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REVIEW ARTICLE

Aptamer and its role in diagnostics

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Abstract: Aptamers are new class of recognizing agents which are being used in diagnostics and therapeutics. They are single strand DNA or RNA molecules and are selected against targets by systematic evolution of ligands by exponential enrichment (SELEX) method. This method was developed in 1990 by Turk and Gold. These days high through put version of SELEX is being used for quick selection of aptamer, working on same principle that was developed in 1990. It is believed that in near future aptamers could replace monoclonal antibody. The biggest advantage of using aptamers is that the process is *in vitro* in nature and does not require the use of animals; further properties of aptamers are comparable or even better than antibodies. Aptamers based sensors can be used for detection of toxic chemicals, pathogens, antibiotics etc. Although they are in the preliminary stages of development, results are encouraging and it seems that aptamer research has a very bright future.

Key words: Aptamer; SELEX; ALISA; Gold nanoparticles; Electrochemical method

Introduction

Aptamer are new class of recognizing agents that came into light in 1990[1]. Turk and Gold developed a method known as systematic evolution of ligands by exponential enrichment or popularly called as SELEX method for selection of aptamers against target. The method is *in vitro* in nature and does not require the use of animals. MonoLEX a new method is also being used which requires single step process as compared to multi step process in SELEX for selection of aptamers. This process is cost effective and requires less time. Aptamers are synthetic single strand DNA or RNA molecules in nature. Due to flexibility and small size they form non-covalent bond with target in specific condition at room temperature. It is also believed that due to this compatibility of aptamers, they can be generated against any target molecules. Aptamers are biggest competitor of monoclonal antibody and it is likely that they will replace antibody in near future for diagnostics and therapeutics purposes. From last 10 to 15 years many research papers have been published giving information on progress of aptamer research. Some of the key companies working on aptamers include Aptagen, Aptamer group, Aptamer Sciences, Somalogic, NOXXAN Pharma and others and it is believed that global market of aptamer research will reach 5.4 billion dollars by 2019.

Aptamer

The term aptamer is derived from Latin and Greak words, "aptus" (means fitting) and "meros" (means particle)[2]. Aptamers are 50-100 base long, ssDNA or RNA molecules, capable of recognizing a wide range of target molecules. These are selected from nucleotide library containing millions to billions random sequences, through a combinatorial approach known as systematic evolution of ligands by exponential enrichment (SELEX). They can bind to nucleic acids[3,4], proteins[5,6] to small organic compounds[7,8].

SELEX (systematic evolution of ligands by exponential enrichment) method

SELEX method is an iterative process which is required during aptamer selection. The method comprises of following steps:

Incubation of aptamer library with target: In this process aptamer library containing more than 10^{15} random nucleotide sequences is incubated with target at room temperature for 30 to 45 minutes duration. Aptamer library contain identical 10 to 15 bases at 5' and 3' ends; middle region contains 40 to 60 random bases, creating multiple ssDNA (aptamers) having same 5' and 3' ends but unique bases at middle region (Figure 1A).

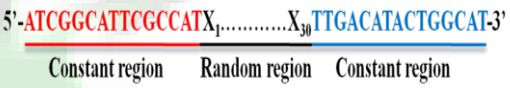

Figure 1A Design of sequences in the ssDNA library; the random region in the middle differs in each of the molecule of ssDNA library, the constant regions in the ends are meant for primer annealing for amplification of enriched pools obtained after each round of SELEX.

Removal of bound aptamer from target: During interaction of aptamers with target, many aptamers fail to recognize target due to less affinity but some of them have high affinity for target and they bind to it[9]. Unbound aptamers are removed by washing steps and are discarded while bound aptamers are released from target by heating at high temperature[10] or incubating with urea solution[11] or using urea, EDTA and high temperature together[12,13]. Quantification of both bound as well as unbound oligonucleotides is carried out in each

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round of SELEX. Aptamers are fluorescently tagged[13,14] or radioactive labelled[15,16], while others measure O.D at 260 nm[17,18] for this purpose.

Amplification of eluted aptamers: Eluted aptamers is amplified by polymerase chain reaction method (PCR). So they can be used for next round of SELEX.

Conversion of dsDNA to ssDNA: The main obstacle of this method is conversion of dsDNA to ssDNA. These days several strategies are employed to get ssDNA from dsDNA for example- Terminator lengthener method- A hexaethyleneglycol (HEGL) spacer and an extension of several adenine nucleotides (poly A) are added at the 5'–end of the primer for undesired strand. The HEGL–spacer acts as a terminator for Taq polymerase. The elongation of the desired strand stops, while the undesired strand grows further. This causes differences in length of both strands of the DNA that can be separated by denaturing Polyacrylamide gel electrophoresis (PAGE) method[19]. Alternatively, ribonucleotide moiety is introduced at 3' end of primer corresponding to undesired strand to allow its cleavage by alkaline hydrolysis and subsequent separation in denaturing PAGE[20]. In another method one of the strands is biotinylated and dsDNA is separated on streptavidin coated surface[21]. Asymmetric PCR uses primer for desired strand in larger amounts to get that strand in single strand form[22]. Citartan and co-workers, 2011[23] have described the method where 5' end of primer corresponding to undesired strand is phosphorylated and undesired strand is degraded by lamda exonuclease to get desired ssDNA after amplification process. In snap cooling method dsDNA is heated at 85°C for 15 minutes followed by quick cooling to get ssDNA[24].

All four steps together referred as one round of SELEX. Each round of SELEX (Figure 1B) gives the chance for selection of high affinity aptamers, removing those having less affinity. A stage is come which is known as saturation point where further enrichment of high affinity aptamers are no more required, ending SELEX process. In general, 10 to 15 rounds of SELEX are performed to get aptamers having high affinity for target. In SELEX not only target molecules are used during selection process but also counter selectors are also being used[12]. These molecules are structurally similar to target but not identical to them. This process further increases the specificity in SELEX for aptamer selection and thus removing aptamers having affinity for counter molecules. Negative selection is also included in SELEX process[25] where solid naked support molecules are incubated with aptamers to remove those one having affinity for support. As most of the target is coated on support molecule, but it is never hundred percent and during SELEX, ssDNA molecules can also bind with support. This selection process is also included in SELEX to increase the specificity.

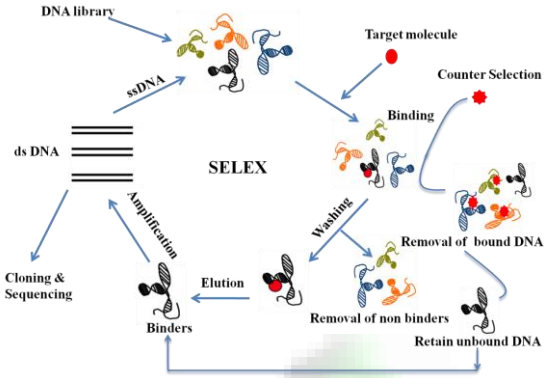

Figure 1B: SELEX process

DNA or RNA SELEX: The basic steps of DNA or RNA SELEX are same. In DNA SELEX, DNA library is same kind of as mentioned in SELEX section. However, in RNA SELEX method, random ssDNA library has to be transformed into a RNA library before starting the first round of SELEX. In this process T7 promoter is added within the constant region of DNA library to produce RNA library by T7 RNA polymerase. This creates randomized RNA library for SELEX. In each round of SELEX eluted RNA is converted to cDNA by reverse transcription process, ssDNA is amplified to dsDNA by PCR and converted back to RNA by *in vitro* transcription with the help of T7 promoter and T7 polymerase[26] (Figure 1C). As RNA is very sensitive to nuclease action, DNA based SELEX is in more demand further it is easy to amplify DNA as compared to RNA.

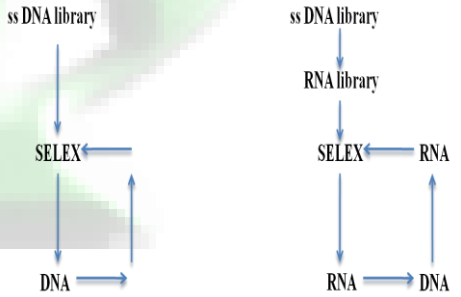

Figure 1C: DNA & RNA SELEX

Cloning, sequencing, characterization and K_d determination of enriched aptamer pool

After the end of SELEX rounds enriched pool of aptamers are cloned into an appropriate vector and are sequenced[27]. Structural motifs are predicted by M-fold or other software to find putative regions in aptamers that might be involved in interaction with target. For binding studies dissociation constant of each selected aptamers is calculated, aptamers having K_d in nanomolar or in picomolar range are selected for biosensor development.

Variants of SELEX

Magnetic bead-based SELEX: Magnetic beads are in demand for SELEX process where they act as solid support system[12,25,28]. This is because of easy separation of bound and unbound aptamers in presence of magnetic field and availability of magnetic beads with different class of functional groups that can covalently bind with different targets.

Capillary electrophoresis SELEX: In this process aptamers are interacted with target in free solution and then injected in gel based system. The mobility of free aptamer, target and aptamer-target will be different and according to speed in gel they form three distinct bands which can easily be elute out. This method is fast and reduces nonspecific binding[29,30].

Primer free SELEX: Aptamers always contain constant regions at 5' and 3' ends for their easy amplification, however it also creates a problem of proper working of aptamers in detection of target in real samples. To overcome this situation truncated versions of aptamers are tried to get optimal result. In primer free SELEX, DNA library contains only few bases at constant regions avoiding truncation process, further it is easy to synthesize chemically, short length of ssDNA (Aptamers). Primer free SELEX method was used to select aptamers against calcitonin gene-related peptide[31].

FluMag-SELEX: This method was developed by Stoltenburg and co-workers in 2005[13]. In this method target is immobilized on magnetic beads while aptamers are tagged at 5' end by fluorescent agent, that's why it is called as fluorescent magnetic bead SELEX or FluMag SELEX. This process virtually eliminates radiolabeling of DNA by fluorescent agent. Immobilization process further helps in requirement of very small amount of target, rapid and efficient separation of bound and unbound aptamers by magnets and rigorous washing steps. Stoltenburg and co-workers selected streptavidin specific aptamers by this method.

Photo-SELEX: In Photo-SELEX, native base of RNA or ssDNA is replaced by a modified nucleotide. The modified base is activated at specific wavelength of light. Activated base of aptamers form a photo cross-links with aromatic amino acids of target protein, making a very strong bond between target and aptamers. Golden *et al.*, 2000[32] used this method to select aptamers against bFGF. They replaced uridine base of RNA aptamers by 5-bromo-2'-deoxyuridine which absorbs UV light at 310 nm. due to presence of bromouracil chromophore.

Cell-SELEX: Aptmares can be selected against particular extracellular protein present on cell surface or even against whole cell having unique structures by cell SELEX method. In future it will be possible to detect cancer cells from normal ones or cell-targeted drug delivery by this method. Apatmer (Wy-5a) has been selected against prostate cancer cell line PC-3 by cell-SELEX method[33]. In 2011 Kunii *et al,*[34] selected a DNA aptamer against SBC3 marker, found in adherent small cell lung cancer (SCLC) cell line by this method. Also, aptamers have been selected that are specific for cancer cells such as liver cancer cell[35] and leukemic cells[36].

Why aptamers?

Aptamers are synthetic DNA or RNA molecules and are the biggest competitor of monoclonal antibody (Table I).

Table I Aptamer Vs Antibody

Aptamer	Antibody
Small in size and chemically synthesized with no batch to batch variation.	Large in size, requires animal for its production, suffers from batch to batch variation.
Less immunogenic, shelf life is unlimited.	Highly immunogenic, shelf life is limited.
Affinity to target can be increased by employing more stringent condition during SELEX.	This cannot be done with antibodies.
Cost effective and requires less time in production.	Costly and production is time consuming.
Aptamer labelling can be done without losing its binding capability to target.	Antibody can lose its binding capability to target during labelling.
Aptamer undergo reversible change during temperature insult.	Antibody loses its activity irreversible during temperature insult.
Aptamers can be generated against molecules producing little immune response or even against toxic molecules.	Limited to molecules that produces an immune response.

Application of aptamers in diagnostics

i) ALISA method- ALISA- aptamer linked immobilized sorbent assay uses aptamer as a recognizing agent for targets. The method is very similar to competitive ELISA where immobilized antibody is used for detection purpose. Biotinylated aptamer is incubated with streptavidin coated plate in presence of NaCl-citrate buffer for their immobilization. After immobilization, target present in sample is added which competes with a constant concentration of target bound HRP for limited number of aptamers. High amount of target gives low intense blue color in 96 well plate while low amount gives intense blue color in presence of TMB. In ELISA both primary and secondary antibodies are required for detection; however, this process is simplified in ALISA which uses only single aptamer as recognizing agent (Figure 2). Thus reducing overall time in detection of target molecules present in sample. This method was

used by Vivekananda and Kiel, 2006[37] for detection of tularemia antigen and claiming that aptamer method was more sensitive to antibody based method. The same method was also used in detection of betacasomorphin-7 peptide[38]. NeXstar Company has developed ELONA (enzyme linked oligonucleotide assay) method (similar to ALISA) for detection of *Leishmania infantum* H2A antigen[39,40].

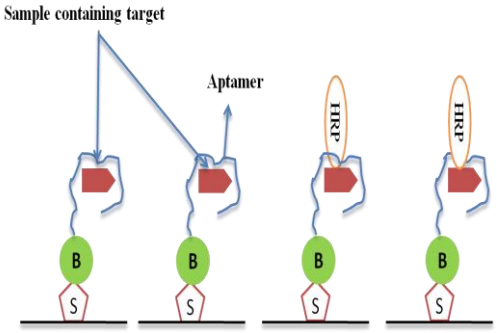

Figure 2: ALISA method

iia) Electrochemical method

As the name suggests this method works on change in electric current in response to electron donating compound. Biotinylated aptamers are immobilized on electrode which has streptavidin on its surface. Electron donor compound such as methylene blue (MB)[41,42] or ferrocene[43]are tagged with aptamer in such a manner that an electrical communication is established between these compounds with an electrode. This produces an electrical current which is detected by detector. As target is bound with aptamer, conformation of aptamer is changed in such a manner that it halts the production of electric current which indicates the detection of target, inhibition in electric current production (turn off) could take place either due to wrapping of aptamer on these compounds during binding with target or removal of electron donor from aptamer or increase in proximity from electrode. In another case binding of target-aptamer complex makes electron producing compound near to electrode. This causes electric current generation (turn on), (Figure 3A). Streptavidin-coated magnetic beads[44], carbon nanotubes[45] and streptavidin/polymer-coated indium-tin oxide electrodes[46] are some the examples which are being used as surface for immobilization of aptamers in this method.

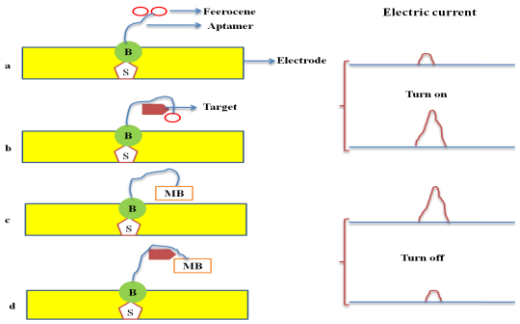

Figure 3A: Turn on/off electrochemical method

iib) Sandwich electrochemical method

The method uses two aptamers specific to target, one is immobilized on electrode surface while second contains platinum (Pt) nanoparticles on its surface. As target present in sample passes to electrode surface, aptamer present on surface interacts with it and inhibit its further movement. Now the platinum tagged aptamer is passed that interacts with target (specific to targets second site), platinum nanoparticles reduces H_2O_2 which produces electrons. Electrons generate electric current in presence of electrode. This method can only be employed with target having large size and aptamers must be generated for two specific sites of that target which a tedious process, thrombin protein was detected by this method[47] (Fig. 3B). In second approach gold nanoparticles tagged aptamers are used. Electrode surface contains immobilized antibody specific to that target. Target interacts to antibody when sample is passed through it, after this process GNP tagged aptamers are passed where aptamer interacts to the available site of target. Aptamers contains unique Poly A bases at their end which do not participate in interaction. Poly A tail can be broken down from aptamer by nuclease action. These adenine bases help in generation of electric current in presence of graphite electrode, which can be detected[48] (Figure 3B). In other approaches antibody is replaced with aptamer specific to one site of target while second aptamer contains Poly A tail which is tagged on GNP detection. Methylene blue is tagged with aptamer which interacts with target in presence of immobilized antibody in electrochemical method[49,50] (Figure 3B). Although the method is very sensitive but the costs of equipment's are very high, further skill persons are required to perform the experiment. Thrombin[51,52], lysozyme[53] and human IgE[54] are some of the examples that are detected by electrochemical method.

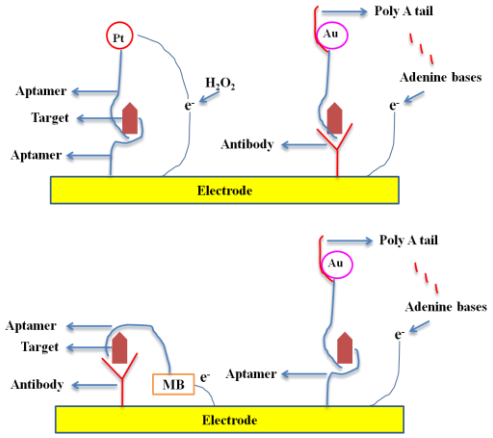

Figure 3B: Different strategies in sandwich electrochemical method

iii) Fluorescence-based optical sensor

Fluorescent labeled aptamers based optical sensors are also very sensitive in detection of target molecules. In this method selected aptamer is tagged with fluorophore at 5' end while 3' is conjugated with quencher. Quencher emits light at longer wavelength and due to proximity with fluorophore it simply quenches the fluorophore which produces light of shorter wave length. The experiment is set as such that at a particular conformation of aptamers, fluorophore does not able to emit any light. But when aptamer binds to target present in sample a conformational change takes place in aptamer making quencher far away from fluorophore that emits light of particular wavelength on excitement (Figure 4A). This method is also known as molecule beacon system. Osborne *et al.,* 1997[55] used this method for detection and measurement of serum amino acid phenylalanine, which is useful in diagnosis of phenylketonuria. Aptamers have also been selected that can distinguish between pathogenic and non-pathogenic forms of prions proteins by using this method[56, 57]. In another method very similar to above method, a compound known as pyrene is used which acts as wave shifter. When tagged to both ends of aptamer, it shows florescence at 400 nm, however as aptamer interacts with target a conformational change takes place in aptamer as a result both pyrene molecules comes closer to each other thus giving florescence at 485 nm (Figure 4B). This method was used in detection of lysozyme in buffer and human serum and could avoid the process of protein purification prior to its detection[58].

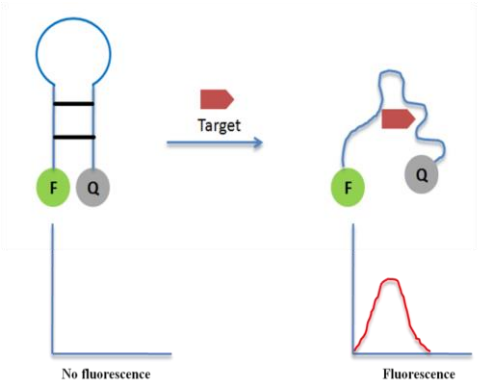

Figure 4A: Molecular beacon method

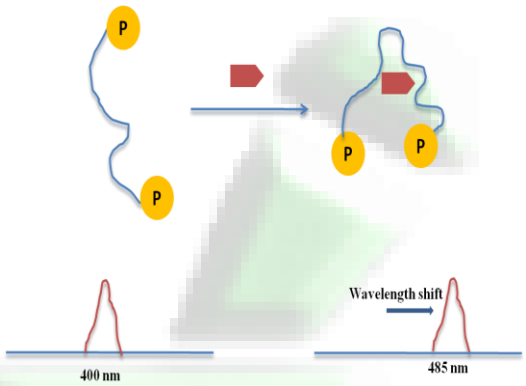

Figure 4B: Pyrene tag aptamer for target detection

iv) Colorimetric-based optical sensor

This method is one of the simplest one that can be used for target detection by aptamers. In general citrate reduction method[59] is used for preparation of gold nanoparticles (GNPs) of size 10 to 30 nm which appears pink in color and gives a characteristic maximum at 520 nm. The charge on GNPs is negative in nature due to presence of citrate ions. The color (pink) of gold nanoparticles (GNPs) changes from pink to purple when sodium chloride (NaCl) is added; this is due to interactions between positively charged sodium ions and GNPs followed by clumping of GNPs together. Aptamers can form noncovalent bond to GNPs increasing overall negative charge on their surfaces and required NaCl concentration for color change which will be higher as compared to naked GNPs (aptamer is not tagged to GNP surface) for clumping. However, as target is added to aptamer coated GNPs solution, a color change takes place from pink to purple at a concentration of NaCl which was used for naked GNPs, clearly showing that due to high affinity of target for aptamers, ssDNA dislodged form the surface of GNPs, making the tolerance for GNPs for NaCl as same as naked GNPs (Figure 5). The color change can be distinguished even by naked eyes. Song *et al.*, 2011 and 2012[60,61] used this method to detect kanamycin and ampicillin antibiotics in milk and food products.

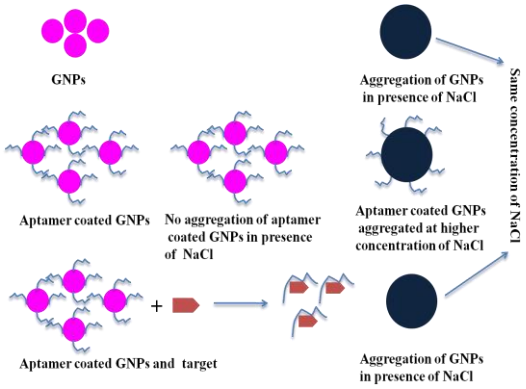

Figure 5: GNP based colorimetric method for target detection

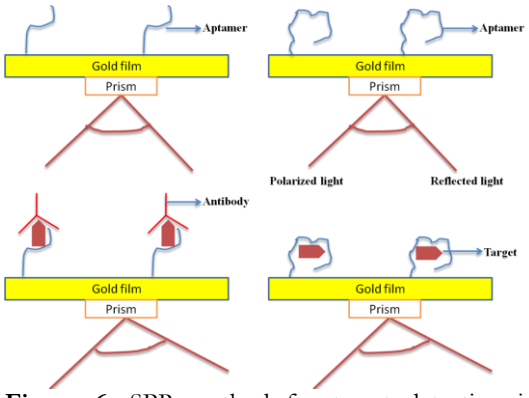

Figure 6: SPR method for target detection in presence of polarized light

v) SPR (surface plasmon resonance) method

This method is highly sensitive in nature and measures change in mass or thickness of the surface on which probe is imprinted. It also contains light reflecting prism that causes reflection of polarized light. Biotinylated aptamer is imprinted on gold film surface by streptavidin -biotin interaction as streptavidin is first imprinted on gold film. A polarized light is passed through the film; light is reflected through prism by forming a resonance angle. When target binds to aptamer, a change in mass or thickness takes place on surface of gold which causes further change in refractive index of the film. This change is recorded by change in angle of resonance (Figure 6). The method was used in detection of neomycin antibiotic where a modified RNA aptamer (2'OMe-RNA) was used and the detection limit was 10 nM[62]. Bruno *et al.*, 2010[63] used fluorescence resonance energy transfer (FRET) and SPR methods to detect crook's strain of *E. coli* by a DNA aptamer and limit of detection was 30, *E. coli* units/ml. Other SPR method uses sandwich SPR that contains either aptamer-antibody or aptamer-aptamer combination for target detection (Figure 6). In one of the approaches α-1 antitrypsin (marker of Alzheimer's disease) sandwiches in between aptamer (tagged to gold electrode) and antibody for its detection from serum[64]. Thrombin protein was detected by this method however here antibody was replaced by second aptamer[65]. Although this method is costly but it is very sensitive, fast and label free in nature.

vi) Lateral flow method

This method works on principal of capillary action which uses nitrocellulose membrane as stating material on which capillary flow takes place. The method works as follows- aptamer is conjugated with gold nanoparticles (GNPs) where 5' end of aptamer contains SH group that binds to GNP. Aptamer also contains few bases at extreme 3' end which is not the part of target binding, also known as linker sequence. Nitrocellulose membrane contains control line which contains imprinted streptavidin on which biotinylated ssDNA having bases which are complementary to variable region of aptamer is immobilized. Test line which is next to control line also contains immobilized ssDNA whose sequence is complementary to bases of linker sequence. When GNP conjugated aptamer flow (in the absence of target) through membrane surface, they encounter with these ssDNA and form two red lines of equal intensity, one indicates control line while next one is a test line (Figure 7A). However, in presence of target most of the aptamers interact with target having no or little affinity for ssDNA present at control line forming a very faint red line while, aptamer-target complex interacts with ssDNA of test line through linker sequence. This incidence forms a very strong red line band on membrane (Figure 7B). Antibody based method is also known as card test method which is very sensitive and reliable and is commercially available for detection of malaria and filarial. A DNA based aptamer lateral flow method was used in detection of Ochratoxin A, a carcinogenic mycotoxin found in cereals and cereal products as a contaminant. The LOD of that system was 0.18 ng/ml[66].

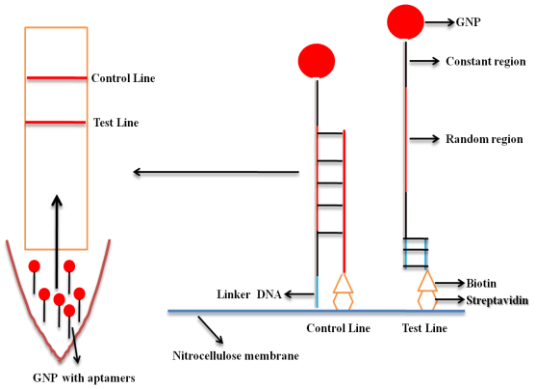

Figure 7A: Lateral flow of aptamer in absence of target

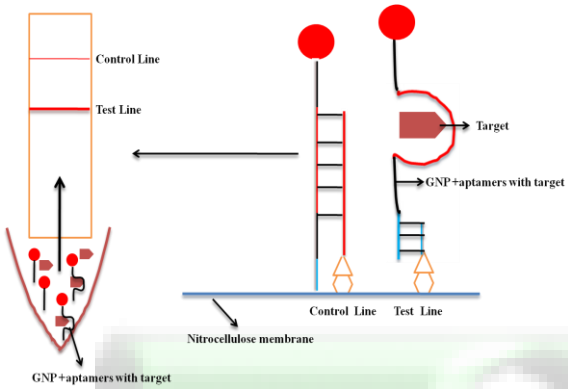

Figure 7B: Lateral flow of aptamer in presence of target

Table II: Aptamers in biosensors

Target	Aptamer	Detection limit	Method/Purpose
Ochratoxin A[73]	DNA	0.07 ng/ml	Electrochemical biosensor for its detection in food products
p53 gene[74]	DNA	10 picomole	SNP detection in p53 gene by GNP method
Toxin B of *Clostridium difficile*[75]	DNA	50 nM	Modified sandwich ELISA method for detection of toxin B in human fecal preparations.
VEGF[76]	DNA	190 pg/ml	Electrochemical biosensor for its detection in blood or serum
IgE[77]	DNA	0.5 nmol/l	Aptamer based quartz crystal biosensor for detecting of IgE
Campylobacter jejuni[78]	DNA	2.5 cfu	Quantum dot sandwich assay for Campylobacter detection in food
Foot and mouth disease[79]	DNA	25-250 ng/ml	Competitive FRET based detection of FMD
Botulinum neurotoxin[80]	DNA	40 pg/ml	Electrochemical biosensor method for its detection
Abrin toxin[81]	DNA	1 nM	Assay developed for its detection in serum using a luminescence switching complex
Cancer cells[82]	DNA	800 cells	GNP based method for detection of circulating cancer cells
Tobramycin[83]	RNA	3.0-72.1 µM	Electrochemical method used during therapy owing to side effects of antibiotic
Daunomycin[84]	DNA	17.1 nM	GNP based method for monitoring its level in food
Kanamycin[85]	DNA	9.4 nM	GNP based method for detection of antibiotic in milk

vii) Imaging by aptamers

Aptamer is conjugated to a fluorophore or other materials such as gadolinium, which is useful in magnetic resonance imaging (MRI). As aptamer is nontoxic and specific in nature this method can be employed in imaging technology. On that basis AS1411, a DNA aptamer is selected against nucleolin transmembrane protein found in cancer cells and tagged with gadolinium for imaging of cancer cells through MRI[67]. RNA aptamer named A10 specific to PSMA of prostate cancer cells was conjugated to iron nanoparticles and was used in cancer detection by MR imaging system[68]. Aptamer has also been tag with ^{64}Cu and used in PET (Positron Emission Tomography) imaging for detection of cancer[69,70]. In another method Indium-111 labeled aptamer was attached to GNP for detection of head and neck cancer by CT (Computed Tomography) scan method[71,72]. Here are some of the examples of aptamers used in biosensor for detection purpose (Table II).

Conclusion

All the aptamer based biosensors are in their developing stages and further investigation is required. We have made lots of progress in understanding the surface chemistry of these new compound, more and more understanding will help in proper DNA immobilization on their surface to get more precise results. All of above mentioned methods are highly sensitive, fast and reliable but at the same time very expensive, required skilled person and they have yet to be commercialized. Using aptamer for diagnosis of target in real sample is always a challenge. Aptamers are yet to show specificity that can match to monoclonal antibody.

Further nuclease sensitivity is always an issue in working with real samples such as serum. Methods have been adopted to make aptamer as serious recognizing agents. It has been seen that immobilizing aptamer from 3' end makes it nuclease resistant further modification at bases of aptamers by chemical functional groups also do the same without losing its specificity. These kinds of approaches increase the specificity, half-life of aptamers thus making it useful in diagnostics and in therapeutics also. New methods have also shown that a very sensitive method can be developed by using both aptamer and monoclonal antibody together. Upcoming methods are also equally good

for other recognizing agents where they can be used alone or in combination with aptamer or antibody. The small size, low cost and easy preparation will further make more investment and research in this field.

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