Identification and Engineering of Aptamers for Theranostic Application in Human Health and Disorders

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Abstract

Aptamer is short sequence of synthetic oligonucleotides which bind to their cognate target specifically while maintaining similar or higher sensitivity as antibody. Small size, chemical synthesis, low batch variation, cost effectiveness, long shelf life and above all low immunogenicity provide advantages over antibody. The in-vitro selection of aptamer applying a conjoining approach of chemistry and molecular biology is referred as Systematic Evolution of Ligands by Exponential enrichment (SELEX). These initial products of SELEX are considered as first generation aptamers, further modified chemically in an attempt to make it stable in biofluid avoiding nuclease digestion and renal clearance. These types of aptamers are called second generation aptamers. While modification is incorporated, enough care should be taken to maintain its sensitivity and specificity. These modifications and several improvisations have widened the window frame of aptamer application that is currently not only restricted to in-vitro system, but have been used in molecular imaging for disease pathology and treatment. In food industry it is used as sensor for detection of different diseases or fungal infections. In this review we have discussed a brief history of its journey, process of synthesis, different types of modifications to improve its characters. We have also focused on its applications and highlighted its role as therapeutic plus diagnostic; theranostic tools. Finally, the review is concluded with a brief discussion on future prospective in immunotherapy, as well as in identification of novel biomarkers in stem cell, and also in single cell proteomics (scProteomics) to study intra or intertumor heterogeneity at protein level.

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ABSTRACT

Aptamer is short sequence of synthetic oligonucleotides which bind to their cognate target specifically while maintaining similar or higher sensitivity as antibody. Small size, chemical synthesis, low batch variation, cost effectiveness, long shelf life and above all low immunogenicity provide advantages over antibody. The*in -vitro* selection of aptamer applying a conjoining approach of chemistry and molecular biology is referred as**S** ystematic **E** volution of **L** igands by**Ex** ponential enrichment (SELEX). These initial products of SELEX are considered as first generation aptamers, further modified chemically in an attempt to make it stable in

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KEYWORDS

Aptamer, Aptasensor, Proximity ligation assay (PLA), Spiegelmer, Systematic Evolution of Ligands by Exponential enrichment (SELEX), Theranostic

INTRODUCTION

The concept of DNA interacting with protein can be dated back to the days of discovery of DNA foot printing experiment in 1978, which is primarily used in search of DNA sequences specifically interact with ligands (proteins or small molecules) (Galas & Schmitz, 1978). Nucleic acid-protein interactions were used to study the specificity of sequences of DNA/ RNA to bind with transcription factors that regulated the expression of gene. The specificity of sequences of nucleotides is also evident in the interaction of recombinant endonuclease, ligase, polymerase (Van Dyke & Dervan, 1984). Electrophoretic mobility shift assay or EMSA is another technology that used the basis of interaction of nucleic acid and protein (Garner & Revzin, 1981) where mobility of DNA is retarded in gel when bound with protein. Hence, it is notable that there is the affinity of nucleic acid sequences to bind with various macromolecules. Based on these observations, it is inevitable that like antibodies, nucleic acid sequences have certain degree of affinity to bind with protein, and which can be harnessed to replace the antibodies in diagnostics and therapeutics applications. Thus, the era of theranostic battle is highly anticipatable between the high affinity nucleotides (*popularly known as aptamer) and the antibodies.

It is conceivable that if the random sequences will be incubated with macromolecules like protein or enzyme, there will be some nucleic acids that will be binding with different dissociation constant. The nucleic acid sequences that have lowest dissociation constant (Kd) will be the highest in proportion in binding (highest affinity) and vice versa. This prompted Gold and his graduate student Turek to devise a technology termed as **S** ystematic **E** volution of **L** igands by**Ex** ponential enrichment (SELEX) in an attempt to identify the nucleic acid sequences (RNA) having high affinity against RNA bound protein (in this case bacteriphageT4 DNA polymerase; gp43) that is responsible for its own translational repression (Tuerk & Gold, 1990). The alternate cycles of binding to the ligand and amplification of the bound nucleotide sequences from the initial pool of random sequences resulted in high affinity sequences. By this approach, the wild type and the most abundant variant form of the sequences that bound the ligand (e.g. gp43) with highest affinity were obtained. These high affinity oligonucleotide sequences are later termed as *aptamer* by Ellington and his group in 1992 (Ellington & Szostak, 1990). The word 'Aptamer' is obtained from two different words, i.e. 'Aptus' (Latin) and 'Meros' (Greek) which denote 'Fit' and 'Part' respectively. They can be developed against any targets e.g. small molecules, proteins (ligands, receptors, etc.), or even the cells using amalgamation of combinatorial chemistry with molecular biology tools using traditional SELEX or its variants (Bock, Griffin, Latham, Vermaas, & Toole, 1992; Hermann & Patel, 2000; Pleiko et al., 2019).

Interestingly, the synthesis of aptamers is completely done*in-vitro* without any requirement of biological system like antibody and thus batch to batch variation is minimized. Ease of chemical synthesis makes these molecules cost effective also. It has long shelf life; therefore, long storage can be possible easily (Ali, Elsherbiny, & Emara, 2019). Small size (10-15 KD for aptamers vs. 150 KD for antibodies; IgG) helps in

efficient tissue penetration, targeting (Xiang, Zheng, et al., 2015) along with reduced immunogenicity helps in vivo applications and a candidate for future precision medicine (B. T. Huang et al., 2017; Xiang, Shigdar, et al., 2015).

APTAMER SELECTION

There is a specialised technology behind aptamer selection called SELEX which was first designed by Gold and Turek (Tuerk & Gold, 1990). Here random oligonucleotide library consisting of variable sequences flanked by fixed sequences at both ends for primer binding is incubated with target (protein, cells or other molecules). The bound sequences having high affinity with the target is collected by elution and further enriched by PCR. After 15-16 similar cycles, the high affinity desired aptamer sequence against the specific target is obtained. The procedure is illustrated below in Figure 1.

Later, variations and modifications on classical SELEX were devised to increase its specificity (Zhuo et al., 2017). For example, in Negative SELEX (Ellington & Szostak, 1992), nonspecific binders are ruled out incubating aptamer pool with purification agarose after few initial rounds of selection (Figure 1). On the other hand, in Counter SELEX (Jenison, Gill, Pardi, & Polisky, 1994), structurally similar target is used to reduce non specificity (Figure 1). Development of cDNA SELEX by Dobblestain, et al. added another milestone to this field (Dobbelstein & Shenk, 1995). Idea of Spiegelmer or Mirror image DNA came at 1996 (Eulberg & Klussmann, 2003; Klussmann, Nolte, Bald, Erdmann, & Furste, 1996). 'Spiegel' is a Germen word which means mirror. Basically Spiegelmer is L ribose based aptamers which are mirror image of normal D ribose based aptamers and thus highly nuclease resistant. These are basically composed of L ribose sugar which is enantiomer of normal D ribose sugar. Being L ribose, these Spiegelmers are highly resistant to nuclease degradation. But these enantiomeric DNA cannot bind with normal protein which is composed of L amino acid. Rather, this L form of DNA binds with D amino acid which is enantiomer of L amino acid. First, protein sequence is chemically synthesized which comprised of D form of the corresponding amino acids. Then normal D aptamer is selected against this D protein. L aptamer of the selected D aptamer is synthesized which is now able to bind normal L protein of the system. Klussmann, et al. generated a 58 mer L-RNA aptamer against D adenosine which has 9000 fold higher affinity towards D adenosine than L adenosine and nuclease resistance as well (Klussmann et al., 1996). First bioactive Spiegelmer was synthesized by Bartel, et al. against the nona peptide hormone Vasopressin (VP). They synthesized L aptamer against VP which showed activity in cultured kidney cell by antagonizing activity of its target (Williams et al., 1997). Later Spiegelmers against various protein target have been synthesized (Vater & Klussmann, 2015), among them some of which are in clinical trial. One of such promising Spiegelmer is NOX A12 against CXCL12 which has been discussed briefly in the later part of this review.

Later, *in-vivo* SELEX, Cell SELEX helped aptamer to bind to its target in its native form, which is more closely mimicking the biological system. Cell SELEX is a specialised SELEX, where the whole cell is used for target incubation instead of purified protein, to develop aptamers against extracellular proteins. In cell SELEX, proteins remain in its native state thereby aptamers generated against the native form; have higher probability to maintain its specificity *in-vivo*or intact physiological systems. Some conditions should be optimized during cell SELEX, like cell detachment, cell viability, reduced nonspecific binding etc.

IMPROVEMENT OF APTAMER STABILTY, SPECIFICITY AND SENSITIVITY

The enhanced affinity, specificity along with stability is the desired qualities required for making a potential aptamers against a particular target (antigen, protein etc.) Linking two or multiple aptamers is a promising method to increase their specificity with reduced background signals. Target protein has multiple epitopes to which different aptamers can bind. When aptamers against different epitopes get linked, they become more specific towards target and their affinity also gets increased. Different methods to link aptamers are as follows:-

Proximity Ligation Assay (PLA): In this assay two different aptamers binding with different regions of same protein or two nucleic acid molecules (ssDNA) attached with two different antibodies which have affinity towards two different epitopes of target protein ligated together with suitable linker when they are in

proximity (Greenwood et al., 2015). The common linker has sequence complementarity to 5' free end of one oligo and 3' free end of other, thus brings the two nucleic acid moieties in close proximity with one having free 3'-OH and another with free 5'-phosphate group to undergo ligation reaction (Figure 2A). Then ligated DNA product is amplified by PCR and detected. This immuno-PCR based approach can lead to enhancement of affinity and specificity than the single counterpart. Addition to this, PLA can omit washing steps too. Primers are designed as such that forward primer is complementary to one aptamer and reverse primer is complementary to the other one, so that PCR can only give product when both of them are linked, and non-specificity gets reduced. Although PLA was performed using the antibodies (protein) -linked to nucleic acid molecules via Biotin-Streptavidin chemistry (Greenwood et al., 2015), but PLA can be performed devoid of antibodies/protein. In place of two antibodies, two DNA aptamers having same sequence and binding affinity for platelet derived growth factor B (PDGF-B), were used against homodimer of PDGF (PDGF-BB) (Fredriksson et al., 2002). The DNA aptamers were further extended using additional nucleotide sequences called proximity probe pairs at 5' of one aptamer and 3' end of the other by Fredriksson, et al. When this probe pair bound with the target, the free ends of their extended sequence came close together to be hybridized to another oligonucleotide which basically served as a connector and allowed the ends to be joined by ligase enzyme. The ligated product was then amplified by PCR, whereas nonligated probes were not amplified (Fredriksson et al., 2002).

Linker length and multimerization of aptamers: Linkers are those molecules which can link between two molecules but do not take part in their secondary structure formation. These linkers play important role in aptamer multimerization and hence in increasing affinity as well as avidity (Kalra, Dhiman, Cho, Bruno, & Sharma, 2018). Hasegawa, et al. connected two aptamers against thrombin protein via poly dT linker. A bivalent aptamer i.e., 15 mer aptamer against fibrinogen binding region and a 29 mer aptamer against heparin binding region of thrombin protein are connected with varying lengths (1 to 20 mer) of poly dT stretch. They have found 5-mer linker is the most suitable length (5nm) and have $1/10^{\text{th}}$ KD value of 29-mer aptamer (Hasegawa, Taira, Sode, & Ikebukuro, 2008) (Figure 2B). Distance between these two domains are about 3.4 nm whereas five consecutive thymines have length of about 5 nm which confers 5x dT linker to fit perfectly and provide required flexibility. The length of linker should vary according to the distance between two aptamer binding sites. Although poly dA linker has been reported (Muller, Wulffen, Potzsch, & Mayer, 2007) but poly dT (pyrimidine nucleotides) is preferred due to small size and flexibility as compared to poly dA (purine nucleotides) (Hasegawa et al., 2008). In case of homodimeric molecule e.g. vascular endothelial growth factor (VEGF165), the homodimeric aptamers binding in the same epitope region of two different subunits of VEGF165 can be used to increase the binding affinity. The 66-mer VEa5 binds to heparin binding domain of VEGF165 (Hasegawa et al., 2008) and they observed the dimerization of VEa5 (VEa5-VEa5) simply reduced the Kd by 18-fold. Thus, dimerization of aptamer will increase the sensitivity and specificity and improve the diagnosis of the disease which involved homodimeric molecule. Another group, Mallikaratchy, et al. made dimer, trimer, tetramer of TD05, an aptamer against an epitope of B cell receptor (BCR) present on B cells and B cell lymphomas. As linker they used polyethylene glycol (PEG) molecules of different lengths (12.6 nm, 16.8 nm, 22.5 nm) and found affinity increment is independent of linker length beyond 16.8 nm in dimeric aptamer construct (Mallikaratchy et al., 2011). Therefore length of spacer molecule is very important in case of multidentate aptamers concatenation and should be flexible to fit perfectly with the distance between different epitopes of target protein (Mallikaratchy et al., 2011), (Kalra et al., 2018). Moreover addition of PEG as spacer or linker molecule is expected to increase the local concentration of multi-valent aptamers by increasing the retention time of aptamers. Hence, increased the binding affinity in physiological condition or biological systems is highly anticipated. *Chemical modifications of aptamer* : Inside biological system aptamers encounter several hindrances to be effective as therapeutic agent. They are highly prone to renal clearance. So their circulation time in biological fluid should be increased via modifications. Being oligonucleotides, these aptamers are also prone to nuclease degradation which can be countered by modifications and make them stable. Modifications can be done at 5' and/or 3' end of sugar phosphate backbone of aptamers or at nucleotides of aptamers or at linker (Figure 3).

1. End modifications of backbone: Sugar phosphate backbone of aptamer molecule can be modified at 5'

and/or 3' end by end capping or bulky moiety conjugation (Figure 3). These are effective to increase aptamer stability and circulation time as well as to inhibit 3' exonuclease degradation in human serum (Maier & Levy, 2016), (Dougan et al., 2000), (de Smidt, Le Doan, de Falco, & van Berkel, 1991).

2. Modifications of sugar: Sugar is modified by changing the bonds (LNA and UNA), adding bulky moiety or using enantiomer (spiegelmer) (Figure 3) which finally result in improved thermostability and nuclease resistance (Mallikaratchy et al., 2011), (Pasternak, Hernandez, Rasmussen, Vester, & Wengel, 2011), (Padilla & Sousa, 1999), (Eulberg & Klussmann, 2003; Klussmann et al., 1996), (Williams et al., 1997), (Vater & Klussmann, 2015).
3. Modifications of phosphodiester linkage**:** i) Triazole linkage replacement at the position of phosphodiester linkage (Figure 3) is another promising approach against nuclease digestion. Phosphodiester bond is also modified by phosphorothioate (substitution of O with –S), Phosphorodithioate (substitution of both O atoms with –S) and methylphosphonate (substitution of O with –CH3) linkage (Sacca, Lacroix, & Mergny, 2005) (Figure 3).
4. Modifications of base: To increase target diversity, functional groups which can mimic structure of amino acid side chains are conjugated, which in turn provide them protein like property as well as diverse secondary and tertiary structure. They can interact with more epitopes of target and have slow dissociation rate (slow off rate). These are recognized as Slow Off-rate Modified Aptamers or SOMAmers (Figure 3) with better binding affinity along with binding kinetics and nuclease resistance activity over traditional unmodified aptamers (K. Y. Lee et al., 2010).

APPLICATIONS

Though aptamer was initially prepared as the replacement of antibody but it shows immense applications in different fields. Aptamers are playing great role in bioimaging, diagnosis and therapeutic purposes.*Bioimaging* Bioimaging is the optical visualization of biological process where two different types of agents are required, one which helps in visual detection or tracer molecule and other for carrying the former one to the target tissue or carrier molecule. Due to its efficient tissue penetration property and high target specificity Aptamer can be widely used in different types of bioimaging. Magnetic Resonance Imaging or MRI needs powerful contrast agents which can increase or decrease relaxation time according to requirements. It may be of longitudinal or transverse (T_1 or T_2) and the change of relaxation time helps in changing the brightness of MRI signal generating three dimensional image of the target (Grover et al., 2015). Gadolinium compounds or Supermagnetic iron oxide nanoparticle is engineered with target specific aptamer in direction of making a smarter contrast agent. A short oligonucleotide coupled with gadolinium-tetraazacyclododecanetetraacetic acid (DOTA-Gd) has been synthesized having complementarity to a part of anti-adenosine aptamer. When aptamer recognized adenosine, it released the DOTA-Gd coupled oligonucleotide which in turn lowered the molecular weight of gadolinium compound. This led to turn the MRI signal off due to increase of its relaxation time or T_1 (W. Xu & Lu, 2011). The same principle can be implemented on T_2 weighted contrast agent like Cross Linked Iron Oxide nanoparticles called CLIOs (Yigit et al., 2007).

In Positron Emission Tomography or PET imaging the decaying property of a radioisotope by positron emission is used. Radioisotope injected intravenously emits positron which interacts with the electron of tissue and generates two photons directed 180 apart. These photons are captured by PET camera and finally converted into electrical signal (Berger, 2003). Among various suitable radioisotopes like ^{12}C,^{18}F, ^{15}O, Fluorine-18 (^{18}F) is mostly used due to its physical properties like convenient half-life, facile production, low emission etc. Being non-invasive imaging, PET is highly recommended for in vivo study of protein expression in tissue specific manner. To quantify the level of protein tyrosine kinase 7 (PTK7) in mouse xenograft model an aptamer was conjugated with ^{18}F-benzylazide with the aid of click chemistry (Jacobson et al., 2015). Although this approach has been used to check the protein expression level in cell lines and mouse tumor model having differential expression of PTK7, it can have a high impact in future clinical application to study or quantify multiple protein expressions in different human malignancies.

Apart from above mentioned methods, aptamers have also been used in SPECT (Single Photon Emission

Computer Tomography), CT (Computer Tomography), US (Ultrasound) and fluorescence imaging for in vitro and in vivo purposes (Dougherty, Cai, & Hong, 2015), (S. Yoon & Rossi, 2018). Broccoli, Spinach, Corn, Mango are some of the fluorescent aptamer candidates studied extensively to enhance fluorescence activity and bioavailability (S. Yoon & Rossi, 2018).

Diagnostics

Aptamer, when works as sensor molecule in detection of its target, is called Aptasensor. Different types of aptasensors are available, like-electrochemical aptasensor, fluorescence based aptasensor, colorimetric aptasensor etc. For fluorogenic detection, a complementary oligo containing quencher molecule binds with aptamer which is released upon target recognition leading to fluorescence enhancement (Figure 4A). Nowadays in place of traditional quencher, nanomaterials like quantum dots or QD (artificial spherical fluorescent nanocrystals having semiconductor property), gold nanoparticle (AuNP), graphene oxide or GO (single atomic layered compound synthesized by oxidation of graphite) etc. are used for their superior quenching property. As an example, a cocaine specific aptamer was tagged with fluorescein (fluorophore) and DABCYL moiety (quencher) (K. M. Song, Lee, & Ban, 2012). In a different study Zhao, et al. detected aflatoxin B1 for which they have used a 29 mer aptamer. For fluorescence they tagged the aptamer with FAM and for quencher they used a complementary 14 mer cDNA tagged with BHQ1. When the target molecule was absent, aptamer paired with cDNA by base complementarity to quench the fluorescence. Fluorescence recovery took place when quencher DNA (qDNA) was released, upon aptamer mediated aflatoxin recognition. This technique will be of great use in medical and food safety purposes (Li, Sun, & Zhao, 2018). This method is sometimes bypassed by direct conjugation of fluorophore to aptamer (Figure 4B). A DNA aptamer was developed against pancreatic ductal adenocarcinoma (PDAC) by Cell SELEX (Wu et al., 2015) and labelled with Cy5. It has been applied on clinical PDAC tissue sample along with PDAC tumor-bearing mice for *in vivo* imaging and detection at which tumor site was illuminated up to 3 hour post-injection (Wu et al., 2015). Later it was found to be specific against CD71, a human transferrin receptor, by mass spectrometry analysis (Wu et al., 2019). As CD71 is overexpressed in PDAC along with other malignancies, this can be a potential diagnostic method for tumor biomarker detection.

In colorimetric assay the aptamer is affixed onto gold nano particle (AuNP), chemical (like salt or cationic detergent) mediated aggregation of which finally helps in target detection by producing visible colour. Single stranded oligonucleotides coat AuNP with their exposed nitrogenous bases which in turn inhibit AuNP aggregation. In presence of proper target, ssDNA aptamer binds to it, leaving AuNP to aggreagate and colour change (A. Dhiman, Kalra, P., Bansal, V., Bruno, J. G., Sharma, T. K., 2017) (Figure 4C). One of such cationic detergent, Cetrimonium bromide (CTAB) mediated AuNP aggregation helped in detection of Bisphenol A (BPA) level in different daily use products where BPA specific aptamer was used. This helps to regulate its level below toxicity (Hwang, 2019). Point of Care Test (POCT) or bed-side test is the diagnostic test which performed near the patient (Quesada-Gonzalez & Merkoci, 2018) among which Lateral Flow Assay (LFA) is considered to be very popular. It is rapid, cost effective, easy to handle and does no requirement of trained personnel. Conventional LFA biosensor requires target specific antibody but aptamer can be a good choice of replacement to overcome disadvantages associated to antibody (Reid, Chatterjee, Das, Ghosh, & Sharma, 2020). For thrombin analysis Liu, et al. devised a biosensor strip with the aid of aptamers and AuNPs which has superior sensitivity than commercially available antibody-based strip sensor. Capillary action helped the sample solution to migrate through the strip after being applied onto sample pad. When it reached to conjugation pad, the target molecule was recognised by primary aptamer coated onto AuNP. In the test zone, biotinylated secondary aptamer was immobilized by streptavidin which was designed against a different epitope of same thrombin molecule. When AuNP-primary aptamer-target complex was captured by secondary aptamer, detection was possible by colour production in the test zone due to AuNP accumulation, as a positive result. In the control zone a complementary DNA probe named control DNA was affixed using same principle which bound with excess AuNP-primary aptamer to develop a second coloured band which confirmed proper functionality of the biosensor strip. In absence of thrombin, the second band was generated only at control line (H. Xu et al., 2009) (Figure 5A). Besides POCT like rapid detection test (RDT), ELISA (Figure 5B) is one of the best plate based protein detection methods in

laboratory where antibody is used as detector, but the same approach can be achieved by aptamer called Aptamer Linked Immunosorbent Assay (ALISA) (A. Dhiman, Kalra, P., Bansal, V., Bruno, J. G., Sharma, T. K., 2017) (Figure 5C). Zeng, et al. have designed two different set ups: one with two different aptamers (2 and 10) against Zika NS1 protein as capturing and detection agents respectively and another with aptamer 2 as capturing and an anti NS1 antibody as detection agent. The hybrid aptamer-antibody assay was superior to the aptamer-aptamer assay due to its low limit of detection (0.1-1 ng/ml). It has shown its efficiency even in human serum sample where proteases, other proteins, ions are present (detection limit >10ng/ml) which indicates its future application in clinical detection (Hwang, 2019). ALISA has been successfully used in detection of different forms of tuberculosis like tuberculous meningitis (A. Dhiman et al., 2018), pleural tuberculosis (Kumari et al., 2019), pulmonary tuberculosis (Lavania, 2018) etc. with equal or even better sensitivity than available diagnostic techniques like ELISA, microscopy smear or chest X ray.

Aptamer has been extensively used in infectious agent detection. As for example, Wang, et al. screened a DNA aptamer where recombinant protein Hemagglutinin (HA) of H5N1 was used as target for first few rounds (4 cycles) followed by screening against an entire virus particle as target (Wang et al., 2013). This mixed mode of selection helped in overcoming the drawbacks of aptamers that were raised against non-native recombinant proteins using the aptamers to bind against the native protein.

Aptamer aided electrochemical sensors are also being used efficiently for pathogen detection. In packaged food and water, E. Coli contamination was detected as low as 10cells/ml using aptamer-nanozyme technology on electrochemical platform. Sharma et.al. exploited the inherent peroxidase property of gold nanoparticle which when present with bare surface oxidises TMB turning its colour into blue. E. Coli specific aptamer turned off the reaction by coating AuNP surface in absence of the pathogen and the reaction was turned on by aptamer-pathogen interaction. Finally H2SO4 mediated quenching of this reaction is detected with electrochemical probe and captured electrical signal was found to be proportional to bacterial load. This application can challenge available detection methods by ease of handling, sensitivity and cost effectiveness (R. C. Das, B; Kapil, A;Sharma, T, K, 2020). The same principle was also applied by this group to detect other pathogens (R. D. Das, A; Kapil, A; Bansal, V; Sharma, T, K, 2019). In a separate study, electrochemical sensor was designed using methylene blue (MB) conjugated aptamer to detect tuberculosis meningitis in patient samples where the electron transfer property of MB was used to design probe and the signal was turned off upon aptamer-antigen binding (R. D. Das, A; Mishra, S, K; Haldar, S; Sharma, N; Bansal, A; Ahmad, Y; Kumar, A; Tyagi, J, S; Sharma, T, K).

Some commercially available aptamer based diagnostic products are listed in Table 1.

Therapeutics

In therapeutics, aptamer can act either as a carrier for therapeutic agents or as an inhibitory molecule itself. Aptamer conjugated with liposome or nanoparticle helps in targeted drug delivery. Nano particles such as QD, AuNP, GO, hydrogels etc. act as vehicle, whereas aptamer coating helps in directing by binding with respective target molecule. Aptamer against a tumor biomarker named prostate-specific membrane antigen (PSMA) was affixed on a 2D structure of GO. This 2D structure was converted into a 3D structure with the aid of PEG to make a sieve where circulating tumor cells (CTC) of prostate cancer were being trapped due to presence of PSMA specific aptamer. Although this construct was employed to detect CTC in blood (Rothlisberger, Gasse, & Hollenstein, 2017; Viraka Nellore et al., 2015), similar approaches can be performed for targeted drug delivery for therapeutic purposes.

Liposome, an artificial, small, lipid bilayer vesicle has been efficiently used since a long time as a cargo for drug delivery system. Huwyler, et al. designed monoclonal antibody conjugated liposomes to encapsulate and deliver daunomycin labelled with radioisotope tritium directly to target cells (Huwyler, Wu, & Pardridge, 1996). But several investigators replaced this antibody coating approach by aptamer. By click reaction, a PEGylated liposome was engineered in such a way to carry cancer stem cell (CSC) marker CD44 specific aptamer onto it. This aptamer-liposome construct acted as an efficient drug delivery system for CD44 positive cells (Alshaer, Hillaireau, Vergnaud, Ismail, & Fattal, 2015). The same approach was earlier taken by Kang,

et al. for drug delivery to leukaemia cell line CEM-CCRF by using sgc8 conjugated PEG functionalised liposome (Kang, O'Donoghue, Liu, & Tan, 2010) (Figure 6A). On the other side, Plourde, et al.exploited the property of binding of nucleic acids to intercalating drugs like Doxorubicin (Dox). Encapsulation of drug-aptamer complex into cationic liposome (Figure 6B) with the aid of electrostatic interaction has shown increased Dox incorporation into liposomes than the negative control. They have also shown that in case of taubromycin, a lipophilic drug usually having low capacity of loading into liposome, aptamer helped to overcome this drawback and finally increased the loading capacity up to six times (Plourde et al., 2017). Tumor targeted delivery of nanomaterial encapsulated chemotherapeutic drug is also achieved by aptamer. PEG functionalised biocompatible nanomaterial is used to encapsulate Docetaxel which has shown potential cytotoxic effects in prostate cancer earlier. Its efficacy is enhanced by coupling PSMA specific aptamer with drug-nanoparticle bioconjugate by carbodiimide chemistry. This RNA aptamer helps in uptake of this bioconjugate specifically to PSMA expressing prostate epithelial cells (Farokhzad et al., 2006).

Besides drugs, aptamer has been used as a carrier for other nucleic acids like miRNA, siRNA, DNAzyme which act as gene regulatory elements. One of such example is aptamer-siRNA chimera (Figure 6C) where efficacy of siRNA was improved as well as targeted delivery was also achieved (McNamara et al., 2006). To construct aptamer-siRNA chimera, a ribonucleic acid aptamer A10 was connected to two different siRNAs against two genes i.e. polo-like kinase 1 (PLK1) and B-cell lymphoma 2 (BCL2). These construct targeted PSMA overexpressed tumors where aptamer bound with PSMA and siRNA helped in silencing their respective genes leading to high therapeutic impact. Jeong, et al. took almost a similar approach to overcome multidrug resistance of breast cancer cell line for which they targeted the underlying anti-apoptotic pathway by BCL2 gene silencing. A polyvalent aptamer-doxorubicin-siRNA chimera was designed to target multidrug resistant MCF7 (MDR-MCF7) with anti Mucin 1 aptamer and BCL2 specific siRNA. Silencing BCL2 mediated anti-apoptotic pathway triggered the sensitivity of tumor cells towards the drug Doxorubicin. This combinatorial pharmacogenomic approach of silencing anti-apoptotic pathway and Doxorubicin release finally helped to accelerate the mortality of cancer cells via activating apoptotic caspase-3/7 pathway (Jeong et al., 2017). Therapeutic agents can also be directly guided to their specific target either by intercalation with aptamer or by complex formation via short linkers (Figure 6D). For an example, Dox was linked to sgc8c-aptamer by a short linker. The linker was connected to aptamer and Dox via click reaction and hydrazone moiety respectively (Y. F. Huang et al., 2009). Unconjugated Dox was found to be more toxic to non-targeted cells than the aptamer-Dox conjugate as the later one specifically killed the target cells only.

Aptamer itself can act as inhibitor by binding and inhibiting the interaction of its target protein with the downstream partners and thus impede signalling pathways. Yang, et al. demonstrated an aptamer molecule against envelop protein E1E2 of Hepatitis C virus. This aptamer molecule was found to inhibit bacterial infection (Yang et al., 2013). In another study, it was reported that Spiegelmer against CXCL12, named NOX-A12, inhibits interaction between CXCR4 and its ligand CXCL12 (Susek, Karvouni, Alici, & Lundqvist, 2018). Clinical trials are ongoing to check the role of NOX-A12 (Olaptesed pegol) in different aspects of pancreatic and colorectal cancers like increasing the infiltration rate of immune cells as well as sensitivity of tumors to checkpoint inhibitors along with inhibition of tumor repair mechanism. CXCR4-CXCL12 interaction triggers tumor cell growth, migration, and invasion. Hence this inhibitory aptamer undoubtedly have a great potential as a drug molecule (Susek et al., 2018).

Aptamers in clinical trials: Macugen (Pegaptanib sodium) was approved as a drug after getting clearance from FDA (U.S. Food and Drug Administration) in the year of 2004 to treat patients suffering from age related macular degradation (AMD). Pegaptanib was developed for anti-VEGF therapy as VEGF plays crucial role in ocular neovascularisation (Ng et al., 2006). Human VEGFA contains several isoforms due to alternative splicing among which VEGF165 is predominant (Ferrara, Gerber, & LeCouter, 2003). VEGF165 carries both of the heparin binding (HBD) and receptor binding (RBD) domains in which HBD is exclusive for VEGF165 and does not present in other isoforms including VEGF121 (Ferrara et al., 2003), (J. H. Lee et al., 2005). Macugen was developed against VEGF 165 with several modifications like PEGylation and base modifications to improve its stability and circulation time in body fluid (Ruckman et al., 1998). Though it cannot bind with RBD region, it inhibits angiogenesis by binding with HBD and thus impeding

receptor binding to RBD through some steric hindrance or inhibiting the interaction of VEGF 165 with Proteoglycan, Heparan sulphate or Neuropilin1 (J. H. Lee et al., 2005). Inspite of its early success, Macugen failed to become the most promising candidate for treating AMD as it cannot inhibit the VEGF-VEGFR interaction by RBD and thus VEGF mediated angiogenesis. mAb based drug Lucentis is now preferred over Macugen as it can bind with RBD of all the isoforms and have broad range of target recognition (Chen et al., 1999), but an aptamer against the same target domain may able to acquire the equal potential. Nonaka, et al. designed an aptamer V7t1 exclusively against RBD which is able to interact with both the isoforms VEGF165 and VEGF121 (Nonaka, Sode, & Ikebukuro, 2010). They have screened this aptamer in order to increase the affinity of detection by constructing a bivalent aptamer using HBD specific del5_1 (Hasegawa et al., 2008) and RBD specific V7t1 (Nonaka et al., 2010). The potential role of V7t1 aptamer as a better substitute of Lucentis should be explored and requires extensive study. Currently, multiple aptamers are in phase I, phase II and phase III clinical trials and waiting for approval, some of which are listed in Table 2.

FUTURE PROSPECT: Aptamers in stem cell research, single cell proteomics and immunotherapy

Stem cells contain some unique features or markers contrasted from differentiated cells. Aptamers against these markers are being used in stem cell recognition in mixed population, stem cell isolation, stem cell targeted therapy and tracking or imaging stem cell differentiation. Iwagawa, et al. developed three aptamers (L1-65, L2-2, and L3-3) against mouse embryonic stem cells. These aptamers' targets are expected to express in the transitional state of differentiation but not in fully differentiated cell lines (NB2a, A9, C2C12). These are expected to be helpful in tracking embryonic stem cell differentiation into ectodermal and mesodermal cells (Iwagawa, Ohuchi, Watanabe, & Nakamura, 2012). An interesting work by Yoon, et al. showed CD31 specific aptamer can isolate endothelial progenitor cell from mixed population which in turn when transplanted to murine hind limb ischemia mice model can restore blood flow (J. W. Yoon et al., 2015).

Tumour initiating cells (TICs) or cancer stem cells (CSCs) are the main culprit behind therapy resistance, cancer recurrence, metastatic spread, etc. Identification of these cells itself is a challenge. Kim, et al. took cell SELEX approach to develop aptamers against Glioblastoma (GBM) TICs where for positive selection they have used TICs and for negative selection human neural progenitor cells (NPCs) has been used. These TICs specific aptamer may have potential for several applications, like drug therapy for GBM, imaging of tumour site, exploring mechanism and subtypes of GBM etc. (Kim et al., 2013). Besides it, EpCAM specific aptamer has been reported to be used to isolate EpCAM positive cells from mixed population of cells (Y. Song et al., 2013). Several aptamers, available against CSC markers (CD44, CD133, ABCG2, etc.), can be used as a bait to trap caner stem cell from heterogeneous population (Figure 7A). Single cell proteomics is basically the snapshot of protein landscape of a single cell for a given time (Minakshi, 2019). Single cell omics is a powerful depiction of tumor heterogeneity at single cell level among which scProteomics data is the most reliable one as it shows the actual translational status of each and every cell. But available technologies (Fluorescence flow cytometry, Mass cytometry etc.) do have several pitfalls like poor sensitivity, inefficient sampling procedure, reduced multiplexing capacity etc. (Minakshi, 2019). Analysis of maximum 50-100 proteins has been achieved till date in a single go which is actually very minute part of the entire proteome (Su, Shi, & Wei, 2017). This drawback can be overcome using nucleotide aptamers. Aptamers can be conjugated with different heavy metal ions and used in Mass CyTOF in place of antibody that could make the technique cost effective. On top of it, hundreds of thousands aptamers, bound to a single cell with their respective protein targets, can be screened simultaneously using Targeted Sequencing. These aptamer should be tagged with a common unique sequence that must not present within human genome. This unique sequence can act as an adaptor and be captured by common complementary sequence. Single Cell Targeted Sequencing can help to sequence only these captured aptamer molecules rather than the whole genome which would reduce the time as well as cost. This approach can finally give the copy numbers of different protein bound aptamers which in turn provides the final status of the expression level of corresponding proteins i.e. proteomics data with single cell resolution (Figure 7B). One of the recent advancement in immunotherapy is the application of bispecific antibody (bsAb), a monoclonal antibody (mAb) designed against two different antigens (Sedykh, Prinz, Buneva, & Nevinsky, 2018), (Labrijn, Janmaat, Reichert, & Parren, 2019). Two

different heavy and light chain variable regions specific for two different proteins or antigens are engineered together into a single molecule in order to construct a bsAb which can bind both of the corresponding targets simultaneously or sequentially (Labrijn et al., 2019). Target molecules can be present in same cell (cis interaction) or in different cells (trans interaction) (Labrijn et al., 2019). This crosstalk helps in synergistic outcome by recruiting effector molecules or cell. Bilantumomab, the first clinically approved bsAB was engineered using VL region from two different mAb against T cell specific CD3 and B cell specific CD19 respectively. It engages CD3+ cytotoxic T cells to interact with CD19+ lymphoma cells and helps in tumor cell mortality at a low dosage (Sedykh et al., 2018). This principle is being applied in making of multispecific antibody (Labrijn et al., 2019). The advantage of bispecific or multispecific antibody is low level of toxicity than simultaneous use of their parent monoclonal counterparts (Sedykh et al., 2018). This idea can lead to a new direction in aptamer therapeutics. Bispecific or multispecific aptamers, synthesized chemically using different available linkers, can be a better choice over complicated in vivo synthesis of bsAb. Linker type and length should be optimized such that proper binding of desired cis or trans mode could be achieved without any steric hindrance. This may have promising implementations in different human diseases including malignancies where aptamers against different target proteins act together in activation or/and inhibition of their respective binding partners to alter different signalling pathways. Synthesis, binding property, affinity, mode of action, therapeutic applications of multispecific or multivalent aptamer demands extensive study that would explore a new horizon in near future. Interpatient and intratumor heterogeneity is the common reason for the failure of prevailing chemotherapy. Thus precision medicine is the need of the hour for the success of cancer treatment. The aptamers identified by SELEX (via biomolecule and/or Cell based) against heterogeneous targets or heterogeneous population of cancer cells is anticipated to strengthen the efficacy of existing cancer therapies with reduced side effects.

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TABLE 1: Aptamer based products in diagnostic pipeline

PRODUCT NAME	APPLICATIONS	DETECTI
Aptocyto	Isolation of biomarker positive cells from heterogeneous population using magnetic bead	Flowcytom
Aptoprep	Aptamer based protein (biomarker) pull down from sample using magnetics bead	Fluorescen
AflaSense	Fungal aflatoxin detection in food industry	Fluorescen

PRODUCT NAME	APPLICATIONS	DETECTI
CibusDx	Food borne and water borne pathogen detection	Electroche
OLIGOBIND	Active Thrombin level detection in plasma sample	Fluorogeni
OTASense	Fungal Ochratoxin A detection in food industry	Fluorescen
SOMAscan	Novel biomarker detection associated with different diseases	SOMAmer

TABLE 2: Aptamers in Different Phases of Clinical Trials

APTAMER	TARGET	MEDICAL CONDITION	CLINICAL STATUS	REFERENCES	TRIAL IDENTIFIER (Clinical Trials.gov Identifier)
ARC1779	von Willebrand factor	von Willebrand's disease	Phase III (Awaiting)	(Gilbert et al., 2007),(Spiel et al., 2009),(Markus et al., 2011)	NCT00432770 NCT00507338 NCT00632242 NCT00694785 NCT00726544 NCT00742612
ARC1905	Complement Factor 5 (C5)	Neovascular age related macular degeneration	Phase I	(Biesecker, Dihel, Enney, & Bendele, 1999)	NCT00709527 NCT00950638 NCT02686658 NCT03362190 NCT03364153 NCT03374670
ARC19499	Tissue Factor Pathway Inhibitor (TFPI)	Haemophilia	Terminated	(Waters et al., 2011)	NCT01191372
AS1411	Nucleolin	Advanced solid tumors, Renal cell carcinoma, Acute myeloid leukaemia (AML)	Phase III (Awaiting)	(Rosenberg et al., 2014),(Reyes-Reyes, Salipur, Shams, Forsthoefel, & Bates, 2015)	NCT00512083 NCT00740441 NCT00881244 NCT01034410
E10030	PDGF	Von Hippel Lindau disease, Age-related macular degeneration	Phase III (Awaiting)	(Jaffe et al., 2016),(Jaffe et al., 2017)	NCT00569140 NCT01089517 NCT01940887 NCT01940900 NCT01944839 NCT02591914 NCT02214628 NCT02859441
EYE001	VEGF	Wet age-related macular degeneration	Phase I (Completed)	(Carrasquillo et al., 2003),(Eyetech Study, 2002)	NCT00021736 NCT00040313 NCT00056199 NCT00150202 NCT00239928 NCT00321997 NCT00736307

APTAMER	TARGET	MEDICAL CONDITION	CLINICAL STATUS	REFERENCES	TRIAL IDENTIFIER (Clinical Trials.gov Identifier)
NOX-A12	CXCL12	Chronic lymphocytic Leukaemia, Multiple myelomas, Metastatic pancreatic and colorectal cancer	Phase II	(Roccaro et al., 2014),(Hoellenriegel et al., 2014)	NCT00976378 NCT01194934 NCT01486797 NCT01521533 NCT01947712 NCT03168139
NOX-E36	CCL2	Type II diabetes mellitus	Phase II	(Oberthur et al., 2015),(Menne et al., 2017)	NCT00976729 NCT01085292 NCT01372124 NCT01547897
NOX-H94	Hepcidin	Anaemia of chronic inflammation	Phase I (Completed)	(Schwoebel et al., 2013)	NCT01372137 NCT01522794 NCT01691040 NCT02079896
NU172	Thrombin	Coronary artery disease	Phase II	(Troisi, Napolitano, Spiridonova, Russo Krauss, & Sica, 2018)	NCT00808964
Pegaptanib (Macugen)	VEGF165	Age related macular Degeneration (AMD), Diabetic macular oedema, Uveitis, Diabetic cystoid oedema, Proliferative diabetic Retinopathy (PDR)	In market	(Ruckman et al., 1998),(Gragoudas et al., 2004),(Manresa, Mulero, Losada, & Zafrilla, 2015)	NCT00406107 NCT00549055 NCT00788177 NCT00790803 NCT01175070 NCT01486771 NCT01487070 NCT01573572
RB006	Factor IX	Coronary artery disease	Phase III (Awaiting)	(Povsic, Wargin, et al., 2011),(Staudacher et al., 2017)	NCT00715455 NCT00932100 NCT01872572
REG1 System	Factor IX	Coronary artery disease, Acute coronary syndrome	Phase III (Terminated)	(Cohen et al., 2010),(Povsic, Cohen, et al., 2011),(Tanaka, Szlam, Rusconi, & Levy, 2009)	NCT00113997 NCT00715455 NCT00932100 NCT01848106 NCT02435082 NCT02797535 etc.

APTAMER	TARGET	MEDICAL CONDITION	CLINICAL STATUS	REFERENCES	TRIAL IDENTIFIER (Clinical Trials.gov Identifier)
Sgc8	PTK7	Colorectal cancer	Recruiting	(Shangguan et al., 2006),(Shangguan et al., 2008)	NCT03385148

FIGURE LEGENDS

Figure 1: Schematic representation of Systemic Evolution of Ligands by Exponential Enrichment (SELEX). Random ssDNA pool, having a variable sequence flanked by two constant sequences, is heated followed by snapcooling. Oligos attaining secondary structure bind with target and is eluted after washing. Eluted oligos are amplified and applied for next round. Same process is repeated for multiple times in cyclical fashion to select only the specific binders ruling out the nonspecific ones. In the modified version of SELEX like Negative SELEX purification matrix is used after few rounds of selection to rule out nonspecific binders whereas in Counter SELEX target analogue is used for the same purpose which finally enhances the stringency of the process.

Figure 2: Schematic representation bivalent aptamer formation. A) Aptamer mediated Proximity Ligation Assay: Aptamer 1 and Aptamer 2 shown in black bind with two different regions of same protein. These two are extended by extension sequences shown in red and purple respectively. These two extensions are ligated upon hybridization with common connector shown in green. B) Aptamer dimerization via linker: Two different aptamers bind with thrombin protein at two different domains i.e. fibrinogen binding exosite (blue) and heparin binding exosite (green) respectively and connected by various length of poly dT linker among which five thymine linker is the most suitable one.

Figure 3: Schematic representation of different modifications in aptamer . Modifications can be done at 5' and 3' ends of sugar phosphate backbone, by substitution of phosphodiester linkage, by using modified bases or by changing configuration of sugar.

Figure 4: Schematic representation of aptamer mediated detection. (A) Target induced structural conversion mode: Fluorophore tagged aptamer binds with a short oligonucleotide sequence having quencher molecule by base pair complementarity. In absence of target molecule, quencher molecule quenches fluorophore. Upon target binding, the conformational change in aptamer helps to release qDNA and finally fluoresce. (B) Direct binding based mode: Fluorophore tagged aptamer directly binds with target. (C) Target induced dissociation mode: Gold nanoparticle (AuNP) are coated onto aptamers. Salt mediated aggregation of AuNPs after aptamer-target interaction finally leads to target detection by a colorimetric assay.

F*igure 5*: Schematic representation of aptamer mediated diagnostic assays. (A) Lateral Flow Assay (LFA): Here an aptamer based strip sensor is used which is more efficient than antibody based strip sensor. After sample loading, target molecule binds with AuNP conjugated primary aptamer. As the sample migrates, target-aptamer-AuNP conjugate binds with biotinylated secondary aptamer. Finally red band is shown at test zone due to AuNP accumulation. In control zone AuNP-primary aptamer binds with control DNA to give the second red band. When target molecule is absent only the second red band is generated. (B) Conventional ELISA: Here antibody is used to detect the target (C) ALISA: Here aptamer is used for target detection in place of antibody.

Figure 6: Schematic representation of role of aptamer in therapeutics. (A) Aptamer coated liposome: Drug containing liposome is conjugated with cell surface marker specific aptamer which helps in targeted delivery

of the drug. (B) Liposome containing aptamer-drug conjugate: Drug like Doxorubicin is intercalated with aptamer and drug-aptamer conjugate is coated by cationic liposome which enhances the delivery of the drug. (C) Aptamer-siRNA-drug chimera: Streptavidin has four binding domains where biotinylated aptamer, drug and siRNA bind. Aptamer helps in targeted delivery of siRNA and drug whereas siRNA and drug help in gene silencing and therapeutics at desired target. (D) Linker joined aptamer-drug conjugate: Short linkers are used to join drug with aptamer by click reaction.

Figure 7: Schematic representation of Future applications of aptamer: (A) Aptamer mediated cancer stem cell isolation: In tumor site there is mixed cell population. CSC specific aptamer can only binds to the particular CSC marker against which it is generated. Aptamer bound CSCs are sorted out from the heterogeneous population. (B) Role of aptamer in Single Cell proteomics: Unique sequence tagged aptamers bind with different protein targets present on a single cell. Targeted Single cell sequencing determines the copy number of each of the bound aptamers which in turn provide the expression level of corresponding proteins or the nature of ScProteomics.