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Review Article

Biochemistry Section

Aptamers in Therapeutics

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ABSTRACT

Aptamers are single strand DNA or RNA molecules, selected by an iterative process known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Due to various advantages of aptamers such as high temperature stability, animal free, cost effective production and its high affinity and selectivity for its target make them attractive alternatives to monoclonal antibody for use in diagnostic and therapeutic purposes. Aptamer has been generated against vesicular endothelial growth factor 165 involved in age related macular degeneracy. Macugen was the first FDA approved aptamer based drug that was commercialized. Later other aptamers were also developed against blood clotting proteins, cancer proteins, antibody E, agents involved in diabetes nephropathy, autoantibodies involved in autoimmune disorders, etc. Aptamers have also been developed against viruses and could work with other antiviral agents in treating infections.

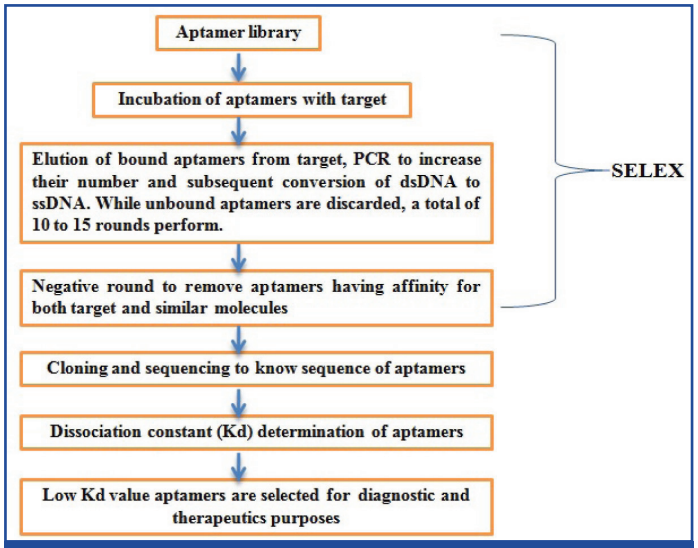
Keywords: Aptamer, Cancer, Diabetes nephropathy, Macugen, SELEX

INTRODUCTION

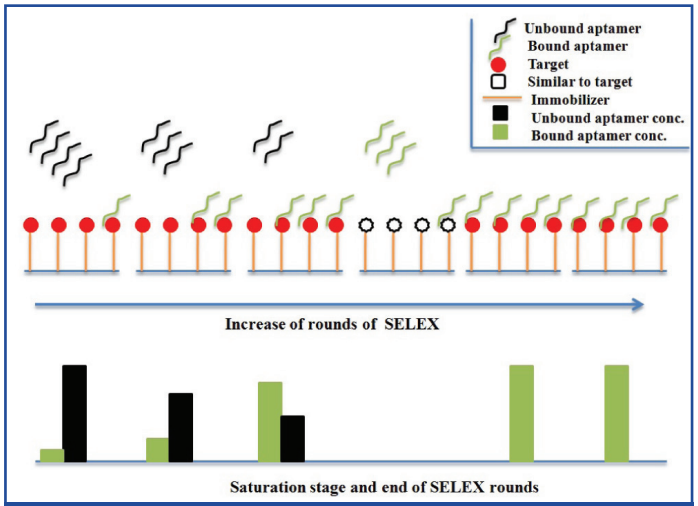
Aptamer are a new class of agents that can be used for both diagnostic and therapeutic purposes. They are synthetic single strand (ss) DNA or RNA molecules. They are selected against target molecules by an iterative process known as SELEX (Systematic Evolution of Ligands by Exponential Enrichment) [1], which was developed in 1990. Due to its various advantages, aptamers are regarded as promising alternatives to antibodies. Aptamers could be massively synthesized using *in vitro* techniques; its production is cost effective and animal free in nature. Aptamers bind specific ligands with high affinity and selectivity. They are more robust at elevated temperatures and thermal denaturation is reversible [2]. In last few years aptamers have been widely used in therapeutics. Macugen was the first FDA approved drug that was used against macular degeneracy disease [3]. Aptamers are also being devolved that can be used in clot buster, cancer therapy, autoantibodies, diabetes etc [3]. Companies such as NOXXON, Anisoma and other are doing cutting edge research on aptamers to be used as drug.

Aptamers and SELEX

Aptamers are synthetic DNA or RNA molecules that are custom made [4]. Artificial DNA has length in the range of 60 to 100 nucleotides. The 5' and 3' end of aptamer contains bases which are common in all ssDNA or aptamers and their ends can be 15 to 18 bases long. Rest of the region is middle region known as random region, contains bases at different positions. These unique DNA molecules together form DNA or aptamer library. A typical library contains more than 10^{15} different ssDNA molecules. Aptamer library is required to start SELEX process for aptamer selection against target molecule. SELEX is an iterative process where DNA library is incubated with target. Due to randomness in library, some of the aptamer binds to target while rest are discarded. The bond between the aptamer and target is broken down by using urea, EDTA at high temperature. Eluted aptamers are amplified by Polymerase Chain Reaction (PCR). This converts ssDNA to double strand (ds)DNA which is converted back to ssDNA for next round of SELEX. This process is known as one round of SELEX. A total of 10 to 15 rounds of SELEX are performed to get aptamers having very high affinity for target. During SELEX, similar but not identical molecules to target are also incubated with aptamers to discard any aptamer having affinity for both target and similar molecules. This process is known as negative or counter selection round [5]. Selected aptamers can be used for diagnostic as well as therapeutic purposes [Table/Fig-1,2].



[Table/Fig-1]: Selection of aptamers.



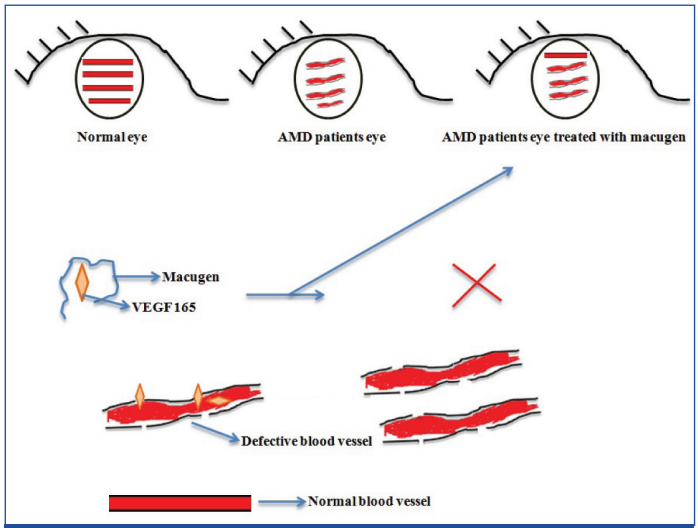
[Table/Fig-2]: SELEX method.

Application of Aptamers in Therapeutics

The first SELEX experiment was carried out by Tuerk and Gold in 1990, when they selected RNA aptamer against bacteriophage T4 DNA polymerase [1]. In literature we can find large number of aptamers specific against wide variety of targets [6-13].

Aptamers Against Age-related Macular Degeneration (AMD)

i) Macugen- It is an RNA aptamer that consists of 28 nucleotides and also known as pegaptanib. Macugen was the first FDA (in 2004) approved drug used in treatment of wet AMD (age-related macular degeneration) [14,15]. It was initially developed by NeXstar pharmaceuticals and in 2000 license was given to EyeTech Company (now OSI Pharmaceuticals) for late stage development and marketing in the United States. From outside of US it is marketed by Pfizer. Its molecular formula is $C_{294}H_{342}F_{13}N_{107}Na_{28}O_{188}P_{28}(C_2H_4O)_{2n}$, (n=900), M.W is 50 kDa and biological half time is 10 days. Macular degeneration is a disease of eyes where excessive leaky blood vessels are formed which causes blindness in patient if untreated. Macugen binds to 165 isoform of VEGF (Vascular Endothelial Growth Factor) and stops its interactions with VEGF receptors present on blood vessels in eyes [Table/Fig-3]. The anti-angiogenesis effect of aptamer not only stops the excessive growth of blood vessels, but also prevents the formation of defective blood vessels which ultimately reduces swelling in eyes. Pegaptanib is given by intravitreal injection into the eyes, more specifically, into the vitreous humour part of eyes [16]. Poly lactic-co-glycolic acid (PLGA) microsphere is used to encapsulate the drug for its release [17].



[Table/Fig-3]: Macugen for AMD treatment.

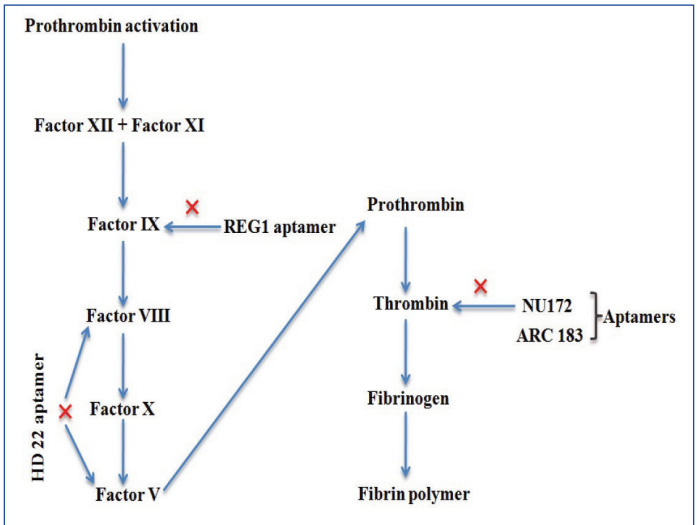
In animal studies it was also found that aptamer can be given subcutaneously and intravenously, while maintaining its desired concentration in blood [17]. In experimental studies macugen has shown inhibition of VEGF mediated vascular leakage which was measured by a corneal micropocket assay in guinea pigs (Eyetech Study Group, 2002). It has shown anti-angiogenesis effect in rat corneal angiogenesis model [18]. Further it has also effectively inhibited blood-retinal barrier breakdown in diabetic rat model [19]. In rhesus monkeys and rabbit models, macugen has shown neither the toxic effects nor change in intraocular pressure. Further, no immune response was shown by those animals [17,20]. In humans it has shown side effects such as anterior chamber inflammation, bleeding inside of the eyes, and infection of eyes or spots in vision. These issues can be minimized by adjusting the dose required by AMD affected person which vary from patient to patient. Pegaptanib has been approved in United States, Europe, Canada, Brazil and Australia [16,17,21].

Although this drug is used for AMD but a new monoclonal antibody (ranibizumab, Novartis) drug which is believed to be more effective than macugen is now available in market and competing with this aptamer.

ii) ARC1905- To fight against AMD, a new approach was developed by Ophthotech Company [22]. They used aptamer to target C5 protein which is involved in breakdown of membrane of cell. It has been known from past few years that AMD could be genetic in nature and caused by hyper action of alternate pathway of complement system. It is believed that mutation in chromosome 1q31 region expresses defective factor H protein of alternate complement system and is involved in causing AMD [23-28]. The over activation of complement system ultimately activates C5 protein that starts a cascade involving more complement proteins and forming Membrane Attack Complex (MAC) which ultimately kills retinal cells.

Clot Buster Aptamers

i) ARC183- It is a 15 bases long single stranded DNA aptamer [29] having G-quadruplex structure that interacts with exosite I of α-thrombin protein [30] [Table/Fig-4]. This interaction inhibits the binding of fibrinogen to thrombin causing anti-coagulation. The interaction is specific as it does not interact with other form of thrombin such as γ-thrombin. Further its half-life is only of two minutes creating rapid reversal of its own effect, thus it can be used in coronary artery bypass graft surgery. A more advanced version of this aptamer which is optimized and called as NU172 (26-mer DNA aptamer) is under phase II trial and soon be commercialized by ARCA biopharma Company [31].



[Table/Fig-4]: Clot buster aptamers in use for cardiac operation.

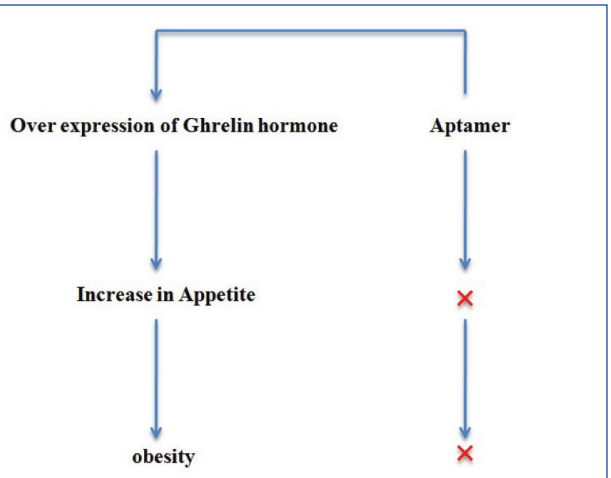
ii) Aptamer HD22- This aptamer contains duplex/G-quadruplex mixed structure which recognizes exosite II of thrombin and is involved in inactivation of factor V and factor VIII proteins of blood clotting system [32] [Table/Fig-4]. Further, the binding affinity of aptamer HD22 for thrombin is low as compared to ARC183.

iii) REG1 aptamer- This aptamer binds with factor IX and stops blood clotting process [Table/Fig-4]. This RNA aptamer is in trial process for its commercialization by Regado Biosciences Company [33].

iv) ARC1779 aptamer- It targets A1 domain of von Willebrand factor and is in phase II of clinical trial (Archemix Company) [34]. Nuclease resistant RNA aptamer selected against factor XII of intrinsic pathway could also be used in preventing pathological thrombosis formation [35]. One of the advantages of using these aptamers is that not only they can be used in therapeutics, but also as sensors for diagnosis of thrombosis by tagging fluorescent molecules with them.

Anti-obesity Aptamer

The NOXXON Company has developed spiegelmers (L-aptamer) against ghrelin, a peptide hormone associated with appetite and weight gain [Table/Fig-5]. This aptamer can serve as an anti-obesity drug and the animal test has already shown positive results in rats [36].



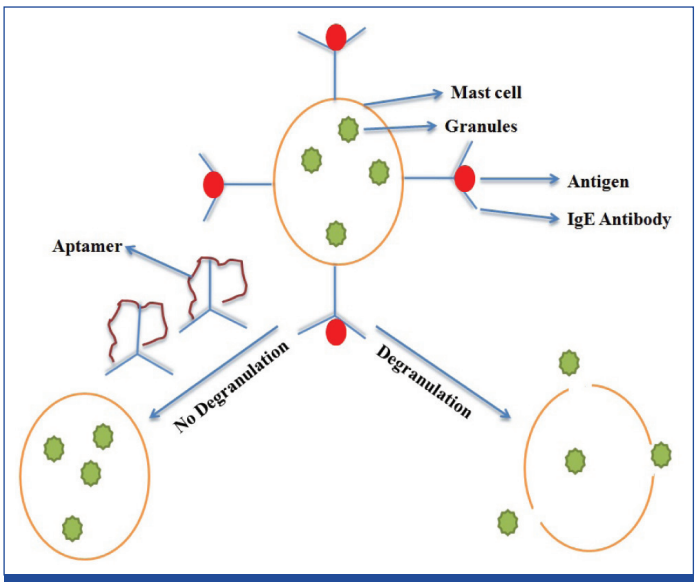
[Table/Fig-5]: Spiegelmers against ghrelin hormone.

Aptamers Against Autoantibodies

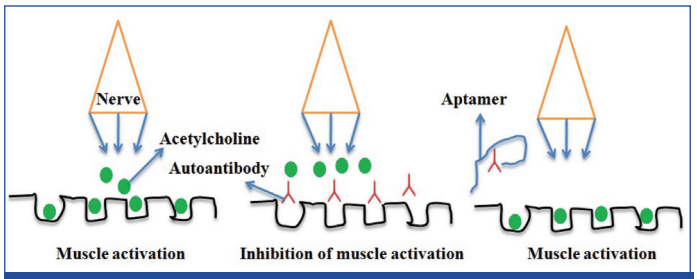
i) Role in allergic response- Immunoglobulin E (IgE) is involved in type 1 hypersensitivity reaction. Type 1 allergens can be due to environment factors (dust, grass, pollens) or food which activates immune system to produce IgE antibodies in excessive amounts. Overproduction of IgE causes asthma, allergies, dermatitis [37]. The molecular mechanisms involves allergens (antigen)-antibody interaction. Fc portion of IgE has receptors on mast cell. When immune complex (antigen+antibody) binds to mast cell, rupturing cell membrane releases large amounts of granules. These granules release mediators such as histamine, serotonin, prostaglandins, cytokines etc. which are involved in smooth muscle contraction, increase in vascular permeability or pulmonary smooth muscle contraction, ultimately causing hypertensive responses [Table/Fig-6]. Allergic responses can be stopped by inhibiting the interaction between antibody and mast cell. A DNA aptamer developed by Mendonsa group [38] in 2004 binds specifically to IgE antibody and masks its effect. Wang et al., developed aptamer sensor which is based on fluorescence protection assay for detection of IgE and the detection limit was 0.1 nM [39].

ii) Role in prevention of autoimmune disorders- Systemic Lupus Erythaematosus (SLE) is very common autoimmune disorder in women as compared to men. Auto-antibodies are generated against patient's own DNA, RBC and platelet membrane. These antibodies cause lysis of RBC causing anaemia and activate complement system that are involved in inflammatory responses causing tissue damage. RNA aptamers have been selected against anti-DNA autoantibodies frequently found in SLE patients. The Kd value of aptamer is 2 nM and is highly specific to autoantibody [40]. In Myasthenia Gravis (MG), autoantibodies are generated against nicotinic acetylcholine esterase receptors (AChRs) found in skeletal muscle. The inhibition by autoantibody prevents the interaction between receptor and acetylcholine hormone, causing muscular weakness and fatigue. A RNA aptamer against AChRs autoantibodies has shown inhibition of autoimmune response in animal models of MG and also showed bioactive protection of AChRs in human cells [Table/Fig-7] [41]. Cho et al., has also selected an aptamer (RNA) specific to AChRs which can be useful for MG patient in near future [42].

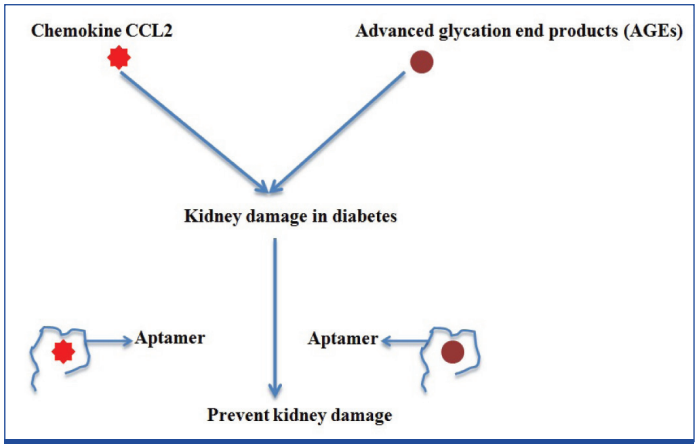
iii) Role in diabetes- Diabetes is life style disease and occurs most probably due to obesity, lack of physical activity; intake of high sugar or it could be genetic. Diabetes 1 is also known as childhood diabetes where patient fails to produce insulin due to destruction of beta cells of pancreas. In diabetes II (adult diabetes), insulin is produced properly but insulin receptors of cell become inactive due to presence of auto antibodies against them, causing inhibition of glucose metabolism. Lee and group selected a nuclease resistant aptamer (RNA sequence) that can bind to murine insulin receptor antibody (MA20). MA20 antibody destroys the insulin receptors of murine, causing diabetes [43]. The use of RNA aptamer has reduced the symptoms of diabetes in animal model and could be used for diabetes II treatment in humans, due to similarity between insulin receptor of murine and human. The main complication associated with diabetes II is damage of kidney which is also known as chronic kidney disease (CKD) which occurs due to action of chemokine CCL2 (MCP-1). NOX-E36 aptamer (RNA aptamer) targets CCL2 and reduces the progression of kidney damage [44-46] and is in phase II of clinical trial, developed by NOXXON pharma. In another work Japanese



[Table/Fig-6]: DNA aptamer binds specifically to IgE antibody.



[Table/Fig-7]: DNA aptamer binds specifically to AChRs autoantibody.



[Table/Fig-8]: Aptamer to prevent diabetes nephropathy.

researcher raised and tested DNA aptamer against advanced glycation end products (AGEs) that cause nephropathy in diabetic patient. The experimental data is very promising and it is believed that in near future aptamer could be used for therapeutic purposes [Table/Fig-8] [47].

Role in Cancer Diagnosis and Therapy

Cancer is unregulated proliferation of cells which occurs due to gain in function or loss in function of genes. The cause of this change can be genetic or by environmental pollutants. Several methods are now available to fight against this deadly disease. [Table/Fig-9] depicts proteins that are overexpressed during cancer and selected aptamer against them that can be used for diagnosis or therapeutic purposes.

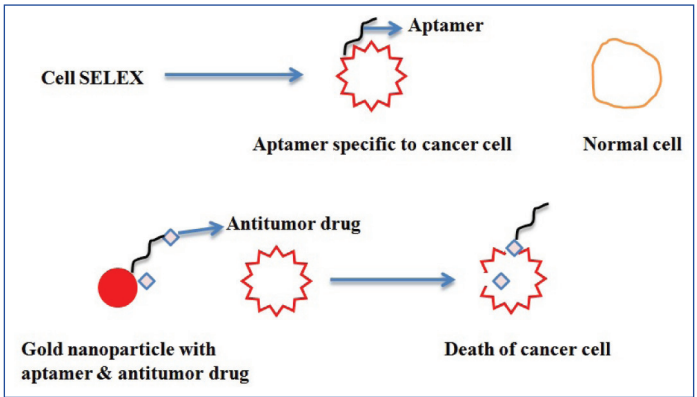
Target	Aptamer	Function of proteins in cancer
Pigpen	DNA	Endothelial protein, expresses on the surface of microvessels which is involved in blood vessel formation in cancer cells [48].
PDGF-r	DNA	Platelet Derived Growth Factor Receptor (PDGF-r) is a tyrosine kinase protein involves in metastasis of tumour [49-51].
Tenascin-C	DNA	This is an extracellular matrix protein that is expressed by chromosome 9 in humans. It is basically involved in cell signaling processes and activated during events such as fetal development, wound healing and in case of tumour growth [52-55].
CTLA-4	DNA	Cytotoxic T cell antigen-4 is a transmembrane protein that is expressed on the surface of activated T cells. Activated T cells further reduce the T cell response by raising threshold response needed for T-cell activation during cancer [56].
Sialyl Lewis X	DNA	It is a tetrasaccharide carbohydrate that is attached to O-glycans on the surface of cells and selectively binds with selectin proteins during cell adhesion and inflammation. Sialyl Lewis X is overexpressed in cancer cells and through selectin helps in metastasis process of cancer [57].
Nucleolin	DNA	It is a nucleolar phosphoprotein and found in nucleolus region of nucleus. Recent studies have shown the presence of this protein on cell surface especially on cancer cells. Thus nucleolin could be used as marker in cancer [58,59].
PMSA	DNA	Prostate specific membrane antigen is a transmembrane protein and found at prostate epithelial cell and involved in hydrolytic cleavage of poly-γ-glutamated folates to produce glutamate. In general PMSA has high expression and activity in prostate tumour as compared to normal one which can be used as possible biomarker as well as target site for cancer treatment [60-64].

[Table/Fig-9]: Aptamers against proteins involved in cancer.

In hepatic neoplasm, Osteopontin (OPN) [65], Alfa-Feto Protein (AFP) and heterogeneous nuclear ribonucleoprotein A1 are involved in tumour growth and metastasis and could be used as target site for cancer treatment. Aptamers specific to these protein have shown drastic reduction in tumour formation in liver cell in *in vitro* conditions [66,67]. Xu and group in 2015 [68] selected seven aptamers through cell-SELEX method that are specific for liver cancer cell (HepG2 cell line) but not to normal cells. Further they also concluded that these aptamers also bind with lung cancer, ovarian cancer and luminal A subtype breast cancer cells, showing multi specific nature of these aptamer in recognizing cancer cells and could be used in cancer therapy. P12FR2 RNA aptamer is specific to pancreatic adenocarcinoma up-regulated factor (PAUF), and could be used in treatment of pancreatic cancer. This aptamer binds with PAUF and inhibit metastasis process in cancer [69]. Other aptamers such as NOX-A50 and NOX-f33 (polyethylene glycol-modified Spiegelmers) are specific to A1 High Mobility Group (HMGA1) proteins which are involved in anchorage-independent growth and epithelial-mesenchymal transition in normal cells making them cancerous one [70]. Epidermal growth factor receptor (EGFR) also found to be overexpressed in pancreatic and other types of cancer. Nuclease resistant RNA aptamer specific to EGFR was selected and it inhibits tumour growth in a mouse xenograft model of human non-small-cell lung cancer [71]. DNA aptamer, called XQ-2d was developed having high affinity and specificity for pancreatic ductal adenocarcinoma (PDAC). Aptamer XQ-2d selectively binds to PL45 cells and can be used in PDAC diagnosis and treatment [72].

Kwak et al., selected a RNA aptamer, specific to peroxisome proliferator-activated receptor delta (PPAR-delta) [73]. PPAR-delta is overexpressed in colon cancer which further helps in overexpression of vascular endothelial cell growth factor-A and cyclooxygenase-2. RNA aptamer has reduced tumourogenecity in HCT116 colon cancer cells by inhibiting expression of PPAR-delta protein. Another protein beta-catenin is involved in transcription and alternate splicing of oncogenic genes. High-affinity RNA aptamer has been developed against this protein which reduces the activation of these genes [74]. In one of the experiment Hungs *et al.,* selected eight aptamers specific to colorectal cancer cells and stem cells by using on-chip cell-SELEX method [75].

One of the interesting way of treating cancer is to select aptamer that are specific to cancer cells but not to normal cell through cell-SELEX. These selected aptamers can act as carrier for antitumour drug or toxin. Reports have shown that aptamer is first coated on carbon nanotubes or quantum dots or gold or iron nanoparticles and then therapeutic drug is either intercalated to aptamer or directly attached to nanoparticles [Table/Fig-10] [76].



[Table/Fig-10]: Aptamer based cancer therapy.

Aptamers as Antiviral Agent

In past few years we have seen that viral infection is major cause of human death. New virus strains are emerging and at the same time antiviral drugs fail to respond, further side effects of these drugs have created need for search of new kind of drug. Aptamer could work in this direction. Viral infection can be inhibited by preventing virus fusion to human cell such as Hepatitis C virus which infects liver cells of human by interaction between E2 glycoprotein of virus and CD81 receptors of liver cell. A DNA aptamer was selected against E2 glycoprotein that inhibits the above interaction in *in vitro* studies [77]. Other approaches uses aptamers specific to viral polymerase enzymes [78], genetic material of virus [79], capsid proteins of virus [80] or other proteins that are involved in virus replication or processing of virus inside the cell. In one of the new approaches of cancer therapy aptamers are conjugated to small interfering (si) RNA to kill cancer T cell [56]. This approach can also be used to prevent viral infection.

Constrain of Using Aptamer in both *in vitro* and *in vivo* Conditions

Despite of its bright future in diagnostics and in therapeutics, aptamers are still in their preliminary stages of developments and there are constrains such as nuclease sensitivity, small size, toxic

effect and transportation of aptamers to inside the cell which needs to be overcome before it can be used for *in vitro* and *in vivo* conditions.

i) Making nuclease resistance- There are many ways in which nuclease effects on aptamers can be reduced. Aptamers contain several sites such as sugar moiety, phosphodiester region where modification can be done without interrupting its activity, one example is capping of 3' end of ssDNA as serum contains nucleases that act on 3' end rather than on 5' end. Substituting natural nucleotides with unnatural ones, such as 2'-F, 2'-OCH_3 or 2'-NH_2 modified nucleotides reduces affinity of nuclease for DNA degradation. Further, L-enantiomers form of nucleotides also known as spiegelmers makes aptamer nuclease resistant.

ii) Increasing renal filtration time- As already mentioned low size (10 to 15 kDa) causes quick filtration of aptamer from kidney, reducing its overall effect. Methods are being in used to increase the size of ssDNA by adding PEG (Poly Ethylene Glycol) or cholesterol. In case of macugen the PEGylation process increased the size from 10 kDa to 40kDa and at the same time half-life was also increased from 1 day to 10 days [81]. The increase in half time in serum shows that the renal filtration process was slow because of large size of modified aptamer.

iii) Reduction in toxicity- Very little information in this regard is available. As it is believed that aptamers are less immunogenic as compared to protein. But it's difficult to predict that they are free from toxic effect, as already macugen has shown its side effects in AMD patient. It is believed that in near future when more and more aptamer based drugs will be used to treat patients then we will face the toxicity issue.

One of the disadvantages of aptamers is that it is difficult to select against those targets that are negatively charged and hydrophobic in nature as ssDNA molecules are also negatively charged. SELEX is the only technology which is being used for aptamers selection while there are well established methods available for antibody generation. Aptamer selection against small molecules is always a big challenge. In most cases those aptamers are selected that have affinity for target as well as immobilizer or linker attached to target, reducing aptamers selectivity. Also, the generation of aptamer is based on combinatorial DNA library which contains random sequences of aptamers but it seems difficult to match diversity that is found in immune system of living beings used for antibody generation.

CONCLUSION

Aptamers are short oligonucleotides of DNA or RNA selected *in vitro* to bind a specific target with high specificity and high affinity which require simple synthesis protocols compared to the *in vivo* development of antibodies. It might become an alternative to monoclonal antibodies due to its flexibility of *in vitro* selection. Yet, despite the advances and the huge body of literature documenting the success of the technology, the commercial application of aptamers remains relatively undeveloped. The fact that there is a vast antibody-based market and a certain degree of hesitation to move to a new type of product, unless aptamers offer verifiably significant improvements on current technologies. In recent years advances are being made to improve the efficiency of selection and to increase the affinity, specificity, biostability and bioavailability of aptamers.

Aptamers are emerging as promising bio-recognition elements for diagnostic, therapeutic and biosensing purposes. It is likely that in near future aptamer technology will increasingly find use in the development of new therapeutic and diagnostic agents.

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