Talanta 216 (2020) 120951

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Selection, characterization, and electrochemical biosensing application of DNA aptamers for sepiapterin

Shimaa Eissa[a,1], Shahad Alkhaldi[a,1], Raja Chinnappan[a,1], Ayesha Siddiqua[a], Mai Abduljabbar[b], Anas M. Abdel Rahman[b,c,d], Majed Dasouki[b], Mohammed Zourob[a,b,*]

[a] Department of Chemistry, Alfaisal University, Al Zahrawi Street, Al Maather, AlTakhassusi Rd, Riyadh, 11533, Saudi Arabia
[b] Department of Genetics, King Faisal Specialist Hospital and Research Center, Zahrawi Street, Al Maather, Riyadh, 11211, Saudi Arabia
[c] College of Medicine, Alfaisal University, Riyadh, Saudi Arabia
[d] Department of Chemistry, Memorial University of Newfoundland, St. John's, NL, Canada

ARTICLE INFO

Keywords:
Sepiapterin reductase deficiency
Aptamers
Electrochemical sensor
Competitive biosensor
Neurotransmitter

ABSTRACT

Sepiapterin reductase deficiency (SR) is a rare inborn disorder of neurotransmitter metabolism. The early diagnosis of SR disease should be achieved through the determination of the sepiapterin level in body fluids of suspected patients. Here, we report the selection, identification, and characterization of DNA aptamers against sepiapterin. The aptamer selection was achieved via the systematic evolution of ligand by the exponential enrichment technique. After ten rounds of selection, high-affinity aptamers were identified. The binding affinities of the selected aptamers were evaluated using fluorescence binding assays showing dissociation constants ranging from 37.3 to 79.0 nM. The highest affinity aptamer was then integrated into a competitive electrochemical biosensor. The biosensor achieved outstanding sensitivity with a detection limit of 0.8 pg/ml which was much lower than the reported chromatographic method for sepiapterin quantification. The aptasensor has also shown a high degree of selectivity against the closely-related compound. The aptasensor was then challenged by detecting the sepiapterin in spiked serum samples where a good recovery percentage was achieved.

1. Introduction

Sepiapterin reductase deficiency (SR) is a rare inborn genetic error of neurotransmitter metabolism [1–3]. The SR is usually characterized by motor and cognitive abnormalities, and usually involves a pattern of involuntary and sustained muscle contractions. Unlike some other disorders of neurotransmitters metabolism, this disease is not associated with hyperphenylalaninemia [4]. The early diagnosis of neurotransmitter diseases is very important to avoid the progressive neurologic symptoms that can be misdiagnosed as a neurodegenerative disorder [5]. The diagnosis of SR is usually achieved by measuring the metabolites of the biogenic monoamines, serotonin and dopamine, and various pterins such as (isoxanthopterin, biopterin, neopterin, monapterin, primapterin, xanthopterin, sepiapterin, and 7,8-dihydrobiopterin) in the cerebrospinal fluid [1]. Typically, decreased concentrations of 5-hydroxyindolacetic acid and homovanillic acid, and elevated levels of 7,8-dihydrobiopterin are found in such patients [1]. However, the significant increase in the sepiapterin level in Cerebrospinal fluid (CSF) is the main confirmatory diagnostic test for SR.

The detection of sepiapterin is challenging and can only be done in a few specialized laboratories worldwide. A High-performance liquid chromatography (HPLC) method has been reported for the detection of sepiapterin showing elevated levels in CSF of patients with SR deficiency compared with healthy controls [6]. However, this method is still costly, time-consuming, and cannot be easily applied for high throughput screening of a large numbers of samples in newborn screening programs adopted mainly by hospitals. Therefore, the development of cost-effective, easy-to-use and rapid biosensor-based methods for the detection of sepiapterin is highly needed.

Aptamers have emerged over the past few decades as excellent alternatives to antibodies in various biosensing platforms [7] particularly in biomedical diagnosis [8–11]. Aptamers are single-stranded DNA or RNA molecules that can bind to their target analytes with high affinity and specificity [12,13]. They offer several advantages over antibodies in terms of stability under various conditions, low synthesis cost, reproducibility, and ease of integration in biosensors. Several aptamers have been successfully selected against various small molecule targets [14] such as toxins [15–17], hormones [18,19], and pesticides [20]

* Corresponding author. Department of Chemistry, Alfaisal University, Al Zahrawi Street, Al Maather, AlTakhassusi Rd, Riyadh, 11533, Saudi Arabia.
E-mail address: mzourob@alfaisal.edu (M. Zourob).
[1] S. E, S. A., R. C contributed equally.

https://doi.org/10.1016/j.talanta.2020.120951
Received 27 February 2020; Received in revised form 15 March 2020; Accepted 17 March 2020
Available online 20 March 2020
0039-9140/

using the combinatorial selection process called Systematic Evolution of Ligands by Exponential enrichment (SELEX).

To the best of our knowledge, only an enzyme-linked immunosorbent assay (ELISA) kit for the sepiapterin reductase protein is commercially available; however, there is no available antibody until now for sepiapterin. Therefore, no immunoassays for sepiapterin detection have been reported so far.

In this work, we report for the first time a selection of a DNA aptamer against the small molecule sepiapterin using SELEX. High affinity and specificity aptamers were identified showing dissociation constants in the nanomolar range. One of the selected aptamers was integrated in a square wave voltammetry-based competitive biosensor for sepiapterin detection. The detection is based on the competition between free sepiapterin molecules in the sample and immobilized sepiapterin for a constant amount of free aptamer. This approach achieved high sensitivity and showed a high degree of selectivity against closely-related molecules. We believe that this aptasensor has several advantages over the existing HPLC method for sepiapterin detection in terms of simplicity, sensitivity, high throughput, and cost-effectiveness.

2. Experimental

2.1. Materials, reagents and instrumentations

All the material, reagents, and instrumentation used throughout this study are described in detail in the supporting information file.

2.2. Procedures

2.2.1. Conjugation of sepiapterin to NHS sepharose beads

To attach the sepiapterin molecules to the NHS-activated sepharose beads, the manufacturer protocol was followed. Briefly, a 250 μg/ml solution of the sepiapterin in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was prepared. Then, 2 mL of the NHS activated beads were rinsed multiple times with 1 mM HCl for 15 min to remove the additives and preserve the activity of the reactive groups. The sepiapterin solution was then added to the washed sepharose beads (1:1 vol ratio) and mixed by end-over-end rotation at room temperature for 3 h. Then, the sepiapterin-coupled sepharose beads were washed several times with a coupling buffer to remove the excess sepiapterin. The conjugated beads were then washed with 01. M Tris-HCl buffer pH, 8 and incubated in the same buffer for 1 h with rotation in order to block the unreacted active groups on the surface of the beads. After blocking, the sepiapterin beads were washed with three cycles of alternating pH buffers. The washing cycle is performed by washing first with 0.1 M acetic acid/sodium acetate, pH 4 containing 0.5 M NaCl, and then with 0.1 M Tris-HCl buffer, pH 8 containing 0.5 M NaCl. Pterin beads were also prepared for the counter selection round by performing the same immobilization protocol. Finally, the sepiapterin and pterin beads were stored in 50 mM Tris-HCl buffer, pH 7.5 containing 0.05% sodium azide at 4 °C until further use in the SELEX experiments.

2.2.2. The design of the DNA library and PCR primers for SELEX

The DNA library and the PCR primers were synthesized following the previously reported design [21]. The library used in the first SELEX cycle consists of around 10^{15} sequences. Each sequence composed of a 60-mer random region and two fixed terminal primer binding sites from both ends of the sequence. Each primer binding site consists of 18 nucleotides, (5′-ATACCAGCTTATTCAATT-N60-AGATAGTAAGTGCAATCT-3′). The two PCR primers used during the selection cycles were labeled with fluorescein and poly-A, respectively. The forward primer was labeled with fluorescein to enable the quantification of the DNA during the selection (5′-fluorescein-ATACCAGCTTATTCAATT-3′). However, the reverse primer was attached to hexa-ethyleneglycol spacer consisting of 18-atom followed by twenty A, polyA20, (5′-poly dA20-HEGL-AGATTGCACTTACTATCT-3). The spacer is designed to stop the PCR extension step during amplification. The PCR amplification using these two primers will result in the formation of a double-stranded DNA product with different lengths for each strand. Then, denaturing polyacrylamide gel electrophoresis (PAGE) is employed to separate the ssDNA from the double-stranded amplified PCR products. After ten SELEX cycles, the eluted ssDNA from the last cycle was amplified using unlabeled primers and the PCR product was used for ligation and cloning.

2.2.3. Selection protocol of the sepiapterin aptamers using SELEX

Each selection cycle was performed by incubation of sepiapterin beads with the DNA library. 100 μl of the sepiapterin beads were washed using the centrifugal filters for five times each with 500 μl binding buffer to remove the sodium azide solution and any leached sepiapterin.

The ssDNA solution is pretreated before each cycle as follows: 1) heating at 90 °C for 5 min, 2) cooling to 4 °C for 10 min and 3) keeping at room temperature for 5 min. In the first SELEX cycle, 3 nmol of the DNA library solution in binding buffer was used, whereas 150 pmol of the DNA solution was used for the subsequent cycles. 300 μl of the pretreated DNA solution was added to the washed sepiapterin beads in the centrifuge filter and incubated with end-over-end rotation at room temperature for 2 h. After incubation, the sepiapterin beads were washed 5 times with binding buffer and the fluorescence intensity was measured for each wash. In the last washes, no fluorescence should be detected indicating the elimination of the unbound DNA to the sepiapterin beads. Then, the DNA bound to the sepiapterin beads were collected by elution using elution buffer containing urea with heating at 90 °C for 10 min. The elution step was performed for 6 times, each with 300 μl elution buffer until no fluorescence DNA is detected in the eluted aliquots indicating the complete elution of the bound DNA from the beads. The eluted aliquots were then collected, concentrated and desalted using desalting filters (3 kDa cutoff) to remove the urea and salts which can inhibit the PCR.

In the counter selection cycle, the pterin sepharose beads were incubated for 1 h with the DNA solution collected from round eight. The beads were then washed 4 times and the collected DNA from the flow-through and the washes were concentrated. The concentrated DNA solution was then pretreated by heating and cooling as described above and then incubated with sepiapterin beads to start the subsequence SELEX cycle.

2.2.4. PCR amplification and separation of the ssDNA from the gel

The steps for the PCR cycles and the purification of the ssDNA from the gel in order to be used for the next SELEX cycles are described in the supporting information file.

2.2.5. Cloning and sequencing of selected sepiapterin aptamers

The selection cycles were stopped at round ten and the collected DNA solution was desalted. The PCR amplification with the unlabeled primers was performed for the DNA solution followed by ligation of the double-stranded PCR product into the pCR2.1-TOPO cloning vector by following the protocol provided with the cloning kit. Then, the ligated product was transformed into E. coli DH5α competent cells via the heat shock method. After that, the cell suspension was moved to a pre-warmed SOC medium and incubated at 37 °C for an hour with end-over-end rotation. Different amounts of the cell suspension in SOC media were spread on Lauria Broth (LB) agar plates supplemented with ampicillin and X-Gal. The colonies were left to grow at 37 °C until blue and white colonies were seen. The Positive colonies (the white or light blue colonies (were then picked up from the plate and colony PCR were performed for each using M13 forward and reverse primers to amplify the ssDNA inserts. 2% agarose gel electrophoresis was run and the observation of single bands with 300 base pairs confirmed the amplification of the inserts. The PCR products were then Sanger sequenced and the identified sequences were subjected to binding affinity testing to sepiapterin.

2.2.6. Determination of the dissociation constants of the identified aptamers to sepiapterin

The binding affinity of the identified aptamers towards sepiapterin was evaluated using a fluorescence assay. Six sequences were tested and the dissociation constant (K_d) was calculated in each case. To perform the fluorescence binding test, fluorescein-labeled aptamers (60-mer) were synthesized after truncation of the primers-binding sites. Different concentrations of labeled aptamers solution were pretreated as described in the selection protocol (by heating to 90 °C for 10 min, 4 °C for 10 min and then 25 °C for 5 min). 40 µl of sepiapterin beads were incubated with various concentrations of each labeled aptamer for 1 h at room temperature with rotation. Afterwards, the beads were washed in centrifuge filters using a binding buffer. Then, elution of the bound DNA to the sepiapterin beads was performed using an elution buffer at 90 °C. The fluorescence intensity was then measured for each eluted fraction and used as a measure of the recovered DNA. Binding affinity curves (the ssDNA aptamer concentration versus the fluorescence intensity) were plotted for each aptamer sequence and the K_d was determined via nonlinear regression analysis of the hyperbolic curve.

2.2.7. Attachment of the sepiapterin molecules to the gold electrode surface

First, the gold electrodes were cleaned by polishing with alumina slurries, immersing in piranha solution, and then subjected to electrochemical cleaning in sulfuric acid as reported previously [18]. The electrodes were then functionalized and the sepiapterin molecules were immobilized following the steps shown in the schematic diagram (Scheme 1A). The clean electrodes were first immersed in a 10 mM aqueous solution of cysteamine hydrochloride for 2 h at room temperature. After that, the electrodes were washed with ethanol to remove the excess cysteamine residues and then immersed in a 10 mM solution of the linker, PDITC for 2 h at room temperature. After incubation with the linker, the electrode was washed extensively with DMF and then incubated in 100 µg/ml solution of sepiapterin in PBS buffer, pH 8.5 for 2 h at room temperature. The modified electrodes were washed and then incubated with 0.1 M ethanolamine solution, pH 9.0, for 1 h to block the free isothiocyanate groups on the gold surface. A final blocking step was performed by incubation in 1 mM MCH for 30 min in PBS buffer, pH 7.4. The sepiapterin-modified electrodes were then stored at 4 °C in tris buffer, pH 7.4 until further use in the sensing experiments.

2.2.8. Electrochemical measurements

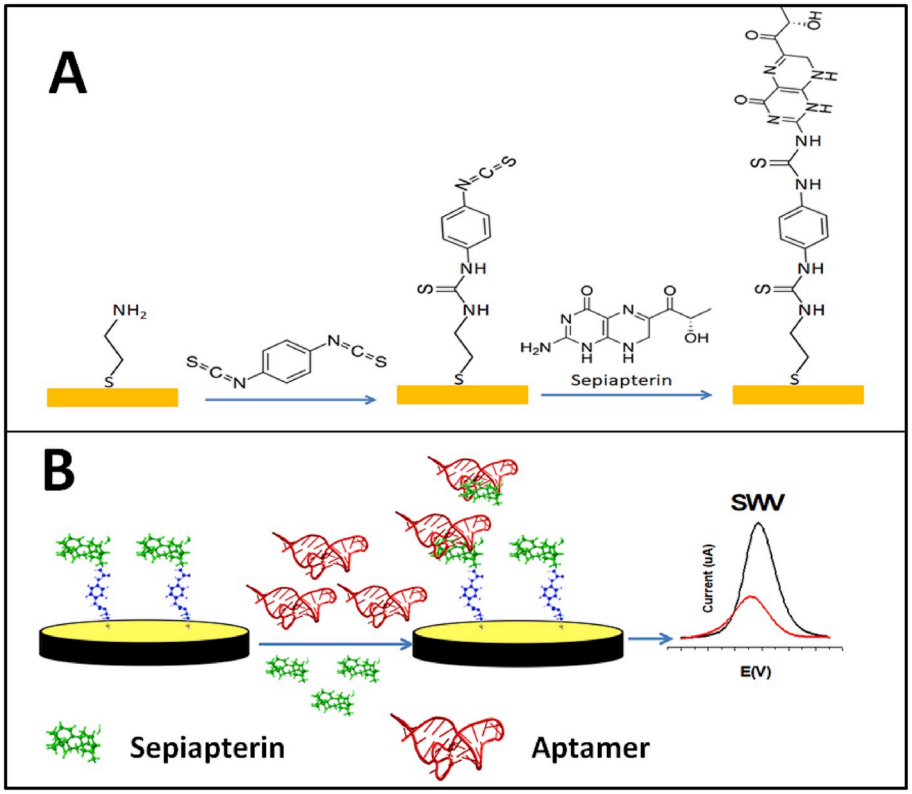
The parameters of all the electrochemical measurements are described in the supporting information file.

2.2.9. Electrochemical competitive aptasensor experiments for sepiapterin

The aptamer sequence SP3 showed the highest affinity to sepiapterin was used to construct a competitive aptasensor for sepiapterin detection. The sepiapterin modified electrodes were incubated with 50 nM of SP3 aptamer solution in binding buffer mixed with various concentrations (1 pg/ml to 100 µg/ml) of the sepiapterin solution for 40 min in order to find out the analytical range of the aptasensor. However, for the cross-reactivity experiments, other nonspecific analytes such as biopterin and azlocillin were mixed with the SP3 aptamer and incubated on the sepiapterin electrodes. After incubation, the electrodes were washed with tris buffer, pH 7.4 and subjected to SWV measurements following the protocol described above. The sensor response signal was calculated as the percentage change in the SWV peak current before and after incubation.

2.2.10. Testing of the developed aptasensor in sepiapterin spiked serum sample

A serum sample from a healthy volunteer (one of the authors) was diluted 1:100 with a binding buffer and then spiked with 1 ng/ml of sepiapterin. The spiked sample was then mixed with 50 nM of SP3 aptamer solution in binding buffer and incubated on the sepiapterin



Scheme 1. Immobilization steps of sepiapterin on the gold electrode surface via cysteamine/phenylene isothiocyanate linkers (A) and the working principle of the competitive voltammetry aptamer-based biosensor (B).

modified-electrodes for 40 min. The electrodes were then washed and SWV was recorded as described above.

3. Results and discussion

3.1. Aptamer selection against sepiapterin using SELEX

Sepiapterin is a small molecule with a molecular weight of 237.22 g/mol. The selection of high-affinity aptamers against small molecules is often challenging and requires careful design and consideration of all the experimental conditions. Among the important steps for a successful SELEX protocol for aptamers is the stable immobilization of the small molecule analyte on a solid matrix. This attachment enables the partitioning of the bound from the unbound DNA to the analyte during the selection. Since the sepiapterin molecule contains a terminal amine group, we have chosen commercial NHS activated sepharose beads as a solid matrix for target immobilization. These beads were used to attach the sepiapterin molecules through the reaction of their amine groups with the activated carboxylic groups on the beads forming an amide bond. Then the free active carboxylic groups on the sepharose beads were deactivated by the reaction with tris buffer to minimize any nonspecific adsorption on the beads.

After the immobilization of sepiapterin on the sepharose beads, they were used for the SELEX rounds. Each round of selection was performed by incubation of the sepipterin beads with a ssDNA library consisting of around 10^{15} random sequence, then removal of the unbound DNA by washing, followed by elution of the bound DNA to the sepiapterin, desalting and PCR amplification of the eluted DNA and finally, purification of the dsDNA PCR product to separate the ssDNA. By performing this selection cycle, we obtain a DNA pool that is more enriched with the high-affinity sequences to the target than the initial library that is then used to start the subsequent SELEX cycle. This process was repeated for 10 rounds as shown in the recovery graph (Fig. 1A). The progress of the selection is monitored during the SELEX by measuring the fluorescence intensity of the eluted DNA solution. As shown in Fig. 1A, the recovered DNA was low in the first 7 rounds until a dramatic increase was observed at round 8 indicating the enrichment of the DNA pool with the specific sequences to the sepiapterin beads. Therefore, a counter selection step was performed after the 8th round by incubating the recovered DNA solution with pterin beads. The washes were then collected from the counter selection step and concentrated by ultrafilteration and then used for round 9.

Pterin is a structurally related heterocyclic compound that represents the parent bicyclic heterocycle called pteridine. Sepiapterin is composed of a pterin molecule with an additional substituent. Therefore, it is very important to perform a counter-selection against this core structure to eliminate the sequences which will commonly bind to all the structurally related pterins. The counter selection was also needed to remove the sequences which were non-specifically bind to the beads matrix and the linker.

As shown in the recovery graph, a significant decrease in the fluorescence intensity of the recovered DNA was observed indicating the elimination of the portion of the DNA that binds to the beads' matrix as well as the pterin core molecule. We then performed one more SELEX cycle and a good increase in the fluorescence intensity was seen implying the enrichment of the 10th cycle DNA with the specific sequences for sepiapterin. Hence, the SELEX was stopped at round 10 and the collected DNA was cloned. The cloning was performed by ligation into a cloning vector and then transformation into *E. coli* competent cells. After the growth of the cells, separate colonies were collected individually, and PCR amplified then Sanger sequenced. The identified sequences showed some identical sequences indicating the enrichment of the DNA pool. The distinctive sequences were analysed by multiple sequence alignment using PRALINE software as shown in Fig. S1. The aptamers shown in Table 1 were then tested for binding to sepiapterin.

3.2. Evaluation of the binding affinity of the selected aptamers towards sepiapterin

Fluorescence-labeled aptamers (60 nu) were then synthesized after truncation of the primers sites and then tested using fluorescence binding assay to evaluate their binding affinity to sepiapterin. The binding affinity experiment was carried out by incubating constant amounts of sepiapterin beads with different concentrations of each fluorescent aptamer. After incubation, the beads were washed and the recovered DNA from the beads was collected after elution using urea and heating. Binding affinity curves for the six aptamers were plotted where an increase in the fluorescence intensity of the eluted DNA solution was observed with increasing the aptamer concentration until saturation was obtained. As shown in Fig. 1B, the dissociation constants (K_d) were calculated for each aptamer-sepiapterin complex by nonlinear regression fitting of the saturation graphs. Six selected aptamers have shown an excellent affinity towards sepiapterin with K_ds ranging from 37 to 79 nM as shown in Table 1 indicating the success of the aptamer selection. The specificity of our binding affinity measurements were also investigated by testing the binding of the sepiapterin beads with scrambled sequence as a negative control. As shown in the graph the scrambled sequence did not show any binding to the sepiapterin indicating good selectivity of the assay.

3.3. Characterization of the immobilization steps for sepiapterin molecules on the gold electrodes using square wave voltammetry

The sepiapterin molecules were attached to a gold electrode surface after two-step functionalization to fabricate the aptasensor as shown in scheme 1A. Fig. 2 shows the SWV signals of the stepwise modification of the gold electrodes in ferro/ferricyanide redox solution (The bare gold surface, after incubation with cysteamine, after adding PDITC, sepiapterin attachment and after blocking with MCH). The bare gold electrode shows a well-defined cathodic signal for the reduction of the redox couple implying that the gold surface was clean. After the incubation with cysteamine solution, an increase in the peak current and a positive shift in the peak potential was

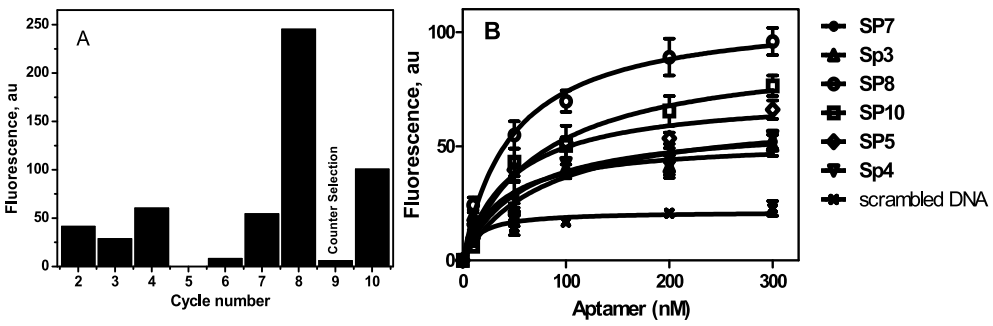


Fig. 1. (A) The fluorescence recovery graph of the DNA during the sepiapterin aptamer selection (The cycle number versus the fluorescence intensity of the eluted DNA). (B) The fluorescence binding affinity curves for six sepiapterin aptamers (a plot of the aptamer concentration versus the fluorescence intensity of the eluted aptamer solution). The dissociation constants were calculated via nonlinear regression analysis of the saturation curves and the error bars represent the standard deviation of the measurements.

Table 1
Sepiapterin binding aptamers and their dissociation constants (K_d).

Sequence Name	Sequence (5'to 3')	K_d (nM)
SP3	AAGGATGACTGACGACGATGGGAATGGAGAGGAAGGGGATATAGGTATGTTGGATGTAG	**37.3**
Sp5	ATGCGGGTACATCTCTCCAACCAGTCAAACTGCTATGATAAGACCTCATTCAACCCCGGTA	**47.0**
SP4	AACCCAACCCCGCACCATTAGTCTAAATTTTCGCGACGGAACACAGCAACAACTCCCCA	**79.0**
SP7	AGCAGTGAAGAGGCGAGAGATTTATGAGGGTAGCGTCCACATATGCCAGGATCGTGTTG	**55.0**
SP8	TCACTGACAGTAGGCCCATACGCACATTCAACAATCGGTAGTCCATACAACCGCGCCCCGTG	**47.0**
SP10	AATCCGACCACGAGAGCAGACCGACCACAGCATTATCTAAACCCATTAGTCTAGTCCTGG	**73.0**

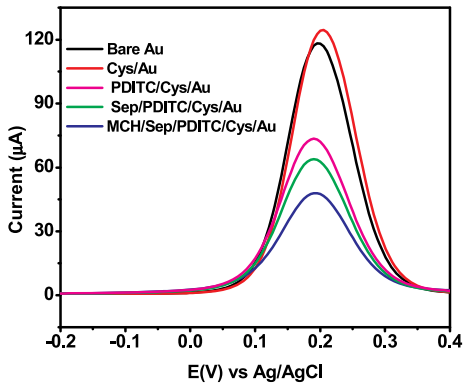


Fig. 2. The characterization of the immobilization steps of the sepiapterin on the gold electrodes using square wave voltammetry in 5 mM $[Fe(CN)_6]^{4-/3-}$ redox solution in PBS buffer, pH 7.4. The black curve is the bare gold electrode, the red curve is the cysteamine modified electrode, the purple curve is after the addition of PDITC, the green curve is after the attachment of sepiapterin and the blue curve is after blocking with MCH. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

observed likely due to the positive charge of the amine groups which attracts the redox anion to the electrode surface and facilitates the electron transfer. However, the incubation with the crosslinker PDITC caused a significant decrease in the peak current due to the negatively charged isothiocyanate groups which lead to electrostatic repulsion with the redox anions retarding the electron transfer process. On the other hand, the incubation with sepiapterin leads to a further decrease in the peak current likely due to the blocking of the conductive electrode surface with the sepiapterin molecules. A final blocking step with MCH was carried out to minimize the nonspecific adsorption by filling the free areas on the gold surface which leads to a further decrease in the SWV reduction peak current.

3.4. Construction of the competitive aptasensor for sepiapterin detection using SP3

As shown in Table 1, the aptamer sequence which showed the lowest K_d was SP3 with a K_d of 37.3 nM, indicating a very good affinity towards sepiapterin. Therefore, this aptamer was chosen for further biosensing applications. A competitive aptasensor was designed using the sepiapterin-immobilized gold electrodes as shown in Scheme 1B. The principle of the aptasensor was based on the direct competition between the immobilized sepiapterin and the free sepiapterin in the sample for a fixed amount of SP3 aptamer added to the sample solution. The higher the concentration of the sepiapterin in the sample, the lower the available aptamer molecules in the solution to bind to the sepiapterin electrodes. The binding event of the aptamer to the sepiapterin-modified electrode was monitored using SWV by following the percentage change of the SWV reduction peak current upon incubation with different solutions.

Before investigating the dynamic range of the aptasensor, it was crucial to optimize the binding conditions between the immobilized sepiapterin and the SP3 aptamer. Two parameters were tested; the SP3 aptamer concentration and the binding time. As shown in Fig. 3A, a dramatic decrease in the SWV reduction peaks were observed upon incubating the sepiapterin modified electrodes with increasing concentrations of the SP3 solutions. This decrease in the SWV peaks is due to the binding of the sepiapterin on the electrode to the SP3 aptamer. The attachment of the aptamer leads to electrostatic repulsion between the negatively charged phosphate backbone of the DNA and the redox anions leading to the shielding of the surface and thus, diminishing the electron transfer rate. Fig. 3B shows the percentage change in the SWV current before (i^0) and after (i) binding with different concentrations of the SP3 aptamer ($(i^0\text{-}i)/i^0\%$). An increase in the current change response was seen when the aptamer concentration was increased until saturation was reached at around 50 nM. Therefore, The SP3 aptamer concentration was fixed at 50 nM for the detection experiments.

Hence, the incubation time is also an important factor in the binding experiments, the binding of 50 nM SP3 aptamer to the sepiapterin-modified electrodes was monitored at different time points. As shown in

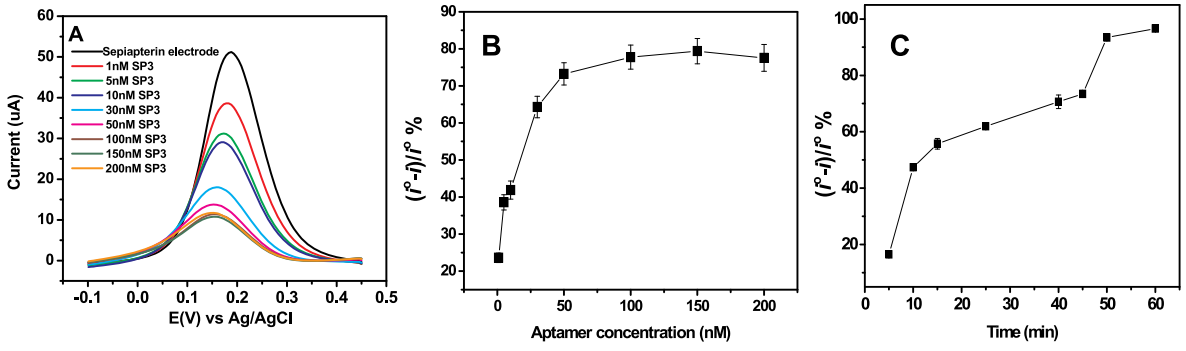


Fig. 3. (A) The square wave voltammograms of the sepiapterin-modified electrodes before and after incubation with different concentrations of SP3 aptamer solution in binding buffer. The measurements were recorded in 5 mM $[Fe(CN)_6]^{4-/3-}$ solution in PBS buffer, pH 7.4. (B) A plot of the SP3 aptamer concentration versus the percentage change in the SWV reduction peak current, $(i^0\text{-}i)/i^0\%$. (C) A plot of the incubation time of 50 nM SP3 aptamer solution versus $(i^0\text{-}i)/i^0\%$. The error bars on the graphs are the standard deviations of two measurements.

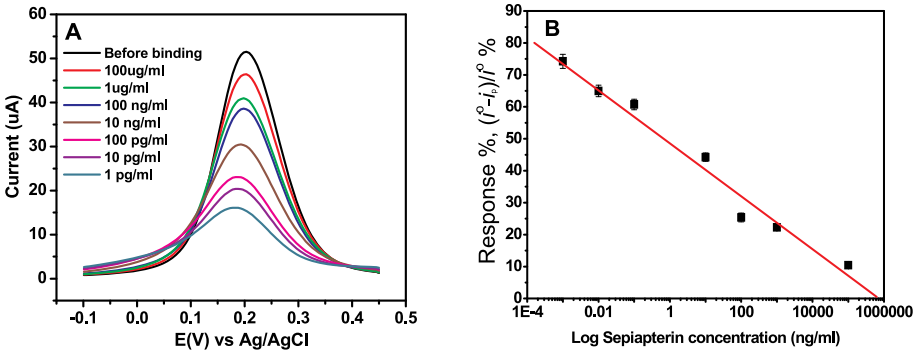


Fig. 4. (A) The square wave voltammograms of the sepiapterin-modified gold electrode before and after incubation with various concentrations of sepiapterin solution mixed with 50 nM of SP3 aptamer. (B) A calibration graph of the sepiapterin aptasensor (a plot of the logarithm of the different concentrations of the sepiapterin versus the aptasensor response ((i^0-i)/i^0%). The error bars are the standard deviations of triplicate measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3C, the current change was increased by increasing the incubation time until it reaches a plateau. Thus, 40 min was chosen for the detection experiments where around 75% response was obtained.

To determine the analytical range of the sepiapterin aptasensor, various concentrations of the sepiapterin solution was mixed with 50 nM of SP3 aptamer and then incubated on the sepiapterin-modified electrodes for 40 min. After incubation, the electrodes were washed and the SWV was recorded in ferro/ferricyanide redox solution as described above.

The SWV signals recorded after incubation of the sepiapterin electrodes with 50 nM aptamer mixed with various concentrations of the sepiapterin solution ranging from 1 pg/ml to 100 μg/ml (Fig. 4A). A significant decrease in the SWV signal was observed when the sepiapterin concentration was low due to a large amount of SP3 aptamer available for binding to the sepiapterin-modified electrode. However, with increasing the sepiapterin concentration, lower signal change was observed indicating the increased competition which led to fewer SP3 aptamer molecules available to bind to the electrode. Fig. 4B shows the calibration graph of the competitive sepiapterin aptasensor (the concentration of the sepiapterin in the sample versus the sensor response calculated as (i^0-i)/i^0%). A wide linearity range from 1 pg/ml to 100 μg/ml was obtained with a regression equation of: $(i^0\text{-}i)/i^0\% = 48.6\text{–}8.3\ C$ [ng.mL^{-1}], R = 0.984. The detection limit (LOD) of the sepiapterin aptasensor was calculated as 3σ/b, where σ is the standard deviation of the blank measurements and b is the slope of the straight line to be 0.8 pg/mL. This LOD indicates excellent sensitivity of the developed competitive aptasensor for sepiapterin detection compared to the reported HPLC method which has a LOD of 0.23 ng/mL [6].

3.5. Cross-reactivity of sepiapterin competitive aptasensor with other small molecules

To assess the specificity of the aptamer SP3 for sepiapterin, other small molecules were tested on the aptasensor. The non-specific analytes were mixed with SP3 apatmer solution and incubated on the sepiapterin-modified electrodes and then the SWV was recorded. Azlocillin was tested as a non-related analyte. The cross-reactivity of the aptasensor was also tested against the structurally related molecule, biopterin. As shown in Fig. 5, the aptasensor response towards sepiapterin was significantly lower than the responses against azlocillin as well as biopterin. These results implied a high degree of selectivity of the aptasensor towards sepiapterin. Moreover, there was no observed cross-reactivity against biopterin which shares the parent pteridine ring with sepiapterin except one different substituent. This indicates the success of the counter selection we performed during the SELEX against the parent pterin compound.

3.6. Application of the sepiapterin competitive aptasensor in a spiked serum sample

The sepiaptrin competitive apatsensor was tested in a spiked serum sample t (o confirm the usefulness of the developed aptasensor in real

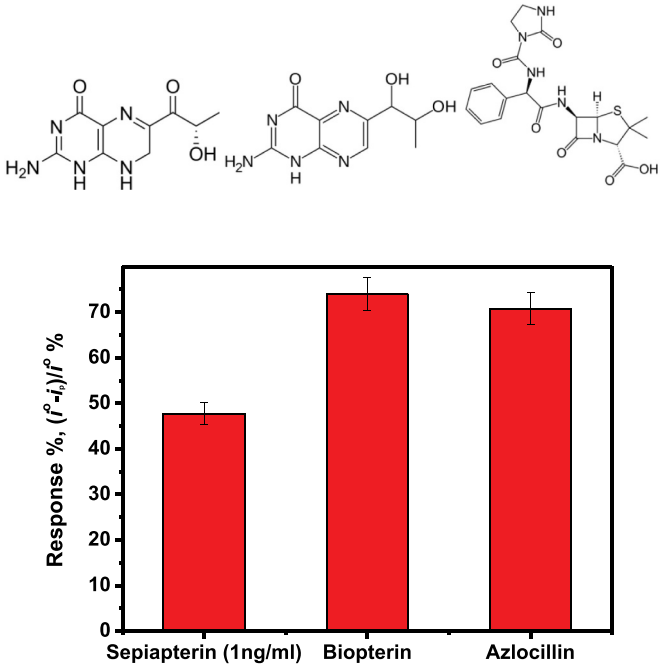


Fig. 5. The sepiapterin aptasensor response against sepiapterin, biopterin and azlocillin. The chemical structures of the three analytes are shown.

applications. The recovery percentage was calculated to be 90% indicating very high selectivity and nonsignificant matrix interference. This implies the possible applicability of our aptasensor in biological fluids. The reproducibility of the method was determined by calculating the relative standard deviations of triplicate measurements (RSD%) showing 5%. This confirms the high reproducibility of the sepiapterin aptasensor.

4. Conclusions

High affinity and specificity aptamers have been successfully selected against sepiapterin for the first time using the systematic evolution of ligand by exponential enrichment. Sepharose beads were used as a solid matrix for the immobilization of sepiapterin and the aptamer selection was achieved through ten cycles. A counter selection was performed against the parent petrin molecules to realize good selectivity against the sepiapterin and to avoid possible cross-reactivity with other pterins with closely-related chemical structures. The selected aptamers showed very low dissociation constants in the nanomolar range. The aptamer SP3 which showed the highest binding affinity to sepiapterin was then used to fabricate a competitive aptasensor for sepiapterin detection. Square wave voltammetry was used for the detection. The electrochemical aptasensor showed very good sensitivity and a high degree of selectivity against other closely related petrins.

This sepiapterin aptasensor offers several advantages over the existing chromatographic method in terms of sensitivity, stability, low cost and capability of miniaturization. Thus, we postulate that with a further selection of aptamers against other pterins, our methods will be highly suitable to the eventual development of multiplexed biosensing platforms.

Author contribution

S.E., R.C., M.Z., M.D. and A.R. designed the study. M.D. and A.R. chose the analyte and provided the relevant clinical information. R.C. planned the SELEX experiments. S.A. and R.C. carried out the SELEX experiments. M.A. helped in the DNA sequencing experiment. S.E. planned the biosensing experiments. S.E. and A.S. performed the biosensing experiments and analysed the data. S.E. wrote the manuscript. All the authors reviewed the final version of the manuscript.

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.

Acknowledgments

The authors would like to thank Alfaisal university for their support of this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.120951.

References

[1] B. Echenne, A. Roubertie, B. Assmann, T. Lutz, J.M. Penzien, B. Thöny, N. Blau, G.F. Hoffmann, Sepiapterin reductase deficiency: clinical presentation and evaluation of long-term therapy, Pediatr. Neurol. 35 (2006) 308–313.
[2] N. Blau, L. Bonafé, B. Thöny, Tetrahydrobiopterin deficiencies without hyperphenylalaninemia: diagnosis and genetics of DOPA-responsive dystonia and sepiapterin reductase deficiency, Mol. Genet. Metabol. 74 (2001) 172–185.
[3] N. Blau, Disorder of Tetrahydrobiopterin and Related Biogenic Amines, the Metabolic and Molecular Bases of Inherited Disease, (2000).
[4] L. Elzaouk, H. Osmani, W. Leimbacher, A. Romstad, J. Friedman, M. Maccollin, B. Thöny, N. Blau, Sepiapterin reductase deficiency: molecular analysis in a new case presenting with neurotransmitter deficiency without hyperphenylalaninemia, in: S. Milstien, G. Kapatos, R.A. Levine, B. Shane (Eds.), Chemistry and Biology of Pteridines and Folates: Proceedings of the 12th International Symposium on Pteridines and Folates, National Institutes of Health, Bethesda, Maryland, June 17–22, 2001, Springer US, Boston, MA, 2002, pp. 277–284.
[5] B. Assmann, R. Surtees, G.F. Hoffmann, Approach to the diagnosis of neurotransmitter diseases exemplified by the differential diagnosis of childhood-onset dystonia, Ann. Neurol. 54 (2003) S18–S24.
[6] G. Zorzi, U. Redweik, H. Trippe, J.M. Penzien, B. Thöny, N. Blau, Detection of sepiapterin in CSF of patients with sepiapterin reductase deficiency, Mol. Genet. Metabol. 75 (2002) 174–177.
[7] S. Song, L. Wang, J. Li, C. Fan, J. Zhao, Aptamer-based biosensors, Trends Anal. Chem. 27 (2008) 108–117.
[8] W. Zhou, P.-J. Jimmy Huang, J. Ding, J. Liu, Aptamer-based biosensors for biomedical diagnostics, Analyst 139 (2014) 2627–2640.
[9] S. Eissa, A. Siddiqua, R. Chinnappan, M. Zourob, Electrochemical selection of a DNA aptamer, and an impedimetric method for determination of the dedicator of cytokinesis 8 by self-assembly of a thiolated aptamer on a gold electrode, Microchim Acta 186 (2019) 828.
[10] S. Eissa, M. Zourob, Aptamer- based label-free electrochemical biosensor array for the detection of total and glycated hemoglobin in human whole blood, Sci. Rep. 7 (2017) 1016-1016.
[11] A.Y. Almusharraf, S. Eissa, M. Zourob, Truncated aptamers for total and glycated hemoglobin, and their integration into a graphene oxide-based fluorometric method for high-throughput screening for diabetes, Microchim Acta 185 (2018) 256.
[12] G. Marrazza, Aptamer Sensors, Biosensors (Basel) 7 (2017) 5.
[13] A. Ruscito, M.C. DeRosa, Small-molecule binding aptamers: selection strategies, characterization, and applications, Front Chem 4 (2016) 14-14.
[14] W. Zhang, Q.X. Liu, Z.H. Guo, J.S. Lin, Practical application of aptamer-based biosensors in detection of low molecular weight pollutants in water sources, Molecules 23 (2018) 344.
[15] S. Eissa, A. Ng, M. Siaj, A.C. Tavares, M. Zourob, Selection and identification of DNA aptamers against okadaic acid for biosensing application, Anal. Chem. 85 (2013) 11794–11801.
[16] S. Eissa, M. Siaj, M. Zourob, Aptamer-based competitive electrochemical biosensor for brevetoxin-2, Biosens. Bioelectron. 69 (2015) 148–154.
[17] S. Eissa, A. Ng, M. Siaj, M. Zourob, Label-free voltammetric aptasensor for the sensitive detection of microcystin-LR using graphene-modified electrodes, Anal. Chem. 86 (2014) 7551–7557.
[18] S. Eissa, A. Siddiqua, R. Chinnappan, M. Zourob, Electrochemical SELEX technique for the selection of DNA aptamers against the small molecule 11-deoxycortisol, ACS Appl. Bio Mater 2 (2019) 2624–2632.
[19] G. Contreras Jiménez, S. Eissa, A. Ng, H. Alhadrami, M. Zourob, M. Siaj, Aptamer-based label-free impedimetric biosensor for detection of progesterone, Anal. Chem. 87 (2015) 1075–1082.
[20] S. Eissa, M. Zourob, Selection and characterization of DNA aptamers for electrochemical biosensing of carbendazim, Anal. Chem. 89 (2017) 3138–3145.
[21] R. Chinnappan, S. Eissa, A. Alotaibi, A. Siddiqua, O.A. Alsager, M. Zourob, In vitro selection of DNA aptamers and their integration in a competitive voltammetric biosensor for azlocillin determination in waste water, Anal. Chim. Acta 1101 (2019) 149–156.