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Development of a quality control test for Diphtheria vaccine based on an electrochemical aptamer-based biosensor

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Abstract

At every stage of the vaccine's development, including research and development together with the routine production (in-process control of batch release), quality control testing is a crucial operation for ensuring the efficacy, safety and consistency of final products. Conventional quality control tests rely on a combination of biochemical, physicochemical, and cell-based assays. In this study, we report a quality control test based on an electrochemical aptamer-based sensor with a specific application to Diphtheria vaccine. First, specific aptamers for Diphtheria are successfully selected and characterised by using SELEX (Systematic Evolution of Ligands by EXponential enrichment) process. A total of 11 rounds were performed to select five sequences with variable affinities to Diphtheria toxoid. The aptamer D1 exhibiting the best affinity towards the Diphtheria

toxoid (K_d= 1.4 nM) was employed to develop an electrochemical aptasensing platform. The selected aptamer was conjugated to a thiol group to enable its immobilization on screen-printed gold electrodes (SPGEs). Then, the determination of Diphtheria toxoid was performed in a label-free mode by monitoring the electrochemical signal before and after the formation of the aptamer-target complex. Excellent analytical performance was obtained within a wide linear range (0.008 to 290 ng/mL) with the low detection limit of 0.0095 ng/mL. Given the combined use of Diphtheria with Pertussis and Tetanus, cross-reactivity tests were centred on these two vaccines. The aptasensor showed high selectivity and specificity for Diphtheria compared to a negligible response for the non-specific toxoids. Furthermore, the applicability of the aptasensor was successfully investigated for the detection of Diphtheria toxoid in real vaccine samples. The developed aptasensor shows a great promise as a fast, cost-effective and accurate method for vaccine quality control testing.

Keywords: Diphtheria, Quality control testing, Vaccines, SELEX, Aptamer, Electrochemistry.

Introduction

The incidence of the bacterial infection from *Corynebacterium diphtheriae* has greatly decreased due to the introduction of Diphtheria vaccines, which are among the most common and successful human immunisations [1, 2]. They are made up of Diphtheria toxin (toxoid) preparations formaldehyde-inactivated and adsorbed onto an aluminium adjuvant [3, 4]. The World Health Organization (WHO) recommends the

integration of Diphtheria vaccines into the national immunization programs of all countries [5]. Indeed, Diphtheria vaccines constitute an essential part of the extended vaccination campaign in Saudia Arabia. According to WHO recommendations, children receive three doses of the vaccine at 6, 10, and 14 weeks of age, followed by a booster dose at 18 months and another one at 4-6 years [6]. Diphtheria vaccines are usually authorised as a combination of paediatric primary dose vaccines or vaccines with low antigen concentrations to boost immunisations. Other antigens such as Tetanus (T), acellular Pertussis (aP), inactivated polio virus (IPV), Haemophilus influenzae type b (Hib), and hepatitis B (HepB) components could be also added to these combinations [7].

The development of consistently high-quality vaccines, rather than optimising product yield or lowering manufacturing costs, is the industry's top priority [8]. To ensure that vaccines can be considered safe and efficient, regulatory bodies have established standards. Vaccines are tested at several stages of the manufacturing process to ensure that they meet standards [9]. Aiming to avoid the variations between vaccines batches and lots tested clinically safe and effective, quality control is accordingly focused on in-process monitoring. Most of the in-process monitoring is based on biochemical, physicochemical, and cell-based assays [10]. This consistency approach is approved from both the European Pharmacopoeia (Ph. Eur.) and the WHO [11]. In the context of routine lot release testing, it has become standard practice for newly developed, well-defined vaccines (such as polysaccharide conjugate

vaccines). This standardization include the use of *in vitro* methods allowing faster batch release while maintaining safety and efficacy standards [12].

The routine quality control procedures of vaccines applied from either manufacturer or National regulatory authority (NRA)/National control laboratories (NCL) of each country are based on different tests. They include but not limited to physical testing (e.g.; visual inspection, extractable volume, PH determination), chemical testing; (identity by immunoassays), potency (*in vivo / in vitro* test), sterility test, adjuvant content and preservative content [13, 14]. Considered as an essential component in immuno-based identification and potency test methods, antibodies are characterized with a high affinity towards their targets [15]. Nevertheless, whether monoclonal or polyclonal, antibodies suffer from major disadvantages such as lack of stability, short shelf-life, lot-to-lot variations and high cost. In addition, their production is time-consuming and evolves animal immunization [16]. In this context, aptamers appeared as an excellent alternative that likely overcome many of these limitations. Aptamers are short segments of oligonucleotides selected by SELEX (Systematic Evolution of Ligands by EXponential enrichment) for their ability of specific binding to a particular target. With dissociation rates in the low nanomolar to picomolar range, aptamers frequently exhibit affinities and specificities that are equivalent to those of antibodies [17, 18]. Moreover, aptamers are characterized by a high selectivity since they can distinguish between proteins that differ by a single amino acid or tiny molecules that differ by a single atom in particular conditions [19, 20]. In contrast to antibodies, aptamers are chemically synthesized, thus

eliminating batch-to-batch differences, enhancing the stability in different environmental changes and lowering the cost and production time [21]. Aptamers have shown a successful applicability in bioanalysis, since they can be easily labelled with a variety of chemical groups, molecules, linkers and dyes [22].

SELEX is based on three main steps: incubation of the random oligonucleotides' library with the targeted molecule, isolation of the bound nucleic acid ligands, and amplification by PCR. These steps, considered as a cycle of selection are frequently repeated 6 to15 times including interval counter selection with non-target substances to remove nonspecific candidates [23]. The resulting enriched pool of nucleic acids exhibiting the highest binding affinity is then cloned, and sequenced. The obtained sequences are examined, and the classified sequence with the highest percentage of occurrence is identified as the potential aptamer [24-26]. In the current study, we employed SELEX process to isolate aptamers specifically recognizing the Diphtheria toxoid. Through multiple rounds of selection and amplification, we selected five sequences, among which the aptamers D1 and D2 exhibited the lowest dissociation constants. The aptamer D1 exhibiting the best affinity towards the Diphtheria toxoid (K_d=1.4 nM) was used to develop an electrochemical aptasensing platform. The sequence was first conjugated to a thiol group and covalently immobilized on a gold transducing surface. The aptasensor ability to quantify Diphtheria toxoid was assessed by using voltametric analysis based on a label-free mode. The applicability of the proposed aptasensor was tested on a combined commercial vaccine, i.e. Diphtheria, Tetanus,

Pertussis (DTP). As far as we know, this is the first report describing the selection of aptamers for Diphtheria toxoid and their application in an electrochemical biosensor enabling the fast and accurate detection of Diphtheria toxoid in vaccines.

Experimental

1. Materials and reagents

Diphtheria toxoid (WHO international standard: 13/212) and Diphtheria antibody (international standard: 10/262) were acquired from The National Institute for Biological Standards and Control, UK. N-hydroxysuccinimide (NHS)-pre-activated agarose beads, used as solid matrix in SELEX process, were purchased from Purolite Healthcare & Life Sciences (King of Prussia, PA, USA). Phosphate buffer saline (PBS), pH 7.4, Ethylenediaminetetraacetic acid (EDTA), sodium bicarbonate, sodium chloride, sodium azide, Tris-base, Bovine serum albumin (BSA), urea, N-dimethylformamide (DMF), magnesium chloride, ethanol, hydrochloric acid, sodium acetate, Tetramethylethylenediamine (TEMED), acrylamide/bis-acrylamide (30% solution), ammonium persulfate (APS), Potassium ferrocyanide ($K_4Fe(CN)_6$), potassium ferricyanide ($K_3Fe(CN)_6$), and mercapto-1-hexanol (MCH) solution were purchased from Sigma Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP)-labelled secondary antibody and 3,3',5,5’-tetramethylbenzidine (TMB) chromogen solution were purchased from Thermo Fisher Scientific. Spin-X with CA membrane, pore size 0.45 µm was purchased from Corning life sciences (Tewksbury, MA, USA). 3K centrifugal filters (Amicon Ultra, 0.5 mL)

utilized for DNA desalting and concentration, were obtained from EMD Millipore (Alberta, Canada). PCR reagents; (10x buffer, dNTPs, Taq polymerase, loading dye, and 100 bp ladder) were purchased from ACE Biotech (Riyadh, Saudi Arabia). The DNA library (5'-TCCCTACGGCGCTAAC-40 N -GCCACCGTGCTACAAC-3'), fluorescent primer carboxyfluorescin (5'-FAM-TCCCTACGGCGCTAAC3'), Poly A primer (5'-poly-dA20-hexaethyleneglycol-GTTGTAGCACGGTGGC-3'), unlabelled primers, M13 primers, and the aptamer sequences were synthesized by Metabion International AG (Planegg, Germany). Cloning Kit with One Shot TOP10 and the DH5α T1R chemically competent *E. coli* cells were purchased from Invitrogen Inc. (New York, USA). The X-Gal utilized in cloning steps was purchased from Bio Basic Inc. (Toronto, Ontario, Canada). Agarose powder, 50X Tris base, and ampicillin were purchased from Bio-Rad (California, USA). Culture media were acquired from Saudi Prepared Media Laboratory (SPML; Riyadh, Saudi Arabia). Milli-Q water was used for the preparation of all the reagents in this study.

2. Instrumentation

A UV-Vis microplate reader from BMG labtech, Germany, was used to measure the absorbance for ELISA (enzyme-linked immunosorbent assay) experiments. NanoDrop 2000C Spectrophotometer from Thermo Scientific (Ottawa, Canada) was used to measure the DNA concentration and the protein absorption. A Powerpack-current Power supply from Bio-Rad (California, United States) was used for the electrophoresis. UVP BioDoc-It Imaging system (UK) was used in the DNA band analyzing. T100

Thermal Cycler was used to carry out the PCR amplification. The NanoDrop 3300 was used to measure the fluorescence intensity of the DNA at 515 nm. Electrochemical measurements were performed by using AUTOLAB potentiostat PGSTAT302N (Multichannel) purchased from Metrohm, Netherlands. The potentiostat was connected to a computer and carried out by Nova 1.11 software. Screen-Printed Gold Electrodes (DRP-C220AT) were obtained from Metrohm DropSens (Spain). Each electrode consists of three parts (gold working electrodes, gold auxiliary electrode and silver reference electrode). The electrodes were linked to the potentiostat via a specific connector obtained from Metrohm DropSens.

3. Coupling of Diphtheria Toxoid to N-hydroxysuccinimide (NHS) activated agarose beads.

The N-hydroxysuccinimide (NHS) agarose beads were repeatedly washed with 2.5 mL of 1 mM HCl to remove the preservative solution and maintain the beads' activity before the conjugation. Then, 0.4 mg/mL (25 LF/mL (Limit of Flocculation)) of Diphtheria toxoid prepared in $NaHCO_3$ (0.2 M, pH 8.3) was incubated with the activated beads at 4 °C overnight while being rotated end-over-end in a 1:1 ratio. After that, toxoid-conjugated beads were washed with 2 mL of 0.1 M Tris (pH 8.5). The free active sites on the beads surface were then blocked by incubation in Tris buffer for 1 hour while being rotated. After that, three cycles of washing with alternate pH buffers: 0.1 M Tris (pH 8.0) and 0.1 M Acetate NaCl (pH 4.0) were carried out. Finally, Diphtheria toxoid-conjugated

beads were stored at 4 °C in TE (Tris-EDTA) buffer containing 0.05% sodium azide, for further use.

The immobilization of Diphtheria toxoid on the beads was confirmed by performing a direct ELISA for the coupled beads and the control negative beads. The ELISA was performed in a 96-well plate. Two samples were prepared: a negative control containing unconjugated beads and the second containing Diphtheria-coated beads. In brief, 30 µL of each sample were incubated on the microplate wells overnight at 4 °C. After washing with PBS, pH 7.4, a blocking step was performed by adding BSA, for one hour at room temperature 2% BSA. The wells were then incubated with 1:1000 Diphtheria antibody prepared in PBS buffer, pH 7.4 for 1 hour at room temperature. After washing with PBS buffer, HRP-conjugated secondary antibody (1:1000 in PBS) was added and incubated for 1 hour at room temperature. Following three washes with PBS buffer, TMB substrate was added, and left to react for 30 minutes in dark. Finally, the absorbance of each well was measured by using a microplate reader at a wavelength of 450 nm. A well containing free Diphtheria toxoid was also prepared and used as a positive control.

4. *In vitro* selection of the ssDNA aptamer

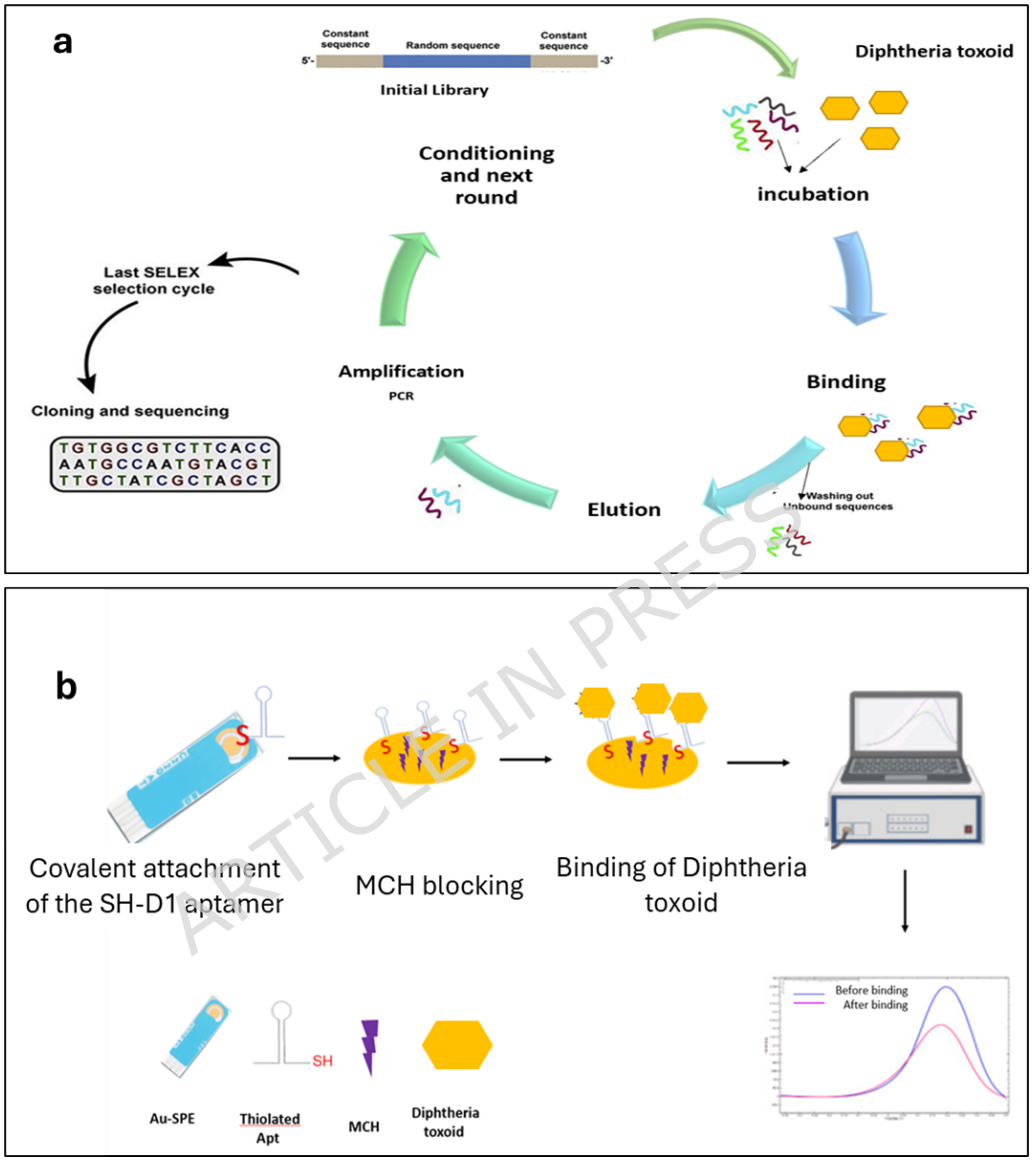
Scheme 1.a depicts the main steps of the aptamers selection process.The ssDNA library utilized in the first cycle of SELEX is composed of a random region and constant primer binding regions of 16 nucleotides at 5′ and 3′ ends (5’-TCCCTACGGCGCTAAC-40 N -GCCACCGTGCTACAAC-3’). Before use, the library was pretreated by heating the DNA solution at

90°C for 5 minutes, cooling at 4 °C for 10 minutes and incubating it at 25° C for 5 minutes. Then, 300 nmol of the library were incubated with 100 µL of Diphtheria toxoid-beads, prepared in binding buffer (BB), under rotation in a centrifuge filter tube for 2 hours. After washing, a denaturation step was used to elute the DNA bound to the Diphtheria toxoid-beads. Then, 400 µL of heated elution buffer (90°C) containing urea was added to the beads and incubated at 90°C for ten minutes. This step was repeated six times until no fluorescence was detected. The DNA solution obtained from the elution step was concentrated and desalted by using a 3 kDa cutoff centrifugal filter (ultrafiltration tube). After cycle eight, a counter selection was performed by using similar steps; the DNA library was incubated with the negative beads instead of the diphtheria-toxoid conjugated beads. After washing the beads four times, the DNA was collected and concentrated using a centrifugal filter with a 3 kDa cutoff. The collected DNA was then subjected to the heating-cooling cycle as described earlier, and the resulting ssDNA solution was incubated with target-specific beads for the next cycle. Subsequent PCR amplification generated dsDNA with a length of 72 base pairs. In each cycle, the dsDNA was denatured into ssDNA using denaturing PAGE (Polyacrylamide gel electrophoresis). SELEX process was terminated after the eleventh cycle and the eluted ssDNA was purified, amplified by PCR and cloned. The master mix consisting of 10x buffer, 10 mM dNTP, 25 mM $MgCl_2$, 0.2 µM primers (forward and reverse), and 2 units of Taq polymerase was applied in 20 reactions for the PCR amplification stage. The PCR thermal cycle started at 95°C for 5 minutes, followed by 15 cycles of 95°C for 30 seconds, 54°C

for 30 seconds, 72°C for 45 seconds, and finished with an additional 10 minutes at 72 °C. The amplified products were verified by 2% agarose gel electrophoresis at 110V for 30 minutes. The amplified dsDNA was then subjected to ethanol precipitation using three volumes of absolute cold ethanol and 0.1 volume of 3 M sodium acetate (pH 5.5). The mixture was centrifuged for 30 minutes at 4 °C after being incubated at -80 °C for one hour. The pellet was then rinsed with 75 % cold ethanol and dried at 37 °C to eliminate any leftover ethanol before the supernatant was removed. The dried pellet was dissolved in a mixture of water and formamide loading dye in a 1:2 v/v ratio, and then incubated for 5 minutes at 90 °C. The FAM-labelled ss-DNA (specific sequence) was separated from the nonspecific one (PolyA-sequence), through a 10% denaturing polyacrylamide gel at 300 V for 2.5 hours. After that, the obtained band corresponding to the fluorescent ssDNA was cut from the gel, and the DNA was extracted by adding TE buffer and using the freeze/thaw method. The DNA mixture was then incubated in a rotator at 37 °C overnight. The eluted ssDNA solution was desalted and concentrated using a 3K filter and was subsequently used to initiate a new SELEX cycle.

To clone the DNA obtained from the last round (round 11), in which the recovery nearly plateaued, the DNA was first amplified using a set of unmodified primers, followed by cloning using the TOPO TA Cloning Kit and the 1-TOPO vector. The colonies were grown on LB-agar medium involving IPTG, X-Gal, and ampicillin. Then the white and light blue colonies were observed. These colonies were picked up one by one, and PCR was run using forward and reverse M13 primers on each colony to

amplify the ssDNA inserts. Finally, PCR products underwent Sanger sequencing analysis, and PRALINE program was used for alignment of the selected sequences (https://www.ibi.vu.nl/programs/pralinewww/).



Scheme 1: Schematic diagram of the mechanism of selection of the ssDNA aptamers towards Diphtheria toxoid by using SELEX process **(a)** and the fabrication and detection steps of the Diphtheria toxoid voltametric aptasensor **(b)**.

5. Determination of the binding affinities for selected Diphtheria aptamers

To study the binding affinities of the selected aptamers for Diphtheria toxoid, the dissociation constants (K_d) were determined by fluorescence binding examinations. After being heated and cooled, various concentrations (0 to 400 nM) of the selected sequences were prepared in binding buffer and incubated with Diphtheria toxoid conjugated-beads for 1 hour at room temperature with end-over-end rotation. Then, the unbound DNA was removed from the beads by washing with binding buffer, while the bound DNA was eluted by using a heated elution buffer. NanoDrop 3300 was used to quantify the fluorescence of the eluted DNA, and GraphPad Prism was used to plot the saturation curve for each aptamer sequence. The dissociation constants were ultimately determined by nonlinear regression analysis.

6. Diphtheria toxoid aptasensing

The aptamer D1 (Diphtheria 1) showing the best affinity was used in the aptasensor fabrication (**Scheme 1.b**). The thiolated sequences were synthesized by Metabion company, Germany. 10 µL of the aptamer solution (1 µM), prepared in BB were dropped onto the working electrode of screen-printed gold electrodes. The SH-labelled ssDNA sequences were left to interact with the gold surface overnight at 4 °C in a water-saturated atmosphere. Then, the functionalized SPGE was rinsed with PBS and incubated with a blocking solution containing 1 mM MCH prepared in PBS

buffer, pH of 7.4, for 30 minutes. After that, the aptasensor was carefully rinsed with PBS and used right away in the electrochemical tests.

For the electrochemical determination experiments**,** the aptasensor was incubated with 10 µL of various concentrations of Diphtheria toxoid, ranging between 0.008 ng/ml and 4 µg/mL, for 30 minutes at room temperature in water-saturated atmosphere. Square wave voltammetry (SWV) was performed for monitoring the electrochemical signal before and after the Diphtheria binding in the redox solution (5 mM ferri/ferrocyanide prepared in PBS buffer pH 7.4) at 25 Hz frequency, 0.04 s interval time, 0.5 to -0.5 V, −5 mV step potential, 125 mV/s scan rate, and 20 mV amplitude.

7. Cross reactivity and real sample applicability

To examine the selectivity of the fabricated aptasensor, Tetanus and Pertussis toxoids were used as potential interfering targets. For that, the three toxoids were, individually, incubated with the aptamer-functionalized electrodes for 30 minutes at room temperature. After washing the surface with PBS buffer, square wave voltammograms were recorded. Finally, the electrochemical responses of the interfering vaccines (Tetanus and Pertussis) were compared to that obtained with diphtheria-specific target.

To demonstrate the feasibility of our technique for Diphtheria toxoid detection in real vaccine samples, the aptasensor performance was tested on a combined commercial vaccine; Diphtheria, Tetanus, Pertussis. First, the preservative was eliminated using chemical desorption step of the

vaccine [27]. In brief, the vaccine was centrifuged, and the pellet was re-suspended in a freshly prepared solution of EDTA (1.12 g/L EDTA, 88.2 g/L Na_2HPO_4). Then, the mixture was maintained at 37 °C for 6 hours and centrifuged again. The clear supernatant was removed and stored for up to 1 week at 4 °C. Following that, 10 µL of the desorbed vaccine was incubated with the designed aptasensor at room temperature for 30 minutes. SWV measurements were then carried out by following the same procedure described above.

Results and Discussion

1. Coupling of Diphtheria Toxoid to N-hydroxysuccinimide (NHS)-activated agarose beads.

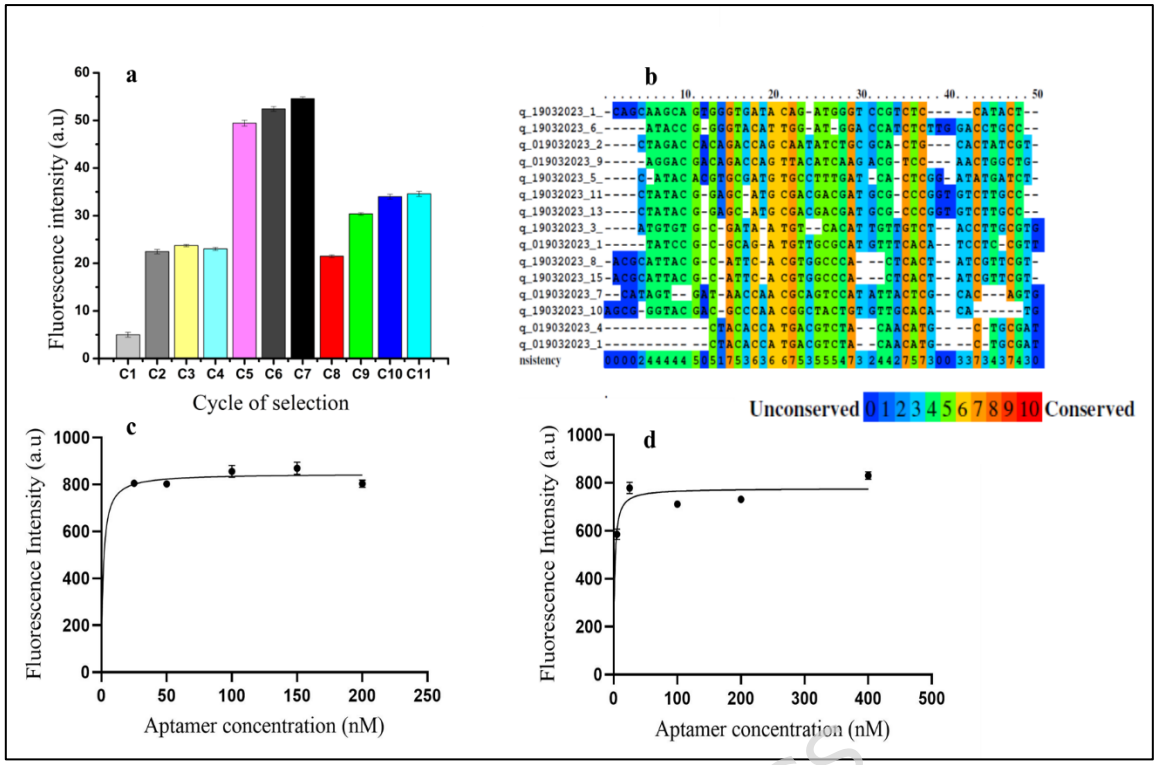
During the SELEX procedure, immobilising the targeted molecule on a solid matrix is a crucial step in separating bound and unbound DNA sequences. Here, the NHS terminal group of the beads reacts with the protein's amine groups through covalent bond, thus conjugating Diphtheria toxoid to agarose beads. After that, direct ELISA was used to confirm the conjugation. To confirm successful conjugation of Diphtheria toxoid to agarose beads, a direct ELISA was performed. This assay utilizes the specific interaction between the target molecule (Diphtheria toxoid) and HRP-labelled antibodies. In parallel, a negative control consisting of unconjugated beads, was prepared to evaluate the non-specific binding or background signal arising from the beads or the used materials. During the ELISA, the addition of TMB substrate resulted in a colorimetric reaction. The HRP enzyme labelling the antibody catalysed the oxidation

of TMB, producing a blue colour in wells containing Diphtheria toxoid. In contrast, a slight colour change was observed in the negative control well, indicating the absence of significant background signal. The presence of blue colour in the wells containing Diphtheria-conjugated beads confirmed the successful covalent conjugation of Diphtheria toxoid to the NHS-activated agarose beads (**Figure.S1**).

2. *In vitro* selection of the ssDNA aptamers specific to Diphtheria toxoid

The different steps of the positive and negative SELEX generating the specific aptamers to the Diphtheria toxoid are shown in **Scheme 1.b**. The principle is based on the incubation of the targeted toxoid with the DNA library pretreated by a heating/cooling procedure (5 min heating at 90 °C, 10 min cooling at 4 °C, and 5 min at RT) to guarantee the proper folding of ssDNA to bind the target. This step is followed by a partioning the bound sequences from the non-bound ones. Finally, the last step consists in amplifying the specific candidates by PCR. Since the interaction DNA-target is noncovalent, the bound DNA can be separated from the complex by applying mild conditions like heat and chaotropic chemicals like urea. Urea causes the proteins secondary structures to be disrupted and the bound DNA to be released from the target, by breaking the native backbone hydrogen and hydrophobic bonds between the proteins and the aptamers [28, 29]. These three steps (incubation, partitioning and amplification) are repeated in iterative SELEX rounds.

The fluorescence intensity of the eluted DNA, obtained from each round, is measured to monitor the enrichment of the specific DNA targeting Diphtheria toxoid via the SELEX process. The fluorescence intensities corresponding to the eluted DNA from the different rounds of Diphtheria toxoid aptamer selection are displayed in **Figure 1.a**. The enrichment of the DNA sequences specific to Diphtheria toxoid can be confirmed by the remarkable increase in the DNA recovery as the number of cycles increased. Aiming to eliminate non-specific oligonucleotides, counter selection was carried out by incubating the DNA pool obtained from the seventh cycle with blank beads. After negative selection, a remarkable decrease in the fluorescence intensity of the recovered DNA was noted, confirming the elimination of nonspecific DNA sequences. After each cycle, the eluted DNA was amplified by PCR and visualized by using agarose gel electrophoresis. The amplification stage was successful when a band of the same size as the original library was observed at 72 bp. Between two cycles, dsDNA was separated and purified through a 10% Polyacrylamide gel electrophoresis, and the resulting ssDNA sequences were used to start the following round. Finally, the ssDNA pool of cycle 11 was collected for cloning and sequencing. 15 clones were randomly chosen from each selection and subjected to multiple sequence alignment analysis using the PRALINE programme. Based on their similarities, we divided the selected sequences into 5 groups (**Figure 1. b**), and one aptamer was chosen from each group to perform further experiments.



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397

398 **Figure 1**: Results of the selection process: Sequence analysis and
399 categorization by using PRALINE Software **(a)**, Plot of the fluorescence
400 intensity corresponding to the recovered DNA after each round of the
401 aptamer selection for Diphtheria toxoid **(b)**, fluorescence binding affinity
402 curves for the aptamers D1 **(c)** and D2 **(b)**. The error bars represent the
403 standard deviation of three independent measurements. Fluorescence
404 measurements were performed at an excitation wavelength of 495 nm and
405 emission wavelength of 519 nm.

406

407 **3. Determination of the binding affinities for selected Diphtheria**
408 **aptamers**

The binding experiments were carried out by incubating 50 µL of increasing concentrations of FAM-modified aptamers prepared in binding buffer (5 to 400 nM) with a fixed amount (100 µL) of Diphtheria toxoid beads. Nonlinear regression analysis was used to determine the constant of dissociation (K_d) by plotting the fluorescence intensity of the eluted DNA for each aptamer concentration. As shown in the binding curves displayed in **Figure 1.c** and **d**, the fluorescence intensity increased by increasing the aptamer concentrations until it reaches a plateau. **Table1** lists the five selected Diphtheria toxoid sequences as well as their corresponding K_d values. As it can be seen from the table, the K_d values of the selected aptamers range from 1.4 to 21.05 nM. The obtained results show that aptamers D1 and D2 exhibit the lowest dissociation constants 1.4 and 2.3, respectively. The aptamer D1 was selected for the fabrication of the electrochemical aptasensor since it demonstrated the highest binding affinity.

Table 1: Sequences of the generated aptamers for Diphtheria toxoid and their corresponding K_d values.

Name	Sequence	K_d (nM)
D 1	CTA GAC CAC AGA CCA GCA ATA TCT GCG CAC TGC ACT ATC GT	1.4
D 2	CTA TAC GGA GCA TGC GAC GAC GAT GCG CCC GGT GTC TTG CC	2.3

D 3	ATG TGT GCG ATA ATG TCA CAT TGT TGT CTA CCT TGC GTG T	16.1
D 4	ACG CAT TAC GCA TTC ACG TGG CCC ACT CAC TAT CGT TCG T	3.3
D 5	AGC GGG TAC GAC GCC CAA CGG CTA CTG TGT TGC ACA CAT G	21.05

4. Electrochemical aptasensing of Diphtheria toxoid

A biosensor is a device that uses biological molecules to detect the presence of a target molecule in a sample. In the case of an electrochemical biosensor, the target molecule is detected by measuring the electrical current generated by the reaction between the target molecule and the biological molecule [30, 31]. Given the sensitivity, accuracy and the cost-effectiveness of electrochemical techniques, we demonstrated the applicability of the selected aptamers by integrating them into an electrochemical biosensing platform. D1 aptamer was immobilized on a screen-printed gold electrode, which served as the transducer for the biosensor. When the target molecule (Diphtheria toxoid) binds to the aptamer, it causes a change in the electrical current generated by the reaction, which can be measured and used to detect the presence of Diphtheria toxoid in a label-free mode. Square wave voltammetry was chosen to monitor the electrochemical response of the aptasensor.

4.1. Electrochemical characterization of the aptasensor fabrication steps

The aptamer immobilisation on the gold surface was characterised by using CV measurements, a potent electrochemical method frequently used to investigate reductions and oxidations phenomena [32, 33]. CV measurements were carried out before and after surface functionalization, at a scan rate of 100 mV/s within a potential range of (-0.6 to 0.6 V) by using a redox solution (5 mM ferri/ferrocyanide prepared in PBS buffer, pH 7.4). As shown in **Figure 2**, the cyclic voltammogram of the bare gold electrode (black line) displayed typical quasi-reversible peaks for the redox couple's oxidation and reduction. This peak separation is characteristic of the strong covalent binding between thiol groups on the redox probe and the gold surface. The addition of negatively charged DNA, which repelled the redox anions, due to electrostatic interactions, resulted in a decrease in the cathodic and anodic peak currents. This decrease is characteristic of the strong covalent binding between thiol groups on the redox probe and the gold surface. This behaviour confirms the successful assembly of aptamers on the gold surface through thiol-gold covalent bonds, as the DNA layer hinders the electron transfer between the redox probe and the electrode. After the blocking step using MCH, a drop in the peak current was noted confirming the elimination of the physically adsorbed DNA from the gold surface [34].

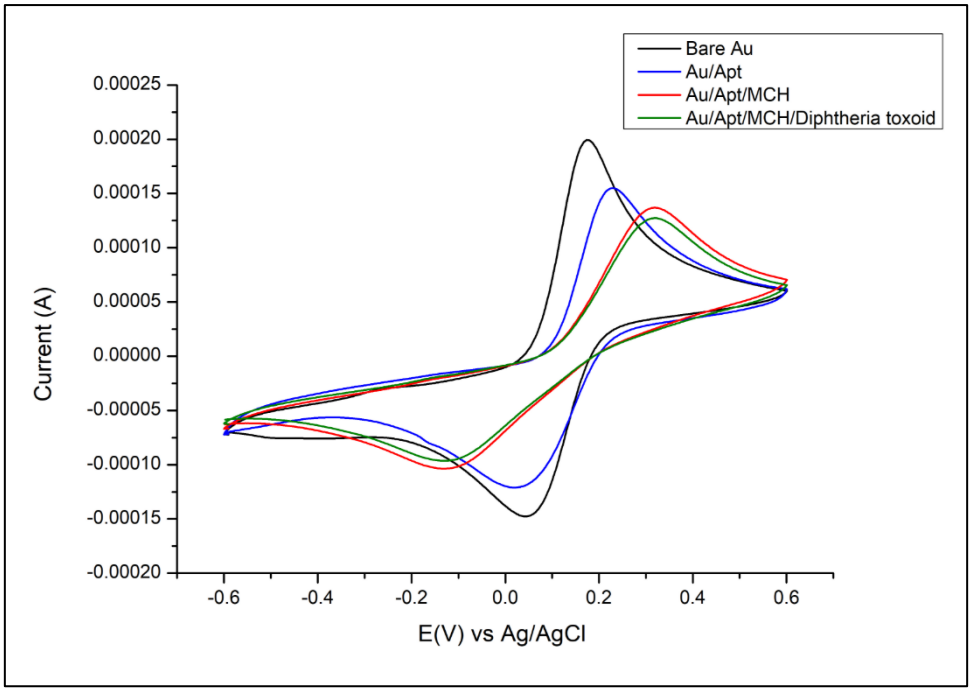

Figure 2: Electrochemical characterization of the aptasensor fabrication steps; bare screen-printed gold electrodes (black line), aptamer-modified SPGE (Red line) and the aptamer-modified electrode after blocking with MCH (blue line), after incubation of the aptasensor with 0.008 ng/mL of diphtheria toxoid. The cyclic voltammograms were performed in [Fe $(CN)_6]^{-4/-3}$ redox couple in PBS buffer, pH 7.4 at a scan rate of 100 mV/s.

3.5 Diphtheria toxoid aptasensing

The analytical performance of the fabricated aptasensor was studied by incubating the aptamer-functionalized electrodes with increasing concentrations of Diphtheria toxoid ranging from 0.008 ng/ml to 290 ng/mL, for 30 minutes. The electrochemical response of the aptasensor was monitored by square wave voltammetry in $[Fe(CN)_6]^{4-/3-}$ redox couple solution. The recorded square wave voltammograms obtained before and after incubation with the toxoid were shown in **Figure 3.a**. we observed

that the recorded peak current decreased gradually by increasing Diphtheria concentration. The interaction between the fabricated aptasensor and Diphtheria toxoid's bulky structure likely hinders electron transfer between the electrode surface and the $[Fe(CN)_6]^{4-/3-}$ redox couple solution, resulting in a decrease in the peak current.

The obtained voltammograms were then used to calculate the aptasensor response as $((i^0 - i)/i^0)$ %, where i^0 and i correspond to the peak currents recorded before and after incubation of the aptasensor with Diphtheria toxoid. Then, the calibration curve was plotted as the aptasensor response versus the logarithm of Diphtheria toxoid concentration (**Figure 3.b**). A good linear relationship was obtained within the range of 0.008 ng/ml to 290 ng/mL with the regression equation $(i^0-i)/i^0$ %=8.48+ 3.45 Log Diph [ng/mL] and a coefficient of determination (R^2) of 0.98. The limit of detection (LOD) for the aptasensor was determined to be 0.0095 ng/ml using the formula 3.3 σ/b, where b represents the slope of the calibration curve and σ is the standard deviation (SD) of the blank. Each set of data was measured three times independently to assess the aptasensors' repeatability.

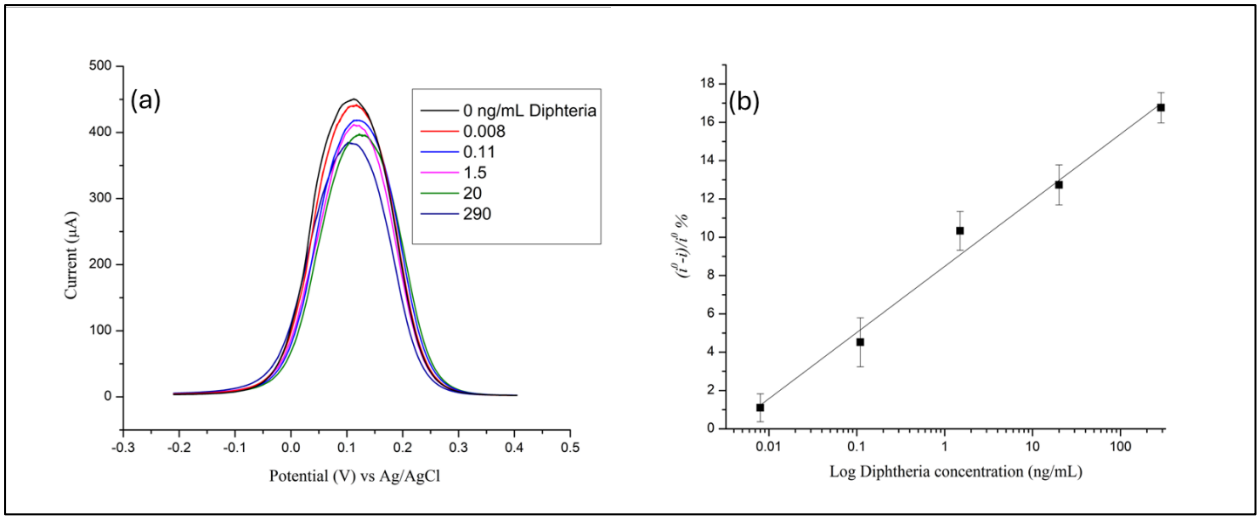

Figure 3: (a) Square wave voltammograms recorded for the D1 aptasensor against different concentrations of Diphtheria toxoid solution. SWV was performed within the potential range (0.4 to -0.2 V), frequency 25 Hz, interval time 0.04 s, step potential −5 mV, scan rate 125 mV/s and amplitude 20 mV. **(b)** Calibration curve plotted for Diphtheria toxoid determination: plots of the sensor response $((i^0-i)/i^0\%)$ versus the logarithm of toxoid concentrations (ng/mL). The error bars represent the standard deviation of three measurements.

3.6 Selectivity and stability studies

To ensure that the selected aptamer is specific to the Diphtheria toxoid, the cross reactivity of the aptasensor was studied against other bacterial toxins. Tetanus and Pertussis toxoids were chosen for this study, since they can be present at the same vaccine combination with diphtheria. Therefore, a fixed amount of Tetanus and Pertussis toxoids (290 ng/ml) were separately incubated with D1 aptasensor in the same experimental conditions described above. The corresponding electrochemical responses were recorded by SWV and used to calculate the percentage $((i^0-i)/i^0\%)$ for

each interfering compound. As it can be seen from **Figure 4,** a significant response signal was observed for the specific analyte, Diphtheria toxoid. In parallel the response obtained after incubation of the aptasensor with the nonspecific toxoids; Tetanus and Pertussis was almost 6 folds lower. These findings confirm the high selectivity of the selected aptamer as well as the designed aptasensor for Diphtheria toxoid.

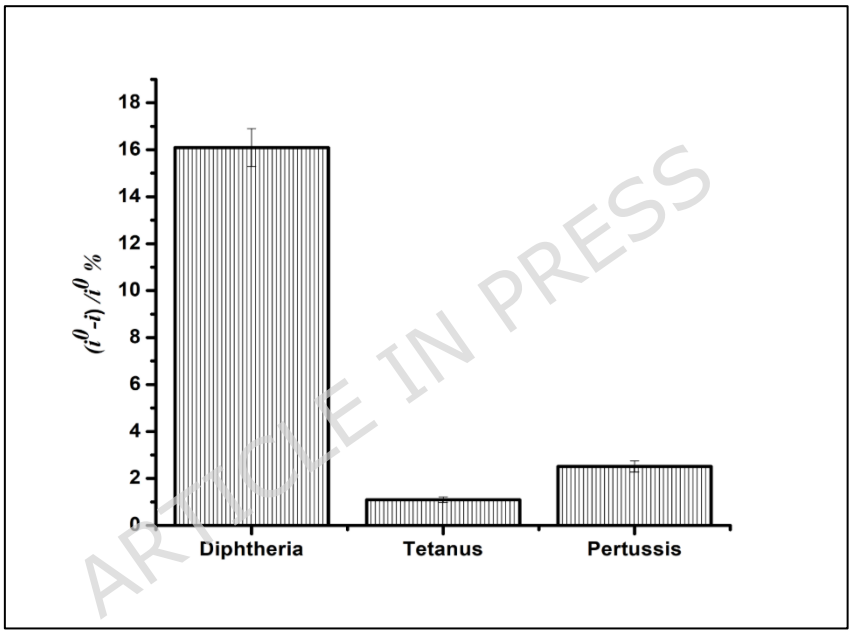

Figure 4: Interference study of the Diphtheria aptasensors towards the potential interfering toxoids Tetanus and Pertussis.

We also evaluated the stability of the aptasensor by assessing its analytical performance at different time intervals after fabrication, specifically after 3 days, one week and one month. The results showed no significant loss of performance over time, confirming its good stability and robustness for prolonged use (data not shown)**3.6 Detection of Diphtheria toxoid in real sample (Diphtheria vaccine)**

To validate the practical applicability of the developed aptasensor for Diphtheria toxoid detection, a commercially available Diphtheria vaccine specimen was tested. This approach mimics real-world scenarios where the aptasensor might be used to analyse vaccine samples or environmental toxins. The vaccine sample was strategically chosen as it represents a matrix likely containing the target analyte alongside potential interferences from other vaccine components. In addition to diphtheria toxoid, this vaccine includes other antigens: hepatitis B surface antigen, inactivated B. pertussis, Tetanus toxoid, along with adjuvants, stabilizers and excipients. To assess the aptasensor's performance across a range of relevant concentrations, the Diphtheria toxoid was desorbed from the vaccine matrix and diluted in PBS. The concentrations 85.7 and 6.3 ng/ml were tested by using the proposed aptasensor. This dilution scheme covers a broad spectrum, encompassing potentially levels of toxoid in the vaccine formulation down to potentially low limits of detection for the assay. Following incubation of these diluted samples on the aptasensor surface, SWV was employed to record the electrochemical response. The corresponding percentages $((i^0-i)/i^0\%))$ were calculated and compared to that obtained in buffer. As shown in **Table 2**, the measured concentrations were very close to the added ones with excellent recovery percentages . The measured response, indicative of the bound Diphtheria toxoid (identified), was then compared to the results obtained from the standard immunodiffusion assay (detectable line), considered the reference method for such analysis (**Figure S.2**). The good agreement between the aptasensor response and the immunodiffusion results signifies the

aptasensor's promising capability for Diphtheria toxoid identification in real-world samples like vaccines. Moreover, unlike the immunodiffusion assay, which provides only qualitative information, our aptasensor offers quantitative measurements of Diphtheria toxoid. This not only allows precise determination of toxin concentration but also enhances sensitivity and reproducibility, demonstrating a clear advantage of our method for real-world vaccine analysis.

Table 2: Analysis results of diphtheria toxoid in vaccine samples.

Sample	**Added concentration (ng/mL)**	**Found concentration (ng/mL)**	**Recovery percentage (%)**	**R.S.D (%)**
Dilution 1	85.7	77.13	90	3.1
Dilution 2	6.1	5.80	95	2.8

4. Conclusion

In this study, we successfully developed a label-free electrochemical aptasensor for Diphtheria toxoid detection. First, Specific DNA aptamers were identified through SELEX, where D1 aptamer exhibited the lowest dissociation constant. D1 aptamer was then immobilized on a screen-printed gold electrode to develop an electrochemical aptasensor for

Diphtheria toxoid. Under optimized conditions, our aptasensor demonstrated an excellent performance for Diphtheria toxoid quantification . In addition, the aptasensor exhibited a high selectivity for Diphtheria toxoid with a negligible signal to Tetanus and Pertussis toxoids. Furthermore, the aptasensor was successfully applied for the determination of Diphtheria toxoid in a commercially available diphtheria vaccine. This label-free aptasensor offers several advantages over existing methods, including simplicity, cost-effectiveness, and high accuracy. This highlights the potential application of the aptasensor for real-world scenarios such as vaccine quality control testing [20, 35]. This work paves the way for the development of a new generation of aptamer-based sensors for rapid, sensitive, and reliable detection of Diphtheria toxoid in various settings, including vaccine quality control, environmental monitoring, and potential clinical applications.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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