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Review Article

Aptamers: An *in vitro* Evolution of Therapeutic and Diagnostic Applications in Medicine

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Abstract

Aptamers are nucleic acid oligomers with distinct conformational shapes that allow binding targets with high affinity and specificity. Selective Evolution of Ligands by Exponential Enrichment (SELEX); an in vitro selection process to develop aptamers, has been invented in 1990. Despite more than 20 years have passed after its discovery, products of SELEX technology are in use in medicine. In this review we discuss why we need aptamers not only in therapeutic but also in diagnostic applications; and also critical points in SELEX technology. Finally; we present the aptamers in use and some patented aptamers awaiting approval.

Introduction

The detection, identification and quantification of molecules play an essential role in basic research as well as in clinical practice. The so called 'magic bullet', proposed by Paul Ehrlich in the beginning of 20th century, is a compound which specifically identifies, targets and kills disease causing microorganisms in the body. Accordingly, several molecules have been invented which interact with microorganisms. At the end of 20th century the same approach was applied to tumor cells to eradicate the cancer. In cancer management, however, the major problem was defining a component that would specifically recognize tumor antigens. The discovery of hybridoma techniques and monoclonal antibodies by Kohler and Milstein in 1985 has resolved this issue and transformed these antibodies into multipurpose toys with a wide range of application in molecular recognition. Monoclonal antibodies react with a single antigenic determinant; the epitope. Thereby, they possess a unique specificity and an extremely high selectivity for the epitope in comparison to polyclonal antibodies. Since their discovery; monoclonal and polyclonal antibodies are considered as promising diagnostic and therapeutic candidates in clinical and basic science applications (1-3). Nevertheless, there are certain limitations associated with antibodies. First; the species source of antibodies limits the use of them. Especially in case of indirect immuno-histochemistry staining paired antibodies should be from different source of species to prevent cross-reactions. Obviously; it is not possible to use an antibody originated from a rodent to identify an antigen on a rodent tissue. Second; the cross-reaction limits not only the effect of antibody but also its wide use in different areas. Third, its biological activity is unpredictable; thus monoclonal antibodies directed against the same epitope can bind with different affinities; can be more or less toxic, and can be more or less useful for staining or ELISA techniques (4-6).

The above mentioned limitations have directed scientists to discover alternatives to monoclonal antibodies. In 1990, specific oligonucleotides, referred to as "APTAMER", with high binding affinity against a given target protein have been identified by the screening of combinatorial oligonucleotide libraries. The term aptamer is derived from the Latin word 'aptus', meaning 'to fit' and the Greek word 'meros' meaning 'particle'. An aptamer is a DNA or RNA oligonucleotide that has

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been selected *in vitro* for specific binding to a target molecule. The process through which these molecules are isolated is called Selective Evolution of Ligands by Exponential Enrichment (SELEX). It starts with a pool of DNA oligonucleoides containing a region of randomized nucleotides (usually 30-100 nucleotides) flanked by conserved sequences that contain primer binding sites for use in the PCR (7).

The SELEX and SELEX Procedure

The identification of nucleic acid sequences with unique properties from a random pool of sequence is achieved through iterative cycles of *in vitro* selection called SELEX. The SELEX technique has been developed in the early 1990s by Larry Gold and Jack Szostak at the same time, in different laboratories (8,9).

SELEX procedure involves three basic steps (Figure 1). The first step includes the synthesis of a combinatorial nucleic acid library (DNA or RNA) with the aim of determining the sequence of that particular library. While random sequences are located in the middle, fixed sequences composed by primers design the binding sites. Oligonucleotide libraries with randomized regions of 20-80 nucleotides are commonly used at that step (10). An issue of uncertainty is about the preference of short or long random sequences. Libraries with short randomized regions are better manageable and cost-effective. Studies have shown that they are sufficient for a successful aptamer selection. On the other hand, long randomized regions, due to their greater structural complexity, are important for targets which are known not to be associated with nucleic acids. Klusmann et al. have shown that extended domains can increase the binding sites of an aptamer to the target that is important at high affinity aptamer selection (11-13).

The second step of the procedure, named as 'selection', includes the incubation of the library with the target molecule under suitable conditions for binding. This step comprises the binding of the target molecules with oligonucleotides, separation of unbound oligonucleotides and elution of bound oligonucleotides. The aim of this step is to identify the molecules with the highest affinity and specificity to the target structure. One of the crucial processes of the second step is the partitioning target binding and non-binding oligonucleotides. This process is performed either by using the affinity chromatography separation technique, in which the target is immobilized by a gel matrix, or by using magnetic beads. The latter one requires only a small amount of target and enables a very simple handling (14,15). Alternatively; cyanogens bromide activated sepharose or Starata-CleaneTM Resin (Stratagene Inc.) and reverse enzyme linked immunosorbant assay is used to separate aptamer-target complex. This process, however, is

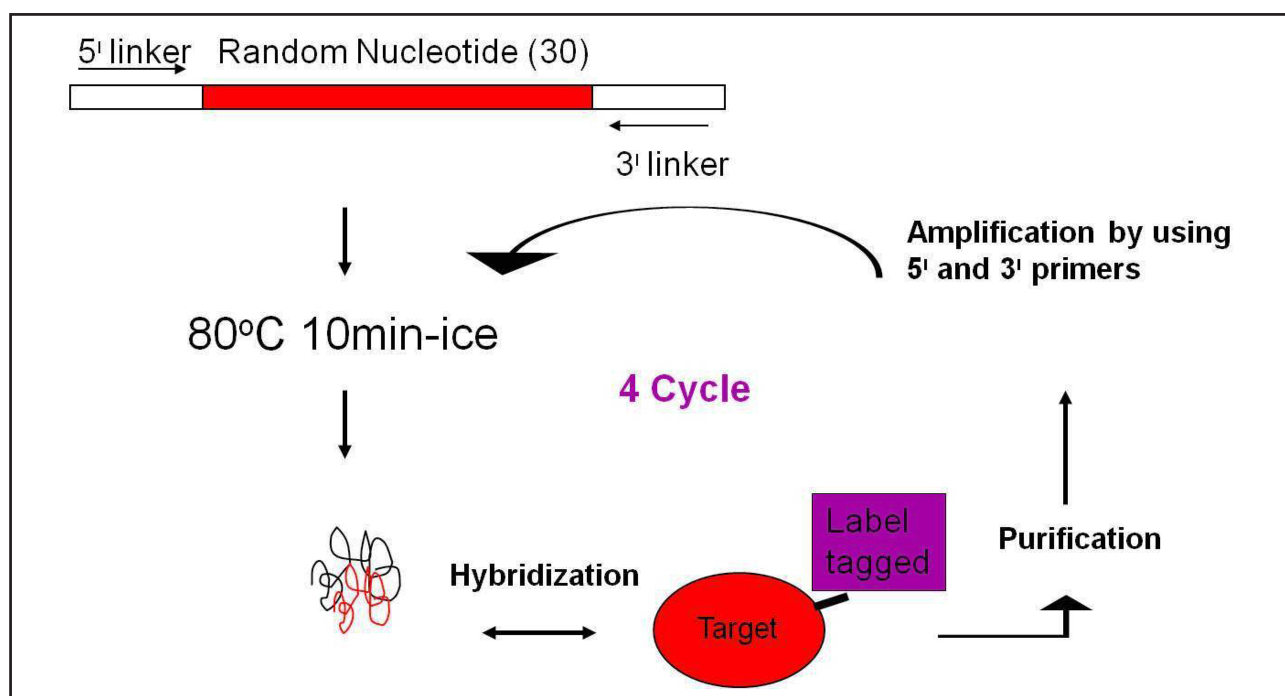

Figure 1. A basic draw of SELEX procedure (Presented in a meeting at Weizmann Institute of Science, Israel in 2003, art work performed by corresponding author).

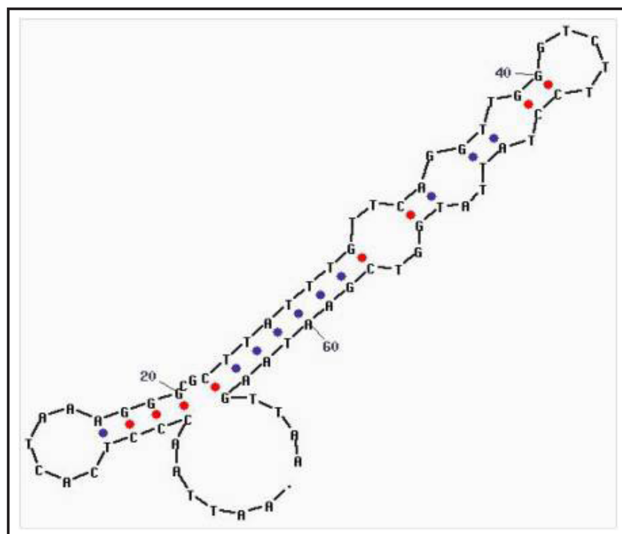

Figure 2. A computerized draw of an aptamer molecule (A21) targets influenza haemagglutinin molecule with low affinity (25).

only used for protein targets and concerning that the protein is selectively adsorbed and therefore any aptamer attached to it, whereas free aptamers do not (16-18). In most cases bound oligonucleotides are separated from the target and prepared for amplification after discarding unbound oligonucleotides, but there are examples in literature where aptamer-target complex are unseparated. Denaturing by heating or adding special substances such as urea, SDS or EDTA, is the simplest method for that purpose. (19-21). The separation of an aptamer from the target increases the efficacy of the third step named as ‘amplification’.

RNA oligonucleotides have to be passed through a reverse transcription process to achieve cDNA before amplification. Subsequently, an adequate amplification process in which one of the primers has to bear a T7 promoter for RNA polymerase at the 5’ end, can be performed. In case of ssDNA aptamers, special primers can be used to achieve modifications such as increasing functional groups since only few functional oligonucleotides are obtained during the selection step.

These three steps are repeated for several rounds to achieve high binding affinity and specificity. Prior repetition, amplified oligonucleotides should be prepared for the next cycle since at the end of amplification step enriched pool is available as dsDNA. Therefore, a transcription process with T7 RNA polymerase should be performed for RNA aptamers. In case of DNA aptamers, dsDNA with only one strand biotinylated primer bind to streptavidin surfaces on plates or beads and separated strands can be isolated by negative selection (22).

After several rounds of SELEX process the final pool is cloned into a bacterial vector and individual colonies are sequenced. At the end of all these procedures, depending on the target itself and the inflexibility of the selection, a number of different aptamers can be obtained. Although the aptamer clones are mostly within identical sequences, their binding efficacy varies, thus the affinity of those aptamers must be analyzed one by one. Eventually, the structural configuration of the selected aptamer should be examined to consider the loops on ssDNA (Figure 2).

Therapeutic Approaches

The use of aptamers as therapeutic agents was first demonstrated by Sullenger et al. in 1990 (23). Unlike antisense oligonucleotides, siRNA, miRNA or monoclonal antibodies; aptamers have several features to modulate the functions of their target proteins, the most significant one being the directly binding to target proteins and modulating their function. This may be accomplished in several ways; first the function of a protein can be inhibited by blocking or distorting the active site of that protein; second, the interaction of a protein with another one may be interrupted by an aptamer; third, a specific domain may be inhibited. The majority of aptamer therapeutics are administered in vivo by either intravenous, subcutaneous or intravitreal routes. A22; a specific aptamer for influenza haemagglutinin awaiting evidence for therapeutic application is administered via intra-nasal route (24,25). During our experience acquired in Prof.Arnon’s laboratory at Weizmann Institute of Science in Israel, we observed that aptamers even at concentrations as low as 12.5 pmole could be effective. We additionally observed that concentrations as high as 200pmole did not show any toxic effects on cell viability, *in vitro* (25). These observations were thereafter confirmed by in vivo experiments.

Although the use of therapeutic aptamers dates back to 1990, it took 15 years for the first therapeutic aptamer to be used in clinics. Pegaptanib, commercially known as Macugen, is the first and only FDA approved therapeutic aptamer in the market. Pegaptanib is a RNA based molecule administered locally aimed at treating age related macular degeneration by targeting vascular endothelial growth factor (VEGF). Pegaptanib specifically binds to VEGF165, a protein that plays a critical role in angiogenesis and leakage from blood vessels, two of the primary pathological processes responsible for the

vision loss associated with age related macular degeneration (AMD). Pegaptanib denotes an anti-angiogenic medicine for the treatment of neovascular AMD (26,27).

Another aptamer used in renal cell carcinoma and non-small lung cancer is AS1411, formerly known as AGRO100. In clinical trials, AS1411 has been administered systemically against human tumor xenografts, in vivo. It binds to the external domain of the membrane protein nucleolin, an eukaryotic nucleolar phosphoprotein involved in the synthesis and maturation of ribosomes. It is over-expressed on the surface of cancer cells that normally influence vital functions such as proliferation, nuclear transport, and transcription of the cell. Although it is administered systemically, it is relatively stable in the circulation. Inhibition of nucleolin activity by binding of AS1411 affects a variety of signaling pathways, including Nf-kB and Bcl-2. Its current status is awaiting at Phase ÎII. Several aptamers have been described in preclinical studies against several cancer targets, such as the antiapoptotic, the transcription factors and signal transducers (28).

NOX-A12 targets and blocks stroma cell-derived factor (SDF-1, CXCL12), a chemokine that promotes stem cell migration to the bone marrow and plays a role in vasculogenesis, tumor growth, and metastasis. In 2009 phase studies were performed and promising results were obtained (29). ARC138, is a DNA aptamers which inhibits thrombin. Phase I studies have been started in August 2004 (30). RB006 is 34 nucleotide aptamer which selectively blocks the factor VIIa/IXa catalyzed conversion of factor X to factor Xa which is an important step in thrombin generation. RB006 is a part of that anticoagulant system together with RB007, called as REG1. REG1 system underwent three phase 1 clinical trials (31).

ARC1779 is a PEGylated DNA/RNA aptamer with 39 nucleotides. It binds to the A1 domain of activated von Willebrand factor therefore; it inhibits its binding to platelet receptor glycoprotein 1b. thereby reducing platelet adhesion, aggregation and thrombus formation in arterial beds. Although it passed the phase 1 trials successfully, phase II studies were terminated for unknown reasons by Archemix Corp. In 2008; a phase II study for ARC1779 was opened to analyze the effectiveness of ARC1779 in comparison with and in conjunction with the drug desmopressin in patients with von Willebrand factor related platelet disorders. However in that trial investigators observed that; ARC 1779 is indeed effective on increasing the amount of platelet formation and it can potentially be used as an anti-bleeding drug for von Willebrand disorder patients (29,32).

Non-coding RNAs, such as small interfering RNA (siRNA) or small hairpin RNA (shRNA) function by specific sequence complementarity to target messenger RNA (33, 34). The efficient and safe delivery of RNA based therapeutics is still one of the major challenges for biologists and clinicians (35-37). Accordingly, combining two therapeutic approaches, aptamer and RNA based therapy could further enhance therapeutic potency of RNA based approaches. Scientists have assessed the use of two separate applications; the first being the non-covalent aptamer-siRNA conjugates and the second one being the covalent aptamer-siRNA or shRNA chimeras. 2’-fluoro modified anti-prostate specific membrane antigen (PSMA) aptamers with low nanomolar binding affinity are successfull examples of the first application (38). In this application two biotinylated A9 anti-PSMA aptamers and two biotinylated 27-mer Dicer substitute siRNAs against lamin A/C or GAPDH are non-covalently assembled on a streptavidin bridge through biotin-streptavidin interaction. Eventually, when the resulting multivalent conjugates were applied to control cells or prostate cancer cell lines obtained from xenograft models, selective cellular uptake and specific RNAi activity was observed (39). In the second approach a 2’-fluoro modified PSMA aptamer is directly fused with the passenger strand of a 21-mersiRNA duplex which subsequently anneals to the complementary guide strand. Truncated version, A10, aptamer –PLK1 or BCL2 siRNA chimeras induced specific silencing of target genes and cell death in prostate cancer cells expressing PSMA (40).

Diagnostic Approaches

Aptamers are also used as biomolecular diagnostic elements in various biosensors wherein they enable detection of small molecules. The enzyme linked oligonucleotide assay,affinity chromatography, affinity capillary electrophoresis, capillary electrochromatography and flow cytometry are examples of aptamers application in analytical systems. However, the routine analysis of biological samples with aptamers is limited because aptamers are relatively unstable in biological fluids; and because non-specific binding in biological samples makes it difficult to quantify the target (41,42).

Despite these limitations aptamers are more suitable than antibodies for in vivo imaging, especially for

intracellular staining. Their small size relative to antibodies with an ease to penetrate into cells and tissues effectively makes aptamers important tools for imaging analysis or flow cytometry. Oligonucleotides produced by using Cell-SELEX approach, is used to label target cells after labelling with fluorophore particles. This process was improved by Avci-Adali et al. to determine the absolute amount of cell bound aptamers by using qPCR based method in order to gain information about enrichment (43). Today; aptamers are used for labelling CD4 positive T-lymphocytes (44); detection of small-cell lung cancer (45), vaccinia virus infected cells (46) and detection of intacellular ATP (47). Additionally, aptamers are used for S. aureus detection (48), food-contamination analysis by C. jejuni (49) and for the environmental water analysis by detcting V. parahaemolyticus (50). Given the potential cytotoxicity of agents such as therapeutic radiolabels, aptamers with faster clearance rate have the advantage of being less toxic relative to antibodies (41,51).

Aptamers play also an important role in biosensors. In this regard, thrombin aptamer and an L-adenosin aptamer used as biosensor receptors are significant examples (52,53). Moreover, Liss et al. have developed an aptamer-based biosensor that specifically and quantitatively detect human IgE in biological samples (54).

Due to their conservative structural stability in the systemic circulation aptamers may also be used as protective or preventive agents. Because we do not know the consequences of aptamer-TLR interactions, the non-immunogenic property of aptamers may negatively influence this feature. Besides; binding of immunogenic substances with aptamers may affect the functional properties of aptamers and may cause non-specific immune system stimulation. Additionally; their limited half-life during both local and systemic applications questions the use of aptamers, particularly RNA aptamers, for vaccination. Although the desired protective effect of aptomers is not long enough as it is for antibodies; the ease in their production and application techniques, proposes the local applications of these molecules in conditions such as seasonal flu outbreaks.

Advantages and Disadvantages of Aptamers

Aptamers are molecules with similar features and functions as antibodies and oligonucleotides. Table 1 provides a summary of the characteristic features of aptamers and a comparison of their advantages and disadvantages with antibodies and oligonucleotides (51,4).

Conclusions

In conclusion; aptamers have shown promising results *in vitro* not only on untreatable diseases such as cancer and AIDS but also on viral infections by either blocking the interaction of pathogens with host cells or by blocking the function of enzymes responsible for pathogen replication. Today there are more than 100 patented aptamers approved for use in treatment and diagnosis of several conditions. Given the fact that the first therapeutic monoclonal antibody anti-CD3 muronomab could reach the market almost after a century of research, it seems too early to anticipate a commercial success for aptamers.

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Table 1. Advantages and disadvantages of aptamers and their characteristic features.

Advantages	Disadvantages
Produced and selected by an in vitro process independent of animals or cell lines	Absence of a standardized SELEX procedure for production
Disregard species specificity during application.	Non-specific binding in biological fluids
Not immunogenic and less toxic	High cost of the SELEX process.
Ability to be regenerated after denaturation	Need for high technical experience and handling.
High shelf-life and structural stability	Limited half-life for in vivo applications, especially for systemic applications.
The ability to be delivered into cells without inducing poration on cells, thereby making them valuable for intracellular staining.	
No variation on their quality between different production charges.	
Rapid reproducibility by an automated system.	

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