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Gel green fluorescence ssDNA aptasensor based on carbon nanotubes for detection of anthrax protective antigen

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ABSTRACT

Bacillus anthracis, the causative agent of anthrax, is a harmful pathogen with potential ability as a biological weapon which persuades scientists to develop novel methods to detect anthrax from infected resources. In this study, a multi-walled carbon nanotube (MWCNTs)-based fluorescence aptasensor was fabricated to detect the recombinant protective antigen domain 4 (rPAD4) of *Bacillus anthracis* as the most important key factor in development of anthrax. First, PAD4 was recombinant expressed in *E. coli* and purified by Ni-NTA column. Second, the affinity of aptamer to rPAD4 was confirmed by ELAA assay. In aptasensor design, the aptamer was labeled with Gel Green and immobilized on MWCNTs. Upon the adsorption of labeled aptamer on MWCNTs, fluorescence emission was quenched. In contrast, by adding rPAD4 to hybridization reaction and incubation for 10 min, the fluorescence emission was significantly recovered to 85% compared to the control. Detection limit for the sensitivity and specificity of the aptasensor was determined 20 ng/ml and 62.5 ng/ml purified and unpurified rPAD4 protein, respectively. Also, applicability of aptasensor was showed in mouse serum sample. Finally, results indicated that nanosensor has the potential to be developed as a high-sensitive, cost-effective and fast-acting system for measuring of PA in anthrax diagnostic tests.

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1. Introduction

Bacillus anthracis, an etiological agent of anthrax, is a gram positive, non-motile and spore-forming bacterium which belongs to the *Bacillus cereus* group [1]. Anthrax is an epizootic, acute and rapidly progressive disease which human infections are caused by direct contact with infected animals or their products, including meat, skin, and hair [2]. The bacterial spores are dormant in the soil for many years and based on their entrance way to mammalian host, three forms of cutaneous, inhalation and gastrointestinal diseases are developed [3]. The most important virulence factors of *B. anthracis* are located on two large extrachromosomal elements. The pXO1 (183 kb) encodes multicomponent bacterial toxin and pXO2 plasmid encodes a poly-γ-d-glutamic acid (γ-DPGA) capsule. Anthrax toxins including protective antigen (PA, 83 KD), lethal factor (LF, 89 KD) and edema factor (EF, 90 KD) are necessary for complete virulence action [4,5]. After infection, toxins encoding genes are immediately transcript in the host cells [6,7]. Protective antigen (PA) with 83 KD molecular weight is the major virulence factor of anthrax which binds to the host cell surface receptor and mediates uptake of EF or LF into the host cell [1,8]. This protein is expressed in the early stages of *B. anthracis* pathogenicity and is found in the bloodstream of infected hosts [6].

Harmful pathogenicity and broad host range of this bacterium and as well as its potential as a biological weapon persuades scientists to study and develop novel methods for rapid detection and eradication of *B. anthracis* from contaminated resources. Current methods for the detection of anthrax are based on conventional approaches which rely on morphological features and growth on selective media like Polymyxin-Lysozyme-EDTA-Thallous acetate (PLET) agar within 1–2 days [9,10], immunological and molecular genetic methods [11,12]. Inoculation of cultured bacteria in guinea pigs or mice and assessing their virulence action form another phase/other phases of detection [10]. In fact, these methods are labor-intensive, slow and work best at high concentrations of *B. anthracis*. Furthermore, they need to be confirmed by biochemical or molecular methods. Therefore, the identification of *B. anthracis* virulence factors in environmental samples in mixed with other *Bacillus* spores is still challenging [12]. In this case, biochemical identification methods based on metabolic properties and affinity tests are used. These tests include flow cytometry assay using fluorescein-labeled antibodies, sandwich based assays, bead-based immunoassay and enzyme-linked immunosorbent (ELISA) assays [12–14]. The value of these diagnostic approaches are considerably hindered due to low specificity or affinity of antibody to pathogen or antigens which observe false positive and negative results in antibody-based detection assays [15]. Molecular genetic-based methods such as PCR are interesting ways to obtain information about the genetic material of pathogens. These methods are dependent on the exact

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identification of the specific genomic sequence of the bacteria to prevent any cross-reaction with other species [12,16]. Since now, several different PCR based methods have been introduced for the detection of *B. anthracis* which specific sequences of PXO1, PXO2 plasmids, rPoB gene, GrA gene, 16S ribosomal RNA and etc. have been subjected to PCR amplification [17–19]. However, some positive signals with *B. cereus* isolates have been reported from these assays [12]. Despite all the advantages, molecular genetic methods from industrial views are not cost-effective and performing of them require to skilled persons.

Given the general concern about the pathogenicity of *B. anthracis*, there is still a need to develop the novel technologies based methods for the detection of these bacteria [11]. Biosensors are diagnostic devices that convert the molecular recognition of a target analyte into a measurable signal via a transducer [20]. In this systems, the biological response signals are converted to a measurable physicochemical signal and related to the concentration of the analyst [21]. Recently, advances in nanotechnology and the use of nanomaterial in the construction of biosensors have developed and introduced a new generation of biosensors which offer interesting features such as high sensitivity, real-time and on-site detection for the detection of cancer biomarkers, pathogens, toxins and etc. [22]. Among these nanomaterials, carbon nanotubes have unique structural, optical and electrical specificity [23,24]. These properties are changed by interactions with different biological molecules like proteins and nucleic acids and monitoring these changes are crucial in sensing device [11]. It have been reported that carbon nanotubes can efficiently quench the fluorescence of nearby fluorescent dyes through fluorescence resonance energy transfer (FRET) mechanism [25].

Gel Green, a sensitive and environmentally safe green fluorescent nucleic acid dye, has been used in molecular biology for various types of electrophoresis. It is an intercalating nucleic acid stain which is structurally related to acridinium orange dyes and based on chemical properties, it can be a good donor carbon nanotubes during FRET process [26,27]. Several safety tests have confirmed that Gel Green is noncytotoxic, nonmutagenic and nonhazardous at low concentrations used in molecular laboratory processes [27]. It is very stable both hydrolytically and thermally, commercially available which has a higher level of fluorescence upon binding to single stranded and double stranded DNA with excitation and emission at 480 nm and 526 nm, respectively [27,28].

Recently, aptamers-based nanobiosensors have attracted more attention. Aptamers are short oligonucleotides with the binding potential to different molecules with high affinity and specificity. They have many advantages compared to antibodies including more stability and without the need for laboratory animals in synthesis [29]. Therefore, the use of aptamer technology in biosensors design can be useful in reducing costs, measurement time and increasing the sensitivity of detection. In this study to introduce a simple, rapid, ultra-sensitive and cost-effective detection method, particular attention was paid on developing FRET between specific PAD4 aptamer labeled with Gel Green and carbon nanotubes in response to the presence of PAD4 in samples. For this purpose, diverse combinatorial strategies such as recombinant DNA technology, immunological methods and nanoparticle conjugate approaches, came together for better constructing Nano-aptasensor scheme. After preparation, the sensitivity, specificity and applicability of novel assay for detection of anthrax protective antigen was shown.

2. Materials and methods

2.1. Reagents

The 5′-amine-modified single-stranded DNA (5′-ATCACTAGTGAATT CGCGGCCTGCA GGTCGACCATAT) as a specific aptamer for *B. anthracis* protective antigen was synthesized by Bioneer, Korea [29]. Nanomaterials including multiwall carbon nanotube and chemical materials including 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide sodium salt (NHS), Nickel-nitrilotriacetic acid (Ni–NTA), Isopropyl β-D-1-thiogalactopyranoside (IPTG) and Gel Green were supplied from Sigma-Aldrich, USA. Other chemicals were purchased from Merck, Germany.

2.2. Recombinant expression and purification of protective antigen (PA) in Escherichia coli

E. coli strain BL21 (DE3) harboring recombinant pET28-PAD4 was prepared for expression of domain 4 of PA (PAD4). Recombinant bacteria were grown in LB medium containing kanamycin (25 μg/ml) under 180 rpm at 37 °C overnight. The overnight grown cultures were diluted to 1/100 (v/v) and cultured at the same condition to an optical density 0.5–0.7. Then, IPTG at a concentration of 1 mM was added to induce expression of recombinant PAD4. After 16 h incubation at 180 rpm and 30 °C, the bacterial cells were harvested and centrifuged at 5000 rpm/10 min. bacterial pellet was resuspended in 5 ml of lysis buffer (50 mM NaH_2PO_4 PH = 8.0, 300 mM NaCl, 10 mM imidazole, 0.2 mg/ml lysozyme) and sonicated. Total protein was extracted by centrifuge at 14000 rpm/20 min and analyzed by SDS-PAGE. To purify the rPAD4 protein, Nickel-nitrilotriacetic acid (Ni–NTA) (Sigma) resin under native condition was used. Extracted total protein from IPTG induced recombinant bacteria was passed through the Ni–NTA affinity column and the flow-through of the soluble fraction was collected. The column was washed with washing buffer containing 40 mM imidazole and bound protein was eluted with 2 ml of 250 mM imidazole followed by 1 ml of 20 mM MES (2-(N-morpholino)ethanesulfonic acid) buffer. The purification procedure was carried out at 4 °C and the purified protective antigen (PAD4) was confirmed by 10% Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE).

2.3. In vitro analysis for evaluation of DNA aptamer interactions with domain 4 of PA protein

To confirm the high affinity of ssDNA aptamer to rPAD4 protein, enzyme-linked aptamer assay (ELAA) was performed to demonstrate the specific binding of ssDNA aptamer to rPAD4 in vitro condition. For this propose, polystyrene 96-well microplate were coated with 200 pM of ssDNA diluted in coating buffer (64 mM Na_2CO_3, 136 mM $NaHCO_3$, pH 9.8) in a 100 μl volume and incubated at 37 °C for 2 h. After washing with phosphate buffered saline containing Tween-20 (PBST), wells were blocked with 3% skim milk in PBST buffer at 37 °C for 1 h. The plates were washed three times with PBST and 5 μg purified rPAD4 protein diluted in 100 μl PBST buffer were added into each well. After incubating at 37 °C for 1 h, the plates were washed three times with PBST then sera against rPAD4 (produced in mice model) diluted to 1:100 in PBST were added to each well. After incubation for 2 h at 37 °C, the wells were washed four times with PBST then diluted 1:50000 anti-mouse IgG (Sigma) was added and incubated for 1 h at 37 °C. The wells were washed four times with PBST, and 100 μl of (*O*-phenylenediaminedihydrochloride) OPD (Sigma) was added as substrate. The plates was incubated at room temperature for 20 min, and colour development was stopped by the addition of 100 μl of 2 mM H_2SO_4. Optical densities were read at 492 nm and 37 °C on a microplate reader (Bio-Rad).

2.4. Optimization and characterization of the Gel Green labeled PA specific aptamer absorption on the surface of MWCNTs

Carbon nanotubes based aptasensor for detection of PA was prepared by adsorbing the oligo green labeled specific PA aptamer on MWCNT. At first, ssDNA aptamer was labeled by mixing of 0.5 μl from Gel Green (diluted with 1:500 distilled water) with 50 μl ssDNA aptamer. Then reaction conditions such as adsorption time and the concentration of MWCNT were optimized. To obtain the optimized absorption time, the reaction was performed by mixing 10 μl aptamer DNA

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(10^4 nM) with 15 μl of MWCNTs (1 mg/ml) in the final volume of 500 μl in 20 mM Tris-HCl buffer solution pH 7.4, containing 100 mM NaCl and fluorescence emission was monitored at different times. Also, to determine the optimum concentration of MWCNTs for complete quenching the labeled aptamer, different concentrations of MWCNTs were mixed with 10 μl of aptamer (10^4 nM) and then fluorescent quenching measured by fluorescence spectrometry at optimum absorption time. After preparation, MWCNT-aptamer conjugates were confirmed by transmission electron microscopy (TEM, Zeiss EM 900 electron microscope), scanning electron microscopy (SEM, Zeiss-DSM 960A microscope) and Energy-dispersive spectroscopy (EDX, Zeiss-DSM 960A microscope). Fluorescence spectra were measured using JASCOFP-750, Rev. 1.00 fluorimeter.

2.5. Hybridization reaction and determination of aptasensor sensitivity for detection of rPAD4

After the preparation of MWCNTs based aptasensor, hybridization reaction was carried out with different amounts of purified rPAD4. At first, 100 ng of purified rPAD4 protein was mixed with 500 μl optimized MWCNT-QD/Aptamer conjugate solution and fluorescence emission was monitored at different incubation times in room temperature. After obtaining the optimized hybridization time, the sensitivity of the aptasensor was determinate with various amounts of purified rPAD4 protein (5 ng, 10 ng, 15 ng, 20 ng, 40 5 ng and 80 ng). The specificity of aptasensor was evaluated with different amounts of total protein (250 ng, 125 ng, 62.5 ng and 31.25 ng) from IPTG induced *E. coli* strain BL21 (DE3) harboring recombinant plasmid of pET28-PAD4. Total protein of other bacteria such as *E. coli*, *Bacillus subtilis*, *Shigella dysenteriae* was used as control. The limit of detection for the sensitivity and specificity of the aptasensor was defined as the minimum concentration of purified and unpurified rPAD4 protein which significantly showed detectable fluorescence emission compared to the control. To demonstrate the applicability of aptasensor for detection of PAD4 in real samples, 1 μg of unpurified rPAD4 (total protein of recombinant bacteria) were mixed with 1 ml of mouse blood sample. Then, the serum was extracted and used to evaluate the performance of the aptasensor. Total protein of other bacteria such as *E. coli*, *Bacillus subtilis*, *Shigella dysenteriae* was used as control. The sensitivity and specificity of the sensor in serum were evaluated by adding different dilutions of the serum to the hybridization reaction. The detection limit for the applicability of the sensor was estimated the minimum serum titer that could indicate a significantly detectable fluorescence emission rate compared to the control. In order to compare the performance of aptasensor with current methods such as ELISA, serum titers were used to detect PAD4 by ELISA assay. In ELISA technique Polystyrene 96-well plates (Nunc) were coated with different rPAD4 serum titers (1:10, 1:20, 1:40; 1:80, 1:160, 1:320 and 1:640) diluted in coating buffer at 37 °C for 2 h. The rest of the ELISA assay steps was performed according to the method of Karimi et al., 2018 [30].

2.6. Statically analysis

All Fluorescence measurement experiments were performed in three replicates. The results presented in each figure were expressed as mean values ± standard deviation. All statistical analyses were performed using the SPSS 18 statistical program. The statistical differences between fluorescence emission mean values of aptasensor in reaction with control and PA protein were determined by Student's *t*-test and statistical significance was set at $p < .05$ and $p < .01$.

3. Results and discussion

3.1. Expression and purification of recombinant protective antigen domain 4

Recombinant PA domain 4 protein after expression in *E. coli*, was purified by HIS-Select nickel affinity chromatography under native condition and the presence of 18 KD single band related to purified rPAD4 protein was detected by SDS-PAGE analysis in flow-through of 250 mM imidazole elution buffer (Fig. 1a). After dialyzing, the purified rPAD4 was used in ELAA assay and biosensor experiments.

3.2. In vitro interaction of PA domain 4 with specific ssDNA aptamer

Protective antigen (83 KDa), a major virulence factor of *B. anthracis* with 4 domain, is a key component in transferring LF and EF to host cell cytosol [31]. Based on reports, in primary stages of intoxication process, elevated levels of PA messenger RNA to EF and LF, highlights its central role in pathogenicity [32]. PA binds to two cell surface receptor,

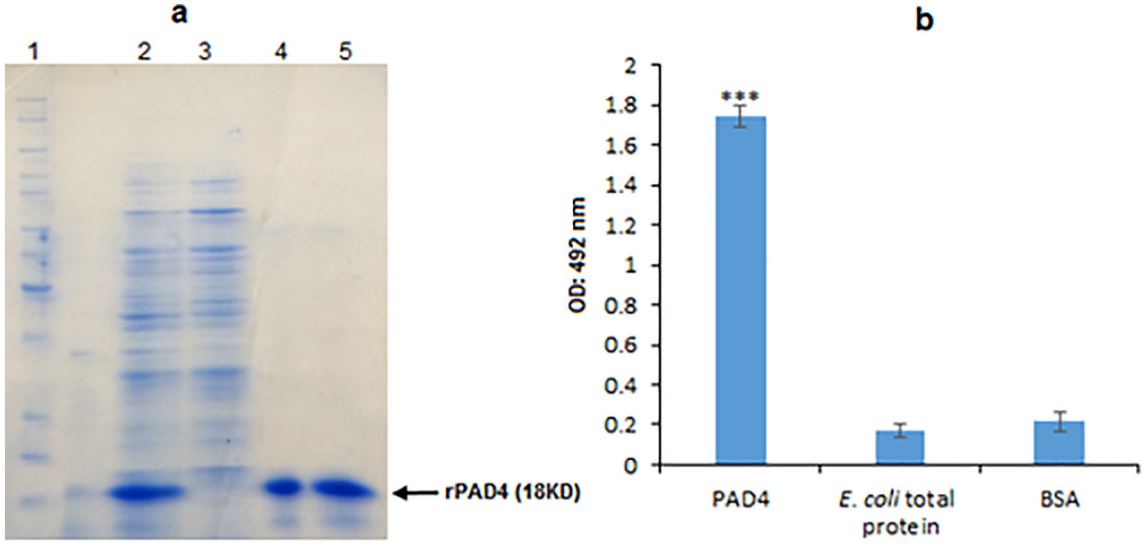

Fig. 1. Recombinant expression of PAD4 and ELAA assay for evaluation of its interaction with ssDNA aptamer. a) SDS-PAGE analysis of rPAD4. Lane1, Protein molecular weight marker. Lane2, induced *E. coli* BL21DE3 cells harboring recombinant pET28a-PAD4 expression vector. Lane 3, Un-induced *E. coli* BL21DE3/pET28a-PAD4 gene as control. Lane 4, 5 purified rPAD4 protein after elution with 250 mM imidazole. b) Results of ELAA assay for rPAD4 and Control proteins. Vertical bars show standard deviation (SD) ($n = 3$). Differences were considered significant whenever $p \leq .001$.

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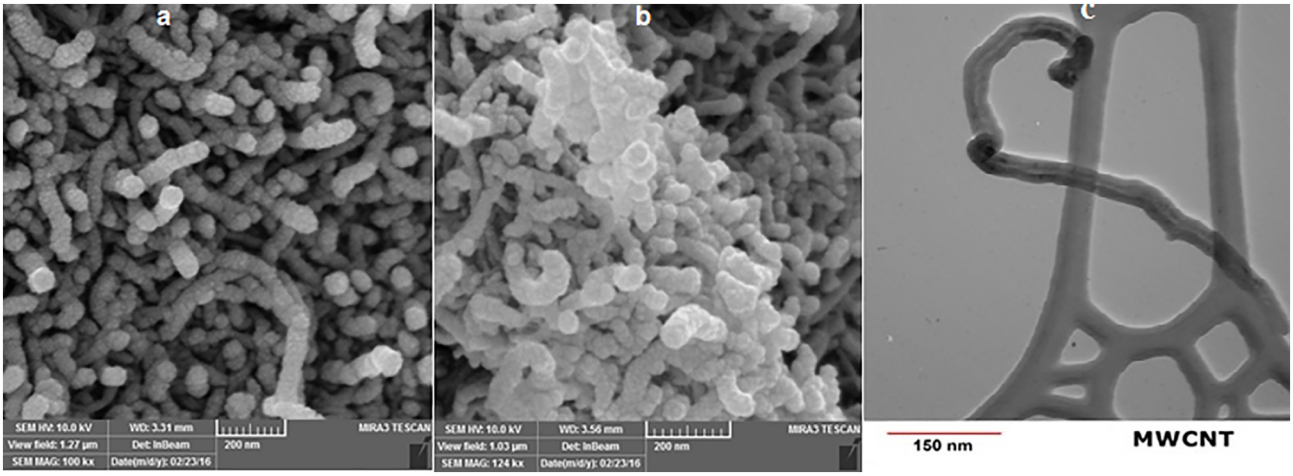

Fig. 2. SEM image of MWCNTs (a) and MWCNTs-ssDNA aptamer (b). TEM image of MWCNTs. Schematic representation of MWCNTs based ssDNA aptasensor for detection of PAD4.

capillary morphogenesis protein 2 (CMG2) and tumor endothelial marker 8 (TEM8), via specific residues in domain 4 [33,34]. This domain is a C-terminal domain of PA that contains specific acidic residues which facilitate the attachment of proteins to receptors [34,35]. Based on online prediction servers (http://web.expasy.org/cgi-bin/protparam/protparam), domain 4 is a stable protein with 37 charged residues that 22 of them are acidic residues. It is flexible, has little contact with other domains, located on the outside of heptamer complex, rarely contact with LF or EF in transferring process and has been demonstrated to be a potent immunogenic factor [36]. Since ungerminated anthrax spores contain significant levels of PA and fourth domain is active in primary connection with host cell receptors [6], its selection as a part of sensing element in biosensor designing can be reasonable. In this study PA specific ssDNA aptamer reported by Mulchandani et al. 2010, was subjected for detection of PA antigen by carbon nanotubes based nanosensor. Before preparation of PAD4 specific aptasensor, affinity and interaction of ssDNA aptamer to rPAD4 was analyzed. For this purpose, enzyme-linked aptamer assay (ELAA) was performed to confirm rPAD4-ssDNA aptamer interaction. The ELAA method has been developed since its first application in 1996 and used for detection of biomolecules and pathogens like respiratory syndrome virus type II and dopamine in serum samples [37,38]. Based on the results of this experiment, high levels of optical density at 492 nm in compared with control were contributed to ssDNA-aptamer interaction with rPAD4 (Fig. 1b).

3.3. Characterization of MWCNTs/PAD4 specific aptamers conjugates

Preparation of PAD4 specific ssDNA aptasensor was performed by absorption/stabilization of ssDNA aptamer on MWCNTs. To confirm MWCNTs-aptamer conjugates, SEM, TEM and EDX analyses were performed. SEM and TEM analysis confirmed the morphological properties of MWCNTs. SEM image of MWCNTs/ssDNA aptamer conjugates showed changes in morphological and diameter of MWCNTs which indicating absorption/stabilization of ssDNA aptamer on MWCNTs (Fig. 2a, b). In TEM image, MWCNTs was observed with an average diameter of 20–30 nm (Fig. 2c). Elemental analysis performed using Energy-dispersive spectroscopy (EDX) confirmed the presence of ssDNA adsorbed/stabilized on of MWNTs. In EDX analysis of ssDNA-MWCNTs conjugates, P, N and elements signal related to Gel Green labeled ssDNA was observed at around 2 and 0.5 KeV respectively (Fig. 3a, b) [39].

3.4. Gel Green labeled PAD4 specific aptamer

In this work fluorescence aptasensor was prepared based on FRET process between Gel Green labeled PAD4 specific aptamer as donor fluorescence parts and multiwall carbon nanotubes as quenching nanomaterials (Fig. 4). Fluorescence emission of Gel Green labeled ssDNA aptamer was confirmed by fluorimeter and significant levels of

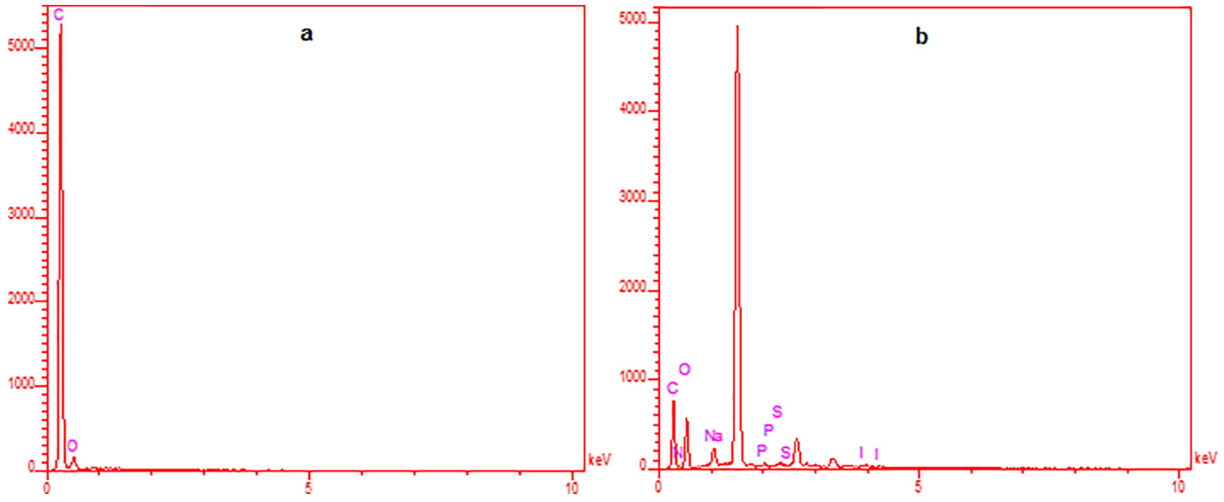

Fig. 3. a) EDX spectrum of MWCNTs, b) EDX spectrum of material containing N, P and I elements in Gel Green labeled ssDNA aptamer-MWCNTs conjugate.

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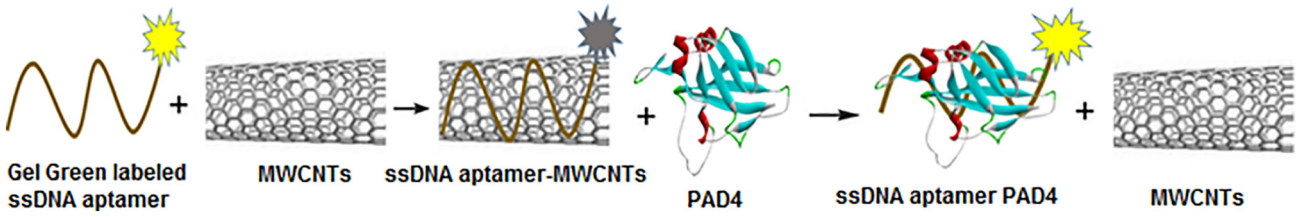

Fig. 4. Schematic representation of MWCNTs based ssDNA aptasensor for detection of PAD4.

fluorescence emission were monitored compared with Gel Green (Fig. 5a). This fluorescence dye is very stable both hydrolytically and thermally, commercially available. Gel Green, a nucleic acid binding cationic conjugated polymer, mostly interacts with phosphate groups of ssDNA aptamers via ionic and other noncovalent interactions [27,40]. In this case, molecular motion slows down and non-radiative energy due to collision with aptamer and solvent components decreases and its structural rigidity increases [28]. This process causes much of the gel green excited state energy at 480 nm to be converted to fluorescence rather than molecular motion. Since nucleic acids do not have specific absorbance in 480 nm, the range of fluorescence emission wavelength of labeled aptamer does not change critically but its intensity rise significantly [40,41] (Fig. 5a).

3.5. Immobilization of labeled aptamer on MWCNTs

In this work, MWCNTs as a quenching nanomaterial were used to design aptasensor. Carbon nanotubes with unique chemical, electrical and optical properties have high potential for use in ultrasensitive biosensors. Their optical features make them a good quencher pair in FRET mechanisms [25]. Recent reports have been demonstrated carbon nanotubes (CNTs) functionalization with nucleic acids and proteins which have highlighted their application in biosensors [23]. Single-stranded DNAs have high interaction affinity for carbon nanotubes due to larger energy between DNA bases and CNT walls than a self-stacking energy of bases [42]. In this case, noncovalent interactions like hydrophobic effects and van der Waals interactions have dominant roles [43]. In this project Gel Green labeled ssDNA aptamer was adsorbed on MWCNTs by noncovalent bonds. In the absence of MWCNTs, a strong fluorescence emission related to Gel Green dye was observed in ssDNA aptamer at 525 nm wavelength. However, adding of MWCNTs lead to decreasing and quenching of the fluorescence emission at room temperature, indicating the strong interaction between MWCNTs and Gel Green labeled ssDNA aptamer and the high fluorescence quenching ability of MWCNTs. The kinetic of fluorescence quenching of green gel labeled aptamer by MWCNTs was studied by measuring fluorescence intensity at different incubation times. In the presence of MWCNTs, fluorescence emission was rapidly decreased in the first 2 min and completely quenched after 20 min. Therefor, 20 min was estimated as a functional quenching time. In another experiment, different amounts of MWCNTs were used and fluorescence quenching was investigated at the optimum time. As shown in Fig. 5b, increasing the MWCNTs concentration results a decrease in fluorescence emission. At a concentration of 30 μg/ml, fluorescence emission

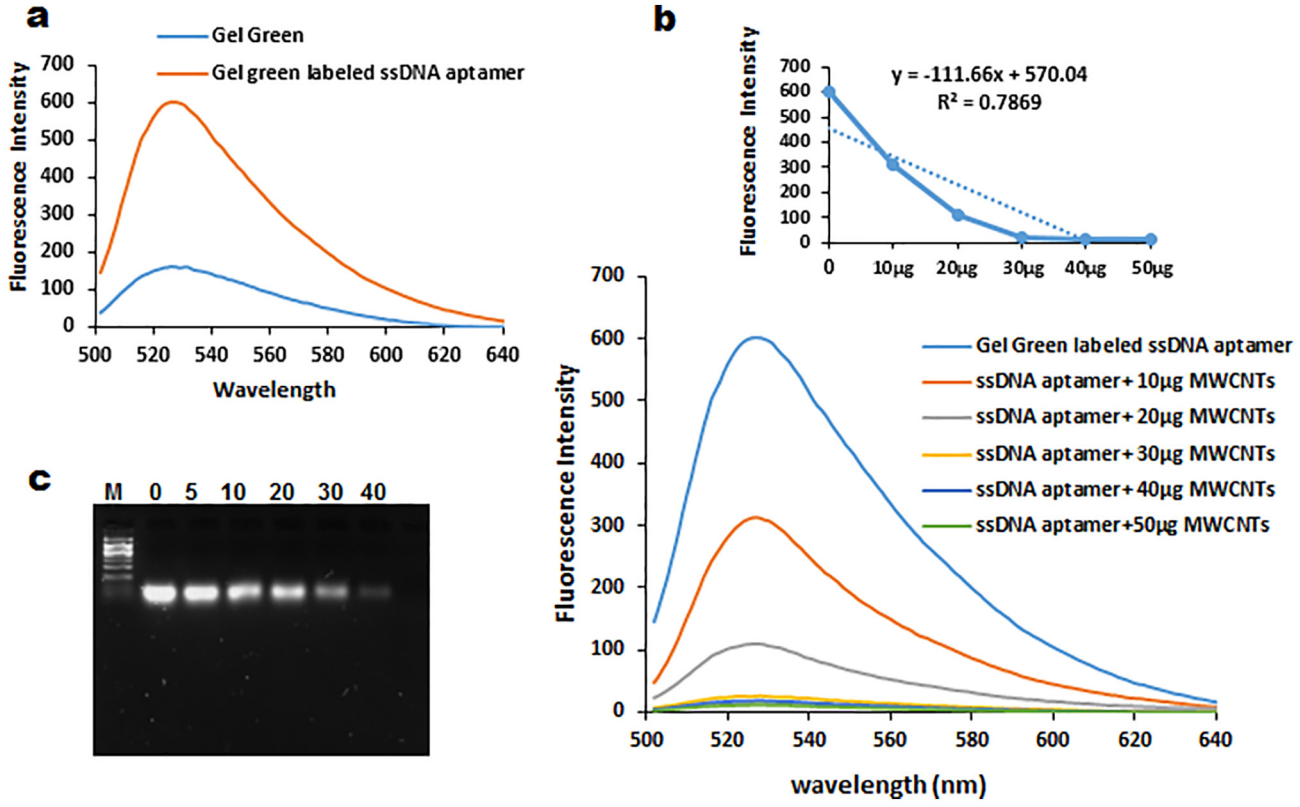

Fig. 5. a) Fluorescence emission spectra of Gel Green and Gel Green labeled ssDNA aptamer. b) Fluorescence quenching optimization of Gel Green labeled ssDNA aptamer in different concentration of MWCNTs. fluorescence intensity of ssDNA aptamer with the increasing of MWCNTs concentration. c) Agarose gel electrophoresis pattern of ssDNA aptamer band intensity in different concentration of MWCNTS.

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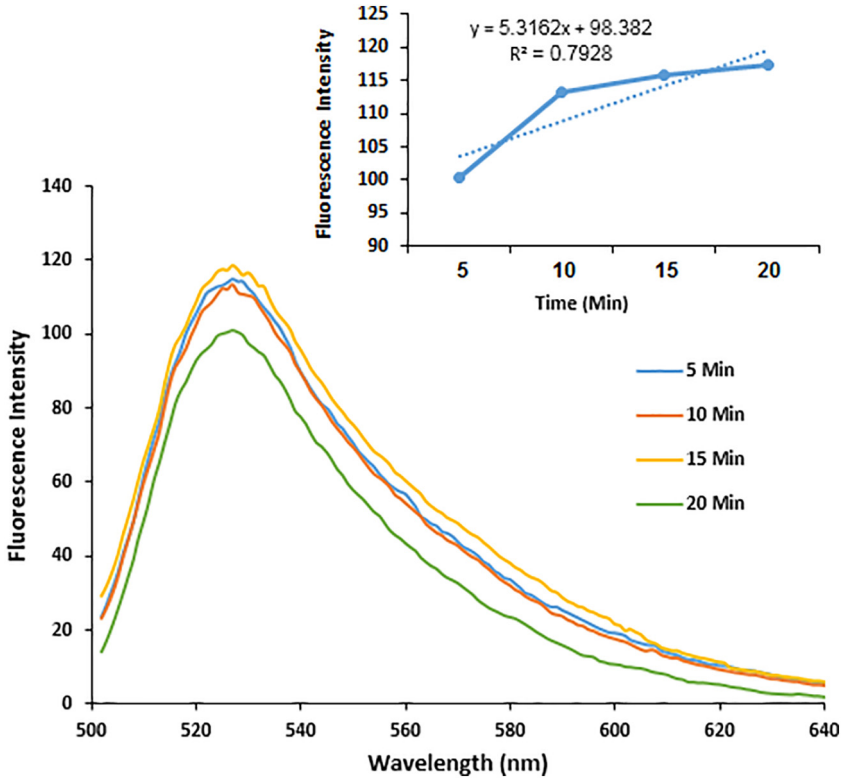

Fig. 6. Fluorescence restoration of ssDNA aptamer-MWCNTs by PAD4 (100 ng) as a function of time. Excited at 480 nm.

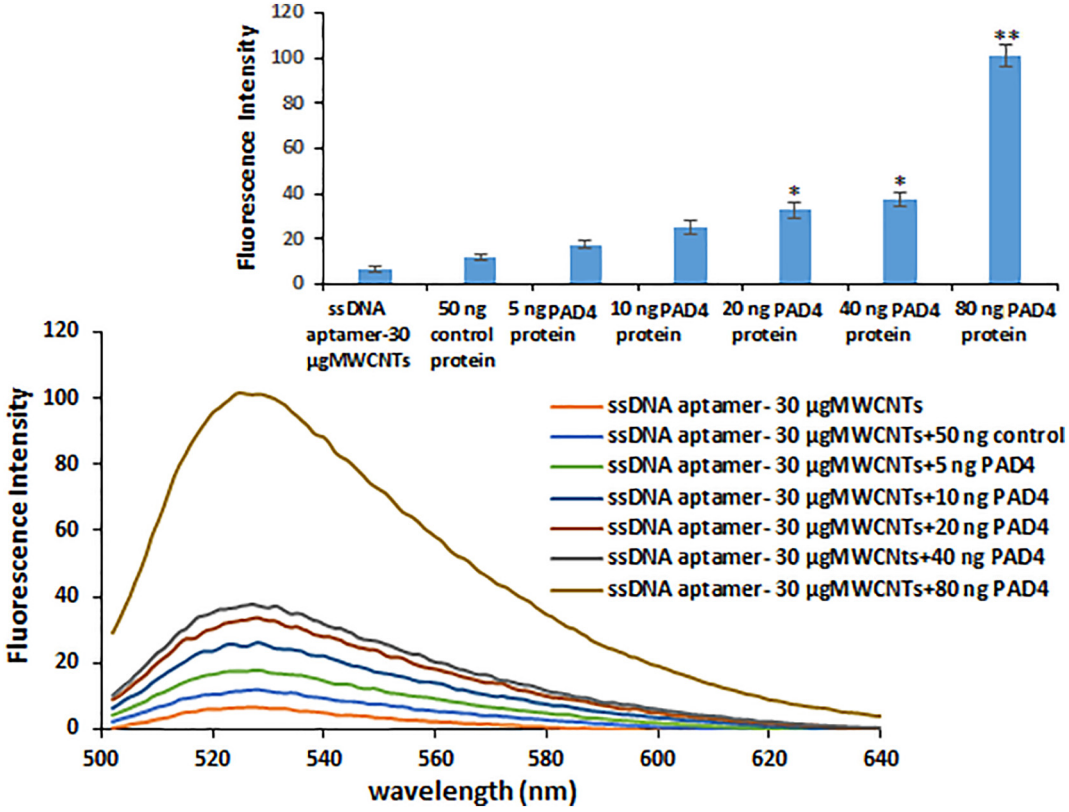

Fig. 7. Fluorescence spectra for determination of MWCNTs based aptasensor sensitivity in the presence of different concentrations of purified rPAD4. Excited at 480 nm. Vertical bars show standard deviation (SD) (n = 3). Differences were considered significant whenever p ≤ .05 and p ≤ .01. *, **present significant difference of fluorescence emission of rPAD4 from control at p ≤ .05 and p ≤ .01, respectively.

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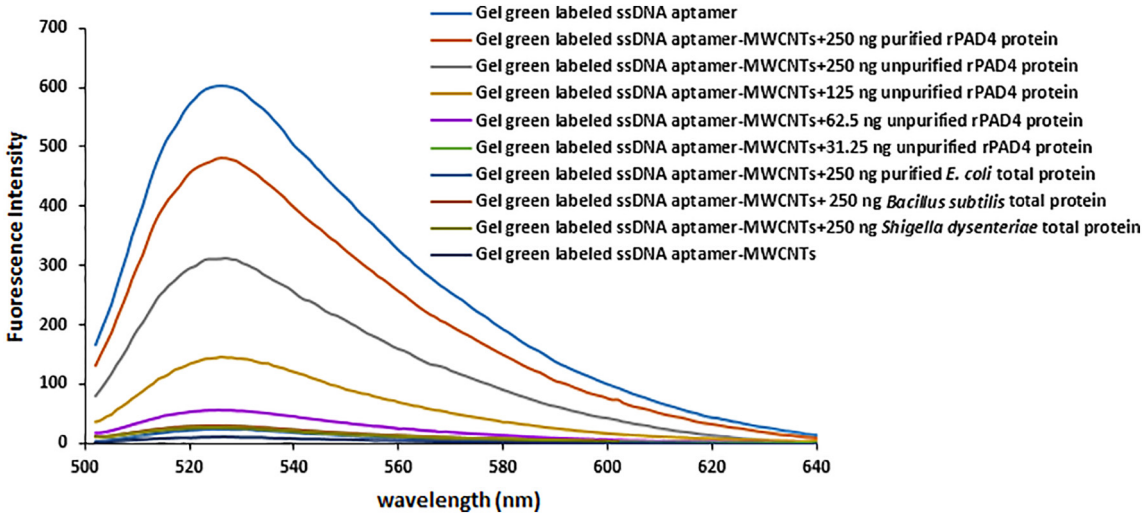

Fig. 8. Fluorescence spectra for determination of the specificity and selectivity of the ssDNA aptasensor in the presence of different concentrations of unpurified rPAD4. At a concentration of 62.5 ng/ml, a high level of fluorescence restoration was observed compared to control bacteria ($P \leq .05$).

was completely quenched. In fact, dye labeled ssDNA aptamer adsorbs onto MWCNTs by π-π stacking interaction between nucleobases and the aromatic MWCNTs side walls. By increasing the concentration of MWCNTs in the functional reaction tube, non-covalent interactions between two parts (aptamer and MWCNTs) increase and result in complete fluorescence decay. Beside the fluorometric analyses, agarose gel electrophoresis confirmed the fluorescence quenching ability of MWCNTs. In the ssDNA-MWCNTs electrophoresis pattern, a decrease in band intensity compared to ssDNA aptamer was observed (Fig. 5c).

3.6. Detection of PAD4 by ssDNA aptamer-MWCNTs based sensor and determination of their sensitivity

In this study detection of rPAD4 by MWCNTs based aptasensor was relied on the unique ability of ssDNA aptamer which binds to rPAD4 with high affinity and specificity. Aptamers are short single-strand oligonucleotides that specifically bind to target molecules. The use of these molecules as a recognition element in biosensor fabrication have opened a new field for the development of the new-generation of biosensors [44]. In this study, PAD4 specific aptasensor was fabricated by adsorption of green gel labeled ssDNA aptamer on MWCNTs. The performance of the sensor was based on MWCNTs ability to bind to the ssDNA aptamer with a greater affinity compared to its binding to well-folded ssDNA or double-strand DNA. During hybridization reaction, by adding the purified rPAD4 protein, the fluorescence emission of gel green was recovered. In this case, by increasing the concentration of rPAD4, the fluorescence restoration enhanced gradually until reach the equilibrium point. It has been reported that fluorescence labeled ssDNA aptamer in the absence of target protein is adsorbed on MWCNT and fluorescence quenched [45]. While, in the presence of target protein and its binding to aptamer changes the spatial conformation of aptamer and leads to the formation of double-strand structures in ssDNA aptamer which results desorption of aptamer from MWCNTs surface. In the hybridization reaction, the kinetic of fluorescence restoration was studied by measuring of fluorescence intensity at different hybridization times. In the presence of 100 ng rPAD4, the fluorescence intensity increased rapidly in the first 5 min, continued with a slow process and remained unchanged after 10 min. Therefore, the hybridization time of 10 min was considered as the optimal hybridization time (Fig. 6). The sensitivity of aptasensor was evaluated with different

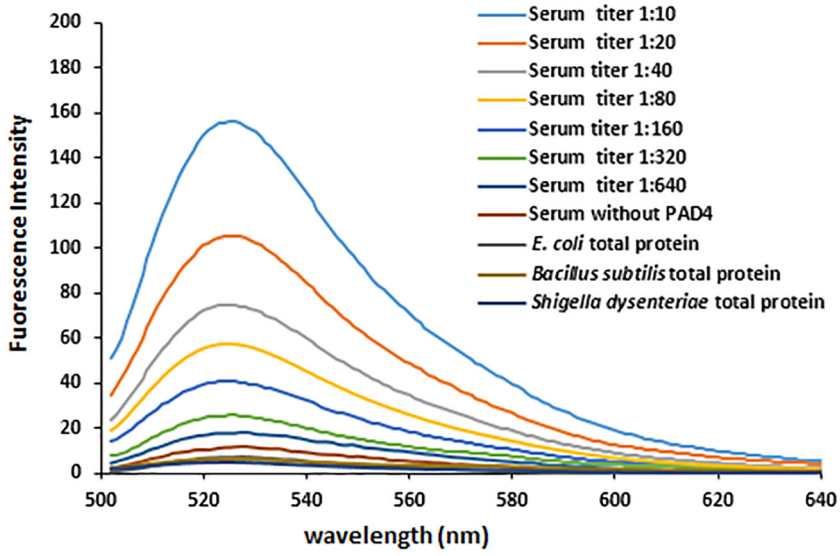

Fig. 9. Fluorescence spectra for evaluation of the applicability of the aptasensor for the detection of rPAD4 in different dilution of mouse serum. In serum titer of 1:640, significant fluorescence restoration was observed compared to the control ($P \leq .05$).

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Table 1
The performance of aptasensor and ELISA assay for the detection of rPAD4.

	Sensitivity with purified PAD4	Specificity with unpurified PAD4	Applicability with unpurified PAD4 in serum	Time assay	Cost assay
Aptasensor	LOD: 20 ng/ml	LOD: 62.5 ng/ml	1:640 serum titer	30 min	Cost effective
ELISA assay	LOD: 20 ng/ml	LOD: 125 ng/ml	1:320 serum titer	6 h	Expensive

concentrations of purified $rPAD_4$ protein and the detection limit of aptasensor was determined 20 ng in 1 ml hybridization buffer. In this concentration, a significant level of fluorescence restoration was observed in comparison with untransformed *E. coli* total protein ($P \leq .05$) (Fig. 7). Range assay for the sensitivity detection of aptasensor was between 5 ng/ml and 80 ng/ml. Investigation of the aptasensor specificity with different concentrations of unpurified rPDA4 indicated high levels of fluorescence restoration in compared to control ($P \leq .05$) (Fig. 8). In hybridization reaction of the aptasensor with total protein from other bacteria, (*E. coli, Bacillus subtilis, Shigella dysenteriae*) no significant fluorescence emission was observed indicating selectivity and specificity of aptasensor to rPAD4. Limit of detection for specificity of aptasensor was determined 62.5 ng unpurified rPAD4 in 1 ml hybridization buffer (Fig. 8). In specificity detection experiments, range assay was between 31.25 ng/ml and 250 mg/ml. In most anthrax diagnosis tests, the levels of PA is evaluated in infected animal serum [46]. In this study to illustrate the applicability of aptasensor, the unpurified rPAD4 was added to the mouse blood samples and then the performance of the sensor was studied for the detection of PAD4 in the serum. In results, significant fluorescence restoration was observed in serum samples compared to the control ($P \leq .05$) (Fig. 9). Detection limit for the applicability of aptasensor was estimated with an endpoint titer, 1:640 in compared with the control ($P \leq .05$) (Fig. 9). After studying the sensitivity, specificity and applicability of aptasensor for detection of PAD4, its performance was compared with ELISA (Table 1). In results of ELISA, detection limit for the sensitivity and specificity of ELISA was determined 20 ng/ml purified rPAD4 and 125 ng/ml unpurified rPAD4, respectively. In this study, comparing the results of the ELISA with the nanosensor showed that the sensitivity of both methods for the detection of purified rPAD4 was almost the same. However, the specificity and ability of the nanosensor for detection of unpurified rPAD4 in bacterial total protein and mouse serum was better than ELISA. ELISA is presently the most effective and prevalent method for detection of PA in serum [47]. The major limitations of this method are labor-intensive and high cost of antibody production [48]. Because the cell culture media are expensive and needed to obtain a specific antibody. While in the aptasensor method, the design and synthesis of the aptamer is inexpensive and cost-effective compared to the production of specific antibodies. In the aptasensor method, the total assay time required to estimate the PAD4 protein levels was 30 min, whereas in the ELISA it was 6 h, indicating that the aptasensor is a fast-acting method compared to ELISA.

4. Conclusion

In conclusion, we developed an aptasensor for detection of *Bacillus anthracis* PAD4 which is the important and earliest key factor in the development of anthrax. Gel Green, as a fluorescence dye was used to label ssDNA aptamer which is safe, thermostable and commercially inexpensive. MTWCNTs and MWCNTs-ssDNA aptamer conjugates were confirmed by SEM, TEM, EDX and molecular biology methods. Fluorescence emission of ssDNA aptamer was quenched in the presence of MWCNTs. in contrast, upon the adding of PAD4 protein and incubation in a 10 min period, fluorescence emission of Gel Green was significantly recovered to 85%. Detection limit of the sensitivity and specificity of the aptasensor were determined 20 ng purified PAD4 protein and 62.5 ng unpurified PAD4 protein in 1 ml Tris-HCl hybridization buffer, respectively. Comparing the performance of the aptasenor with the ELISA assay in real sample showed that the aptasensor is more sensitive than the ELISA method. Finally, results of this study indicated that ssDNA aptamer-MWCNTs based nanosensor has the potential to be developed as a high-sensitive, cost-effective and fast-acting system for measuring of PA in anthrax diagnostic tests.

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