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Electrochemical SELEX Technique for the Selection of DNA Aptamers against the Small Molecule 11-Deoxycortisol

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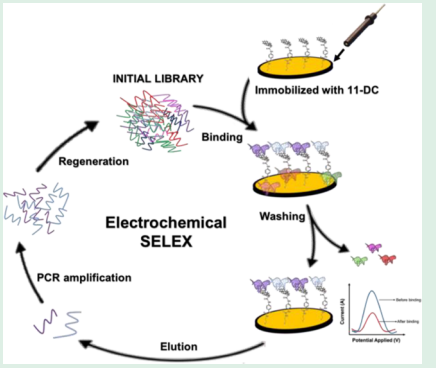
Supporting Information

ABSTRACT: Aptamers have received considerable attention due to their numerous advantages as biorecognition receptors. However, the tediousness and high cost of the conventional selection process of aptamers remain major challenges that limit their widespread use. To overcome these limitations, here we designed a new electrochemical SELEX platform that enables a rapid, efficient, and cost-effective enrichment method for the identification of aptamers, particularly against small molecules. The selection platform is based on the immobilization of the target analyte on gold electrodes that leads to improved selection efficiency and enables in situ monitoring of the enriched DNA using square-wave voltammetry. Compared with the conventional SELEX method, this selection protocol avoids the use of beads as a solid matrix and fluorescently labeled DNA, which is usually required for small molecules, thus offering simplified aptamer/target incubation, washing, and elution steps. Moreover, the DNA recovery can be detected by simply measuring the current of the electrodes in a ferro/ferricyanide redox couple. This would eliminate the need for fluorescence labeling, which significantly reduces the cost of the procedure. The approach was successfully applied for the selection of high-affinity aptamers that bind specifically to the small molecule 11-deoxycortisol hormone using eight rounds. One of the selected aptamers that showed a dissociation constant at the subnanomolar level was used to construct a competitive voltammetric aptasensor exhibiting high sensitivity, selectivity against other steroid analogues, and possible applicability in serum samples. Thus this SELEX approach offers great promise for a faster, simpler, and more economic aptamer identification method for various targets including small molecules for a wide variety of applications.



KEYWORDS: *electrochemical SELEX, aptamers, 11-deoxycortisol, competitive biosensor, voltammetry*

1. INTRODUCTION

Aptamers are single-stranded DNA or RNA molecules that have emerged as a new class of bioreceptors that are analogous to antibodies in terms of binding to their target with high affinity and specificity.[1] The aptamer sequence is capable of forming a unique 3D structure owing to the presence of the intramolecular attractions between the nucleotides, such as van der Waals, hydrogen bonding, and hydrophobic interactions.[2] Aptamers have been identified for several analytes such as proteins, viruses, toxins, drugs, hormones, bacteria, metal ions, peptides, cells, ions, and even tissues with high affinity that can reach the picomolar dissociation constant (K_d).[3] They have shown several advantages over antibodies that make them better candidates for many affinity-based applications, particularly in the development of biosensors.[4] Aptamers are selected by an in vitro process that does not require the use of experimental animals, and thus it is possible to acquire aptamers for both toxic and nonimmunogenic molecules. An indefinite number of aptamers can be made easily and at low cost once the aptamer for the target is identified and its sequence is obtained.[1] Aptamers have high thermal stability and can be effortlessly modified by numerous chemical tags with negligible effect on their binding affinity and specificity, allowing them to be easily immobilized on different solid supports. However, despite the advantages and the great promise of aptamers in different therapeutic and diagnostic applications, only a limited number of aptamers has been successfully identified, particularly for small molecules.[5,6] This is majorly attributed to the limitation of the conventional selection process of the aptamers known as the systematic evolution of ligands by exponential enrichment (SELEX).[7,8] The SELEX process usually involves two major steps: multiple iterative rounds of selection (10−20) from a pool of a large library of DNA sequences and then amplification of the selected subpool. In each round, the DNA library is incubated with the target immobilized on a solid matrix (usually sugar-based or magnetic microbeads). Then, several washing steps for the unbound DNA are performed, followed by elution, PCR amplification of the bound DNA, and then purification of the single-stranded DNA pool for further rounds. Moreover, the evaluation of the aptamer enrichment is usually done by monitoring the fluorescence of the eluted DNA after each

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round. Thus the SELEX process is expensive, labor-intensive, time-consuming, and sometime inefficient.

To overcome these limitations, some innovative selection methods have been developed over the last 20 years combining various techniques to accelerate and improve aptamers selection.[9,10] Capillary electrophoresis (CE)−SELEX[11−13] and nitrocellulose filter binding SELEX[10] have been reported. However, these methods can be used only for large molecules such as proteins. Flow cytometry−SELEX[14] and fluorescence-activated cell sorting−SELEX[15,16] have been also developed for selecting aptamers against cells. Microfluidic technologies[17−21] also have been integrated with different SELEX methods such as CE,[12] sol−gel,[22] and magnetic beads[23−25] to increase the separation efficiency. However, in these methods, the aptamer enrichment is not monitored in real time, leading to selection blindness, longer selection time, and a higher number of failed selection trials.[26,27] Therefore, it is highly demanded to develop a rapid and low-cost selection approach that possesses real-time evaluation capability and remarkable efficiency.

To this end, we have designed an electrochemical-based SELEX method for the efficient, low-cost, and rapid selection of DNA aptamers against small molecules. In our electrochemical−SELEX, the target analyte is covalently immobilized on a gold electrode surface. The interaction of the single-stranded DNA library and the target-modified electrodes can be directly monitored voltammetrically by measuring the current of the electrodes in the ferro/ferricyanide redox couple. The nonbound or weakly bound sequences can be easily and effectively removed through a washing process within 1 min. The platform is used to perform the negative and positive selections to improve the specificity of the selection. Compared with the conventional SELEX, the electrochemical−SELEX approach can eliminate the need for DNA labeling, which significantly reduces the cost of the procedure. Moreover, the use of electrodes as a solid matrix for target immobilization reduces the time of the washing and elution steps and allows efficient control of the selection via the label-free, in situ, and real-time monitoring of the enrichment process.

To demonstrate the success of our new method, we used 11-deoxycortisol (11-DCL) hormone as a small-molecule target. 11-DCL is a hormone that is converted to cortisol by an enzyme called 11ß-hydroxylase.[28] Thus the detection of 11-DCL in blood is used to diagnose 11ß-hydroxylase and other adrenal gland abnormalities.[29] After eight rounds of selection, high-affinity and specificity aptamers against 11-DCL were obtained, showing dissociation constants in the sub- to low nanomolar level. One of the selected aptamers has been successfully used to develop a voltammetric competitive aptasensor for 11-DCL, showing high sensitivity and possible applicability in serum samples. We believe that our method paves the way for the rapid, simple, low-cost, highly efficient selection of aptamers against various targets including small molecules.

2. EXPERIMENTAL METHOD

All of the materials, reagents, and instruments used in this work are described in detail in the Supporting Information.

2.1. Immobilization of 11-Deoxycortisol on the Gold Electrode. The cleaning of the electrode and the subsequent immobilization steps are described in the Supporting Information.

2.2. In Vitro Selection of the Aptamers for 11-Deoxycortisol. A diagram of the SELEX process used to select aptamers for 11-DCL is described in Scheme S1. In this process, 11-DCL was immobilized on the surface of gold electrode and then exposed to a ssDNA library pool. For this purpose, a chemically synthesized random ssDNA library (10^{15} sequences) was utilized. This oligonucleotide library comprises a medial random region of 60 nucleotides flanked by two constant regions of primer binding sites (18-nucleotide sequences) for PCR amplification at the 3′ and 5′ ends (5′-ATACCAGCTTATTCAATT-N60-AGATAGTAA-GTG-CAA-TCT-3′).

Prior to incubating the electrode with the ssDNA library, the square-wave voltammetry (SWV) reduction current of the 11-DCL-modified electrode was measured in ferro/ferricyanide solution. For the first cycle, a 3 nmol concentration of ssDNA library in binding buffer was heated to 95 °C for 5 min, then cooled at 4 °C for 10 min and kept at 25 °C for 5 min. Next, the electrodes were incubated with the treated ssDNA library for 2 h. After that, the electrodes were washed with aliquots of binding buffer three times. Then, the SWV reduction current of the electrode was measured again in ferro/ferricyanide solution. The elution buffer was then heated to 40 °C, and the electrodes were incubated in 200 μL aliquots of elution buffer five times for a time interval of 10 min. After that, the eluted DNA was collected and added to an ultrafiltration device (3 kDa cutoff filters) and then desalted with water six times. Starting from the second cycle, the fluorescence of the eluted DNA was measured by a NanoDrop fluorospectrophotometer after each round for a comparison purpose. Negative selection was performed after five rounds of SELEX, in which the electrode surface was modified with the linkers, blocked, and then incubated with the DNA pool in the absence of the target (negative electrode). Next, the washes were collected, desalted, and subjected to a heating and cooling treatment, then incubated with the positive electrode (immobilized with the target), and the cycle was continued. The ssDNA pool selected after each round was amplified by PCR in 23 equiv reaction tubes consisting of 50 μL of reaction mixture. Each tube consisted of two units of Taq Plus and polymerase buffer, 2 mM $MgCl_2$, 200 μM dNTP, and 0.2 μM of forward and reverse primers. The forward primer was modified with a fluorescein isothiocyanate (FITC) fluorophore label to enable the fluorescence measurement for comparison with the electrochemical measurements (forward primer: 5′-fluorescein-ATACCAGCTTAT-TCAATT-3′), whereas the reverse primer was modified with hexaethylene glycol linker, followed by a long poly(A) chain (reverse primer: 5′-poly dA20-PEG6-AGATTGCACTTACTATCT-3′). The PCR was carried out under the following conditions: 94 °C for 10 min, subsequently followed by 25 cycles of 94 °C for 1 min, 47 °C for 1 min, 72 °C for 1 min, and a last extension step of 10 min at 72 °C. The volume of the PCR product was reduced by performing ethanol precipitation. To the PCR product, 3 M sodium acetate and 100% chilled ethanol were added. This mixture was thoroughly vortexed, then kept at −80 °C for 1 h and centrifuged at 13 000 rpm for 30 min. After centrifugation, the supernatant was discarded, and the pellet was allowed to dry to remove any traces of ethanol. Next, the pellet was resuspended by adding water and formamide (1:2 v/v) and heated to 90 °C for 10 min. The desired ssDNA was separated from the dsDNA PCR product by using 12% denaturating PAGE. The fluorescent bands were cut from the gel and then added to TE buffer. The ssDNA was eluted by a freeze−thaw cycle in which the gel and TE buffer mixture were cooled to −80 °C for 30 min, then heated to 90 °C for another 30 min, and then kept rotating overnight. The following day, the eluted ssDNA in TE buffer was concentrated, desalted by an ultrafiltration device, and then UV-quantified for its use in the next selection round.

2.3. Cloning and Sequencing of the Selected Aptamers. After eight rounds of selection were performed, the ssDNA obtained after the eighth round was amplified by PCR and then ligated to a pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). Next, LB-agar media supplemented with ampicillin were prepared and added to the Petri dishes. After the media dried, X-gal was added to the plates. The ligated product was added to the competent cells, kept on ice for 30 min, then subjected to heat shock for 30 s on a water bath (42 °C) and then back at ice for 1 to 2 min. Next, SOC media were added to the competent cells tube, and the tube was kept

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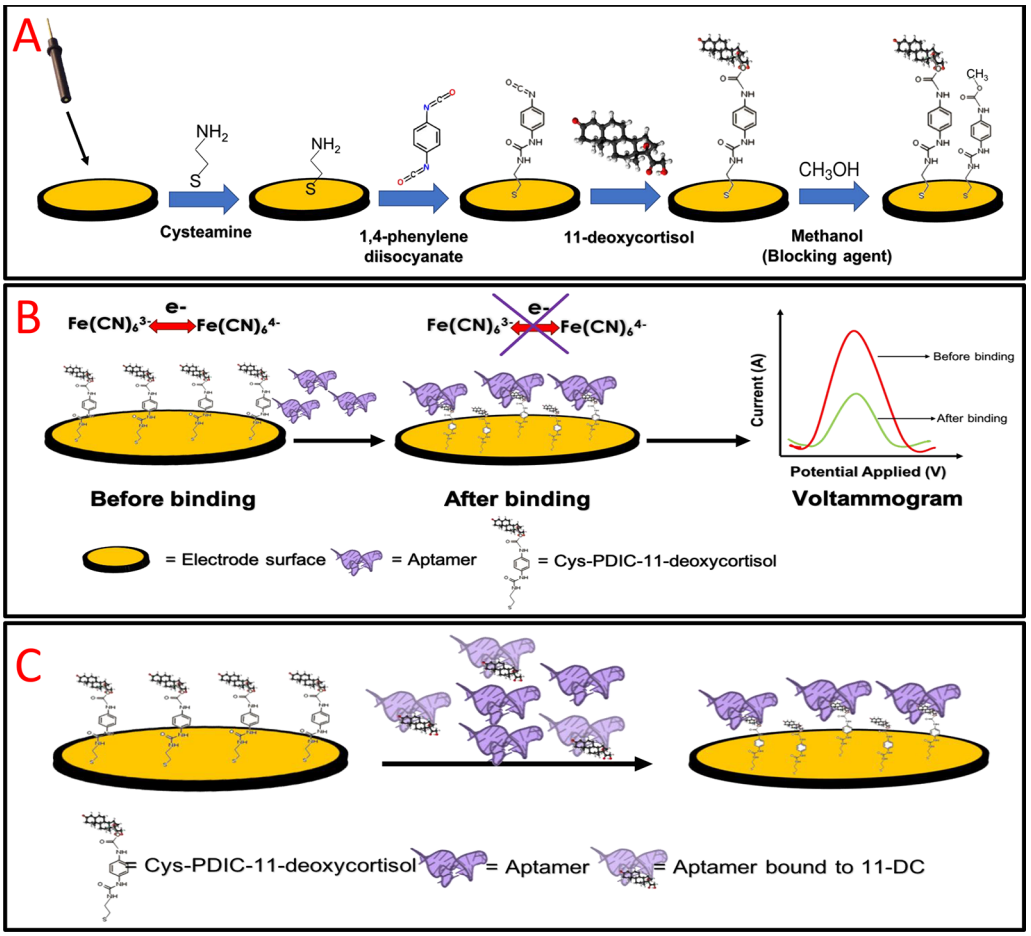
Scheme 1[a]



[a](A) Immobilization of 11-deoxycortisol on the gold electrode surface. (B) Label-free voltammetric monitoring of the bound DNA. (C) Competitive aptasensor working principle.

rotating for 1 h at 37 °C. After 1 h of rotation, the cells were spread on the Petri dish, and the colonies were allowed to grow overnight. Only white colonies were then picked up and allowed to grow in a liquid medium overnight. Then, colony PCR was performed to amplify the ssDNA inserts using M13 forward and M13 reverse primer sites inside the vector. Finally, these inserts were sequenced, and the sequences obtained were aligned using PRALINE (Scheme S2).

2.4. Dissociation Constant Determination of the Identified Aptamers to 11-Deoxycortisol. After cloning, sequencing, and alignment analysis of the selected sequences against 11-DCL, we picked up some sequences for a binding study to determine their affinity to 11-DCL. The binding assay was performed by incubating different concentrations of each aptamer individually (0.1 to 50 nM) on 11-DCL-modified gold electrodes prepared as described in the Supporting Information with 100 μg/mL hormone. After each incubation, the electrodes were washed; then, the SWV was recorded. Then, the DNA bound to the electrode was monitored by calculating the percentage change in the peak current ($(i° - i)/i°$%). Saturation binding curves were plotted for each sequence, and the dissociation constants (K_d) were determined by nonlinear regression analysis of the curves.

2.5. Electrochemical Competitive Aptasensor for 11-Deoxycortisol. From the binding analysis, the DC17 aptamer showed the highest affinity (lowest K_d) to 11-DCL; therefore, it was chosen to develop a competitive aptasensor. For the 11-DCL detection experiments, the 11-DCL electrodes were incubated for 20 min with 5 nM DC17 aptamer mixed with specific concentrations of 11-DCL. For the cross-reactivity testing, a similar experiment was performed using 1 ng/mL of cortisol, corticosterone, or pregnenolone instead of 11-DCL. The electrodes were then washed with 0.1 M Tris buffer, pH 7.4 and subjected to SWV measurements.

2.6. Serum Samples Analysis. A serum sample obtained from healthy volunteer was diluted 1:10 with binding buffer. The sample was then mixed with 5 nM DC17 aptamer and then incubated on the 11-DCL-modified electrode for 20 min. After incubation, the electrode was washed and subjected to the SWV measurement, as described above.

3. RESULTS AND DISCUSSION

3.1. Immobilization of 11-Deoxycortisol on the Gold Electrodes. One of the most important steps in SELEX is the choice of the immobilization matrix of the small-molecule target, which allows the efficient partitioning of the bound and unbound DNA. Here we chose the standard gold electrode as the solid matrix for target immobilization because of its high conductivity, intrinsic stability, and capability of regeneration. The 11-DCL molecules were attached to the gold surface via their hydroxyl groups. The gold surface was first functionalized using a two-step process. First, cysteamine hydrochloride was incubated on the gold surface to form a self-assembled monolayer, exposing a terminal amine group. Then, the Cys/Au electrodes were incubated with the PDIC cross-linker. One of the isocyanate groups was used to react with the amine group of the cysteamine, yielding the carbamide moiety, and the other bound to the hydroxyl group of the 11-DCL, yielding

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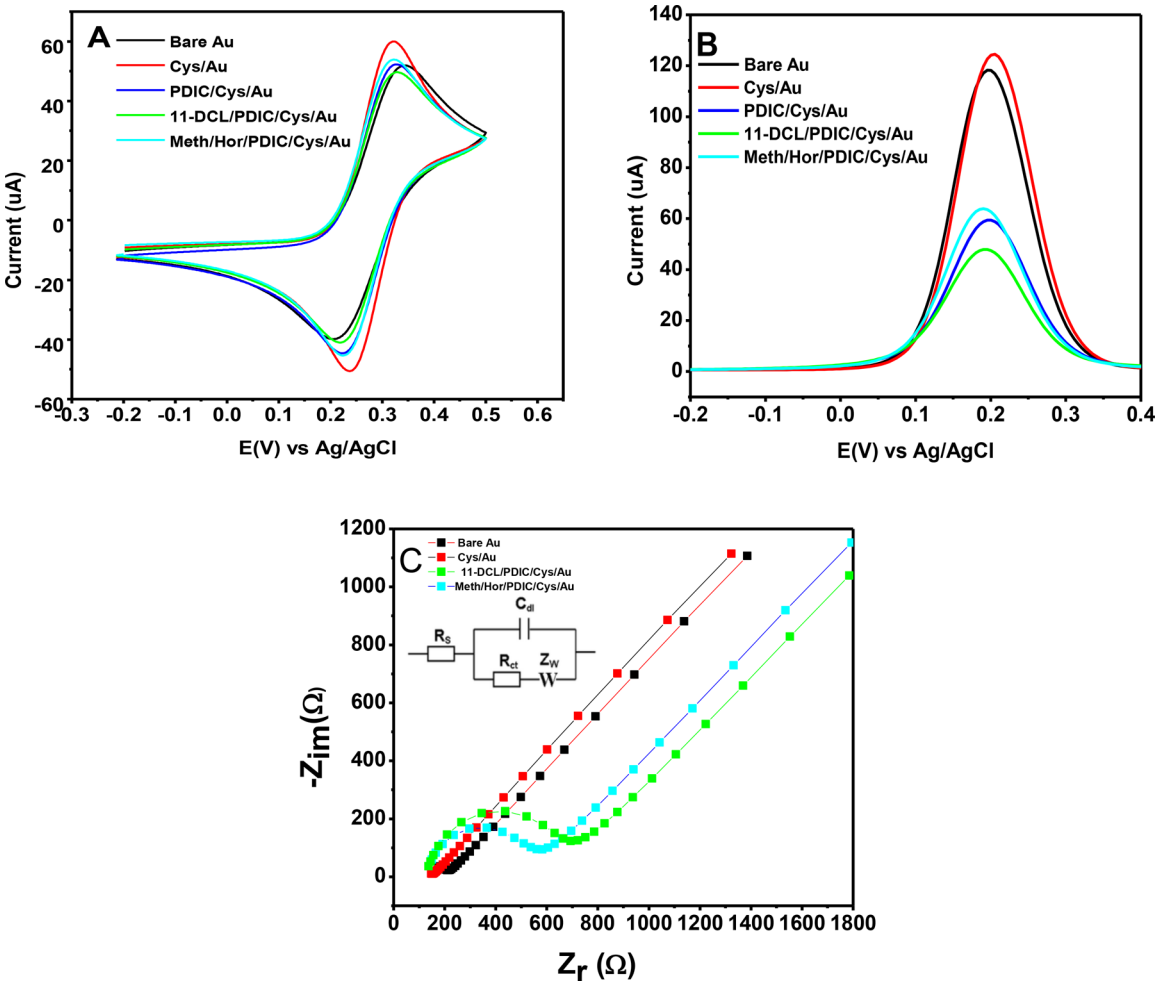

Figure 1. (A)Cyclic voltammetry, (B) SWV, and (C) Nyquist plots of 5 mM $[Fe(CN)_6]^{4-/3-}$ redox solution in PBS, pH 7.4 for the different modification steps of the electrode (bare Au electrode (black), Cys/Au (red), PDIC/Cys/Au (blue), 11-DCL/PDIC/Cys/Au (green), and methanol/11-DCL/PDIC/Cys/Au (cyan)). The scan rate of the CV was 100 mV/s, and the EIS was carried out in a frequency range of 10^4 to 0.1 Hz at an AC amplitude of 10 mV and a DC potential of +0.20 V. The circuit applied to fit the impedance spectra is shown in the inset of Figure 1C.

the carbamate (urethane) moiety[30−32] (Scheme 1A). This linker was previously shown to be very efficient and highly stabile and have a low toxicity. Finally, the unreacted cyanate groups were blocked with methanol to minimize any nonspecific adsorption.

With the aim of confirming the success of the immobilization steps of the target on the electrode surface, we used CV and SWV as well as electrochemical impedance spectroscopy (EIS) measurements. Figure 1A shows the cyclic voltammograms of the gold electrode before and after each modification step measured in $[Fe(CN)_6]^{3-/4-}$ redox couple solution in PBS buffer at pH 7.4. The bare gold electrode showed the characteristic reversible CV behavior of clean gold, exhibiting well-defined anodic and cathodic peaks with peak-to-peak separation (ΔE) of ∼90 mV. The cysteamine-modified gold surface then showed a slight increase in the peak current and a decrease in the ΔE, which are attributed to the positively charged amine groups of the cysteamine, which attract the redox anions, indicating the successful formation of the self-assembly monolayer. On the contrary, after the incubation with the PDIC linker, the anodic and cathodic peak currents slightly decreased again, likely due to the negative charge of the cyanate groups, which repelled the $[Fe(CN)_6]^{3-/4-}$ molecules from the surface. However, after the addition of the 11-DCL to the modified electrodes, the peak current was further decreased, indicating the successful attachment of the target molecules on the surface, leading to the retardation of the electron transfer. After the surface with was blocked methanol, the current increased again due to the blocking of the remaining free negatively charged cyanate groups. Because only slight changes were seen using CV, we have also utilized SWV as a more sensitive technique for characterization. A shown in Figure 1B, the SWV reduction peak of $[Fe(CN)_6]^{3-/4-}$ showed the same trend after each modification step, with more pronounced differences in the peak currents.

Figure 1C shows the EIS Nyquist plots of the electrodes after each modification step. Each spectrum consists of two parts: the semicircle part at high frequency and the straight line part at low frequency, which correspond to the electron-transfer and diffusion processes, respectively. To fit the experimental data, we used a modified Randles equivalent circuit (inset of Figure 1C) that consists of a charge-transfer resistance (R_{CT}), a solution resistance (R_S), a Warburg impedance (Z_W) that results from the diffusion of the redox couple from the bulk of the solution to the electrode interface, and a constant phase element (CPE), representing the electrical double-layer capacitance. Among the electrical parameters, R_{CT} (represented by the diameter of the

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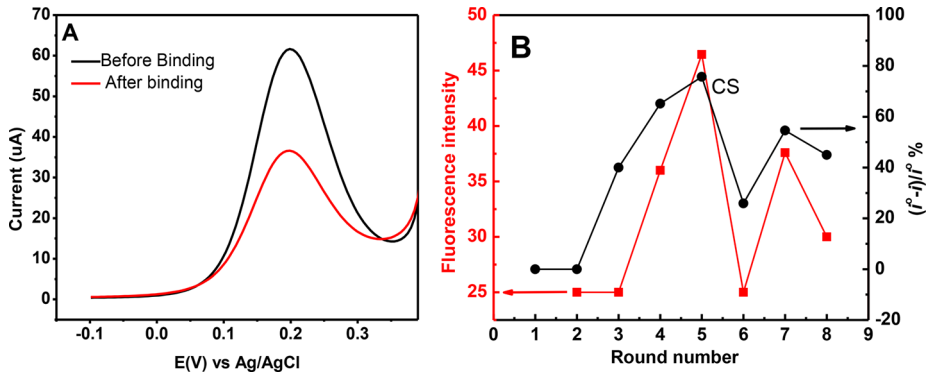

Figure 2. (A) Representative example of square-wave voltammograms of the 11DCL-modified electrodes in 5 mM $[Fe(CN)_6]^{4-/3-}$ redox solution in PBS, pH 7.4 before (black curve) and after binding with the DNA pool (red curve). (B) Binding of ssDNA pools to 11-DCL after each SELEX cycle monitored by calculating the % change in the reduction peak current ($(i° - i)/i°\%$) of the 11-DCL electrodes after binding with the DNA (black plot) and the fluorescence of eluted DNA after each cycle (red plot).

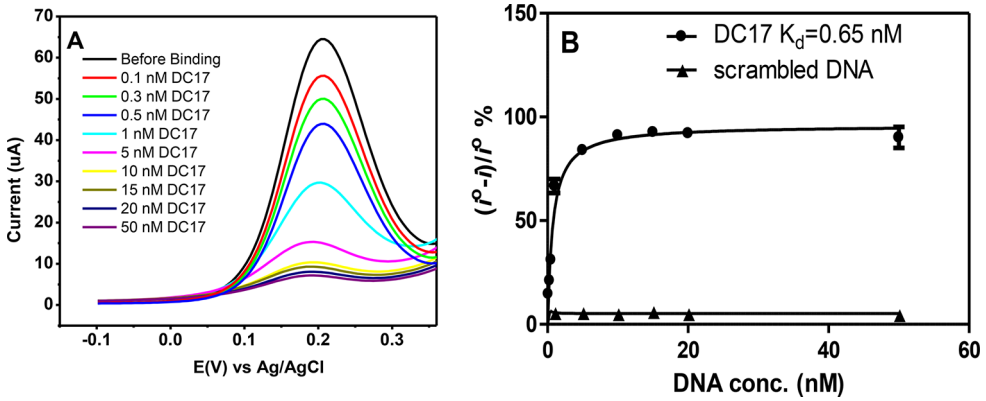

Figure 3. (A) Square-wave voltammograms of the 11DCL-modified electrodes in 5 mM $[Fe(CN)_6]^{4-/3-}$ redox solution in PBS, pH 7.4 before and after binding with different concentrations of DC17 aptamer ranging from 0.1 to 50 nM. (B) Electrochemical binding curve of 11-DCL to DC17 aptamer and a scrambled DNA sequence (a plot of the aptamer concentration versus $(i° - i)/i°\%$). The error bars represent the standard deviation obtained from duplicate measurements.

semicircle) is usually the parameter of choice used to characterize the modification of the electrode−solution interface. As shown in Figure 1C, the bare gold electrode showed a small semicircle and a straight line, indicating the fast electron transfer on the bare gold surface and thus very low R_{CT}. The semicircle is decreased even more after the cysteamine modification due to the attraction of the amine groups to the redox anions, leading to a faster electron-transfer process. However, after the activation with the linker and the attachment of the target, the resistance was dramatically increased due to the blocking effect of the target molecules. Then, a slight increase in the R_{CT} was observed after blocking the unreacted cyanate groups with methanol. Thus the CV, SWV, and EIS results confirmed the successful immobilization of 11-DCL on the gold electrodes.

3.2. In Vitro Selection of 11-Deoxycortisol-Binding Aptamers. Here we used a library that consists of 1.8×10^{15} random 60-nucleotide sequences. Each SELEX cycle was performed as follows: (1) incubating the target electrode with the library, (2) simple washing of the electrodes three times each for 30 s, (3) elution of the bound DNA to the electrodes, (4) desalting and PCR amplification of the eluted DNA, and (5) purification of the PCR product to obtain the ssDNA aptamer pool via PAGE. A counter-selection step was also carried out after the fifth round using functionalized electrodes prepared the same way without target to eliminate any non-specific DNA that binds to the gold surface as well as the linkers.

As explained above, our goal is to develop a label-free-based selection protocol. However, here, we used a fluorescence-labeled primer for amplification to validate the method by comparing the recovery obtained using both electrochemical and fluorescence techniques. Obviously, this method can be applied with unlabeled DNA for any target analyte.

To monitor the progress of the SELEX and evaluate the enrichment process after each cycle, we measured the SWV reduction peak current of the electrodes in the ferro/ferricyanide redox couple before and after incubating the electrodes with the DNA pool. Figure 2A, shows a representative example of the SWV reduction peaks of the electrodes before and after incubation with the DNA. The binding of the target-modified electrodes with the DNA leads to a drop in the peak current. This is attributed to the shielding effect of the surface with the DNA as well as the negative charges of the DNA phosphate backbone, which hinders the access of the redox anion to the surface and retards the electron transfer. Figure 2B shows the binding recovery evaluated by calculating the percentage change in the peak current $(i° - i)/i°\%$ (black plot) for each cycle. As can be seen in the figure, no decrease in the current was obtained in the first two cycles, indicating the very low amount of bound DNA to the surface. However, after that, a progressive increase in the

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Table 1. Sequences and Dissociation Constants (K_d) of the Selected Aptamers toward 11-Deoxycortisol

sequence number	aptamer sequence	K_d (nM)
DC15	ATTACAGGAACGGGGACAAGGGCGAATAGAAATTGTGAAACATAAAGTCACGGGGTATGG	38.12
DC27	AGGGAGCCAAGACAGCGCAATAATTTCTGCAGTGTGGAGCTTTGTTTAATTTAGTTGTTA	24.3
DC21	ATCAGTCGGACCGGCGGTAGAAGAGGAGTTCTATCACCAAAGGGAAAAGTAGTCAGCCG	12.8
DC19	ACACGCACGAGTTCGTACAAAGAGACAAGATCAGCATAAAACCAAGACGGACGCCAACCA	11.87
DC7	GCGAAGCCCTCAACGAACAACACAGATCTAGATCTTTATGGTAAGGTCCGTGCTGCCC	5.4
DC13	TGCGAAGCCCTCAACGAACAACACAGATCTAGATCTTTATGGTAAGGTCCGTGCTGCCC	5.5
DC3	CCGAGCATACTTAAACCATACTACAGTGTACCACTGGCAAAACCATTGATCCGCGAGCTC	3.5
DC28	CACACGACGAAGGACCTATAACACCGTACAGCAATCGACACTTAGTAGACACCGCGGCCA	3.5
DC14	AGTGAGGAACACATAACCGCGTAACGGGTAGCTTACAAGGGCTATTGGGGGATGCAAAGG	2.10
DC23	TTCATGGCAACTGGGTAAGTTACTTTCACAATCCACTTCTAATATGATCGATCAGGCGCGG	1.94
DC17	TAACAGACGTCTCCCAAGCCATGAAAATTAGCCCAACTCATAGGACCATAACGCCCTACC	0.65

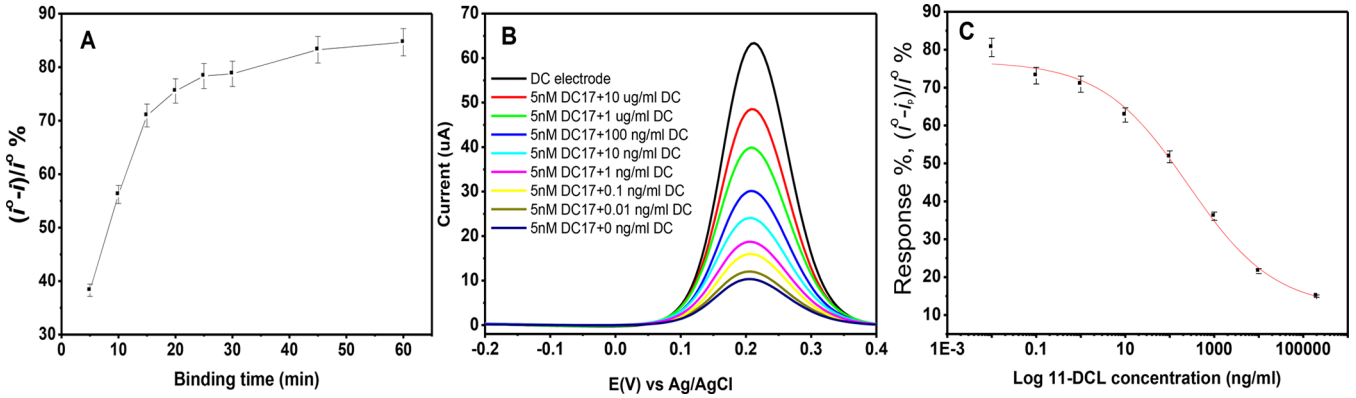

Figure 4. (A) Effect of incubation time on the aptamer/anlayte binding (a plot of the current change $((i^\circ - i)/i^\circ\%)$ after incubating the 11-DCL electrode with 5 nM aptamer at different time points (5−60 min). (B) Square-wave voltammograms of the 11-DCL electrode before and after incubation with different concentrations of 11-DCL mixed with 5 nM of DC17 aptamer. (C) Calibration curve for 11-DCL. Plot of analytical response $((i^\circ - i_p)/i_o\%)$ versus logarithm of the 11-DCL concentration. The error bars represent the standard deviations of two repetitive measurements.

$(i^\circ - i)/i^\circ\%$ was seen, implying the enrichment of the specific sequences. After counter selection (after round 5), the recovery was diminished due to the removal of the nonspecifically bound sequences to the target. Then, in the subsequent rounds, the recovery significantly increased again, suggesting sufficient enrichment in the specific aptamer pool. For comparative purposes, we have also performed an elution step of the bound DNA, and the fluorescence intensity of the solution was measured after each cycle (red plot). We observed a good agreement between the recoveries monitored using both electrochemical and fluorescence methods. Therefore, the selection was stopped, and the DNA pool from round 8 was cloned; then, 15 white colonies were randomly picked up for sequencing. The sequences were then identified and analyzed by the multiple sequence alignment program PRALINE[33] (Scheme S2).

3.3. Determination of the Dissociation Constants of the Selected Aptamers. To evaluate the binding affinity of the selected aptamers, the K_d values of the aptamers were determined using the SWV assay. After sequence alignment analysis of the aptamers was performed, the sequences were grouped, and 11 aptamers (DC3, 28, 7, 13, 19, 21, 27, 15, 17, 23, and 14) were chosen for the binding analysis testing. The SWV binding assay was performed using unlabeled aptamers after eliminating the primer binding sites. The primer binding site was removed to shorten the sequence because it is well established that the shorter sequence is more favorable for the biosensing applications. The assay was performed by incubating the hormone-modified electrodes with different concentrations of each aptamer (from 1 to 50 nM). After incubation, the electrodes were washed and subjected to SWV measurements in ferro/ferricyanide redox solution. The binding was then evaluated as the percentage decrease in the reduction peak current $(i^\circ - i)/i^\circ\%$ upon binding of the electrodes with the aptamer. Figure 3A shows a representative example of the SWV reduction peak current of the hormone electrode before and after binding with increasing concentrations of aptamer DC17. A significant decrease in the current was observed upon incubation with higher concentrations of the aptamer due to the negative charge of the DNA, as explained above. We then plotted the binding affinity curves for each aptamer as the percentage change in the current versus the DNA concentration, and the K_d values were calculated by nonlinear regression analysis of the curves. Figure 3B shows a representative example of the binding curve of sequence DC17, where saturation was obtained. A scrambled DNA sequence was also tested as the control, and no significant change in the current was observed, indicating the selectivity of the electrodes to the bound DNA. As shown in Table 1, the aptamers have shown very high affinity to the 11-DCL hormone target with K_d values in the sub- to low nanomolar range (from 0.65 to 38 nM). These results indicate the success of our protocol.

3.4. Application of the Selected Aptamer in Voltammetric Competitive Biosensor. As shown in Table 1, aptamer DC17 showed the lowest K_d and thus the highest

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affinity to the target. Therefore, we selected this aptamer to develop an aptasensor for the detection of 11-DCL. The aptasensor is based on a competitive assay where a competition is established between the free analyte in the solution and immobilized analyte on the electrode surface for a fixed concentration of aptamer added to the solution. When the aptamer alone is added to the hormone-modified electrode (aptasensor), a significant drop in the SWV reduction peak signal is obtained, whereas adding increasing concentrations of the analyte would lead to fewer available aptamer molecules bound to the electrode surface, and thus less of a current change would be obtained.

With the aim to obtain the optimum sensor response, we first optimized the binding time of the aptamer and hormone. Figure 4A shows the % change in the reduction current before and after incubating with 5 nM aptamer at different time points from 5 to 60 min. As shown in the figure, the peak current continued to decrease with the increase in the incubation time until it reached a plateau at almost 20 min. Therefore, 20 min was chosen for the further experiments.

The concentration of the aptamer is also important in the competitive assay because a low concentration of aptamer can lead to a decreased sensor response (less current change), and a very high concentration would lead to low sensitivity. Therefore, from Figure 3B, 5 nM aptamer, at which almost 80% current change was obtained, was chosen for the competitive assay to strike a balance between the two effects.

Figure 4B shows the decrease in the SWV reduction peak upon the incubation of the sensor with different concentrations of the hormone (from 0.01 ng/mL to 10 μg/mL) mixed with 5 nM of the aptamer DC17. The sensor response was calculated as the percentage change in the current $(i^\circ - i_p)/i_o\%$, where i° is the current of the 11-DCL before any incubation and i_p is the current after incubation with a different concentration of the analyte mixed with the aptamer. As shown in Figure 4C, the calibration curve of the aptasensor plotted as the sensor response versus the logarithm of the hormone concentration, exhibited a sigmoidal dose response.

As the concentration of the analyte in the sample increases, the sensor response decreases due to the competition leading to fewer available aptamers that bind to the electrode. The calibration curve was fitted to a sigmoidal logistic four-parameter equation

$$Y = A + \frac{(B - A)}{1 + \left(\frac{x}{\mathrm{EC50}}\right)^b}$$

where A and B are the minimum and maximum sensor responses, respectively, EC50 is the concentration leading to 50% of the maximum signal, and b is the slope at the inflection point. The fitting values of the experimental data are as follows: $y = 11.8 + (76.9 - 11.8)/(1 + (x/259.3)0.45)$, with a coefficient $R^2 = 0.997$.

The limit of detection of the aptasensor was calculated as the concentration corresponds to the difference between the mean blank signal (signal at zero analyte concentration) and three times the standard deviation of the blank. The limit of detection (LOD) of the aptasensor was 11 pg/mL, indicating the very high sensitivity of our method. This LOD is much lower than that obtained using the commercial ELISA kit (0.34 ng/mL). This high sensitivity is attributed to the high binding affinity of the selected aptamer.

3.5. Cross-Reactivity Study of the Aptasensor with Other Steroids Analogues. To investigate whether the selected aptamer binds to other steroid hormones that have similar chemical structures such as cortisol, corticosterone, and pregnenolone, we have incubated the aptasensor with the aptamer mixed with 1 ng/mL of each analogue instead of 11-DCL. The response here is calculated as the % change in the current at zero analyte (Δi_{max})—the % response signal in each case. Figure 5 shows a high signal for 11-DCL compared with

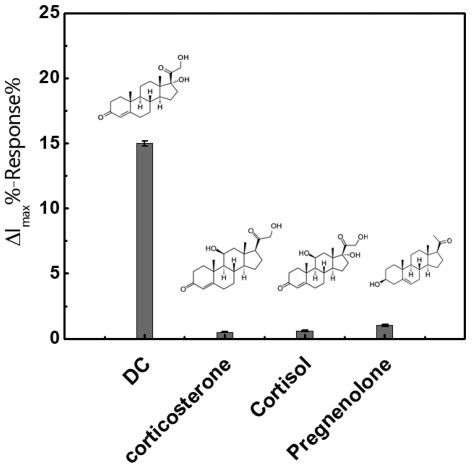

Figure 5. Comparison of the response of the aptasensor to 1 ng/mL 11-DCL, cortisol, corticosterone, or pregnenolone. The chemical structures of the hormones are shown.

the nonsignificant response obtained with the other analogues. This indicates the high degree of selectivity of our aptamer toward 11-DCL. This could be attributed to the presence of the hydroxyl moiety in the hydrophobic core of the cortisol, corticosterone, and pregnenolone molecules, which created some hydrophilic nature that was not favorable for the aptamer binding, unlike the nonhydroxylated core part of the 11-DCL molecule.[34]

3.6. Application of the 11-DCL Competitive Aptasensor in Serum Sample. With the aim to investigate the possible applicability of our competitive aptasensor for the detection of 11-DCL in human biological fluids, we tested the sensor in a serum sample obtained from a healthy volunteer. We found that the concentration of the 11-DCL was 1.1 ng/mL in the serum sample, which falls within the normal reference range of 11-DCL in healthy individuals.[35] The aptasensor was then tested in 0.1 ng/mL spiked serum sample, showing a good recovery percentage. The standard deviation calculated from duplicate measurements was ~5%, indicating the good reproducibility of the assay.

4. CONCLUSIONS

We have developed an electrochemical selection protocol for aptamers using gold electrodes. The method is superior to existing SELEX protocols in terms of reduced cost, high efficiency, simplicity, and capability of in situ monitoring of the DNA enrichment. We have applied this method for selecting the aptamer against the small-molecule hormone 11-DCL using eight cycles. Unlike the conventional SELEX approaches for small molecules, which usually require several days to perform each selection cycle, the current protocol can shorten the selection cycle to only a few hours. The selected aptamers

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have shown very high affinity with dissociation constants in the sub- to low nanomolar range, indicating the success of our method. We then applied the aptamer in a competitive voltammetric aptasensor for 11-DCL detection. The aptasensor showed very high sensitivity and selectivity against other steroid analogues. Therefore, we believe that this SELEX approach offers great promise for a simpler, faster, and low-cost aptamer selection method for small molecules. Moreover, the method can be adopted for other targets using different known immobilization chemistries that are suitable for each specific compound.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsabm.9b00294.

Materials and reagents. Instrumentation. Methods: immobilization of 11-DCL on the gold electrode; electrochemical measurements. Scheme S1: Overview of the electrochemical SELEX protocol using electrode. Scheme S2: Multiple sequence alignment analysis of the selected aptamers using PRALINE program (PDF)

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Notes

The authors declare no competing financial interest.

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