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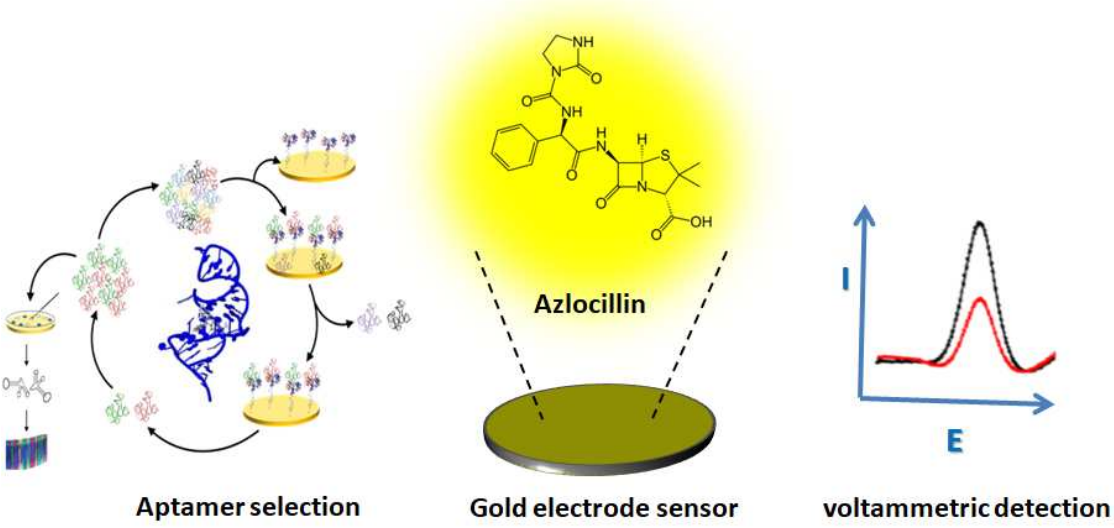

In vitro selection of DNA aptamers and their integration in a competitive voltammetric biosensor for azlocillin determination in waste water

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Abstract

The uncontrolled usage of veterinary antibiotics has led to their widespread pollution in waterways and milk products. Potential impact of antibiotic residues on the environment and human health such as increased antibiotic resistance of microorganisms and triggering allergic reactions in humans have been reported. In this work, we developed a highly selective and sensitive voltammetric aptasensor for on-step, sensitive and low cost detection of azlocillin antibiotic, one of the broad spectrum β-lactam antibiotics. The successful selection of DNA aptamers against azlocillin was accomplished using systemic evolution of ligands by exponential enrichment (SELEX) method. Fluorescence-binding assays showed dissociation constant of 55 nM for one of the selected aptamers (Az9). This aptamer was used to construct a competitive voltammetric aptasensor for azlocillin. A limit of detection of 1.2 pg/mL as well as remarkable selectivity against potential interfering agents, including amoxicillin, were achieved. This signal-off competitive sensor takes 30 to 50 minutes to complete the quantification of the target antibiotic. The sensor was challenged by detecting the target directly in complex environments such as tap and waste water where good recovery percentages were achieved.

Key words: Antibiotics, azlocillin, aptamers, electrochemical sensor, waste water, tap water

1. Introduction

Azlocillin is a member of a wide class of antibiotic family named β-lactam, where β-lactam ring is the center in their molecular structures, with a distinct derivative for each member. They have a broad spectrum effect and usage in both human and veterinary medicine, but are more frequently used in high quantities for prevention and treatment of various diseases (e.g., mastitis) and as growth promotors in industrial livestock farming [1]. Only small portion (~ 10-20%) of the administrated dose is metabolized, while the major quantity of the parenterally administered antibiotic is excreted unmodified through urine and milk in milk-producing cattle. β-lactams are persistent chemical compounds, where they resist the thermal pasteurization treatment during milk production [2]. Uncontrolled practice leads to significant widespread contamination of such compounds in the surrounding environments (wastewater and tap water). Exposure to antibiotics alters microbial ecosystems of humans, animals and the environment, which may lead to the development of antimicrobial resistance [3]. Notably, they cannot be completely eliminated by the conventional water treatment methods. Additionally, the lack of strict adherence to drug-withholding periods leads to trace amounts of these antibiotics in milk products which could trigger allergic reactions in susceptible individuals and disrupt the native microbial system [4]. Maximum residual limits (MRL) for this class of compounds was established in the lower and upper ranges of ppb concentration for milk and water, respectively, considering that designated baby food should be free of antibiotics [5].

There are two main approaches to detect antibiotic residues in various sample types. **A)** The qualitative screening method that includes bacterial growth inhibition and immunochemical techniques such as enzyme-linked immunosorbent assays (ELISA) [6]. The major limitations of these methods are the lack of specificity in the case of microbial method while ELISA requires a specific antibody, previously isolated *in vivo*, with limited flexibility

of signal transduction and detection buffer choice. **B)** The accurate and precise quantification where high performance liquid chromatography (HPLC) [7], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [8], and gas chromatography- mass spectrometry (GC-MS) [9] are utilized. Despite the wide applications, accuracy, low detection levels of these techniques, they are time consuming, expensive, require complex pre-analysis treatment, and qualified-well-trained technicians. Thus, there is still an urgent need to develop fast and reliable methods for quantifying antibiotic compounds in different real samples.

Biosensors could circumvent the problems encountered with the conventional methods and provide fast one-step and on-site analysis of antibiotics. Recently, aptamers have emerged as new class of recognition elements with superior properties (including high affinity and stability, smaller size, low-cost and ease of synthesis with high reproducibility) over the old generation antibodies. They are single-stranded (ss) DNA or RNA oligonucleotides, which can selectively bind to a wide range of targets including metal ions, small molecules (such as hormones [10], toxins[11-13], and pesticides[14]), proteins [15], and even for entire cells [16]. The desired aptamers can be identified by a combinatorial selection process called Systematic Evolution of Ligands by Exponential enrichment (SELEX).

Although, several aptamers were selected for various antibiotic classes such penicillin G [17], aminoglycoside [18], anthracyclines [19], chloramphenicol [20], quinolones [21], and tetracyclines [22]; the detection of different antibiotics present in different sample matrix requires the availability of highly specific and selective aptamer sequences for other common antibiotics. In this work, and for the first time, the selection, characterization, and aptasensor performance of high affinity aptamers for azlocillin antibiotic are reported. The SELEX approach was employed for aptamer screening and a number of aptamer sequences were isolated, and cloned. The binding affinities of the selected aptamers to azlocillin were tested

in solution by fluorescence based approach and the dissociation constant (K_d) values were found to be in the nanomolar range. The highest affinity aptamer was further integrated in a competitive electrochemical aptasensor for azlocillin detection. The competition between the free azlocillin in the sample and the immobilized azlocillin for a fixed concentration of aptamer was realized. The formation of aptamer- azlocillin complex sequentially alters the amount of the aptamer molecules available for binding to the electrode surface. This allows the construction of highly sensitive square wave voltammetry (SWV)-based sensor showing remarkable selectivity against the azlocillin's counterpart, amoxicillin. The performance of the developed aptasensor was challenged by directly measuring azlocillin in spiked tap and wastewater samples showing excellent recovery percentages. Thus, the proposed platform offers several advantages over current methods including the lower cost and higher stability of the aptamer as well as the ease of use of the electrochemical transducers and their availability as hand-held devices. These features make our aptasensor of great value for the detection of emerging environmental contaminates.

2. Experimental section

2.1. Materials and Reagents

Azlocillin and amoxicillin were purchased from Alfa Aesar, (Ward hill MA, USA https://www.alfa.com/en) CarboxyLink Coupling Resin was purchased from (Thermo Fisher Scientific, Waltham, MA, USA) hydrogen peroxide (H_2O_2), potassium ferrocyanide ($K_4Fe(CN)_6$), cysteamine hydrochloride, potassium ferricyanide ($K_3Fe(CN)_6$), phosphate-buffered saline: pH 7.4 (PBS), mercaptohexanol (MCH), sulphuric acid (H_2SO_4), magnesium chloride ($MgCl_2$), sodium chloride (NaCl), Acrylamide/bisacrylamide (30% solution), urea, tris-base, boric acid, EDTA disodium dehydrate,Tetramethylethylenediamine (TEMED) and ammonium persulfate (APS)and ethanol were purchased from Sigma Aldrich (St Louis, MO, USA). N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

hydrochloride (EDC) were obtained from Fisher Scientific (Ontario, Canada). The aptamer sequences, the ssDNA library, labeled primers for polymerase chain reaction (PCR) amplification during SELEX and the unlabled primers for the amplification of the clones were custom-manufactured by Metabion International (Plangg, Germany). Amicon Ultra-0.5 mL centrifugal desalting filter units with a 3 kDa molecular cut-off filter were acquired from EMD Millipore (Alberta, Canada). 0.45 µm centrifuge tube filters with a cellulose acetate membrane were obtained from Corning life sciences (Tewksbury MA, USA). PCR reagents including Taq plus polymerase, Taq Buffer, dNTPs and a 100-base pair ladder were purchased from ACE Biotech (Riyadh, Saudi Arabia). Agarose powder acetic acid and 50X Tris base and EDTA for the TAE buffer used for the agarose gel electrophoresis were bought from Bio-Rad (California, United States). The binding buffer which is used during the binding and washing steps of the aptamer selection and aptasensor development is prepared by mixing 50 mM Tris, pH 7.5, 150 mM NaCl and 2 mM $MgCl_2$. The elution buffer used for eluting the azlocillin bound aptamer sequences is 7 M urea in binding buffer. TOPO TA Cloning Kit consisting of the pCR2.1-TOPO vector and the E-coli competent cells were purchased from Invitrogen Inc. (New York, USA). Milli-Q water was used to prepare all the solutions.

2.2. Instrumentations

An auto lab PGSTAT302N (Eco Chemie, The Netherlands) potentiostat was used to carry out all electrochemical measurements in this study. The data was acquired from the potentiostat via Nova 1.11 software. A standard three electrode system was used throughout the study. The working electrode was a conventional solid gold electrode, Ag/AgCl was used as a reference electrode and a Pt wire was used as a counter electrode.

The Fluorescence measurements were carried out using Nano Drop 3300 Fluorospectrometer and the UV quantification measurements were performed using Nano Drop 2000C Spectrophotometer obtained from Fisher Scientific, Canada.

2.3. Procedures

2.3.1. Preparation of azlocillin conjugated agarose beads

The sodium salt of azlocillin was conjugated with carboxylink coupling gel which is diaminodipropylamine (DADPA) functional groups linked to agarose beads according to the manufacture protocol. Briefly, about 1mg of sodium salt of azlocillin was dissolved in 250µl of coupling buffer (0.1 M phosphate buffer pH 5.0). 2 ml of DADPA beads was washed with coupling buffer several times. 25 mg of EDC was in 500 µl of coupling buffer was mixed with azlocillin solution then transferred into the agarose beads. The final volume of the mixture was fixed to 4 ml. After 3 hours of mixing, the beads were washed with 1M NaCl solution several times to remove the unreacted azlocillin. The conjugated beads were suspended in 2M carbonate buffer pH = 8.5. Then 15 mg of sulpho-NHS was added to the beads to block the reactive amine groups in the DADPA beads. The mixture was washed with binding buffer (50mM Tris+150mM NaCl+2mM $MgCl_2$, pH = 7.4) after one hour of rotation. Similarly the blank beads were prepared by mixing 30 mg of sulpho-NHS with 500 µl of DADAP beads. Positive and the blank beads were stored in 10 mM Tris, pH = 7.5 containing 0.02% sodium azide at 4°C until further use.

2.3.2. Library and primers design

A set of library and primers were designed from a previously reported protocol[23]. The random single strand DNA (ssDNA) library consists of 10^{15} oligonucleotides. Each sequence

contains 60 nucleotides and two fixed 18 nucleotides-sequences at 5'and 3'ends. The constant regions are primer binding sites which are utilized for the DNA amplification by PCR of the DNA library (5′-ATACCAGCTTATTCAATT-N60-AGATAGTAAGTGCAATCT-3′). Denaturting polyacrylamide gel electrophoresis is used to separate the ssDNA from the double stranded (the amplified PCR products). Fluorescein-labelled forward primer was used (5′-fluorescein-ATACCAGCTTATTCAATT-3′). A reverse primer with 18-atom hexa-ethyleneglycol spacer (to block the PCR amplification) followed by twenty A (polyA_{20}) is designed to form double stranded PCR product with different length for each strand. (5′-poly dA20-HEGL-AGATTGCACTTACTATCT-3). After twelve rounds of SELEX, the eluted DNA from the last round was PCR amplified with unlabeled primers and the dsDNA was used for cloning.

2.3.3. In Vitro Selection of the aptamers against azlocillin

The selection process has been carried out by washing 100 µl of azlocillin-conjugated agarose beads for 5 times each with 500 µl of binding buffer. A pretreatment of the DNA library was performed as follows: 150 pmol solution of ssDNA library in binding buffer (3 nmol for first round) was heated at 90°C for *5* min, cooled to 4°C for 10 min and then kept at room temperature for 5 min. This pretreated library was then added to the washed azlocillin beads and left to rotate for 2 h at room temperature, followed by washing with binding buffer for few times until no fluorescence is detected in the washes. Then, the DNA oligonucleotides bound to azlocillin beads were eluted with 300 µl aliquots of elution buffer which consists of 7 M urea dissolved in binding buffer with heating for 10 min at 90°C until no fluorescence was detected in the last elution. Then, the eluted DNA was collected, concentrated and desalted by ultra-filtration device with a 3 kDa cutoff membrane filter.

In the counter selection cycle, the DNA pool from 7^{th} round was used in which the ssDNA was incubated with blank agarose beads (i.e., sulpho-NHS blocked agarose beads

without conjugated azlocillin). The flow-through and the unbound DNA from the blank beads were collected and concentrated followed by heating and cooling treatments as described above, then incubated with azlocillin beads for the next round of SELEX.

The target binding DNA sequences were amplified by PCR in 20 tubes each containing 50 µl reaction mixture consists of 2 units of Taq Plus polymerase, 1x taq buffer, 2 mM $MgCl_2$, 200 µM dNTP, 0.2 µM of forward and reverse primers. The thermo cycling of the PCR mixture is as follows: 94 °C for 7 min, followed by 25 cycles of 94 °C for 45 sec, 47 °C for 45 sec, 72 °C for 45sec, and a final extension step of 10 min at 72 °C. The amplification of dsDNA and the size of the product were confirmed by running 2% agarose gel electrophoresis in TAE buffer using ethidium bromide as fluorescent marker under 110V for 30 minutes. The amplified ds DNA PCR product was precipitated by adding 0.1 volume of 3 M sodium acetate and 3 volumes of cold ethanol and kept at -80°C for an hour, then centrifuged for 30 minutes at 4°C. The supernatant was discarded and the precipitate was washed with 75% cold ethanol and re-suspended in 50:50 water and mixture of loading dye in formamide and heated to 90 °C for 5 min. The DNA strands labeled with fluorescein and PolyA were separated by 10% denaturing PAGE at 300V for 2.30 h. The fluorescent ssDNA band from the gel was sliced and treated with freeze−thaw cycle in TE buffer followed by shaking at 37°C overnight. Eluted ssDNA in TE (10 mM Tris, pH 7.4, 1 mM EDTA) was concentrated by ultrafiltration and used for the next round of selection.

2.3.4. Cloning, sequencing and alignment of the selected aptamers against azlocillin

After twelve rounds of SELEX, a significant increase in the DNA was observed as shown in the fluorescence recovery graph (Fig.1A). The selection was stopped at this round and the DNA was collected, desalted and PCR amplified with the unlabelled primers. The PCR product was then ligated into pCR2.1-TOPO cloning vector using the TOPO TA kit by following the manufacture protocol. Briefly, the ligated DNA was transformed into E. coli

DH5α competent cells through heat shock approach. The cells were transferred into pre-warmed SOC medium, incubated for an hour at 37°C with rotation. Different volumes of the cells solution were spread on Lauria *Broth* (LB) *agar* medium supplemented with ampicillin and X-Gal and the colonies were left to grow. Positive colonies) white or light blue colonies)were picked and ssDNA inserts were PCR amplified using M13 forward and reverse primers by colony PCR. The PCR inserts were confirmed by observing the 300 bp bands in the 2% agarose gel electrophoresis. The colony PCR products were sequenced and aligned by using *PRALINE* program (Scheme S1).

2.3.5. *Fluorescence dissociation constants determination of the identified aptamers to* azlocillin.

Representative sequences obtained after alignment were used for the determination of dissociation constants with the target (K_d). Fluorescein-labelled aptamers were synthesized after eliminating the primers-binding sites and the K_ds of the selected aptamers were calculated by performing fluorescence binding assays. Each fluorescein-labeled sequence was pretreated before binding by heated to 90°C for 10 min, 4°C for 10 min and then 25°C for 5 min. Various concentrations of labelled aptamers were then incubated with azlocillin positive beads for 1 hour. After washing with binding buffer, the bound DNA was eluted. The DNA recovery from each sample was determined by fluorescence measurement. For each aptamer sequence, a saturation curve was plotted and the K_d was obtained by nonlinear regression fitting of the curve.

2.3.6. Immobilization of azlocillin on the gold electrode surface

Before the immobilization, the solid gold electrodes (2 mm in diameter) were manually polished and cleaned with piranha solution as well as electrochemical cleaning as described previously [24].

After that, 10 mM of cysteamine hydrochloride was prepared in distilled water. The clean electrodes were immersed in the cysteamine solution for 2 hr at room temperature. After the incubation, the gold electrodes were washed with water and then absolute ethanol to remove any excess cysteamine residues. 30 mg of EDC and 6 mg of NHS were then used to activate 100 µg/mL of azlociline solution in PBS buffer pH 5.0. The activated analyte was then incubated on the cysteamine modified electrodes for 3 hr at room temperature. The electrode were then washed with PBS buffer pH 7.4 and incubated with 1mM MCH in PBS pH 7.4 to block the free gold surface. The electrodes were then stored at 4^{o}C in binding buffer PH 7.4 until further use.

2.3.7. Electrochemical measurements

All the electrochemical measurements were performed at room temperature in 5 mM ferro/ferricyanide $[Fe(CN)_6]^{3-/4-}$ mixture solution prepared in PBS buffer, pH 7.4 (1:1 molar ratio). The square wave voltammetry measurements were carried out using an autolab potentiostat at a scan rate of 125 mV/s and within the potential range of 0.4 V to -0.2 V. The parameters for SWV measurements were as follows: a frequency of 25Hz, time interval of 0.04s, amplitude of 20 mV and a step potential of 5mV. For the SWV detection graphs, baseline correction was performed.

2.3.8. Voltammetric competitive aptasensor for azlocillin

According to the binding affinity study, the Az9 aptamer showed the highest affinity (lowest K_d) to azlocillin, therefore, it was selected to develop a competitive aptasensor. For the azlocillin detection experiments, the azlocillin-modified electrodes were incubated for 50 min with 50 nM Az9 aptamer mixed with different concentrations of azlocilllin ranging from 1 pg/mL to 100 µg/mL in binding buffer. For the cross reactivity experiment, the aptasensor

was treated as above but with amoxicillin solution instead of azlocillin. The aptasensors were then washed with 0.1 M Tris buffer, pH 7.4 and SWV measurements were recorded.

2.3.9. Tap and waste water samples analysis

A tap water and wastewater samples collected in Riyadh city were spiked with different concentrations of azlocillin. Then the samples were incubated on the aptasensor surface for 50 min after mixing with the 50 nM Az9 aptamer. After incubation, the sensors were washed and subjected to SWV measurement as explained above.

3. Results and Discussion

3.1. Selection of aptamers against azlocillin

The attachment of the small molecule to a solid support in SELEX is a major step to enable the partitioning of the bound and unbound DNA. Here, the azlocillin was immobilized on DADPA agarose beads through its terminal carboxylic groups. The carboxylic groups on the antibiotic molecules were first activated using EDC/NHS chemistry and then used to react with the terminal amine groups on the beads via amide bond formation. After the covalent attachment of the azlocillin molecules on the agarose beads, sulfo-NHS acetate was used to block the remaining unreacted amine groups on the bead in order to minimize the non-specific electrostatic attraction of the DNA to the amine groups[11].

These azlocillin beads were then used for all the aptamer selection cycles which consisted of : 1) binding of the azlocillin beads with the DNA library composed of 10^{15} random 60-nucleotide sequences, 2) partitioning of the bound and unbound DNA via washing the beads with binding buffer, 3) elution of the bound DNA to the agarose beads and 4) amplification of the eluted DNA using PCR and purification of the PCR product to obtain a new enriched DNA pool to be used for the subsequent SELEX cycle. As shown in Fig. 1A, the recovery of the DNA after each cycle was monitored by measuring the fluorescence of the eluted DNA. A gradual increase in the recovered DNA was observed from round 1 to round 7 indicating

the enrichment of the DNA after each cycle. Then, a counter selection cycle was performed by incubating the DNA collected from the seventh round with negative agarose beads. After the counter selection, a significant decrease in the fluorescence intensity was seen at rounds 8 and 9 indicating the removal of the DNA which was non-specifically bound to the beads matrix or to the linker. Then, a dramatic increase in the fluorescence intensity was observed at round 10 and 11 implying the enrichment of the DNA pool with the specific sequences to azlocillin. Thus, the selection cycles were stopped at round 11 and the recovered DNA was cloned into E coli competent cells. Eighteen colonies were then picked up for amplification with the unlabeled primers and sequenced. The identified sequences shown in Scheme S1were grouped into 6 groups using the multiple sequence alignment software PRALINE. One sequence was selected from each group for further binding affinity testing.

3.2. Binding affinity studies of the selected aptamers towards azlocillin

The binding of the aptamers to the azlocillin were investigated using fluorescence assay where fixed amount of azlocillin beads were incubated with different concentration of each aptamer individually. After washing, the fluorescence of the eluted DNA was measured and binding curve is plotted for each aptamer sequence. A representative binding curve for the aptamer that showed the highest affinity (Az9) to azlocillin is shown in Fig. 1B. The dissociation constants (K_d) of the various aptamer-azlocillin complexes were calculated by non-linear regression analysis of the binding curves. A scrambled sequence was also tested for comparison and no significant binding was observed as shown in Fig. 1B indicating the selectivity of the assay. Table 1 shows the different K_{ds} obtained for 6 different aptamer sequences. As show in the table, the K_{ds} of the aptamers were ranging from 55 to 315 nM with Az9 exhibiting the lowest K_d indicating the success of the aptamers selection towards azlocilin with high affinity.

3.3. Characterization of the attachment of azlocillin molecules to the gold electrode surface for the aptasensor fabrication

Fig. 2 shows the SWV reduction peaks in ferro/ferricyanide redox couple of the various steps used during the construction of the aptasensor. The bare gold electrode showed a pronounced reduction peak that was enhanced after the reaction with cysteamine due to the positively charged amine groups which attracts the redox anions to the surface. The formation of terminal amine groups on the electrode surface indicating the success of the self-assembly step of the cysteamine on gold. However, the reaction of the amine groups with the EDC/NHS-activated azlocillin molecules lead to a decrease in the SWV peak current due to the blocking of the electrode surface with the attachment of azlocillin molecules. Further decrease in the SWV signal was also seen after the blocking of the free gold surface with MCH.

3.4. Competitive aptasensor construction using Az9 aptamer

The highest affinity aptamer (Az9) with the lowest K_d was then selected for further application in a competitive aptasensor. The aptasensor was designed where the azlocillin was immobilized on the sensor surface as explained above and a fixed concentration of Az9 aptamer was added to the sample containing azlocillin and incubated on the electrode surface. Then, the competition of the free and the immobilized azlocillin molecules to bind to the aptamer molecules leads to the generation of the sensor response which can be monitored using SWV. High concentration of azlocillin on the sample leads to less available aptamers to bind to the electrode surface and therefore, less sensor response and vice versa.

In order to obtain the best sensor response, it was necessary to optimize the aptamer concentration as well as the binding time of the aptamer to azlocillin. Fig. 3A shows the SWV signals of the azlocillin electrodes after incubation with different concentrations of the

Az9 aptamer. It can be clearly seen that the SWV decreased dramatically after the binding with the DNA aptamer. This is attributed to the negatively charged phosphate backbone of the DNA aptamers which causes shielding of the electrode surface and electrostatic repulsion with the redox anions. The sensor response was calculated as the percentage change in the SWV signals $(i^0\text{-}i)/i^0$ % where i^0 and i represent the reduction current signals of the azlocillin electrode before and after binding with the aptamer, respectively. Fig. 3B shows the gradual increase in the sensor response with increasing the concentration of the Az9 aptamer until a plateau is reached. Thus, 50 nM aptamer concentration was chosen for further experiments. Fig. 3C shows the increase in the sensor response upon incubation of the sensor with the aptamer for longer periods. Here, 50 minutes were used as the optimum binding time for further experiments.

At these optimum conditions, the detection experiments were performed by incubating the sensor with different concentrations of azlocillin mixed with 50 nM of Az9 aptamer for 50 minutes and the SWV measurements were recorded after washing. Fig. 4A shows the SWV signals after incubating the sensor with various concentrations of the analyte ranging from 1 pg/mL to 100 µg/mL. These results were used to construct a calibration plot as shown in Fig. 4B. A linear response of the aptasensor was obtained within the tested concentration range with a linear regression equation of $(i^0\text{-}i)/i^0$ %= 34.9 – 5.2 C [ng.mL^{-1}], R = 0.997, with a detection limit (LOD) of 1.2 pg/mL. The LOD of the azlocillin 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. This LOD indicates very good sensitivity of our aptasensor for azlocillin detection compared to the commercial ELISA which has a LOD of 0.8 ng/mL.

3.5. Cross reactivity testing of azlocillin aptasensor against other non specific drugs

It was very important to check the cross reactivity of our aptasensor with other related antibiotics such as amoxicillin, penicillin and drugs such as estradiol. As shown in Fig. 5 the sensor response was much higher when the aptasensor was incubated with either amoxicillin, penicillin or estradiol compared with azlocillin indicating the high selectivity of the aptasensor.

3.6. Application of the azlocillin aptasensor in tap and wastewater samples

The azlocillin competitive apatsensors were tested in spiked tap and wastewater samples in order to confirm the usefulness of the aptasensor in real applications. As shown in Table S1, the recovery percentages were very high indicating the success of our aptasensor platform for the detection of azlocillin in real samples. The relative standard deviations (RSD%) of the measurements were always less than 5 % indicating good reproducibility of the method.

4. Conclusions

In this work, high affinity and specificity aptamers against the antibiotic azlocillin have been selected. The aptamers have shown very low dissociation constants indicating the success of the aptamers selection. The highest affinity aptamer Az9 showed a K_d of 55 nM and was exploited in the development of an aptasensor for azlocillin detection. The developed aptasensor based on electrochemical competitive assay showed very high sensitivity compared with other previously reported methods as well as good selectivity against other antibiotics such as amoxicillin. Our aptasensor shows several advantages over current methods including the lower cost and higher stability of the aptamer as well as the ease of use of the electrochemical transducers and their capability of miniaturization. Therefore, we believe that these types of aptasensors are of great value for the detection of emerging environmental contaminates.

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Figure captions

Figure 1 A) The recovery profile of the eluted DNA during the SELEX cycles (a plot of the fluorescence intensity versus the cycle number) (B) Binding affinity curves obtained using fluorescence assays of azlocillin with the Az9 aptamer versus scrambled sequence. The Dissociation constant (K_d) was calculated by non-linear regressing analysis to be 55 nM. The error bars represent the standard deviations of triplicate measurements.

Figure 2 square wave voltammetry of 5 mM $[Fe(CN)_6]^{4-/3-}$ redox solution in PBS, pH 7.4, for the different fabrication steps of the aptasensor (the bare Au electrode (black line), Cys/Au (red), Azlocillin/Cys/Au (blue) and MCH/Azlocillin/Cys/Au (cyan)).

Figure 3 Square wave voltammograms of the Azlocillin-modified electrodes in 5 mM $[Fe(CN)_6]^{4-/3-}$ redox solution in PBS, pH 7.4 before and after binding with different concentrations of Az9 aptamer ranging from 1 nM to 200 nM. (B) a plot of the Az9 aptamer concentration versus $(i^0-i)/i^0$ %. (C) a plot of the binding time of the aptamer to the azlocillin versus $(i^0-i)/i^0$ %. The error bars represent the standard deviations obtained from triplicate measurements.

Fig. 4. (A) Square wave voltammograms of the Azlocillin modified electrode before and after incubation with different concentrations of azlocillin mixed with 50 nM of Az9 aptamer. (B) The calibration plot for Azlocillin, $(i^0-i)/i^0$ %. *vs.* logarithm of the azlocillin concentration. The error bars represent the standard deviation of triplicate measurements.

Fig. 5. Comparison of the azlocillin aptasensor response to azlocillin and amoxicillin, penicillin and estradiol. The chemical structures of the two antibiotics are shown.

Scheme 1 Immobilization of azolcillin on the gold electrode surface (A) and the competitive aptasensor working principle (B).

Table 1. Sequences and dissociation constants (K_d) of the selected aptamers towards Azlocillin

Scheme 1

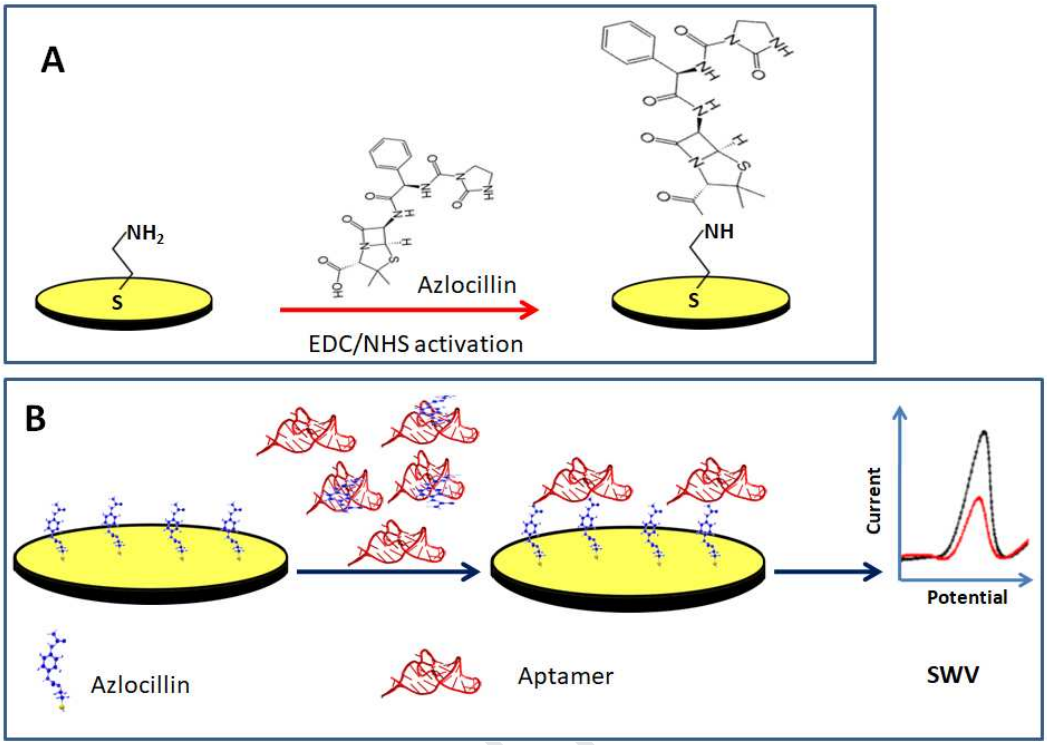

Figure 1

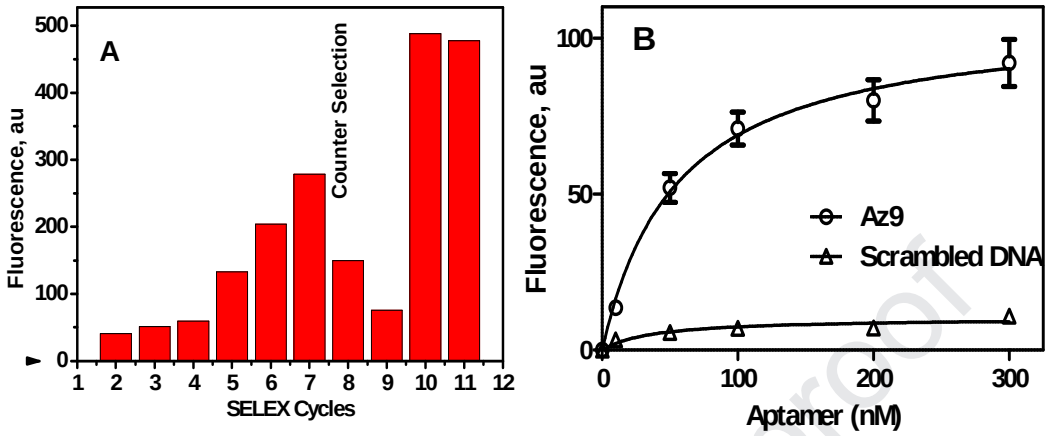

Figure 2

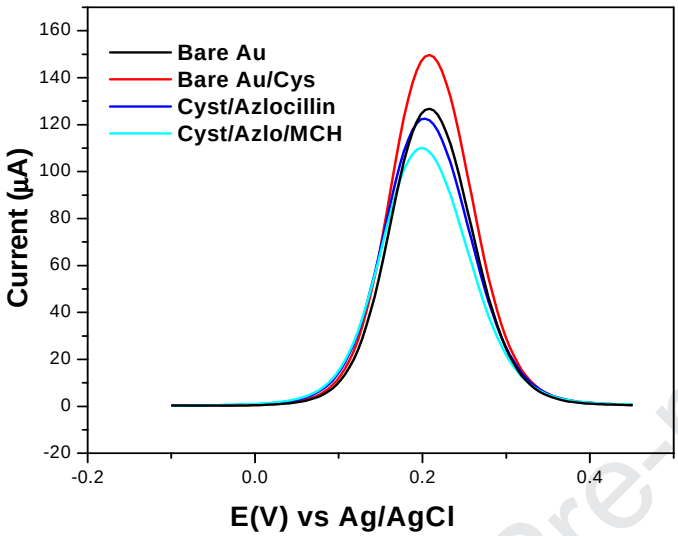

Figure 3

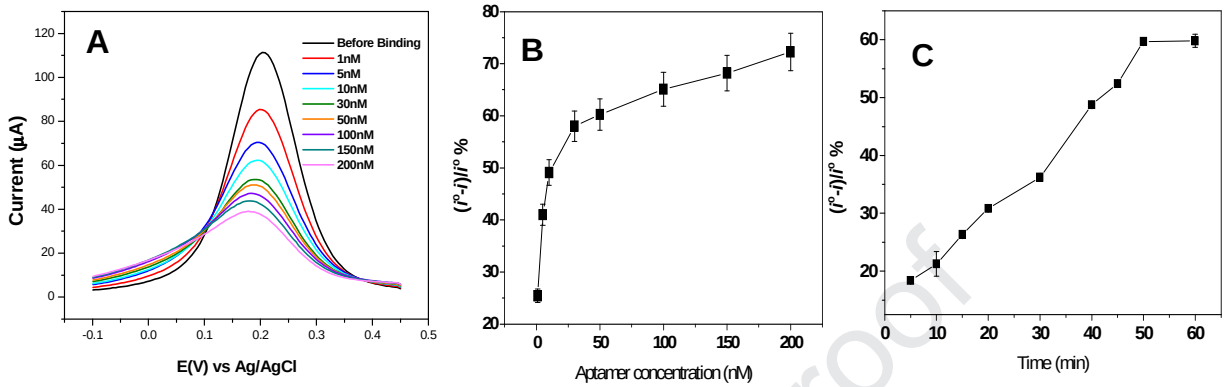

Figure 4

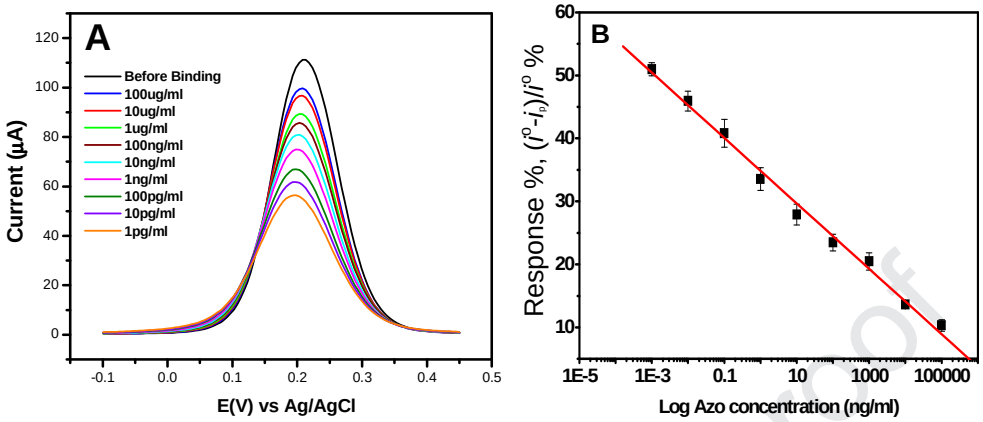

Figure 5

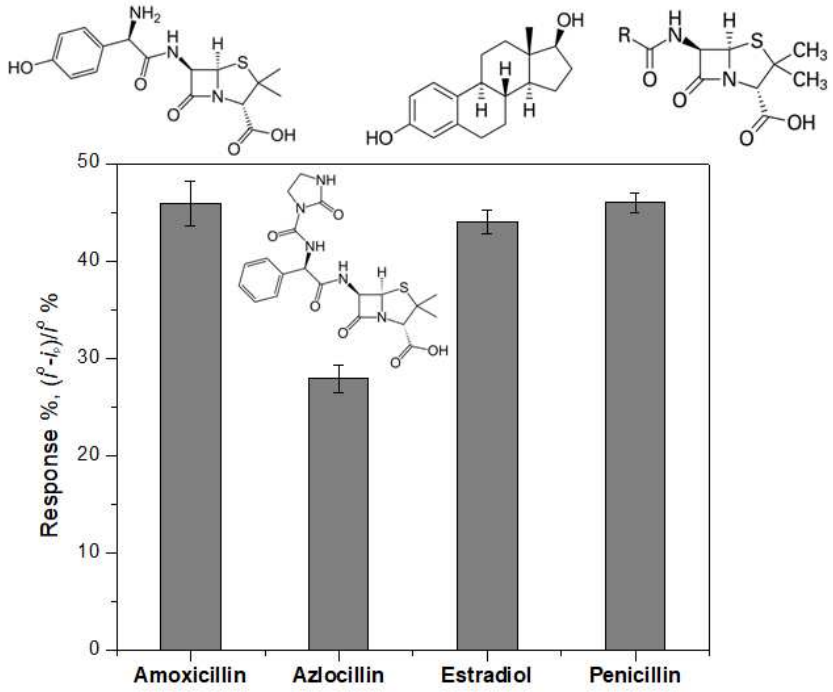
Response %, $(i^o-i_p)/i^o$ %
50
40
30
20
10
0
Amoxicillin
Azlocillin
Estradiol
Penicillin

Table 1

Name	Sequence 5"to 3"	K_d (nM)
Az5	GACGAGGAGAAACGAGGAATACAAAATTCACACCAACCGTCGTACAGTAATGCCTCGG	135
Az2	AGGGCCGTGGAGTGAGCAAAGAATCACGTGAAAGTGGACACGTAAGTTGATCGCGGCG	163
Az9	CAGGAAGACAACTCCGACTAGAATTGATAATCAAGAATTCGTCTGGGGGGAATGTGCG	55
Az28	TTAGGTAGCGTTTGCGAGTAAGCGAACCCTCCGGTGATAGCAAGGTAGAGACGTTGG	151
Az31	GAACCGTAATCTGACGAGATCATAGCCTCAAGGATACCGATACTCCGAGCAGTGGCCA	303
Az36	GTTATCTGGTCTCCACTTGGGTCGAATTGAATAATCTGGTATAAGGGCGAATTCTGC	315

Highlights

- The uncontrolled usage of veterinary antibiotics has led to their widespread pollution in waterways and milk products.
- We developed a highly specific and sensitive voltammetric aptasensor for on-step, sensitive and low cost detection of azlocillin antibiotic
- The successful selection of DNA aptamers against azlocillin was accomplished using systemic evolution of ligands by exponential enrichment (SELEX) method.
- Fluorescence-binding assays showed dissociation constant of 55 nM for one of the selected aptamers .
- This aptamer was used to construct a competitive voltammetric aptasensor for azlocillin. A limit of detection of 1.2 pg/mL

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: