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ORIGINAL ARTICLES

Aptamer as a proper alternative instead of monoclonal antibody in diagnosis and neutralization of menacing biological agents

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Abstract: *Of the major threats to contemporary mankind, is the use of very dangerous and lethal biological agents as the biological weapons. The first step in confronting with this serious threat after prevention, is the accurate and rapid detection of this agents and neutralization of them. In this article, the role of molecules known as aptamer, has been studied in biological defense against these menacing biological agents. Traditional methods for detection of these agents are based primarily on immuno-affinity assays and the use of antibody molecules. While the modern methods, based on aptamer-affinity assays, are being replaced with traditional methods, due to the abundant advantages of them. The selection and preparation method of specific aptamer with high binding affinity to these biological agents is known as SELEX and the use of magnetic nanoparticles to perform this procedure (Mag-SELEX) is very common. The isolated aptamers with high specificity can also be used in neutralization and inhibition of menacing agents function, in addition to, quick and accurate diagnosis of these agents, utilizing them in nano-biosensors, based on aptamers (as the nano-aptasensors).*

Keywords: *Aptamer, SELEX, nano aptasensor, diagnosis and neutralization, menacing biological agents*

INTRODUCTION

Nowadays virulent and dangerous biological agents which can be used as biological weapons are becoming a considerable threat to us [1]. Some of very important agents used in bioterrorism are namely botulinum neurotoxins which are some of the strongest toxins known to men and produced by the anaerobic bacteria called clostridium botulinum [2]. Botulinum neurotoxins, especially three types of A, B and E, have been known as dangerous biologic weapons. Among these three serotypes, serotype A is more potentially virulent [3]. The first action against these very dangerous biological agents, after prevention is to identify and detect the agent accurately and rapidly and then to neutralize it [4]. Modern methods of identification and neutralization is based on molecules called aptamer [5]. In this article we will first introduce aptamer and chemical modifications possible to make on this molecule to approach certain purposes, and compare them to antibody molecules as a conventional tool in diagnoses and neutralization of the biological and viral agents or their exclusive products (such as toxins and etc.) and we will interpret concisely the works done around the world.

APTAMER, AS A NEW MOLECULAR DEVICE

Aptamer is generated from the Latin word of “Aptus” which means “to fit”. In fact, aptamers are oligopeptides or single stranded oligonucleotides (DNA or RNA) with high affinity for binding to target molecule (Figure 1). They create a bond with high specificity through their third structure to a target molecule [6]. In this article we discuss about oligonucleotide aptamers, especially DNA aptamers. By development of

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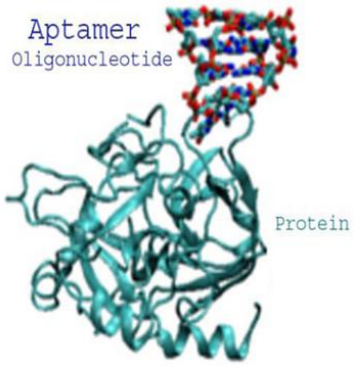
molecular sciences and setting up the SELEX technique, the specific DNA/RNA aptamers have been separated for binding to many molecules. These molecules are consisted of nucleic acids, amino acids, sugars and their polymers, and also mineral compounds, enzymes, growth factors, antibodies, the molecules binding to cells, chitins and even viral components [7].

Figure 1. A schematic representation of the binding of aptamer molecule to the target protein



Natural aptamers in living systems

Naturally, aptamer is found in bacteria, archaea bacteria, fungi and plants, and acts as a part of riboswitch structures and often plays a role in regulation of expression of the genes involved in production of vitamins (co-enzymes) and in some cases is efficient in regulation of amino acids' production [8].

Evolution of aptamers in non-living systems

In 1990, Gold and Tuerk attained RNA fragments which were able to bind to T4 bacteriophage DNA polymerase. These oligonucleotide fragments were selected by a new combinatory technique called SELEX (systematic evolution of ligands by exponential enrichment). The meaning of this expression is that among a collection of aptamers with various sequences, it is possible to attain the specific sequence with high binding affinity to target molecule through some screening steps [9].

Aptamer library (AptLib)

The initial library for performing the SELEX process includes single stranded DNA (or RNA) oligonucleotides with a central core (approximately 40 bases) containing random and variable sequences (more than 1,015 different fragments), in which every oligonucleotide is consisted of certain flanking sites in both ends that are fixed positions for primer attachment [10]. In the middle of these constant regions (i.e. the central core), sequences of 15 to 75 (e.g. 40) nucleotides are randomly situated and every four base (A, G, C, T) may be situated in that position with the same probability [11]. These fragments are prepared by frequent synthetizations, with mixture of four types of nucleotides, coincidentally and by synthetization machine (will extendedly be discussed in results and discussion section) [12].

Aptamer library screening by SELEX cycles

In this stage, fragments with the highest affinity and specificity for target protein is selected from the aptamer library, through several SELEX cycles (usually 13-15 cycles). Repetition of these cycles is required for attaining the suitable affinity [13].

Using magnetic nano-particles for screening by SELEX cycles (Mag-SELEX)

Some of Mag-SELEX benefits include:
1) No requirement for special facilities or equipment,
2) Easiness of gathering and separation of aptamers by SELEX process only using a magnet or a simple centrifugation,
3) Possibility of using nano particles for two purposes: stabilization of target protein and also making aptamers single stranded in each cycle,
4) Possibility of performing the methods such as flow cytometry for evaluation of affinity,
5) Needing to little amount of protein in this technique (around 100 micrograms),
6) Possibility of coating nano particles with streptavidin for very strong interaction with biotin and thus prevention of wastage of stabilized protein during the washing process in which the conditions becomes tougher by rising cycle number of SELEX,
7) Expansion of protein contact surface with existent aptamers in the solution and rise in SELEX efficiency and abate of errors,
8) Easiness of stabilizing protein on these Nano particles.

It is worth mentioning that the main procedure of SELEX in different methods such as cell SELEX, CE SELEX, microfluidic SELEX (M-SELEX) and etc. are the same [14-16].

Corrections and chemical rearrangements of aptamer molecules

For increasing the stability and resistance of these molecules in clinical use, labeling or increasing the performance of these molecules, some modifications are made on these aptamer oligonucleotides.

The most important modifications include:

1) Corrections on carbon 2' of sugar including: a) Adding fluorine and amino group to pyrimidine bases. b) Adding hydroxyl group to purine bases. c) Adding o-methyl group to purine and pyrimidine bases. In clinical applications, 2'-O-

methyl oligomers are in priority, for they are less toxic than other corrections on carbon 2' (these structures exist naturally in ribosomal RNAs) [17].

2) Capping of 3'-terminus of oligonucleotide including: a) Attachment of inverted thymidine (3'-idT) to 3'-end of oligonucleotide and creation of 3'-3' connection. b) Attachment of biotin to the 3'-end [18]. 3) Capping of 5'-terminus of oligonucleotide including: a) Adding alkyl-amine group to 5'-end phosphate. b) Attachment of 5'-end of oligonucleotide to polyethylene glycol or on surface of liposome. c) Adding cholesterol, fatty acids and protein [19]. 4) Correction and chemical rearrangement of phosphate group which means replacement of sulfur group instead of the oxygen that doesn't attend in phosphodiester bond (thio-aptamer). 5) Adding signal peptides to aptamers such as penetrating peptides including Tat and antennapedia and fusogenic peptides [20]. 6) Labeling aptamers with fluorescent molecules (for example derivatives of fluorescein such as FITC, FAM and etc.) for flow cytometry or with biotin molecules for stabilizing aptamer on solid surface coated with streptavidin (for performing ELISA or in biosensors design) [21].

It's rarely seen that the corrections and chemical rearrangements made for resistance improvement are effective on the main features, such as binding affinity and third structure. That's while in performance most of these modifications are possible in primary library before SELEX [22].

Comparison of aptamers with antibody molecules

1) Antibodies' half-life is limited, while aptamers are very stable despite of the chemical rearrangements of their nucleotides. 2) Antibodies unlike aptamers are sensitive to environmental conditions such as temperature, pH and etc. 3) Antibodies have irreversible denaturation, that's while aptamers have denaturation and renaturation capacity because of their nucleic acid structure. 4) Antibodies unlike aptamers are immunogenic and this causes their rapid elimination in the body in clinical applications. 5) Size of antibodies unlike aptamers is massive (around 150 KDa) which causes long-term pause of them in blood and slow penetration in tissues and as a result, causes bone marrow toxicity in radioactive form [23]. 6) The procedure of antibody production is very difficult and for aptamers it is only difficult in the first time, during the panning steps of SELEX and then by sequencing of the aptamer selected from library, it is possible to synthesize the sequence attained easily. 7) Possibility of using aptamers in the chips that are based on DNA, so that in protein staining process aptamers do not get color. 8) Impossibility of antibody production for little non-immunogenic molecules; and possibility of aptamer production regarding to these molecules. 9) Factors like pH variation, salt concentration, heat, and chelating agents may help the process of aptamer reactivation and creation of functional structures in them. But for antibodies these agents would cause denaturation of them. 10) Stabilization of aptamers and labeling them are easier than antibodies.

These prominences have caused them to be widely used as molecular detectors in biosensors based on aptamer molecules (apta-sensors) and nanobiosensors based on aptamer molecules (nano-apta-sensors) [24].

AN OVERVIEW OF THE RESEARCH WORKS DONE UNTIL NOW, IN RELATION TO APTAMERS AND THEIR APPLICATIONS

Selection of aptamers for binding to antibodies, proteins and growth factors

- In 1996, Wiegand et al. succeeded in isolating aptamers) single stranded DNA and also RNA with 2'-amino chemical arrangement) against human IgE to inhibit the interaction of this molecule with "FcεRI" receptor on surface of basophilic leukemia cells of rat (as a new drug for the treatment of allergic diseases) [25].

- In 1995, Doudna et al. succeeded in selecting a RNA aptamer to detect the main epitope of insulin receptor antigen on surface of human cells [26].

- In 1995, Geling et al. selected RNA aptamers (with 2'-amino chemical arrangement) to inhibit the activity of fibroblast growth factor [27].

- In 2002, Lupold et al. isolated the RNA aptamer detecting prostate specific membrane antigen (PSMA) on surface of prostate cancer cells and labeled that aptamer by fluorescent molecules to observe its ligand-binding function in condition of "in vivo" [28].

Disruption of translation pathway signs of lymphocytes by Aptamar

- In 1997, Kubic et al. separated the binding and inhibiting RNA aptamer against the interferon gamma (INF-γ) which caused the inhibition of MHCI and ICAMI expression induction by INF-γ [29].

Selection of anti-viral aptamers

Some of these aptamers resulted in the elimination of virus cell cycle by interaction with the proteins necessary for virus proliferation and some of them were able to block surface

binding proteins and consequently resulted in the prevention of attachment and entry of viruses into cells [30].

- In 1995, Pan et al. separated the detecting and neutralizing RNA aptamer against Rose Sarcoma virus to inhibit the binding of the virus [31].

Selection of specific aptamers for binding and inhibiting the function of enzymes

- In 1998, Brindonau et al. succeeded in isolating 2'-amino RNA aptamer by SELEX (as a new drug) for inhibition of phospholipase A2 enzymatic activity in patients [32].

- In 2011, Tzu-Wang Chang et al. succeeded in isolating the RNA aptamer specified for inhibition of enzymatic activity of botulinum neurotoxin type A [33].

Selection of aptamers for detection of small molecules

- In 1998 and some years later, one DNA aptamer for Sulforhodamine B, two RNA aptamers for sulforhodamine B and fluorescein and one RNA aptamer for malachite green were separated by Wilson and Szostak [34].

Aptamers for cell diagnosis

- In 1999, Bruno et al. detected Bacillus anthracis spore by a single stranded DNA (ssDNA) aptamer using magnetic beads [35].

- In 2005, Qin Pan et al. isolated the RNA aptamer detecting the strains of Solmonella enterica serovar Typhi containing type IVb pili [36].

- In 2007, Fan Chen et al. isolated the ssDNA aptamer detecting a virulent strain of Mycobacterium tuberclosis called H37Rv [37].

- In 2006, Vivekananda et al. isolated 25 sequences of the ssDNA aptamer identifying surface bacterial antigens of Francisella tularensis japonica [38].

- In 2007, Ikanovic et al. designed an optical biosensor based on aptamer for detection of Bacillus thuringiensis bacteria [39].

Aptamers as tools for drug delivery

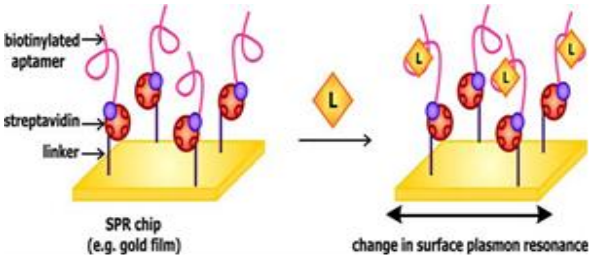
- In 2006, Farrokhzad et al. prepared nano-capsules of poly lactic acid (PLA) containing drug and linked to them the special aptamer related to prostate cancer cells through the poly-ethylene glycol linkage (PEG) [40].

Using aptamers for designing biosensors (apta-sensors) and nano-biosensors (nano-aptasensors)

- In 2011, Lakshmi et al. designed a chemical-resistant nano-aptasensor, for detection of anthrax PA toxin based on single wall carbon nanotubes (SWNT) [41].

- In 2012, Jeffery Degrasse et al. isolated a selective DNA aptamer for detection of Staphylococcus aureus enterotoxin B (SEB) for designing nano-aptasensor on the basis of surface plasmon resonance (SPR) (Figure 2 – as seen in the figure, biotinylated apatamers have been stabilized on streptavidin-coated gold nanoparticles, and the above nanoparticles are also fixed onto gold discs and by use of corresponding device, signals from surface plasmon resonance (SPR) changes are measured after detecting the target ligand by aptamer.) [42].

Figure 2. A schematic representation of aptamer molecule application in the design of nano-aptasensor.



APTAMER AS A PROPER TOOL IN MODERN METHODS OF DIAGNOSIS

Nowadays traditional methods based on immune-affinity assays, in which antibody molecules are used, while transposing with new methods based on aptamers-affinity assays [43].

Functions and features of modern determination methods based on immune-affinity assays:

1. Aptamers are produced in vitro conditions and they do not require any living organisms, to which they allow us to use any molecules regardless of it being toxic for the organism and separate exclusive aptamers for determination.

2. After separation and selection of exclusive aptamers by SELEX, we could easily reproduce and mass-produce it in high purity of specified aptamers.

3. Mass production expenses are very low and they have proved to be cost effective.

4. Nucleic acids (including DNA-aptamers) by having some functional groups can more easily be adapted to their target exclusive ligand. Without any malfunctions or lessening in tendency for binding with exclusive ligand in the specified domain.

5. Aptamers are originally stable in a broader domain of conditions.

6. Aptamers have very high tendency to synthesize and dedicate compared to antibodies.

7. Aptamers with high affinity can be used for determination and detection of different sorts of ligands such as toxins, allergens, sugars (that are weak immunogens), haptens, nucleic acids, proteins and different kinds of little biological and non-biological molecules like morphine and etc. can be separated and used. It is while in previous methods there were many limitations regarding to this matter [44].

USING APTAMERS MOLECULES IN DETECTION AND NEUTRALIZATION (INHIBITION) OF BOTULINUM NEUROTOXINS

As latterly mentioned, until today there have been many researches around the globe on these oligonucleotides with detection and neutralization purposes specifically on botulinum neurotoxins (such as type A).

Reports have indicated that aptamers molecules separated from library are suitable and proper for this matter [45].

Diagnostic purposes

In diagnosis of botulism agent as the one of biologic threat factors, we can separate some aptamers with high binding tendency with SELEX method. Aptamers separated to interact with botulinum toxin and also with different neurotoxin regions including catalytic domain can be very effective in diagnosis purposes. But catalytic domains are more efficient and useful in diagnosis purposes for neurotoxin types specially type E [46].

Inhibitory goals

In inhibiting and neutralizing this extremely lethal factor we can get our hands on molecules with inhibiting and neutralizing effects on operational sites, by investigating inhibiting effects of separated aptamers for exclusive connections with high tendency to catalytic site [47].

These aptamers might hinder the activation of this operational site by connecting to de-activated catalytic site of the toxin's body or by binding themselves to the freed catalytic site and cause the enzyme activity to stop [48]. In fact, inhibiting this position's enzymatic function leads to restraining the toxin's function. But antidotal aptamers can do the neutralizing process by connecting to other sites like connector sites and actually prevent the toxin from connecting to the cell and entering it [49].

Separating the aptamer against the catalytic domain of botulinum neurotoxin for recognizing and counteracting this toxin

Choosing the catalytic site from other operational sites of botulinum neurotoxin is important according to reasons mentioned below:

1. Results gained from conducted studies around the world show that this operational site is very suitable for getting the recognizing and counteracting goals.

- In 2011, Tzuu-Wang Chang et al. separated RNA-aptamer exclusively for inhibiting botulinum neurotoxin type A enzymatic activity [50].

- In 2009, Fang Wei et al. used separated aptamer for recognizing botulinum neurotoxin type A in designing electrochemical sensors [51].

- In 2009, Xinhul Lou et al. separated the special conjugative aptamers to catalytic domain of recombinant botulinum neurotoxin type A for recognizing this neurotoxin [52].

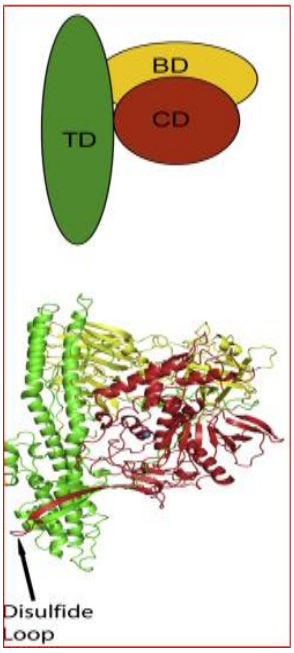
2. The main and operational part of this neurotoxin is the catalytic part and the quality of this neurotoxin is basically related to this part, in a way that said toxin will have no effect or special toxic function without this part [53]. So in conclusion the diagnosis and neutralizing value of this location will be much higher than other ones. And recognizing this location will lead to the certain recognition of infection to this type of neurotoxin, whereas recognizing other locations won't necessarily mean that and it's possible that other locations be used as recombinant with other factors [54].

3. The unique structure of botulinum neurotoxin type E and the importance of epitopes situated on this location and symmetric bond to the connector site, in a more general sense epitope makes the selection of this site for the toxin for this serotype exclusively more suitable (Figure 3 – the red represents the catalytic region, the green color indicates the translocating region and the yellow color indicates the binding region of the neurotoxin. As can be seen, both the catalytic and binding regions are located on one side of the translocating region, not on two sides as seen in the other types.) [55].

RESULTS AND DISCUSSION

Studies have shown that aptamers with central core of 75-100 base length are not suitable aptamers and on the other hand aptamers with the central core of lesser than 15-20 base also cannot be suitable aptamers [56]. If the length of aptamers fragments is longer than the above:

Figure 3. A schematic representation of second structures of neurotoxin botulinum type E and a symmetric relationship between catalytic and binding domains of toxin.



1. The possibility of creating similar sequences with primer in the central core is increased and with binding primer to this central parts and amplification, no particular fragments with shorter length will be the outcome.

2. On the other hand the possibility of connecting flanking sites of 2 sides (primer binding sites) with central sequences of various fragments, is increased and will cause an unhindered length increase and generation of fragments with larger sizes and PCR product of these two processes will be seen as smear.

3. Also the larger of these fragments be their particular operationally will also be decreased. Because after the formation of the 3rd structure there will be more binding sites to bind to target protein that each of which will have different affinity and can bind to our protein from different sites and positions and one of these position might have a high infinity to our target protein but no other position. So in conclusion the average affinity will drop and this means the decrement of specificity (affinity and specificity are two completely separate parameters) [57-59].

If the length of aptamers fragments is shorter than the above:

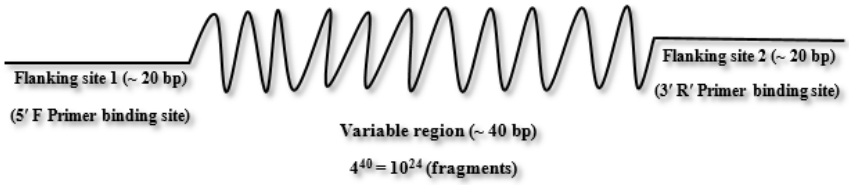
1. We'll witness a significant decrease in genetically variety of aptamer fragments and this will significantly limit our screening stages.

2. On the other hand the 3rd structures and conformation of aptamer which is the basis of aptamers binding to biomolecules will not form properly or won't form at all and this means the inefficiency of aptamer.

3. Another problem is the drawback in performing PCR and evaluating the target binding which distinguishing it from dimer primer is not easily achievable.

4. On the other side we'll face up to a huge problem in the purification of a production with such dimensions. Studies have shown that lengths about 40 base will be adequate and as we advance to larger or smaller numbers we will encounter the problems mentioned above more frequently although we are free to choose between 15-75 (20-100 according to some sources) (Figure 4) [60-62].

Figure 4. A schematic form of the single-stranded oligonucleotide structure of aptamer with a central core of 40 nucleotides. Scientifically and statistically, there is the probability of having a variety of 440 aptamer fragments (which is 1024 fragments) with a central core of 40 nucleotides, but optimistically virtually this variation is about 1015 aptamer fragments.

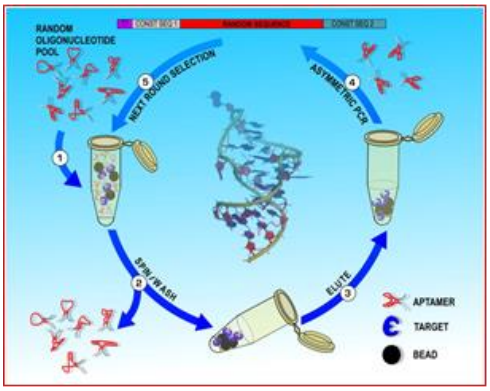


In Figure 5 the schematic form of SELEX cycle on RNA aptamer library is shown. The primary library made of DNA is firstly turned into a RNA library during a transcription process and after being added to nano particles covered with target proteins and performing the stages of washing and reunifying by performing the process of "asymmetric RT-PCR", is transformed to c-DNA and after single threading and removing the non-original strand the next cycle is again continued by transacting on the product mentioned above and creation of a RNA aptamer library [63-65]. It is necessary to perform a stage of negative selection in the presence of similar proteins.

Figure 6 shows the increment of aptamers' affinity as with the progress of SELEX stages, which is evaluable using the

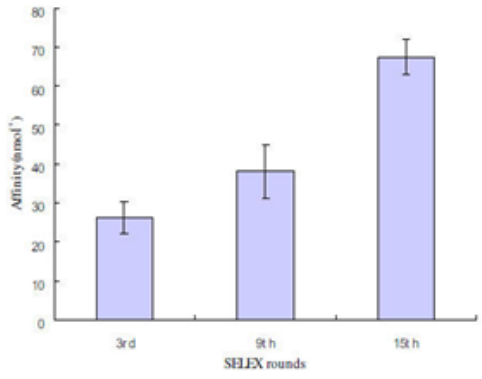
ELASA method (aptamer-based ELISA). Then we can perform a software based test on aptameric sequences using M-fold software.

Figure 5. A schematic representation of SELEX cycle on RNA aptamer library



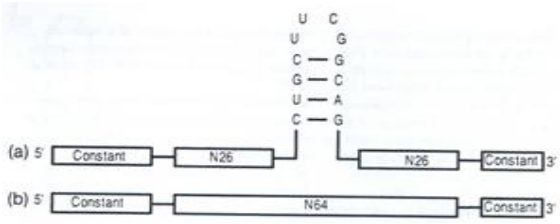
This investigation will give us the chance to understand how nucleotide changes in the final aptameric sequences causes a change in operational structure [66]. On the other hand, the software itself will predict different kinds of possible operational structures for that aptamer molecule and it will also suggest the best possible form. Sometimes the result of such investigations will show us the existence of a necessary operational site in the central core, which will have a key role in binding to the target protein [67].

Figure 6. Binding affinity of aptamers increases with the progress of the SELEX steps



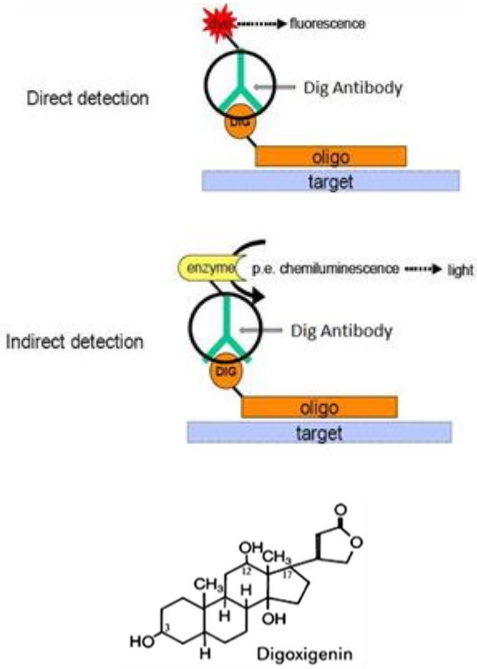
In a more accurate investigation for getting aptamers with higher affinities and higher specificity for binding to target protein we can put this specified operational site in the central core of a new aptamer library and perform the SELEX stages on that library again (Figure 7a and 7b) [68].

Figure 7. A schematic picture of two types of aptamers. 7a: An aptamer library with a central core containing a fixed region that is obtained by software evaluating by the M-Fold software. 7b: A simple aptamer library with a completely variable core



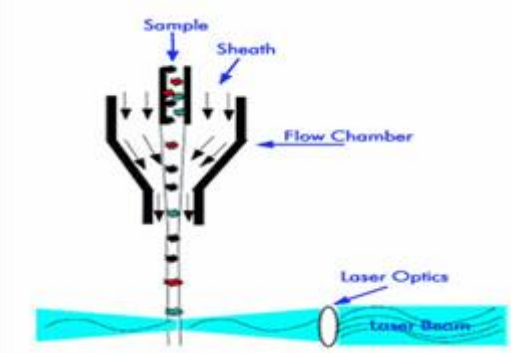
In Figure 8 the pattern of aptamer nucleotides labeled with digoxigenin (DIG) recognizing the target molecule is shown, so in part A the flow cytometry is used for identifying the interaction between aptamer and target molecule and in part B the chemiluminescence is used. Part C shows the chemical formula of digoxigenin [69].

Figure 8. A) Digoxigenin labeled aptamer identifying the target molecule and measuring the binding by flow cytometry B) Digoxigenin labeled aptamer identifying the target molecule and measuring the binding with the ELISA method C) The chemical formula of digoxigenin.



In Figure 9 an image of the function of flow cytometer machine is shown. In a way that the sample containing the aptamers marked with fluorescent (marked aptamers bound to nanoparticles) is passed through a fluorescent sensor and after radiating laser light on that the number of marked nanoparticles are counted [70].

Figure 9. Flow cytometer device operation



CONCLUSION

The most recent discoveries related to aptamer molecule shows the existence of particular properties and unique attributes in this biomolecule for the means of getting to the considered goal and the beneficial role of this molecule as a powerful tool for recognizing and neutralizing (inhibiting) the threatening factors shows great promise. These days the traditional methods for recognizing the biological factors based on immuno-affinity assays in which antibody molecules are used are being replaced with the new methods based on aptamer-affinity assays. The selected aptamers during the different stages of SELEX, with the ability of creating exclusive bonds and high tendency to interact with operational sites of biologic factors, in addition to having precise and fast recognition of this factors e.g. utilizing them in biological nano-sensors based on aptamer (nano-aptasensor), they could also be used in neutralizing and inhibiting the function of these threatening biological agents.

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