**Review Title: Aptamers As A Drug Future**

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1. **Abstracts:**

 Aptamers are nucleic acid molecules that are designed for their clinical applications. These are DNA/RNA molecules that forms particular structures which are of secondary & tertiary types and having the ability to specifically binds to proteins or other target molecules. They are chemically similar to antibodies. These special nucleic acid molecules are developed by a specialized technique which is known as SELEX i.e. systematic evolution of ligands by exponential enrichment. This technique utilizes single stranded nucleic acids (DNA/RNA). Aptamers shows the advantages over the antibodies. The selection of aptamers by cell-SELEX process improves their affinity against live disease causing organisms like bacteria & viruses and also made them capable of identifying specific target cells such as in cancer diagnosis.

**Key Words:** Nucleic acid,cellular targets,single-stranded DNA or RNA,cell-SELEX

**2.Introduction:**

 Aptamers are nucleic acid molecules like RNA & ssDNA or peptide molecules & due to their specific 3D structures, they are capable of binding specifically to their target molecules with more affinity. The research on Human Immunodeficiency Virus (HIV) & adenovirus gives the concept of binding of nucleic acids with proteins in 1980s. It shows that this viruses has ability to encode the sequence of nucleotide bases in RNA that binds with more affinity to specific viral or cellular proteins. Thus aptamers are the functional oligonucleotides that are capable to bind specifically to target molecules with high affinity.Aptamers shows advantages in recognation of molecules which has many use in biotechnology & biomedicine such as bioimaging & biosensing. Cell-SELEX is the process that made aptamers more promising for their use in molecular medicine. Shortly,aptamers are oligonucleotides or nucleic acid molecules that binds specifically to the target molecules with high affinity & shows aids in diagnosis & treatment of various diseases.

**3.Formulation of Aptamers:**

 Aptamers are nucleic acid molecules that have the ability of binding to specific target molecules with high affinity due to

* Three dimentional structural conformation of aptamers.
* Combination of other forces includes hydrogen bonding,van der Walls forces of interaction & stacking of aromatic rings.

 The binding affinities of this single stranded DNA or RNA oligonucleic acids are in the low picomolar & nanomolar ranges, the binding of this oligonucleotides (aptamers) to their target molecules is specific & highly sensitive. The number of the nucleotide bases in the aptamers varies, but generally ranges from 25 to 90 bases & the smallest aptamer which known is of 12 nucleotide bases in length.The process of SELEX (systematic evolution of ligands by exponential enrichment) conventinally develops the aptamers.

We have to increases the stabilility as well as half life of both the DNA & RNA aptamers by adding or introducing the appropriate functional groups in vitro & in vivo. The solid-phase oligonucleotide synthesis is the simplest process by which the synthesis & modification of aptamers can be done & the aptamers synthesized by this process shows improved effectiveness in the diagnosis as well as treatment of the various diseases.

## **Developmet of Cell Specific Aptamer by Cell-SELEX:**

 The process of conventional SELEX is selection procedure which utilizes a large library of oligonucleic acids of unique nucleotide base sequences that undergoes in series of cycle of selection against a specific target molecules of choice.

 To obtaining the aptamers with high affinity the processing steps such as binding,partitioning & amplification are repeated for several times.

 The target molecules such as proteins,peptides,viruses & whole cells against which there is a development of aptammers. The aptamers can binds either intracellular or extracellular sites of this target molecules.

The difference in the Cell-SELEX & traditional SELEX process is that Cell-SELEX process utilizes the live cells as target moleclule for the selection of aptamers.

 The traditional SELEX requires recombinant proteins as target for the aptamer selection but,the Cell-SELEX process conservs the native conformation of proteins which is the major advantage of this Cell-SELEX technique.

  **Stages of Development of cell specific Aptamers:**

1. **Selection:**

The procees of Cell-SELEX is mainly utilized for the selection of Aptamers. The selection cycle is starts with the incubation of target cell with any random sequence of the single stranded oligonucleic acid library ,it generally involved in the general SELEX process.

The sequences from the random sequence that forms binding with target cells are then undergoes in recovery phase for enrichment & regeneration of the Aptamer sequence with high specificity & affinities.

This random sequences become able to forms bonds with target cells only by forming the three dimentional unique structures. Then this aptamer library after enrichment is sequenced for the identification of induividual aptamers which are bounds to the target cells.



**Figure 1.** Schematic representation shows the steps involved in Cell-SELEX. **A**. cell Incubation with an aptamer library; **B**. Isolation of bound sequences from an unbound sequences; **C**. Elution of the bound sequences; **D**. Negative selection to for the removal of non-specific sequences; **E**. Amplifying target specific sequences; **F**. Sequencing of the selected aptamer pool.

## **Enrichment and Amplification:**

By continuously amplifying the aptamers that shows more affinity,there is enrichment in aptamers sequencing in the oligonucleic acid pool after each selection cycle. The process of optimization is required for amplifying selected aptamers to enhance the yield without the amplification of non-specific sequences of oligonucleotides due to the mispriming.

 The gel electrophoresis technique is utilized for analyzing the products of PCR at different amplification cycles & it is helpful in the optimization of the amplification cycles number. Since,amplification cycles more than required number would leads to the amplification of non-specific seuence products,while cycles less than required number would results in the insufficient yield which is also not desired.

## **Regeneration of Selected Aptamers:**

The following are the methods of regeneration of selected aptamers:

1. Magnetic Beads Separation
2. Electrophoresis Based Separation Of DNA Strands
3. Lambda Exonuclase Digestion
4. Asymmetric PCR

#### *3]1. Magnetic Beads Separation*

 In this technique there is the rapid seperation of a molecule from mixture by using magnetic beads that have the magnetic properties & by exposing to magnetic field it congregate, which will allows this seperation. Then there is immobilization of the previously amplified aptamer library (double stranded DNA) on magnetic beads coated with streptavidin. The sepearation of DNA strands is done by elevating temperature or by the treatment of alkali which is generally allowed through biotin-streptavidin interaction.

In the next step after the immobilization, by the exposure of magnetic beads to a magnetic field there is seperation of immobilized strands from their complementary strand. Then by utilizing recommended buffers multiple washings are followed.

The method of magnetic beads seperation is successfully utilized in Cell-SELEX.



**Figure 2.** separation of DNA strands using magnetic beads. **A**. double stranded sequences are immobilized on the magnetic beads; **B**. double stranded DNA denaturation by treatmentof NaOH; **C**. unbound sequences purification.

#### *3] 2. Electrophoresis-Based Separation of DNA Strands*

  The separation of double stranded DNA is also achieved by the process of electrophoresis namely polyacrylamide gel electrophoresis (PAGE). This method involves denaturation of this dsDNA with the denaturing polyacrylamide gel containing urea. The urea plays an important role of breaking of hydrogen bonds between the two strands , and if both the strands to be separated are of unequal size then they can be separated on denaturing polyacrylamide gel electrophoresis (PAGE).

 Then the UV or blue light transilluminator is used to identify the sequences of enriched aptamers & then they are subsequently extracted and purified.

#### *3] 3. Lambda Exonuclease Digestion*

 The technique involves the formation of non-phosphorylated single stranded DNA intact along with the digestion of phosphorylated DNA strand from 5′ to 3′ end by the lambda exonuclease.

 This lambda exonuclease shows difference in their activity on different stranded DNAs. i.e. they are inactive on gapped or nicked DNA & less active on non-phosphorylated double stranded DNA and ssDNA ,which makes it an efficient method for development of aptamers by regenerating ssDNA.

 However, exposure of even non-phosphorylated single stranded DNA to lambda exonuclease can leads to the digestion.



**Figure 3.** ssDNA generation with lambda exonuclease. **A**. Addition of enzyme to double stranded DNA which has a phosphate group; **B**. Incubation of enzyme with dsDNA; **C**. Extraction and purification of ssDNA.

  The process of purification is required for ssDNA product of lambda exonuclease digestion ,in which traces of lambda exonuclease enzyme & digested oligonucleotides are removed for progression of Cell-SELEX.

#### *3] 4. Asymmetric PCR*

 The simplest method of formation of ssDNA from dsDNA is known as Assymetric PCR (Polymerase Chain Reaction). In this method there is preferential amplification of aptamer sequences without amplifying their complementary strand & this proess of amplification is carried out with different ratios of primers.

 According to principle of PCR , at the time of early PCR amplification cycles the aptamers selected are amplified into double stranded DNA. The amount of primers is responsible for enrichment . i.e. the enrichment of desired ssDNA is facilitated by higher amount of primers that extends the sequence of aptamers.

 For removal of any traces of non-specific amplified DNA & extra primers and also for purification of PCR product with the process of gel extraction, the Assymetric PCR amplification method is highly recommended.

**4]  Evaluation of Aptamers Binding:**

 The binding efficiency of the aptamers which are newly formed is check by binding assays & it is first step in confirmation of efficiecy. This binding assays should be performed at the different stages of the process of aptamer development for confirming the target-binding enrichment.

 The following are the some known aptamer binding assays that confirms the binding of cell-specific aptamers:

1. Enzyme-linked assay
2. Fluorescence or confocal microscopy
3. Flow cytometry

These methods shows same steps in determination of binding specificity & affinity, even they are different in their principle.

 The first step involves that similar environment such as temperture, incubation time & buffer composition is used in process of aptamer development for initial aptamer binding affinity tests.

Then, in second step the cells are recommended to be blocked with non-specific oligonucleic acid sequences , which is critical step in determination of binding specificity.

In third step the target cells are incubated with increasing concentrations of aptamers (between 10 to 500 nM) for determining binding affinity,& dissosiation constant (Kd) of aptamer can be determined by plotting the non-linear regression curve of it.

The dissosiation constant (Kd) determination also requires cells withot aptamer treatment i.e. blank treatment.

In final step, the efficacy of binding assay being used is confirmed by using positive controls such as antibodies or aptamers that are known to bound to their targets.

#### *4] 1. Enzyme-Linked Assays*

 It was first reported by Drolet in1996 as a highly quantitative & sensitive method for aptamer binding assay. This methos is same as the ELISA (enzyme-linked immunosorbent assays) which is used for detection of specific substance. The binding affinities of aptamers can be determined by incorporating aptamers into enzyme-linked assay.

Enzyme-linked assays were also applied to study binding affinities to whole cells, cell lysates, or cell excretions/lysates & developed specifically for confirmation of Cell-SELEX developed aptamers binding affinities.

#### *4] 2. Radioactive Scintillation Counting*

 It is powerful method of detection of the binding reaction of aptamer to its target cells because of high sensitivity detection of radiolabled samples offered by scintillation counting.

 Then filtration or centrifugation can be recommended for separation of aptamer-bound targets from non-bound aptamers which is essential for accurate measurements of binding affinities of aptamers.

#### *4] 3. Fluorescence and Confocal Microscopy*

  It is also the most popular method to screen aptamer binding to cells , & can also screens the aptamers specificity towards their target site.

 This both the methods shows similarity in principle where confocal microscopy offers reduction of background,ability for depth field control & ability to take serial optical sections and also same in the excitation & emission of fluoroscence light.

 The specificity & binding affinity of aptamers were confirmed by this method on various human cells

E.g. leukemia cells,HPV-associated cervical cancer cells, SK-BR3 expressing human epidermal growth factor receptor-2,HEK cells expressing TrKB,HepG2 & pancreatic cancer stem cells.

 Fluorescence and confocal microscopy also confirms the development of aptamers against the microorganism.

E.g.  *Campylobacter jejuni*

#### *4] 4. Flow Cytometry*

 This laser-based method does the physical and chemical characterisation of molecules with high accuracy & reproducibility. The characterisation involves the passage of stream of single cell suspension through a detection apparatus for detecting tagged cells. Hence,it is most common method of aptamer binding characterisation.

 It also do the demonstration of binding affinity of selected aptamers various cells.

E.g. *Campylobacter jejuni* , acute myeloid leukemia,CD133-expressing HEK293T cells,lymphoma & small cell lung cancer.

**4. APTAMERS DEVELOPMENT FOR THEIR THERAPEUTIC USE:**

 The aptamers are special class of oligonucleic acid that acts by recognising the structure of both primary structure & 3D confirmation of the target,which is different from RNA tools,such as ribosomes,oligonucleotides & SiRNAs. This structural recognition provides high specificity than their counterparts. Thus aptamers shows more advantages over other therapeutic agents such as antibodies.

 It was discovered in 1990 that aptamers has potential use as therapeutic agent. The *in vitro* obtaining procedure gives more clear idea about efficiency & manufacturing process.

**4.1. Antiviral aptamers:**

 The aptamers has good antiviral activity & it is produced by interfering with the various stages of viral life cycle including attachment, penentration, translation, replication, packaging and budding. Viral targeting is one of the most popular therapeutic application of aptamers.

 The viral polymerase enzyme is firstly tageted by the aptamers , because this enzyme plays a key role in viral development in host body, mainly by interacting with nucleic acid of host.

 The pioneer study performed in 1990 by Tuerk & Gold inform about the efficiency of Aptamers for T4 phage DNA polymerase & also gives the idea about protein domains composition & nature involved in replication initiation. It is helpful in investingating the potential of human viral proteins as a targets.

**4.2. Aptamers in cancer treatment (oncology):**

Hanahan and Weinberg, 2000 discovered that cancer is a genetic alteration affects to the invasive & proliferative properties of carrier cell by affecting to essential signaling pathways. This feature exhibited by cancer cell is utilized by the aptamers for specifically targeting the malignant cells & inhibiting the progression of tumor.

 Aptamers achieves direct action over desired target cell by acting against cell-surface receptors , & on the basis of this cell-type specific aptamers can be isolated (Cerchia and de Franciscis, 2007) . Thus aptamers are developed against variety of tumor cells , including intracellular factors, cell surface proteins and extracellular ligands.

 For wide variety reagents & drugs specific delivery can be provided by aptamers. When active accumulation of anticancer drugs are combined with cell-specific aptamers , it enhances the treatment efficinency of cancer.

**4.3. Anticoagulant aptamers:**

 For treatment of various cardiovascular diseases & prevention of loss of blood during certain clinical conditions , the major pharmaceutical agents used are anticoagulant agents. There are certain major & serious side effects such as certain allergies, hemorrhage & decrease in number of platelets by the use of most commonly used anticoagulant agents like heparin.

Thus, it is essential to discover the new anticoagulant agents to resolve this problem. Therefore, aptamers are excellent alternative to classical anticoagulant drugs.

Thrombin is protein that amplifies the coagulation reaction by activating the procoagulant factors & as a serine protease plays an important role in maintainance of hemostasis. Thus, it is most recommended to target the thrombin in the development of good anticoagulant agent. In early 1990s development of anticoagulant aptamers involves the DNA aptamer isolation which can be interfere with thrombins proteolytic activity.

**5.Advantages of aptamers over the antibodies :**

 common differences between antibodies and aptamers.

|  |  |  |
| --- | --- | --- |
|  | **Antibody (IgG)** | **Aptamer** |
| Size range | 10 to 15 n | Approximately 3 nm |
| Target  | Antibodies targets only immunogenic tissues. against non-immunogenic or toxic proteins it cannot be raised | Targets both immunogenic and non-immunogenic cells or tissues (e.g., metal ions, dyes, small peptides, toxins etc.) targets |
| Specificity | Highly specific | Highly specific |
| Affinity | High affinity | High affinity |
| Tissue Penetration | Tissue penetration is low due to large size | Tissue Penentration is high due to small size |
| Stability (pH, Temperature) | Low | High |
| Shelf life | Few Months (loss of function due to freezing at low temperature) | No. of months to several years at normal temperature & low temperature respectively. |
| Nuclease susceptibility | Absent | Presece of nuclear succeptibity ( nuclease susceptibility is minimized by modified nucleotides) |
| Immune response | High (except humanized Ab) |  Low or absent (rare cases) |
| Batch-to-batch variation | Present | Absent due to synthetic manufacture |
| Conjugation |  Possibilities are less. | Chemical nature of aptamers provides functunilization with widw variety of molecules leads to the easier conjugation. |
| Synthesis |  In the physiological conditions only | Synthetic |
| Cost of synthesis | Cost is high for synthesis. Facilities such as reactors and animal house are required. | Cost is low. Expensive instuments are utilized for synthesis. |
| Scale-of-synthesis | Low | Scalable |

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

**6.Future perspectives of aptamers:**

 Aptamers are structured oligonucleic acid ligand which are either artificially selected or naturally occuring molecules that binds properly & specifically to their targets with high affinity. The artificially selected aptamers has functions similar to antibodies including specific detection, inhibition, and characterization of proteins. For the development of pharmaceutical aids & detecting specific target molecules, these characteristics of aptamers are very important.

 The aptamers are developed by the process of Cell-SELEX “**S**ystematic evolution of **L**igands by **Ex**ponential **E**nrichment” which is aptamer selection process contrained by patent protections. According to recent expiration of patent of SELEX, the aptamers are used for developing human therapeutics & diagnostics.

 “Marketsandmarkets” has published one market research report in 2015 according to which, aptamers global market is expected to reach $244.93 million by 2020.

**6.1 Aptamers for precision medicine :**

 Precision medicines are the class of agents that formulating with the goal of targeting specific cells & thereby minimizing the damage to other normal tissues. It also focuses on disease variability of individual by identifying the diagnostic & treatment platforms.

 An emerging concept initiated in 2002 in the field of precision medicine that combines both therapeutics & diagnostics known as “theranostics” and it mainly developed for more specific & individualized treatment of diseases. Aptamers are functionalized against the cell-surface receptors as an active targeting moities. Thus, for targeted therapeutics, macromolecular delivey & even for bioimaging this functionalized aptamers shows excellent theranostic effet as a tool.

 The patient specific somatic mutations are identified by the researchers through genomic sequencing of an individual patient. The various biomarkers like differentially expressed proteins, mutated gene products, & altered cell surface antigens are identified by this technologies. Aptamers may be serve as a personalized treatment that affects therapeutic responses by targeting misfolded proteins & point-mutated proteins.

 The programmed death 1 (PD-1,) and programmed death 1 ligand (PDL-1) for use in clinical treatment of cancer, and immune checkpoint antibodies against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are recently approved by USFDA. However,immune checkpoint blockade administration along with antibodies may leads to the development of resistance in patient & may assosiated with immune-related adverse toxicities. The aptamers introduced in such systems offers desired specificity & low immunogenecity as an emerging immunotherapeutic platform.

**6.2. Chemically modified Aptamers:**

 As Aptamers are oligonucleic acid ligands, their physicochemical characters mostly affects its therapeutic potential. Therefore, for improving the serum stability there is development of various modifications in chemicals. Modifications are made in most of aptamers by replacing the hydroxyl (-OH) group at the 2nd position with 2'-amino (2'NH2), 2′-O-methyl (2'OMe), or 2′-fluoro (2′F) groups.

 Deoxyuridine which is modified by positive charge at 5’position shows the therapeutic potential in the cynomolgus monkeys & this modified deoxyuridines are present in **S**low **O**ff-rate **M**odified DNA **A**pta**mers** (SOMAmers). The development of tolerable polymerase have been accompanied during the SELEX for incorporating the modified nucleotides into the aptamers. In the RNA aptamers the nucleotides such as 2'F, 2'OMe, and 2'NH2 nucleotides have been incorporated along with Y639F mutant T7 RNA polymerase.

 The other chemically modified nucleotides have been incorporated into aptamers along with engineered polymers are : 2'-O,4'-C-methylene bridged/locked nucleic acid (2',4'-BNA/LNA),2'-deoxy-2'-fluoroarabinonucleotide (FANA), and C2'-O-methyl(C2'-OMe)/ C2'-Fluorine (C2'-F). The other one nucleotide which is incorporated by a wild-type T7 RNA polymerase is 2'-O-carbamoyl uridine (Ucm).

## 7.**Conclusions:**

 There are many significant diagnostic & therapeutic potentials of cell-specific aptamers. The ease of chemical modification in aptamers helps in rapid molecular detection by permitting the longer systemic circulation & high binding affinities, and also the aptamers having small size allows the efficient penentration of tissues. However, as compared to traditional SELEX the cell-SELEX shows more complications such as they use only pure forms of targets. Otherwise, the use of non-purified target leads to the development of non-specific aptamers because in such cases it is very difficult to direct aptamer binding towards specific parts of the target cell.

 In negative control selection the healthy cells that surrounding the targeted tumor cells can also serve, in the tumor cell-SELEX process. For success of cell-SELEX binding assays which are the optimized methods for aptamer library regeneration, plays a significant role. The enrichment can be lead by the non-specific binding of dsDNA to the cell surfaces & for the selection of aptamer as oligonucleotide fragments,the critial step is the efficient efficient regeneration and purification of RNA or ssDNA pool. But, in non-optimized binding assays, false positive results can be given by the enrichment of non-specific binding oligonucleotides.

 The high affinity aptamers can be preferably selected by eliminating the non-specific binding from selection process, & by efficient regeneration of enriched aptamer pool. The highly recommended method is gel electrophoresis for ssDNA product to confirm their purity & yield and other methods of separation of DNA strands are efficient in generation of single stranded DNA for development of DNA aptamers, which are described in this review.

 The selection of binding assay method is very critical step in the development of aptamers. In additionally, the internalized aptamers are detected by the methods such as enzyme-linked assays & Quantitative PCR through the analysis of cell lysates & excreted products.

 Cell-SELEX is a powerful tool that made advancement in cell biology research by

* Identification of new cellular markers.
* Develoment of high affinity aptamers with diagnostic and therapeutic potential.

 But, successful implementation of Cell-SELEX is also critical step in aptamer development.

 For taking full advantage of aptamers high specificity, the aptamers should be selected against the somatic mutated antigens.

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