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From SELEX to the Clinic: Evolution, Engineering, Manufacturing and Clinical Translation of Therapeutic Aptamers

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ABSTRACT: With advances in selection technologies, molecular engineering, chemical modifications, and delivery approaches enhancing their therapeutic capabilities, therapeutic aptamers represent an emerging class of molecular recognition compounds. Advances in aptamer technology from conventional Systematic Evolution of Ligands by EXponential Enrichment (SELEX) to advanced SELEX platforms, including cell-SELEX, tissue-SELEX, in vivo SELEX, microfluidic SELEX, capillary electrophoresis-SELEX, and modified nucleotide SELEX, are described in this review. Aptamer identification, optimization, conjugation, engineering of structure and chemistry, and generation and characterization of therapeutic candidates are also described. Through analysis of the approved, suspended, discontinued, and current therapeutic efforts and the factors that shape them, a particular emphasis on the clinical application of aptamers is provided.Also discussed are issues related to regulation and CMC considerations, which include manufacturing consistency, stability, chemical modifications, and quality. Finally, innovative approaches to improving the effectiveness of selection process, physiological relevance, and clinical translation are reviewed, which include next generation sequencing, bioinformatics, artificial intelligence, microfluidic automation, and engineering. In summary, next generation development of therapeutic aptamers could benefit from the advancement of translational development, molecular engineering, and selection approaches.

1. INTRODUCTION

Aptamer technology's remarkable specificity in identifying molecular targets has made it a valuable tool in contemporary biotechnology and pharmaceutical sciences.[1] Advances in aptamer selection, engineering, chemical modification, and targeted delivery have coincided with the growing use of aptamers in therapeutic development, extending their potential beyond molecular identification to therapeutically significant applications.[2,3] However, the identification of appropriate target-binding sequences is the first of multiple technological and translational steps that must be completed before an aptamer therapy may be successfully developed.

Target-specific aptamer discovery continues to be primarily supported by Systematic Evolution of Ligands by EXponential Enrichment (SELEX). High-affinity oligonucleotide sequences from sizable combinatorial libraries can be iteratively enriched using SELEX by repeating cycles of target binding, partitioning, elution, amplification, and reselection.[4,5] Since its inception, SELEX has undergone significant improvement in order to address the drawbacks of traditional target selection and to enhance the effectiveness, specificity, and biological significance of aptamer production.[5,6]

Translation from SELEX-derived candidates to therapies that are clinically effective is still a significant problem, despite significant advancements in aptamer engineering and discovery. This translational gap is demonstrated by the clinical

development history of aptamers, where candidates move through various phases of development and end up in clinical programs that are approved, ongoing, finished, withdrawn, or terminated.[7,8]

Thus, this review highlights the technological to clinic approach towards aptamer therapeutics starting from the development of SELEX technology and covering aptamer selection, optimization, engineering, production, and clinical development. It also addresses the aspects behind clinical translation of aptamers and novel technologies such as high-throughput sequencing, microfluidics, bioinformatics, artificial intelligence, and aptamer engineering that can facilitate development of novel generation aptamers.

2. SELEX TECHNOLOGY: FOUNDATION OF APTAMER DISCOVERY

2.1 Principle of SELEX

In an in vitro SELection method known as SELEX (Systematic Evolution of Ligands by EXponential Enrichment), nucleic acids capable of binding a target can be discovered. The process involves rounds of selection, isolation, and amplification of the desired nucleic acids from a highly diverse oligonucleotide library.[4,5]

In the successive cycles, selection pressure is imposed in order to produce a collection of sequences that have favorable binding characteristics and from which aptamer candidates will ultimately be isolated and characterized.[9] Though modifications in the selection and amplification processes have allowed SELEX to evolve and become able to deal with complex target molecules, the underlying concept of the method remains unchanged.[4]

2.2 Construction of the Initial Oligonucleotide Library

SELEX begins with a highly diverse library of synthetic single-stranded DNA or RNA sequences containing a randomized central region flanked by constant primer-binding regions required for amplification. The randomized region provides extensive sequence and structural diversity from which target-binding molecules can be selected.[5,10] Library composition and sequence length are important because they influence the structural diversity available for target recognition and the subsequent enrichment process.[9,10]

2.3 Target Binding and Partitioning

In order to enable individual sequences to fold and interact with the target, the oligonucleotide library is incubated with the target under certain circumstances. An appropriate partitioning strategy, such as affinity-based separation, filtration, magnetic separation, or electrophoretic techniques, is then used to separate target-bound and unbound sequences.[5] Because inadequate separation can allow nonspecific sequences to stay in the chosen pool, efficient partitioning is essential.[5,10]

2.4 Elution and Amplification

The target-bound oligos are then amplified after separation in order to prepare the library for the next selection cycle. The DNA library is usually amplified through PCR, but the RNA library is amplified through RT-PCR and transcription.[4,5] This process ensures that the representation of the oligos captured in target selection is increased through amplification from one selection cycle to the other.[10]

2.5 Iterative Enrichment and Selection Stringency

This cycle continues through several rounds and each time sequences having beneficial properties of target-binding are enriched. Through the manipulation of factors such as concentration of the target, the process of incubation, washing, and competitor concentration, selection pressure can be increased, leading to the enrichment of sequences with greater binding abilities .[5,10] Selection condition optimization is very important because too much stringency will result in the loss of useful rare sequences.[10]

2.6 Counter-Selection and Negative Selection

The removal of oligonucleotides which have sequences capable of recognizing undesired elements instead of recognizing the desired target can be achieved through a process called counter-selection. To remove the unwanted sequences, the library is subjected to the non-target substances, related targets, biological environment, or selection matrix before or during positive selection.[6] This process ensures the specificity of the enriched pool and is important for differentiating similar targets.[6]

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2.7 Monitoring Enrichment and Candidate Identification

Enrichment can be measured based on the alterations seen in terms of quantity and diversity of the sequences during each iteration or based on the binding affinity of the selected pool. [4,5] Next Generation Sequencing (NGS) technique allows for deeper analysis of the sequence abundance, enrichment profile, sequence families, and conserved motifs, while conventional techniques may include cloning and Sanger sequencing following enrichment.[4] The affinity and specificity of the candidates in the enriched pool have to be confirmed experimentally.[5]

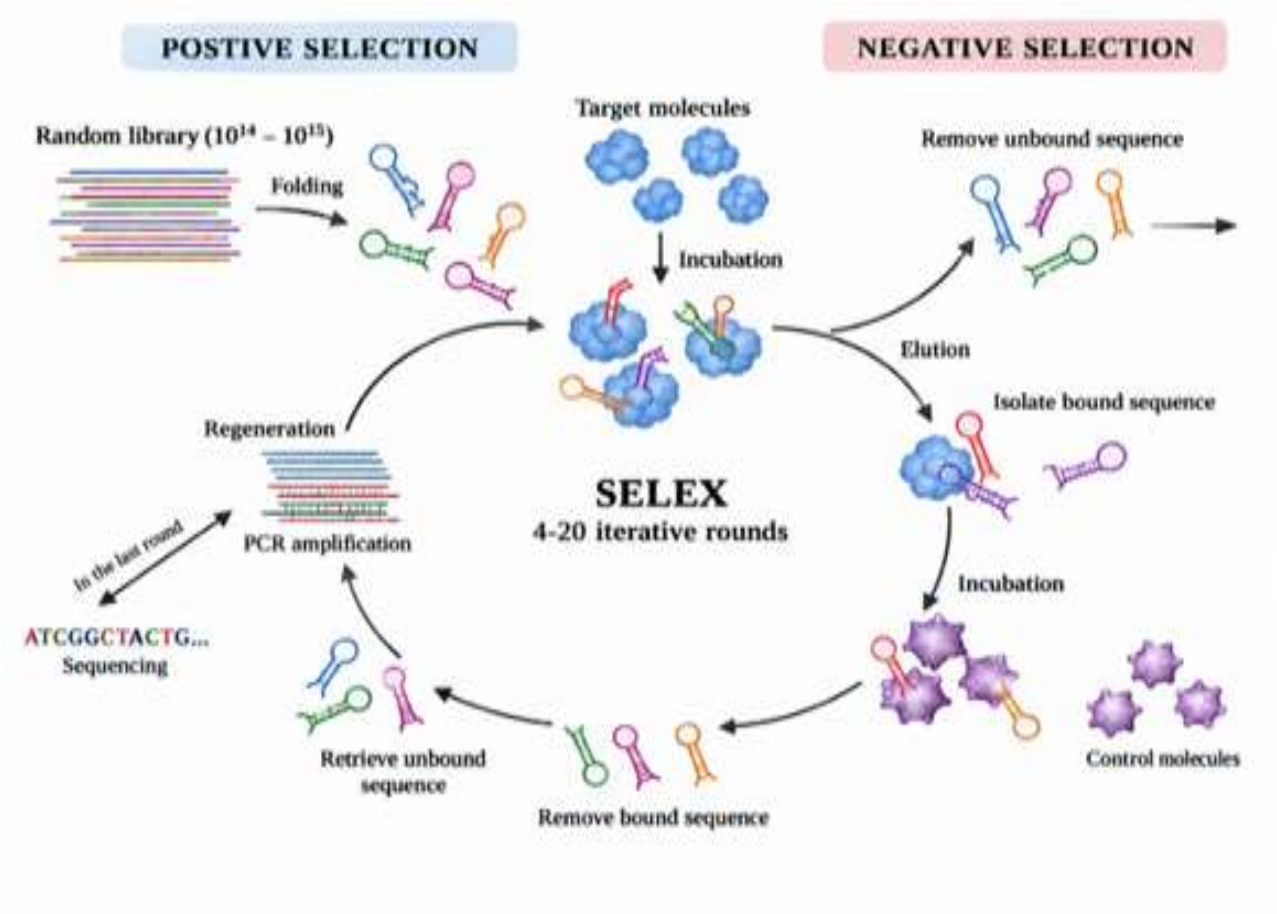

Figure 1. Schematic workflow of the SELEX process illustrating positive and negative selection for the identification and enrichment of target-specific aptamers.

3 EVOLUTION OF SELEX: FROM CONVENTIONAL SELECTION TO ADVANCED PLATFORMS

3.1 CELL SELEX

The cell-SELEX technique is an approach to the selection process, where the aptamers can recognize particular molecular properties of the cell membrane using live cells as targets, rather than just the protein molecule itself. It is because the process of selection can be performed without prior isolation and alteration of the structure of the protein molecule on the cell surface, thus making this technology suitable for membrane proteins.[6] Furthermore, the cell-SELEX does not require any knowledge of the target molecule, thus making the selection process possible for surface-specific cell markers.[6] According to some recent studies, Cell-SELEX is an effective method that can be used for the production of aptamers that can selectively bind to particular target molecules on the surface of cells. This can then be used for targeted drug delivery as well as in molecular recognition in the case of cancer patients.[11] Counter-selection or negative selection in Cell-SELEX is done using cells that do not have the target on their surface. The sequences that have a high selectivity for binding to the target are selected while the ones that bind to the non-target are eliminated.[11] Cell viability and nonspecific binding control are important considerations in Cell-SELEX since the complexity of the cell surface can lead to nonspecific binding, while dead cells can also nonspecifically bind nucleic acids.[6,11]

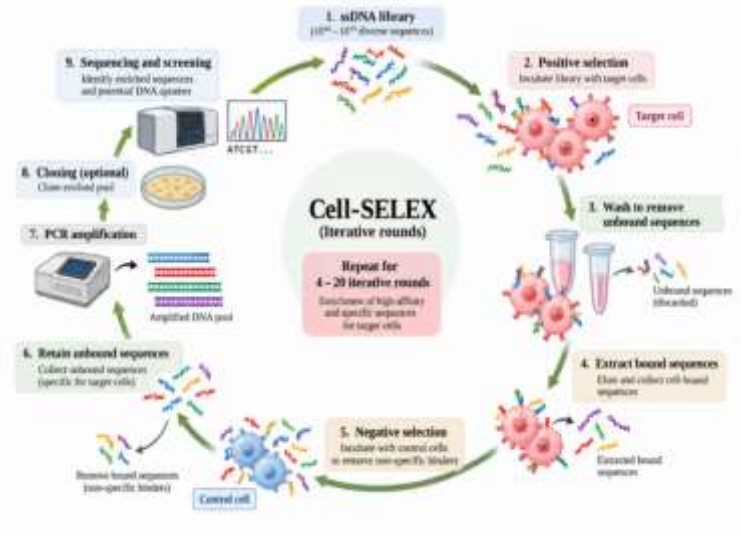

Figure 2. Cell-SELEX workflow for the identification of cell-specific DNA aptamers.

3.2 Tissue-SELEX and In Vivo SELEX

Through the use of tissues or targets associated with tissue, Tissue-SELEX enhances cell-based SELEX by allowing aptamer selection in an even more complex biological environment. This is made even more sophisticated through the use of in vivo SELEX whereby aptamer selection occurs within a living animal where the oligonucleotide pool is exposed to the physiological conditions and allows for the selection of aptamers that can access and bind to their targets within their natural biological environment.[11] Various biological components that cannot be mimicked when doing selections against purified targets or isolated cells can be included using tissues or whole organisms.[3,11] In consequence, this makes the discovery of aptamers with specific recognition properties related to their natural biological milieu an easy task, although this may be complicated due to the greater biological complexity of the system involved.[11]

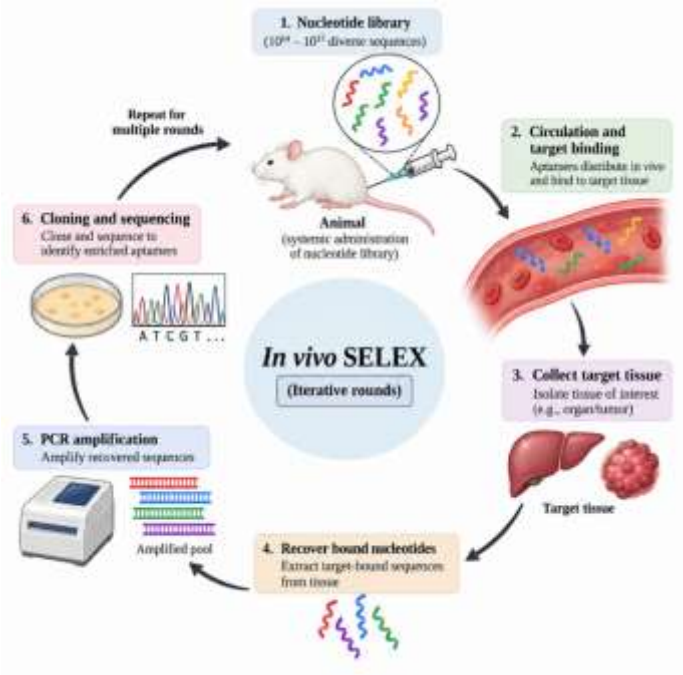

Figure 3. Schematic workflow of in vivo SELEX for the selection and enrichment of tissue-specific aptamers.

3.3 Microfluidic SELEX

The use of microfluidic technology allows for the reduction of reagent and target consumption and enhanced selection stringency through selective binding and partitioning.[12] Microfluidic devices that include electrokinetics/hydrodynamics sorting, bead-based selection, and continuous flow separation have been introduced to enhance the efficiency of each step of SELEX protocol.[12,13] Microfluidic SELEX is a combination of SELEX and microfluidic devices in order to perform the selection within the microchannel with accurate control of fluidics, sample amount, incubation time, and molecular transport.[12,13] For example, electrokinetic manipulation of nucleic acids at the time of selection can be achieved through microfluidic selection with free-solution electrophoresis providing yet another way to differentiate between the target-bound and unbound nucleic acid sequences.[13] Also, microfluidics can be used for automation or continuous SELEX and integration of selection and amplification steps reducing human intervention and simplifying the process of selection.[12]

3.4 Capillary Electrophoresis SELEX

Capillary electrophoresis-SELEX (CE-SELEX) involves the separation of target-bound oligonucleotides from unbound oligonucleotides on account of differences in the electrophoretic mobility of the molecules by employing capillary electrophoresis as the partitioning technique. As opposed to traditional partitioning techniques, CE-SELEX can result in a reduction in the number of cycles required for enrichment, as well as the successful separation of samples in very small amounts.[3] CE-SELEX may prove useful in the selection of aptamers for difficult targets where traditional partitioning methods are not feasible owing to the fact that separation is done under controlled electrophoretic conditions. This technique has been employed for proteins as well as small molecules.[3,4]

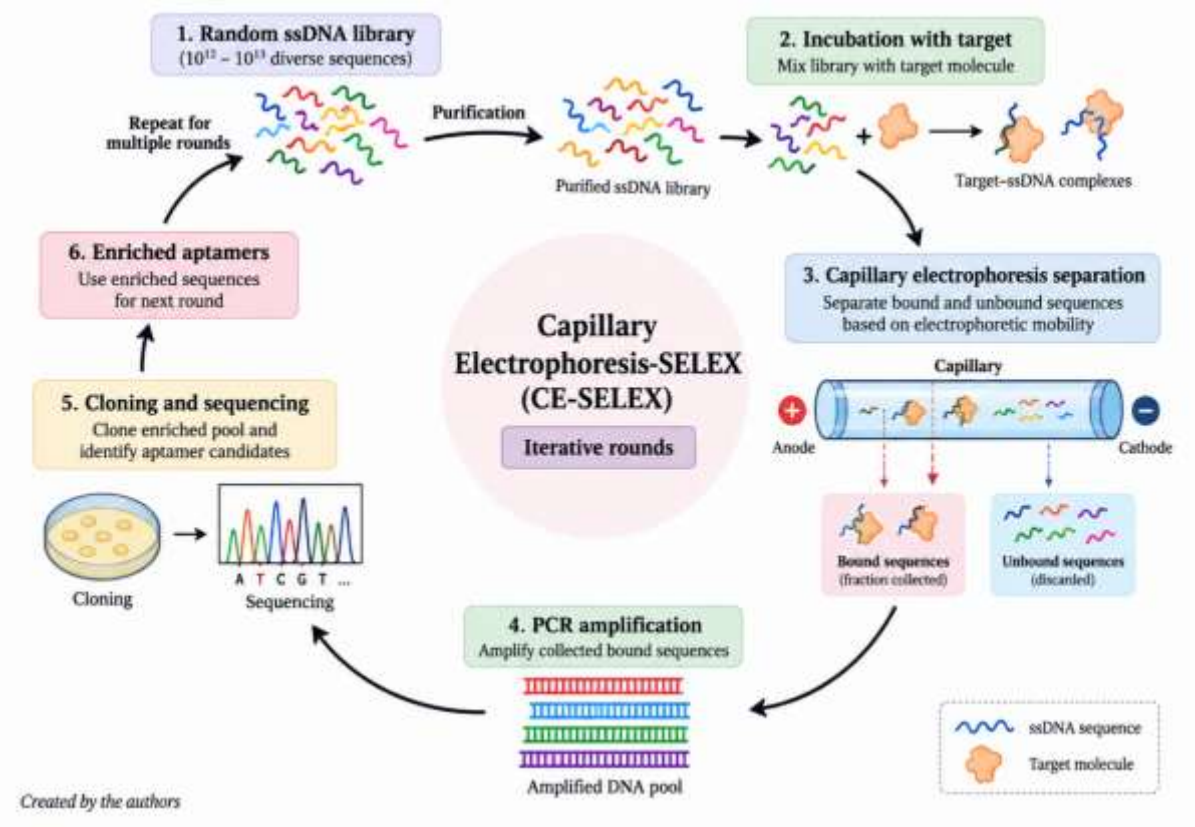

Figure 4. Schematic workflow of capillary electrophoresis-SELEX (CE-SELEX) for the selection and enrichment of target-binding aptamers.

3.5 Modified-Nucleotide and Chemical SELEX

The modified nucleotide SELEX technique adds to the chemical diversity of conventional nucleic acid libraries through the addition of chemically modified nucleotides during the selection process, and thereby provides functional groups that cannot be found on the four native nucleobases. The increase in chemical diversity provided by this procedure may enhance the ability of these molecules to interact with the target proteins and lead to the selection of aptamers for challenging targets where conventional nucleic acid libraries would fail to identify them.[3] A particular strategy would be the development of SOMAmer-like aptamers through the incorporation of modified nucleotides with protein-like functional groups that increase the physicochemical diversity of the selection pool and may improve target recognition.[3,14] It can thus be seen that chemical SELEX approaches not only help in identifying binding sequences but can also be used for incorporating desirable properties into the aptamer molecule itself.[3]

Table 1 Comparative Overview of SELEX Variants Used in Therapeutic Aptamer Discovery

SELEX type	Selection target	Principle	Major advantage	Major limitation	Application
Cell-SELEX	Whole living cells	Selection against intact target cells with counter-selection against non-target cells	Preserves native cellular target presentation and allows selection without prior target identification	Complex cellular surfaces can cause nonspecific binding; dead cells may interfere with selection	Cell targeting, biomarker discovery, targeted drug delivery
Tissue-SELEX	Intact tissues or tissue-associated targets	Selection against targets within intact tissue architecture	Provides a more physiologically relevant selection environment	Complex tissue environment can complicate selection, recovery, and sequence identification	Tissue-specific targeting and identification of tissue-associated targets
Microfluidic SELEX	Molecular targets processed within microfluidic systems	SELEX steps are performed in miniaturized channels with controlled fluid flow and separation	Low reagent consumption, precise control, rapid selection, and potential automation	Requires specialized microfluidic platforms and technical optimization	Rapid and automated aptamer selection
Capillary Electrophoresis-SELEX	Small molecules and proteins	Electrophoretic separation of target-bound and unbound sequences based on mobility differences	Efficient separation with small sample volumes and potentially fewer selection rounds	Requires specialized electrophoretic instrumentation and controlled conditions	Small-molecule and protein aptamer selection
Modified-Nucleotide/Chemical SELEX	Protein and other challenging molecular targets	Selection using chemically modified nucleotides with expanded chemical functionality	Expands chemical diversity and can enhance molecular recognition	Increased chemical and methodological complexity	High-affinity aptamer development and SOMAmer-type molecules

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4 CLINICAL TRANSLATION OF THERAPEUTIC APTAMERS

Table 2. Clinical development landscape of therapeutic aptamers: approved, discontinued, and ongoing programs

Aptamer / drug	Clinical status	Aptamer target / type	Developer	Disease / indication	Key clinical or regulatory outcome	Source
Pegaptanib (Macugen)	Approved by FDA (2004); EU marketing discontinued (2018)	RNA aptamer targeting VEGF-165	Eyetech → Pfizer	Neovascular/ wet AMD	First aptamer approved as a therapeutic; subsequent European discontinuation occurred after the changing anti-VEGF treatment landscape	FDA NDA; PMID: 15505186
Pegpleranib (Fovista)	Discontinued after Phase III	PEGylated RNA aptamer targeting PDGF	Ophthotech	Neovascular/ wet AMD	Phase III studies failed to demonstrate the intended additional clinical benefit when combined with standard anti-VEGF therapy	PMID: 27889071; PMID: 28186566
REG1 system (Pegnivacogin/Anivamersen)	Development terminated	Pegnivacogin: Factor IXa-inhibiting RNA aptamer; Anivamersen: complementary antidote oligonucleotide	Regado Biosciences	Anticoagulation during PCI	Late-stage development was terminated following serious hypersensitivity reactions; anti-PEG antibodies were implicated in the safety issue	PMID: 25882242; PMID: 27354037
AS1411	Development halted after Phase II	G-quadruplex DNA aptamer targeting nucleolin	Antisoma plc	Solid tumors; AML	Early studies showed acceptable safety, but subsequent clinical development was limited by insufficient/inconsistent efficacy	PMID: 24242861; PMID: 30093448
Emapticap pegol (NOX-E36)	Not advanced to Phase	L-RNA Spiegelmer targeting	NOXXON Pharma	Diabetic nephropathy	Phase II studies demonstrated biological activity but did not provide sufficient evidence	PMID: 28186566; PMID: 29269357

	III / not approved	CCL2/MCP-1			for progression to pivotal development	
Rondaptivon pegol (BT200)	Clinical development / Phase II	PEGylated RNA aptamer targeting the A1 domain of VWF	Freedom / Archemix / BioInvent and partners	Type 2B VWD; hemophilia A; related bleeding disorders	Phase II clinical studies have evaluated its effects on VWF/FVIII biology and clinical outcomes	Chion et al., 2024; NCT04677803
Olaptesed pegol (NOX-A12)	Phase I/II clinical development	L-RNA Spiegelmer targeting CXCL12/SDF-1α	TME Pharma (formerly NOXXON)	Glioblastoma; pancreatic cancer	Evaluated in combination treatment strategies, including approaches targeting the tumor microenvironment	NCT03168139; recent GLORIA clinical reports
AON-D21	Early clinical development	PEGylated L-aptamer targeting complement C5a	Aptarion and partners	Severe community-acquired pneumonia; inflammatory conditions	First-in-human/early clinical studies evaluating safety, pharmacology and anti-inflammatory effects	NCT05018403; NCT05343819
AM003	Phase I	Proprietary intratumoral tumor-targeting aptamer immunotherapy	Aummune	Solid tumors	First-in-human intratumoral clinical evaluation investigating local immune activation	NCT06258330; Aummune Phase I report

5 REGULATORY AND CMC CONSIDERATIONS

The development of regulatory and CMC (chemistry, manufacturing, and controls) for therapeutic aptamers necessitates product identity, sequence identity, chemical makeup, structural characteristics, and purity because any small change in synthesis and formulation can result in a different aptamer structure and target binding capability.[15,16] Critical quality attributes for aptamers not only include sequence but also encompass length variability, higher order structure, chemical modification uniformity, impurities and functional binding activity, which makes it necessary to have appropriate analysis and reproducibility in the manufacturing process.Synthesis can give reproducible aptamers, but conjugated and chemically modified aptamers add to other considerations during manufacturing and analysis.[16,17]

Another factor that needs to be taken into account in CMC and regulatory assessment is the stability of the aptamer and its pharmacokinetic properties due to the potential nuclease degradation and fast renal clearance of unmodified aptamers in vivo.[16] Chemical approaches to increase stability, such as 2'-fluoro, 2'-O-methyl, phosphorothioate, or PEG modification, can be used for the improvement of nuclease resistance and increased circulation time of aptamers, but at the same time, these modifications may affect the interaction with the target, molecular stability, and pharmacokinetics and need to be properly characterized.[18] For instance, SOMAmer molecules are constructed from chemically modified nucleotides and are synthesized

using the solid-phase DNA synthesis with subsequent purification and characterization in order to achieve reproducibility of product quality; additionally, the chemical stability reported allows them to be used analytically.[18] In terms of drug development, it is essential that manufacturing procedures and conjugation chemistry are controlled enough to provide consistent composition of the end-product, while stability studies should prove the maintenance of its chemical integrity and activity during the desired shelf life.[17,18]

6 FUTURE PERSPECTIVES

The future of the advancement of aptamer technology is likely to involve the incorporation of more sophisticated SELEX methods alongside high-throughput sequencing, bioinformatics, and computing.[12,19] Specifically, the further advancement of in vivo and combined SELEX methods will likely help create aptamers with physiological relevance through considerations such as accessibility of the targets, biological barriers, resistance to nucleases, and tissue specificity during the process of selection.[19]Combined SELEX, a process that involves in vitro enrichment followed by in vivo selection of the candidates, could be an effective method for decreasing selection time as well as the functional ability of the aptamers.[19] Concurrently, computational techniques involving bioinformatics, structural studies, crystallography, and three-dimensional modeling may enhance the predictions of aptamer-target interaction and contribute to better optimization of the sequence design.[12] Another promising area is that of developing automated platforms integrating the stages of aptamer selection, optimization, screening, and analysis.[12] Integration of SELEX in microfluidics has already proved to be a promising technique for reducing the time of selection, decreasing the amount of reagents used and minimizing the human intervention while providing highly efficient and well-controlled selection procedures.[12] Subsequent research should focus on overcoming the difficulties of technical complexity, variability of biological samples and the absence of standardization which prevents the widespread clinical application of such platforms.[12] From the molecular perspective, further advancements in areas like chemical modifications, multimerization, PEGylation, backbone engineering, and structural stabilization could help increase resistance to nucleases, pharmacokinetics, and stability. [19] The combined efforts of these advancements could pave the way for more physiological aptamers that can be used for therapeutic and diagnostic purposes.[12,19]

7 CONCLUSION

Aptamer therapeutics have seen great improvement compared to conventional SELEX approaches in terms of aptamer selection, engineering, and manufacturing. More advanced SELEX techniques like Cell-SELEX, Tissue-SELEX, in vivo SELEX, microfluidic systems, and nucleotide modifications have enhanced the ability to obtain aptamers with better recognition and physiological relevance. The history of development of aptamers for clinical use shows that just because an aptamer binds to its target does not mean that it will become a useful therapeutic agent, as aptamers face several challenges concerning efficacy, safety, pharmacokinetics, stability, manufacturing, and clinical use. Further development will require the combination of advanced SELEX approaches, computational technologies, high-throughput methods, molecular engineering, and CMC strategies throughout the discovery and development processes.

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