

Review Article

Design of Aptamer-Based Detector for Trinitrotoluene (TNT) and Review of Its Performance

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ABSTRACT

Background: The aim of this work is to achieve a DNA sequence of the aptamer that is specifically able to detect the trinitrotoluene (TNT).

Materials and Methods: Amplification of nucleotide fragments (PCR) was performed using special primers and a library of random nucleotides (two determined sequences of 19 and 21 nucleotides for connecting to primers and 78 randomized nucleotides in the center). In order to perform SELEX¹ study using magnetic nanoparticles and the EDC² reagent, TNT³ as binds to albumin (TNP-BSA) was immobilized. After initial screening, PCR were repeated on isolated pieces of nanoparticles using digoxigenin (DIG)-labeled nucleotides. In order to evaluate the aptamer function, using ELONA⁴ technique (Enzyme Linked Oligonucleotide Assay) and a specific anti-DIG antibody which is conjugated to peroxidase, the performance of aptamer for TNT detection was studied. Finally, this sequence is cloned into pBluescript plasmid and sequenced.

Results and Conclusion: The cloned aptamer has good efficiency for detection of TNT and could be used as aptasensor for detection of TNT in future studies.

Keywords: Aptamer, TNT, Clone, SELEX, ELONA.

INTRODUCTION

RNA or DNA functional oligonucleotide strands were called aptamer derived from Latin word aptus meaning appropriate. They take a special three-dimensional shape based on the placement of nucleic

¹ Systematic Evolution of Ligands by Exponential Enrichment

² 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride

³ Trinitrophenol-Bovine serum albumin

⁴ Enzyme Linked Oligonucleotide Assay

acids. Aptamers often have high tendency for their targets which is due to their folding capability and can specifically and tightly bind to their target. They can put small molecules within themselves and also bind to large molecules such as proteins, [1-5]. Aptamers are named also chemical antibodies because their production process is artificial and is carried out in laboratory conditions, using the SELEX cycle. SELEX is a chemical combinatorial method in which from a library containing 10^{15} to 10^{16} single-stranded oligonucleotide that are generated randomly, only those exclusive for their purpose are connected and selected and the others are separated in washing stage and then the **binding** aptamers are used for next amplification [6-9]. On the other hand, detecting explosive compounds, especially at points of entry such as airports, is a security priority for most countries. On this basis, designing simple, accurate and specialized equipment in order to detect explosives is of great importance. Today, in addition to the use of new chemical and physical techniques and technologies to detect Nitro-aromatic compounds, biotechnology is also used for this purpose. Many techniques have been developed to identify Nitro-aromatic compounds that are not affordable in terms of cost and response time, and therefore it is required to use methods that can operate with very little cost and time.

Biosensors are facilities need that has a major role in detecting and identifying Nitro aromatic compounds. These structures are able to detect these compounds with high accuracy. Hence, after the studies conducted on aptamers,

Table 1- List of primers and successions

Forward -T90 (21 mer)	5' - GGTATCGATAAGCTTGATATC- 3'
Reverse -T90 (19 mer)	5' - GCTGCAGGAATTCGATATC- 3'
Standard (126 bp)	5' - GGTATCGATAAGCTTGATATC GGCAATCGGCCGACGAGGGCATTATGCCGGCCCCCGGTGCATGGTCTCT TATCAGATGGTCAACACAATACCATGGCTCGAGCT GATATCGAATTCCTGCAGC-3'
Random (118 bp)	5' - GGTATCGATAAGCTTGATATC78(N)GATATCGAATTCCTGCAGC- 3'

Amplification of random and standard samples was performed using thermocycler and PCR technique and 1.5 percent agarose gel (with TBE) was used for control test. Also for identifying presence of aptamer in detection

by making aptamer specific for the related analyte and using auxiliary tools such as electrochemical kits, time and cost of these experiments can be reduced.

The aim of the present study is to design and manufacture specialized aptamer and TNT detector.

MATERIALS AND METHODS

-Predicting aptamer structure with the help of computer simulations

In order to design an aptamer, a library of random strand with the right length needs to be produced. Then it is the turn to synthesize this strand. Strand synthesis stage is a time-consuming and costly process. Thus, simulation software can be used. In order for synthesis simulation and prediction of aptamer structure, Rosetta software was used. Also, for obtaining the best interaction between the aptamers and TNT structure, (performing molecular mooring operations), the Auto Dock software was used.

-Amplification of sequences using specific primers

After conducting studies on detector aptamers of Nitro aromatic compounds, the aptamers that was reported with RNA origin in previous studies [10], was prepared by changing into DNA form, as a standard succession (with 86 bp) as well as random succession (with 78 bp). Kown sequences bind able to specific primers (19 and 21) along with cutting site EcoRV, were designed on two sides of both succession (Table1). The structure of standard succession regarding interaction by TNT was studied with the help of computer simulations.

stage, Digoxigenin labeled nucleotides (dUTP) were used.

-Screening using magnetic nanoparticles

In order to perform process of SELEX, given that TNT is a small and volatile chemical and its

stabilization is difficult, therefore TNT bind ed to albumin (TNP-BSA, T-5050, Sigma) was used, so that can be stabilized through albumin. Auxiliary method to speed up and increase the accuracy in obtaining specific succession (optimizing conditions) is stabilization of TNP-BSA through its protein section to the amine magnetic nanoparticle using EDC chemical reagent. Under the influence of magnetic field with aptamers that is added to it after being added to the medium, the collection is drawn towards the magnet and washing will be done better .In this method also, the volume of TNP-BSA, nanoparticle was added to a medium with copper buffer 0.1M (pH 5.5). Then, to activate amino group of nanoparticle and binding it to TNP-BSA, it was incubated for 1 hour along with EDC (50 mg / ml). After this stage, it was subjected the aptamer solution for 3 hours. 35 ml of sterile water was added to the micro-tubes and intense pyptazh was performed. It should be noted that wash is done by copper buffer under magnetic field. One of the advantages of this method can be amplification of aptamer without separation from set (aptamer-analyte-nanoparticle).

-Evaluating performance of aptamer using ELONA technique

For controlling performance of screened aptamer in binding to target molecule, ELONA technique was used [11].This technique is like ELISA in which oligonucleotide is used instead of antibody. In this method, first TNP-BSA (1 mg / ml) was stabilized in 8- Streep wells. Then, the stages were performed as following:

One hour incubation with blocking solution (BSA 3%), three hours incubation with labeled aptamer solution, one hour incubation with specific solution of monoclonal antibodies against DIG (with of dilution 1/1000) which is connected to peroxidase enzyme and finally detection steps by TMB (Tri Methyl Benzamidine) chromogenic peroxide substrate. At this stage, formation or lack of formation of green color in the samples was evaluated. Then, to stop the reaction, H₂SO₄ was added and its absorption was read at 450 nm. It should be noted, after each of the above steps, washing

was done by buffer TBS (pH 7.5) containing Tween for 20, 3 to 5 times.

Also, for labeling the aptamers, labeled nucleotides Dig (dUTP) were used. Dioxigenine is a steroidal compound that can alter oligonucleotides. These probes can be detected using anti-DIG antibody and these antibodies themselves are also visible with fluorescent colors.

-Aptamer clone in plasmid

After screening and investigating performance of the resulting aptamer, the related specific sequence was cloned (ligation) in plasmid and then resulting plasmid was transferred to bacteria. For this purpose, pBlueScript plasmid was used that was cut exposed to the EcoRV enzyme (making two blunt ends). After cutting, to inactivate the enzyme, it was kept in bainmarie at 80 degrees for 20 minutes. After ligation, for transferring new plasmid (pBlueScript-RT) into the bacterial cell, bacteria must be susceptible. In this experiment the XL1-Blue strain E. coli bacteria was used. Then, new circular plasmid was transformed into the bacterial cell. The product was concentrated and developed in LB-Agar medium containing Amp¹/X-gal/IPTG (Blue-White Screening). Next, among white colonies (Indicating presence of a plasmid containing aptamers pieces), one colony was cultured in LB-Broth medium and using plasmid extraction kit, extraction was done. Finally, after molecular confirmation of the cloned fragment using enzyme digestion and reaffirmation of aptamer performance with ELONA method, for determining sequence with M13 universal primers, cloned aptamer sequences was obtained.

RESULTS

-Analysis of the simulation results

The results of investigating simulated structure based on the standard aptamer sequence (with DNA origin) showed that the predicted aptamer structure is well able to detect TNT and bind to it. In studies performed for other family members of benzene, other structures of this

¹ Ampicilin

family also (of course with less intensity), are able to interact with this aptamer.

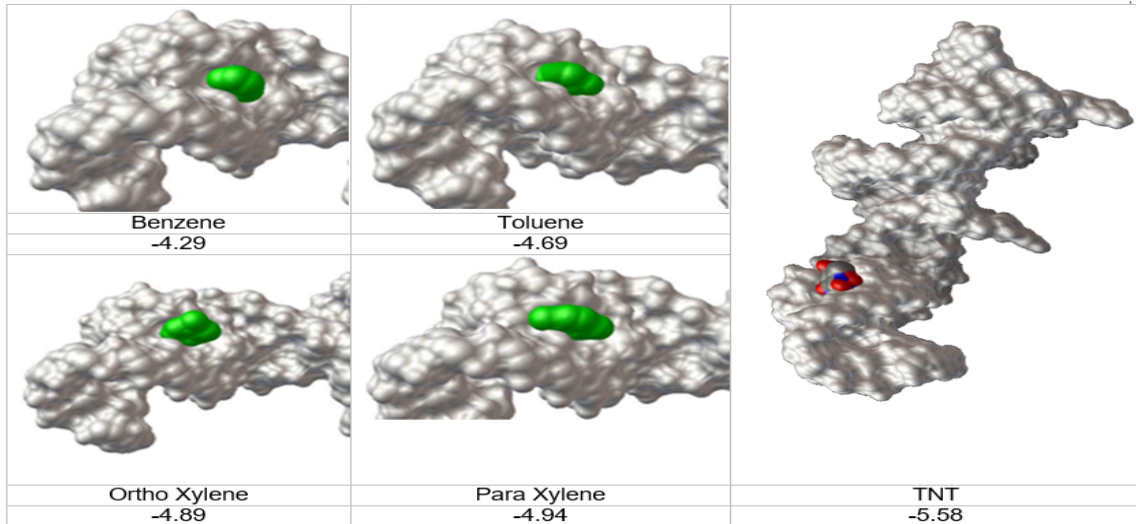


Figure 1- Investigation of the interaction and comparison of binding energy (in kcal / mol) of benzene family members with the anticipated aptamer

-Amplification of aptamer sequences

Similar results were also labeled amplified sequences.

In the studies performed after amplification of sequences using specific primers, an aptamer piece in the desired size was observed in agarose gel, and so the authenticity of this piece is approved (Figure 2). Similar results were also obtained in amplification of labeled sequences.

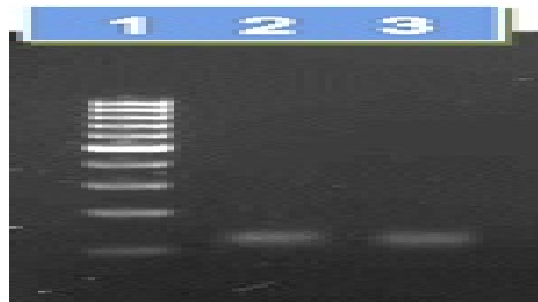


Figure 2- Analysis of PCR products using electrophoresis, (1) DNA (100 bp) marker, (2) PCR product with a standard oligonucleotide sequence as a template, (3) PCR product with random oligonucleotide sequence as a template

-Screening using magnetic nanoparticles

Screening was performed by binding TNP-BSA to nanoparticles and in proximity of aptamer library (with a length of 118 bp). It should be noted that, with regard to the use of nanoparticles in the screening path, a cycle of SELEX was performed.

-Analysis of amplification of plasmid containing screened sequences

After cloning the specific aptamer to plasmid, it is required to evaluate performance of the aptamer. To this end, pBlueScript-RT plasmid

(with specific primers) was amplified. According to the results obtained using agarose gel, aptamer fragment exists within the plasmid.

- Investigating permorance of the aptamer

The aptamer screened with standard aptamer sample (as positive control) and also along with two negative control modes (without aptamer and TNP-BSA), was used (in controlled mode without TNP-BSA, the condition is like positive controls and only, instead of TNP-BSA, BSA has been established in the pit floor, so that performance of the aptamer is evaluated).

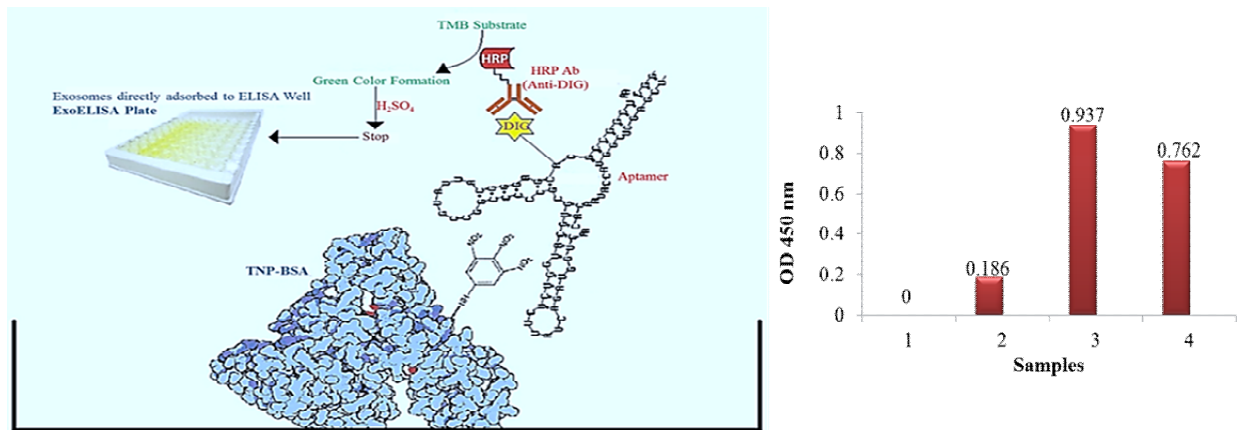


Figure 3-Left side shows ELONA technique strategy and right side shows the samples absorption curve.

In the above curve; (1) is the first negative control, without presence of aptamer, (2) is the second negative control, with BSA stabilization instead of BSA-TNP (in the pit floor) (3) in the presence of screened aptamer, and (4) in the presence of standard aptamer as a positive control.

According to the results obtained (Figure 3), the resulting sequence is highly specific compared to the considered analyte and even is more specific than positive control. The results also show that the second negative control sample (i.e., in stabilization state of BSA instead of analyte) tends a little to absorption by aptamer that can be ignored. Also assessing sensitivity of the used aptamer showed the least amount of TNP-BSA identified by the aptamer is about 1ng.

- Determining sequence of specific aptamer

Plasmid pBlueScript-RT was sent to determine sequence that the following sequence was obtained after investigations:

5'-

GGTATCGATAAGCTTGATATCCATGACG
CCGGTTCGTTGGGATATCTCTTTCAAGA
CGACATGGGGATAGAAGC
GATAATTGTATATTGATTTTGCATGATAT
CGAATTCCTGCAGC-3'

DISCUSSION

Very high performance of the aptamer has caused researchers and scientists to have a positive attitude toward them. For this reason, in this material various diagnostic methods are used in form of biosensors [12-15]. On the other hand, several classical analysis methods have been

used to detect explosive combination so far, such as colorimetric methods, HPLC, GC-MS, which often contain different sensor methods. Today, selecting a wide range of aptamers capable of binding to small molecules, peptides, proteins, cells and so with specificity, selection and tendency, has developed equal to and often more than the antibodies.

In the studies by Lan et al (2000) was performed, TNT antibodies were enclosed in the sol-gel (derived from silica) to be used in safety assessment. These antibodies are able to detect TNT [16].

The research work carried out by Ehrentreich et al in 2008, specific RNA sequences and TNT compound detector were obtained after 13 times of screening. In the above experiment, the related analyte was stabilized in stationary phase of the chromatography column. The mentioned sequence; RNA is single-stranded and is able to bind to relevant analyte with high specificity. Using optical fiber, safety tendency reactions were used for the detection of sensitive explosives [10].

Also, in an experiment by Shankaran research group (2008), quick and labelless safety assessment methods were created for detection of TNT based surface plasma resonance (SPR). Safety sensor with stabilization of TNPh-KLH¹ was created on gold plate with absorption power. Among the four tested antibodies, Anti-tri nitro phenyl polyclonal antibody has the highest sensitivity with detection limit of 002/0 ng ml (2 ppt) TNT [17].

¹ 2,4,6-trinitrophenyl-keyhole limpet hemocyanin

CONCLUSION

In the present study, the method of aptamers (in the form of DNA) was used to detect TNT and also ELONA technique was used to speed up target molecule detection tests. In this method, instead of using a large amount of random library, screening and detection in very small quantities can be performed. Also in this study, magnetic nanoparticles were used for optimizing the screening and therefore screening was performed with a cycle.

In order to achieve aptamer sequences with more specificity, it is required to increase screening cycle and repeat experiments on similar compounds of TNT such as DNT or toluene and benzene.

The results of sensitivity of this method also reflect relatively favorable performance of aptamer in the detection of TNT that is required to be developed in future studies.

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REFERENCES

- Menger M, Glöckler J, Rimmel M. Application of aptamers in therapeutics and for small-molecule detection, *RNA toward medicine* 2006; 173: 359-373.
- Bang, G. S., Cho, S., & Kim, B. G. A novel electrochemical detection method for aptamer biosensors. *Biosensors and Bioelectronics*, 21(6) (2005); 863-870.
- Hermann, T., & Patel, D. J. Adaptive recognition by nucleic acid aptamers. *Science*, 287(5454) (2000); 820-825.
- Mairal T, Cengiz-Ozalp V, Lozano-Sánchez P, Mir M, Katakis I, O'Sullivan CK. Aptamers: molecular tools for analytical applications. *Anal Biochem* 2008; 390: 989-1007.
- Song S, Wang L, Li J, Fan C, Zhao J. Aptamer-based biosensors. *Trac-Trend Anal Chem* 2008; 27: 108-117.
- Stoltenburg R, Reinemann C, Strehlitz B. SELEX—A (r) evolutionary method to generate high-affinity nucleic acid ligands. *Biomol Eng* 2007; 24: 381-403.
- Luo, X., McKeague, M., Pitre, S., Dumontier, M., Green, J., Golshani, A., et al. Computational approaches toward the design of pools for the in vitro selection of complex aptamers. *RNA*, 16(11) (2010); 2252-2262.
- Djordjevic M. SELEX experiments: New prospects, applications and data analysis in inferring regulatory pathways. *Mathematical Biosciences. Biomol Eng* 2007; 24: 179-189.
- Tuerk, C., & Gold, L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science*, 249(4968) (1990); 505-510.
- Ehrentreich-Förster, E., Orgel, D., Krause-Griep, A., Cech, B., Erdmann, V. A., Bier, F., et al. Biosensor-based on-site explosives detection using aptamers as recognition elements. *Analytical and bioanalytical chemistry*, 391(5) (2008); 1793-1800.
- Drolet DW, Sumedha J, Gold L. Enzyme linked oligonucleotide assays (ELONAs), United States Patent 1998; 5789163.
- Kawde AN. Label-free bioelectronic detection of aptamer-protein interactions. *Electrochem Commun* 2005; 7: 537-540.
- Xie S, Walton SP. Development of a dual-aptamer-based multiplex protein biosensor. *Biosens Bioelectron* 2010; 25: 2663-2668.
- Wang W. Aptamer biosensor for protein detection using gold nanoparticles. *Anal Biochem* 2008; 373: 213-219.
- Pultar J. Aptamer-antibody on-chip sandwich immunoassay for detection of CRP in spiked serum. *Biosens Bioelectron* 2009; 24: 1456-1461.
- Lan, E. H., Dunn, B., & Zink, J. I. Sol-gel encapsulated anti-trinitrotoluene antibodies in immunoassays for TNT. *Chemistry of materials*, 12(7) (2000); 1874-1878.
- Shankaran, D. R., Kawaguchi, T., Kim, S. J., Matsumoto, K., Toko, K., & Miura, N. Evaluation of the molecular recognition of monoclonal and polyclonal antibodies for sensitive detection of 2, 4, 6-trinitrotoluene (TNT) by indirect competitive surface plasmon resonance immunoassay. *Analytical and bioanalytical chemistry*, 386(5) (2006); 1313-1320.