2 through the binding of an aptamer to the RBD (receptor binding domain) protein, blocking its interaction with the ACE2 receptor on human cells and preventing viral infection. **B**. Detection of the Zika virus using an aptamer-based biosensor that specifically binds to the virus, enabling rapid and accurate detection.

Table 2
Representative aptamers targeting cancer biomarkers for clinical applications.

Disease	Target	Highlights	SELEX method	Aptamer type	Binding Affinity (Kd)	Reference
Non-small cell lung cancer (NSCLC)	WT EGFR and Mutant EGFRvIII	Prevention of tumor cells proliferation, targeted therapy and drug delivery development	Magnetic beads SELEX	RNA	2,4 nM	79
NSCLC and breast cancer	Mitogen-activated protein kinase-interacting kinase 1	Inhibition of proliferation, colony formation, migration, and invasion in breast and lung cancer cells	Conventional SELEX/ELONA	DNA	1.79 nM	14,112
Lung cancer	Histone acetyltransferase 1	Reduced cell viability, induced apoptosis and cell cycle arrest, and inhibited colony formation in lung cancer cell lines	Conventional SELEX/ELONA	DNA	28.11 nM	73
Breast cancer	HER2	Internalizes rapidly into breast cancer cells overexpressing HER2 but shows no uptake in the HER2 receptor-deficient breast cancer cell line, potentially enabling selective delivery of therapeutic agents or imaging for HER2-positive breast tumors	Cell-SELEX	DNA	260 nM	193
Ovarian cancer	HE4 protein	Due to its binding affinity, it is a promising tool for application in diagnostics and the future development of urine tests or biosensors for ovarian cancer	Hi-Fi SELEX	DNA	87 nM	48
Colorectal cancer	Cell migration inducing hyaluronidase 2 (CEMIP2)	Development of an effective aptamer-based drug delivery system. Treatments with this aptamer carrying doxorubicin significantly reduced CEMIP2-mediated tumorigenesis *in vitro*	Cell-SELEX	DNA	93.80 nM	194
NSCLC, breast cancer, lung adenocarcinoma and pharyngeal squamous cell carcinoma	Oncogenic mutant p53	A specific aptamer carrying a PROTAC drug selectively degrades p53-R175H via ubiquitin–proteasome pathways while sparing wildtype p53, offering a novel strategy to address oncogenic p53 mutations in various cancer therapies	Iterative molecular docking-guided post-SELEX	DNA	290 nM	195

applied to predict and demonstrate that it is possible to identify different stages and subtypes of ovarian cancer.[1] Other works have achieved cancer cell line cytotoxicity *in vitro* and in xenograft models with aptamer-based therapeutics.[54,127] The challenges and advances in the treatment of cancer with the aptamers approach have been explained by Jin et al.[63] and Aller Pellitero et al.[4]

5.3. Aptamer-based drug delivery systems

The low yield of medicines in terms of pharmacodynamic, pharmacokinetic, tissue distribution, pharmaceutical, and pharmacotherapeutic features with conventional delivery systems has led to the development of targeted drug delivery systems (TDDS).[156] Among the ligand substances used in the current TDDS are vitamins, saccharides, bile salts, lectins, oligopeptides, antibodies, and aptamers.[44] Aptamers-based targeted drug delivery could be used for treating tumors, immunological disorders, genetic disorders, infectious diseases, and cytopathic diseases, among others.[44] Some of these concerns include the physicochemical characterization, control of *in vivo* activities, target interaction, tissue distribution and pharmacokinetics.[76]

They have been demonstrated to be particularly useful as carriers of several antitumor drugs for cancer treatment. Epirubicin has been administered via poly-aptamer nanocarriers for breast cancer *in vivo* and *in vitro*, being safe, stable, efficient, and cytotoxic for target cells.[96] Similarly, according to *in vitro* studies, doxorubicin immobilized on mesoporous nanoparticles and covalently attached to aptamers is safe and, *in vivo*, improves the antitumor effects of the drug.[50] In another example, different studies have created aptamer-conjugated systems for the delivery of doxorubicin used in colon cancer treatment and breast cancer,[58,83] so its use could be expected in the clinical management of these types of cancer soon. Furthermore, the use of aptamer-functionalized nanomaterials is proposed for targeted therapy in several types of tumors, including ovarian cancer, glioma, lung cancer,

breast cancer, liver cancer, colon cancer, pancreatic cancer, prostate cancer and oral cancer.[178]

Pegaptanib, an aptamer anti-vascular endothelial growth factor (VEGF) for the treatment of age-related macular degeneration, has received the approval of the Food and Drug Administration (FDA).[159]

On the other hand, Ommen et al.[106] tailored an aptamer-conjugated antibiotic system for local accumulation and administration of rifampicin and vancomycin. Their results indicate that aptamers facilitate the antibiotic-loaded liposome accumulation around *S. aureus* cells inside biofilms, with the subsequent elimination of the bacteria after 24 h of treatment *in vitro*. Additionally, a multifunctional aptamer-navigated particulate delivery system (DPAP) for MgO targeting diabetic cells was tested by Tan et al.[153] DPAP-MgO formulations show an encapsulation efficiency of 93.69 % and a loading capacity of 0.03 mg MgO/mg PLGA, enhancing cellular uptake and insulin resistance reversal *in vitro*.

Likewise, flow cytometry has been used in combination to digital-PCR to develop aptamers targeting drugs in a rapid, simple, and efficient aptamer selection platform.[38]

Aptamer-conjugates have also improved the delivery of antiobesity drugs. Emodin-loaded nanoparticles were coupled with DNA aptamers (Ap-EMO-NPs) with high affinity for adipocytes. Using confocal microscopy and flow cytometry, a significant increase in internalization of the medicine was observed with the Ap-EMO-NPs, as compared to emodin nanoparticles functionalized with nonspecific aptamers.[179] Finally, some studies have explored the possible use of aptamers for the oral delivery of macromolecules and as carriers of drugs for important conditions such as epilepsy, brain damage, inflammation, viral diseases, and neurodegenerative diseases.[2,53,68,180]

5.4. Aptamers and pesticides

5.4.1. Pesticides detection

Recent advancements in polymer-based biosensors have further extended the application of aptamers. For instance, polymer-based biosensors have been shown to effectively detect food contaminants like pesticide residues, helping to prevent their accumulation in humans and mitigate related health risks.[165]

Moreover, the integration of molecularly imprinted polymers (MIPs) on screen-printed electrodes (SPEs) has been explored for pesticide detection. This approach offers a simple and affordable method compared to traditional detection techniques, potentially broadening the application of aptamer-based sensors in various settings.[100] For instance, an electrochemical aptasensor based on carbon nanotubes has been used for the detection of carbendazim (CBZ) in food samples, demonstrating rapid, sensitive, and selective quantification capabilities, essential for preventing adverse health effects caused by pesticide residues.[161]

Similarly, aptamers have been employed in the development of a universal method based on layer-by-layer assembly for the detection of small molecules like glyphosate, a commonly used pesticide. This method achieved a detection limit of 0.3 μM, showcasing its potential application in food safety and environmental monitoring.[128]

5.4.2. Inhibition of adverse health effects

Aptamers role in mitigating adverse health effects is particularly significant in ensuring that agricultural products contain pesticide residues within permissible levels. Aptamers can be utilized in the detection of organophosphate pesticides, thus helping to inhibit the health effects associated with pesticide exposure.[93]

Additionally, aptasensors have been highlighted as effective tools for determining organophosphorus pesticides (OPPs) in fruits and vegetables, which is crucial for environmental safety and human health.[27]

6. Challenges in the therapeutic uses of aptamers

The limited availability of aptamers for diagnostic and biosensor applications in biomedicine remains a significant challenge. Among the reasons for this scarcity are aptamer degradation, rapid filtration by the kidneys, low control of action time, off-target activity, and the lack of a standardized global aptamer production protocol.[74,141]

The short half-life of aptamers is primarily due to degradation by nucleases and other enzymes present *in vivo*, which are not encountered during *in vitro* selection, especially in the bloodstreamNi et al.[103] To enhance the resistance and stability of aptamers in the physiological milieu of the body, post-SELEX modifications have been applied. These modifications typically involve the addition of functional groups at the 2′-position of the sugar and phosphate groups of nucleotides, such as fluorine atoms (2′-F), O-methoxy groups (2′-OMe), or amino groups (2′-NH2).[117] Additionally, large molecules like polyethylene glycol (PEG) and cholesterol can be attached to the 5′ end of aptamers.[74] However, these modifications could be counterproductive because they may alter the negative charge of the phosphate groups, reducing the stability of the G-quadruplex structures of the aptamer.[129] In the same way, additional modification of the sugar ring can be achieved by the introduction of modified nucleotides, including 2′-F-ribose, 2′-NH2-ribose, or 2′-OMe-ribose, into oligonucleotides using the mutational T7 RNA polymeraseNi et al.[103]

Other modifications can also be valuable tools to increase the target specificity of aptamers. In this regard, Tanaka et al.[154] focused on developing aptamers with high affinity and enhanced internalization through conjugation with cell membrane-interacting molecule. They demonstrated that hydrophobic modification of oligonucleotides significantly improves aptamer internalization compared to normal DNA libraries.[154] Increasing the molecular weight of aptamers has been recognized as a strategy to mitigate rapid kidney filtration. For example, PEGylation, where Polyethylene glycol (PEG) is coupled to proteins or nucleic acids, has been recently utilized to design an aptamer for treating osteogenesis imperfectaWang et al.[164] In this study, researchers employed PEG40k (PEG with a molecular weight of 40,000 Daltons) to enhance the serum stability and prolong the elimination half-life of an aptamer targeting sclerostin *in vivo*. As a result, they demonstrated that the conjugated aptamer promoted bone formation, increased bone mass, and improved bone microarchitecture integrityWang et al.[163]

Some researchers postulate that with the growing interest in SELEX against live targets such as living cells, tissue samples, or even whole organisms like bacteria or viruses, aptamers need to be developed in a physiologically appropriate environment to ensure their therapeutic success *in vivo* and subsequently in the clinic.[141] This approach highlights the advantages of Cell-SELEX, peptide SELEX, protein-SELEX, and whole organism/*in vivo*-SELEX, making it an interesting and promising direction. The strategies for modifying and conjugating aptamers with other molecules have been thoroughly reviewed by other works.[74,105,117]

Despite the remaining challenges, recent clinical trials have demonstrated promising results with aptamers. For instance, Hernández-Jiménez et al.[55] reported the first clinical use of ApTOLL, an aptamer that recognizes Toll-Like receptor 4 and improves outcomes in models of ischemic stroke and myocardial infarction. The study involved a Phase I trial with 26 healthy adult volunteers to assess the effects of ApTOLL. They found excellent safety and an adequate pharmacokinetic profile that, along with the efficacy demonstrated in nonclinical studies, provide the basis to initiate clinical trials in patients.[55] Similarly, a phase I/II dose-escalation trial combined radiotherapy with an aptamer that neutralizes CXCL12, a chemokine promoting glioblastoma recurrence post-radiotherapy.[43] The treatment was found to be well-tolerated at all tested doses, with no dose-limiting toxicities or treatment-related deaths, indicating the safety of this novel aptamer-based therapy.[43]

Noteworthy, Avacincaptad Pegol is the second RNA aptamer to receive FDA approval and is available for the treatment of geographic atrophy (GA).[61] In 2021, a phase 2/3 clinical trial involving 286 randomized participants demonstrated a significant reduction in vision loss over 12 months of treatment.[61] Besides, Danzig et al.[26] showed that Avacincaptad Pegol administration could delay the progression to persistent vision loss in a phase 3 trial over 12 months. These advancements highlight Avacincaptad Pegol as one of the most recent and promising tools in clinical applications.

7. Conclusions

Although aptamers offer numerous advantages over antibodies, they are a tool not to replace them but to explore new approaches and fields in which conventional antibodies are not effective. Both DNA aptamers and RNA aptamers have applications in various fields, particularly in pathogen detection and inhibition, drug delivery systems, and targeted therapy for conditions such as cancer, diabetes, and cardiovascular diseases. Therefore, it is crucial to consider the final application and the unique characteristics that each type of aptamer offers before initiating the selection process.

Additionally, each SELEX variant presents its own set of advantages and disadvantages, emphasizing the importance of optimizing library design, SELEX rounds, aptamer recovery, and validation of aptamer-target interactions across all variants. Also, post-SELEX modifications are essential to improve *in vivo* half-life, resistance to nuclease degradation, and avoidance of rapid kidney filtration. Finally, based on current *in vitro* and *in vivo* evidence, targeted therapy using aptamer-based systems is poised to become a robust and widely adopted strategy for treating various diseases.

CRediT authorship contribution statement

Danny Jair Chinchilla-Cárdenas: Writing – review & editing, Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Juan Sebastian Cruz-Méndez:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Julieth Michel Petano-Duque:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Ramón Ovidio García:** Conceptualization. **Lyda R. Castro:** Conceptualization. **María Jesús Lobo-Castañón:** Conceptualization. **Giovanni Orlando Cancino-Escalante:** Writing – review & editing, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Danny Jair Chinchilla Cardenas reports financial support was provided by Laboratorio de genética animal MASCOLAB S.A.S. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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