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An electrochemical aptasensor for chiral peptide detection using layer-by-layer assembly of polyelectrolyte-methylene blue/polyelectrolyte-graphene multilayer

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ABSTRACT

Here we demonstrate for the first time that by physically adsorbing aptamer onto conductive film assembled via alternate adsorption of graphene/polyelectrolyte and methylene blue/polyelectrolyte, a label-free electrochemical aptasensor with high sensitivity and selectivity for peptide detection is constructed. Graphene multilayer derived from layer-by-layer assembly has played significant roles in this sensing strategy: allowing accumulation of methylene blue, facilitating electron transfer and providing much more adsorption site. As compared to previous electrochemical aptasensors, the current sensor based on graphene multilayer alternated with electroactive molecule layer offers extremely high capability for sensitive detection of target without interference of environmental surrounding. This electroactive probe-confined graphene multilayer confers great flexibility to combine with differential pulse voltammetry (DPV) together. In the presence of target D entiomer of arginine vasopressin (D-VP), the binding of peptide to aptamer block the electron transfer process of MB, leading to decreased current peak of DPV. By this way, this electrochemical aptasensor based on electroactive molecule-intercalated graphene multilayer provide highly sensitive and specific detection of D-VP with the lowest detectable concentration of 1 ng mL^{-1} and a wide detection range from 1 to 265 ng mL^{-1}.

1. Introduction

Peptide biomarkers are very paramount indicators of disease progression [1–3], bacterial infection [4,5], hormone level [6], etc. Nevertheless, only limited methods in previous literatures are available for detection and quantification of peptide biomarker [7–16]. Among these methods, liquid chromatography/mass spectrometry (HPLC/MS) is the mainstream tool for analysis of peptide biomarker. However, for point-of-care purpose, the bioanalytical method based on biosensor is highly desirable due to its operation convenience as compared to complicated operation procedures and multiple analysis steps required by HPLC/MS.

Unfortunately, although biosensor field has been expanding at an astonishing rate, the biosensors for detection of peptide are still in the very early stage [2,6,8,17–20]. Therefore, it is highly demanding to develop new biosensor platforms by which various peptide targets can be analyzed and quantified. Provided that arginine vasopressin peptide has played a very important role in the human body, we seek to take it as a prototype. Arginine vasopressin, also known as vasopressin (VP), is a peptide hormone which is secreted and stored by the posterior lobe of the pituitary gland. The major function of VP is to control the adsorption of molecules into the tubules of the kidneys by affecting the tissue's permeability. In case of patients in hemorrhagic shock, the progression to late phase hemorrhagic shock can be indicated by a marked decrease plasma VP levels. It has also been suggested that replenishing VP can serve to rapidly increase peripheral vascular resistance and thereby stabilizes the patient. Although it has played a significant role in the human body, unfortunately until now only few studies [6,15,21,22] have been reported on the detection of VP.

In this study, by utilizing layer-by-layer assembly of electroactive molecule-confined graphene multilayer as signal transducer, aptamer [23,24] as molecular recognition agent and the differential pulse voltammetry (DPV) as characterization tool, a new label-free electrochemical aptasensor has been developed to selectively detect D enantiomer of arginine vasopressin (D-VP). In the past 2 years, electrochemical aptasensor has attracted rapidly increasing attention due to its fast response, low cost, high sensitivity and the potential for miniaturization [25–38]. In order to build this new sensing platform, three pronounced sensing elements have been implicated: graphene multilayer, methylene blue and aptamer specific for D-VP.

Single layer of graphene as a two-dimensional network of sp^2 hybrid carbon atom has tremendous interesting characteristics [39] including high conductivity, large surface area, etc. Graphene multilayer derived from layer-by-layer assembly has played significant roles in this sensing strategy: allowing accumulation of methylene blue, facilitating electron transfer and providing adsorption site

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for aptamer. As compared to previous electrochemical aptasensors [40], sensor based on graphene multilayer alternated with electroactive molecule layer offers extremely high capability for sensitive detection of target without interference of environmental surrounding. Until now, there is no paper reporting on the layer-by-layer assembly of graphene multilayer alternated with polyelectrolytes, by which electrochemical aptasensor can be built up.

Another big advantage of this sensing platform, built from layer-by-layer assembly of graphene layer alternated with electroactive molecule, allows its integration with DPV, leading to construction of a label-free biosensor platform. As compared to another popular electrochemical method named electrochemical impedance spectroscopy (EIS) [30,41,42] which entails redox probes ($Fe(CN)_6^{4-/3-}$) in the electrolyte to detect target of interest, DPV eliminates this disadvantage. In the presence of D-VP, the aptamer on the outermost layer of the multilayer can catch the target onto the electrode interface to form aptamer-target complexes, which form a barrier for electrons and inhibit the electro-transfer, resulting in the concentration-dependent decrease of DPV signals of MB. Using this strategy, we could use DPV signal change to sensitively detect D-VP with the lowest detectable concentration down to 1 ng mL^{-1}.

2. Experimental

2.1. Chemicals and materials

L-Vasopressin (L-VP) and D-vasopressin (D-VP) were bought from Ai-gene, Inc (Shanghai, China). The vasopressin-binding aptamer (VBA) sequence (5′-TCACGTGCATGATAGACGGCGAAGCCGTCGAGTTGCTGTGTGCCGATGCACGTGA-3′) was synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). The concentration of the oligonucleotide was quantified using UV absorbance at 260 nm and the corresponding extinction coefficient. Lysozyme and α-thrombin were bought from Sigma (Missouri, America). Glutathione (GSH) and bovine serum albumin (BSA) were purchased from Dingguochangsheng Biotechnology Company (Beijing, China). Graphite was purchased from Alfa Aesar. Hydrazine solution (50 wt%) and ammonia solution (25 wt%) were obtained from Beijing Chemical Reagent Factory (Beijing, China). All reagents were used as received without further purification. Before use, the oligonucleotide was dissolved in the PBS buffer (25 mM PBS containing 25 mM KCl and 1.5 mM $MgCl_2$, pH 7.6) and quantified by using UV–vis absorption spectroscopy with the following extinction coefficients at 260 nm (ε, M^{-1} cm^{-1}): A = 15,400, G = 11,500, C = 7400, T = 8700. The stock solution of peptide and protein were prepared in the PBS buffer (5 mM PBS containing 100 mM KCl and 3 mM $MgCl_2$, pH 7.0). DNA and peptide/protein solutions were stored at 4 °C before use. All stock and buffer solutions were prepared using Milli-Q water (>18.3 MΩ cm^{-1}).

2.2. Apparatus

UV–vis absorption spectra were recorded on a Cary 500 UV–visible spectrometer (Varian, USA). A normal three-electrode configuration consisting of a modified ITO working electrode (3 mm in diameter), an Ag/AgCl reference electrode, and a platinum wire auxiliary electrode was used. The cell was housed in a homemade Faraday cage to reduce stray electrical noise. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were both carried out on an Autolab PGSTAT30 (the Netherlands, controlled by Gpes and Fra software) in the 5 mM PBS buffer (pH 7.0). All measurements were carried out at room temperature (20 °C).

2.3. Synthesis of PSS–graphene hybrid nanosheets and pure graphene

Graphene oxide (GO) was synthesized from natural graphite by Hummers' method with little modification [43]. PSS–G were synthesized as followed: 20.0 mL of the homogeneous graphene oxide dispersion (0.2 mg mL^{-1}) was mixed with 20.0 mL of PSS aqueous solution (80 mg) and 80.0 μL of ammonia solution, followed by the addition of 7.0 μL of hydrazine solution. After being vigorously shaken or stirred for a few minutes, the vial was put in a water bath (60 °C) for 4 h. The stable black dispersion was obtained.

2.4. Fabrication of the sensing interface

The ITO electrodes were cleaned by ultrasonication in aqueous ethanol solution saturated with NaOH, acetone, ethanol and Milli-Q water, respectively, to provide a negatively charged clean surface. The PDDA–MB/PSS–G multilayer grew on the electrode by alternately immersing the modified ITO electrode into the MB–PDDA mixture aqueous solution (0.5 mg mL^{-1} MB, 5 mg mL^{-1} PDDA) and PSS–G aqueous solution as prepared before for 20 min, respectively. The resulting membrane was washed thoroughly with distilled water and dried in a nitrogen stream. This process was repeated until the desired number (n = 5) of PDDA–MB/PSS–G multilayer was obtained. To assemble the DNA on the multilayer, the PDDA–MB layer had to be the outermost layer to make sure an effective electrostatic interaction. The final multilayer of $(PDDA–MB/PSS–G)_5$(PDDA–MB) was thus obtained. The as-prepared LBL multilayer was covered with 2 μM VBA and kept over night to achieve saturation. Then the modified electrode was covered with 1% BSA for 1 h to reduce the nonspecific adsorption. The final sensing interface was obtained after rinsing with distilled water and being dried by nitrogen.

2.5. Detection of D-VP using electrochemical measurements

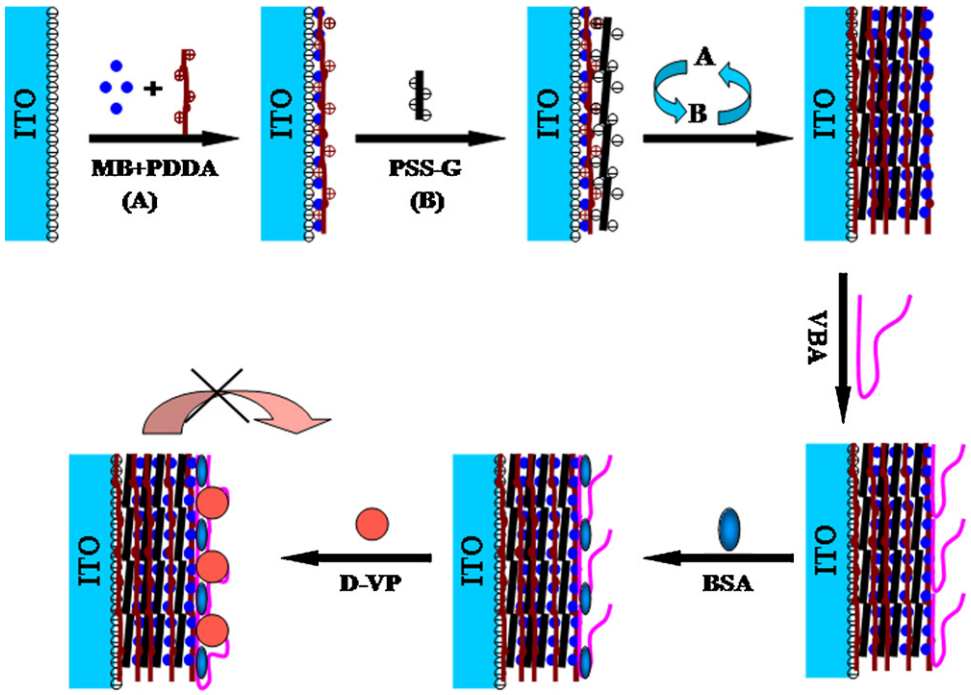
The as-prepared sensing interface was assessed using DPV to collect the electrochemical signal of MB, and then DPV signals corresponding to different concentrations of D-VP were collected after the sensing interface was equilibrated in each PBS buffer solution containing target peptide for 30 min. In order to confirm the selectivity of our electrochemical aptasensor, the DPV signals corresponding to 1 μg mL^{-1} L-VP, 1 mg mL^{-1} BSA, 1 mg mL^{-1} lysozyme, 1 mg mL^{-1} GSH and 600 μg mL^{-1} α-thrombin solutions were recorded under the otherwise same conditions as described above.

3. Results and discussions

3.1. Layer-by-layer assembly of electroactive probe-embedded graphene multilayer and electrochemical characterization

Compared with monomolecular layer, the multilayer with three-dimensional structure assembled from layer-by-layer (LBL) technique [44–47] can not only bring in more electroactive probes to amplify current signal, but also increase the density of molecular recognition agent to improve the sensitivity of the detection. In order to fully realize these potentials of multilayer films based on LBL assembly, graphene as a single layer of carbon atoms network could be the optimal choice due to its the extraordinary electrical property and high surface-to-volume ratio [39].

The detailed assembly process is illustrated in Scheme 1. After the ITO surface with negative surface charge was immersed into solution containing MB and PDDA, MB and PDDA were gradually coadsorbed onto the negatively charged ITO surface, leading to the formation of first layer. Since the graphene coated with PSS



Scheme 1. Schematic diagram of building electrochemical aptasensor for D-VP detection.

polyelectrolyte was negatively charged, electrostatic interaction between PDDA and PSS will induce the deposition of PSS-coated graphene layer as the second layer. Repeating step A and step B will eventually construct the graphene multilayer intercalated with methylene blue. In order to confirm the successful assembly of redox molecule-confined graphene multilayer, the whole assembling process was characterized by cyclic voltammograms. It has to be noted that these experiments were done without saturation

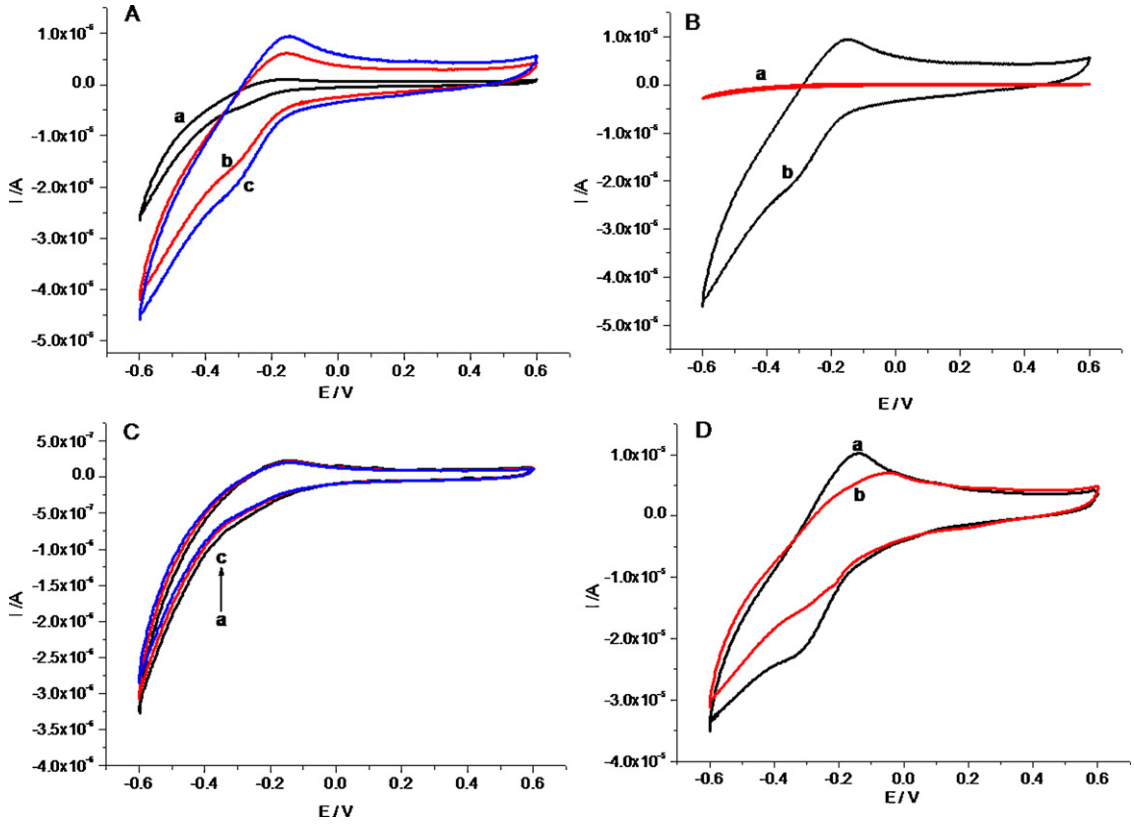


Fig. 1. (A) Cyclic voltammograms (CVs) of the self-assembled layer (PDDA–MB/PSS–G)n on the ITO electrode. The CVs: (a) $n = 2$, (b) $n = 4$ and (c) $n = 5$. (B) The CVs of the self-assembled layer (a) (PDDA–MB/PSS)n and (b) (PDDA–MB/PSS–G)n. (C) The CVs of the self-assembled layer (PDDA–MB/PSS)n on the ITO electrode. The CVs: (a) $n = 2$, (b) $n = 4$ and (c) $n = 5$. (D) The CVs of the self-assembled layer (PDDA–MB/PSS–G)n before (a) and after (b) combination with D-VP (85 ng mL^{-1}).

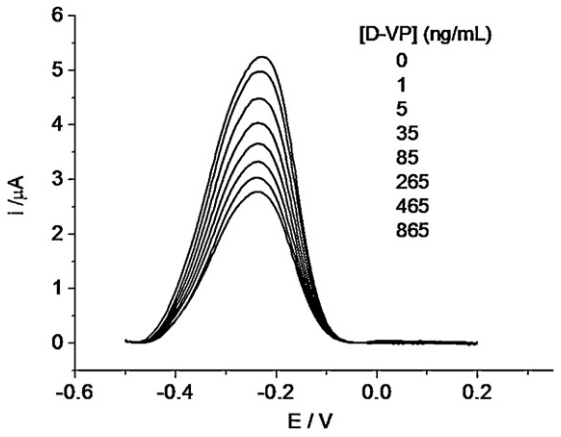


Fig. 2. The DPV responses of the sensing interface to D-vasopressin at different concentrations. The relative responses of the sensor to D-vasopressin at different concentrations (from 1 ng mL^{-1} to 865 ng mL^{-1}). Inset: a linear range from 1 ng mL^{-1} to 265 ng mL^{-1}.

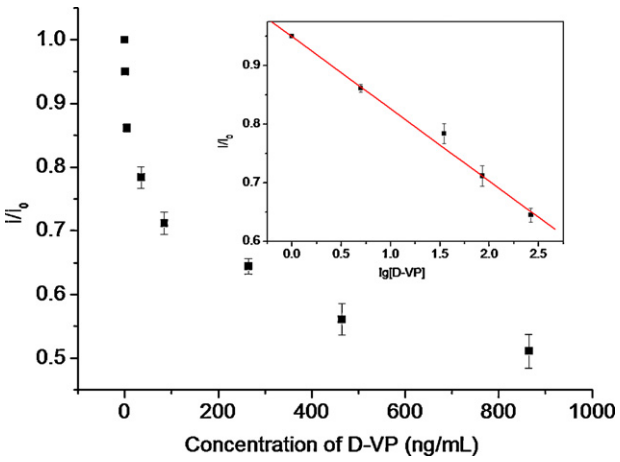


Fig. 3. The relative responses of the sensor to D-vasopressin at different concentrations (from 1 ng mL^{-1} to 865 ng mL^{-1}). Inset: a linear range from 1 ng mL^{-1} to 265 ng mL^{-1}.

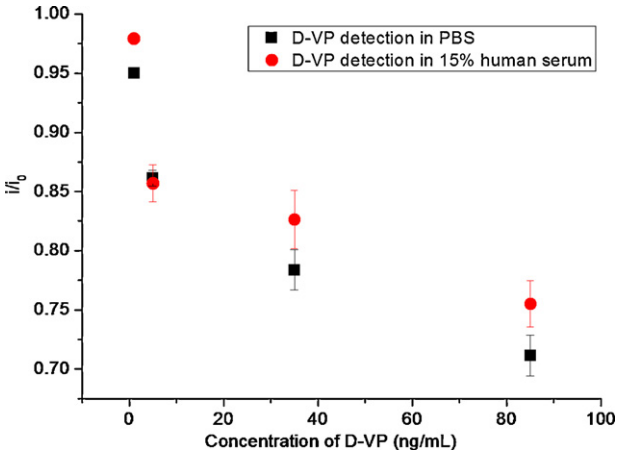


Fig. 4. The relative responses of the sensor to D-VP at different concentrations in PBS (■) and in 15% human serum (●).

of nitrogen gas, which has been claimed to stabilize methylene blue-involved electrochemical system. Notwithstanding this concern, these experiments still can very well justify the layer-by-layer assembly of graphene multilayer in terms of increase in oxidation current peak concomitant with the increase in bilayer number as shown in Fig. 1A. Graphene was expected to provide adsorption sites for methylene blue and facilitate the electron transfer capability of multilayer film, which can be confirmed in Fig. 1B. Without utilization of graphene, the current redox peak was much lower than that for the multilayer film containing graphene. Furthermore, we also conducted electrochemical measurement on a serial of multilayers with different number of bilayer composed of PDDA-MB/PSS. The curves depicted in Fig. 1C show that the redox peak did not change along with the increase of the number of bilayer. On basis of these results, graphene has played significant roles in constructing electroactive multilayer.

In order to construct an aptasensor, the aptamer (VBA) specific for D-VP has to be assembled on the top surface, which can be achieved by the electrostatic interaction between the aptamer and the PDDA–MB coated as the outermost layer of the multilayer sensing interface (PDDA–MB/PSS–G)$_5$(PDDA–MB). In order to remove the unspecific interaction between sensing interface and the positively charged D-VP (isoelectric point = 10.9), the BSA (isoelectric point = 4.7) which was slightly negatively charged was used to occupy the free positively charged site of PDDA–MB to reduce the electrostatic repulsion and other unspecific interaction. The specific interaction between aptamer (VBA) and D-VP has been confirmed, as indicated in Fig. 1D. In the presence of D-VP with concentration of 85 ng mL^{-1}, the redox current peak of methylene blue was distinctly decreased, disclosing that the formed D-VP/VBA complex still remained on the electrode and blocked the electron transfer.

3.2. Detection of D-VP

Aptamer grafted graphene multilayer was incubated with target peptide for 30 min to allow sufficient interaction between D-VP and solid-state probes. As we have described above, the complex formation will suppress the electron transfer between MB and the electrode surface, leading to observed decrease of oxidation current peak of DPV signal as shown in Fig. 2.

In order to establish that this new electrochemical aptasensor can quantitatively assay the target molecule, a series of solutions containing different concentration of D-VP have been prepared. As depicted in Fig. 2, the DPV oxidization signal of MB decreased along with increasing the concentration of D-VP due to the specific interaction between aptamer (VBA) and D-VP. The complex formed from VBA strand and V-DP could interrupt the electron transfer process, which was indicated by the gradual decrease of current peak of DPV signal concomitant with the increasing concentration of D-VP. The lowest detectable concentration of D-VP is 1 ng mL^{-1}, which is compared with the previous work for D-VP detection [22]. The current peak of the sensing interface in the absence of target was defined as I_0, In the presence of certain concentration of target (C_i), the peak current of DPV signal was decreased to I_i. As shown in Fig. 3, we have plotted the current ratio of I_0/I_i as a function of concentration of target peptide. A linear relationship between the current ratio and the concentration of target peptide existed from 1 ng mL^{-1} to 265 ng mL^{-1}. The D-VP concentration over 865 ng mL^{-1} will no longer induce the decrease of DPV signal, indicating the coverage of D-VP on the VBA modified electrode had reached a maximal capacity.

Human serum was used to confirm the practical applicability of our aptasensor. In 15% of human serum, different concentrations of standard solutions containing D-VP were detected by DPV. Also, i/i_0 was also used to assess the D-VP detection ability. In Fig. 4, the DPV responses of the sensing interface to D-VP with different concentrations in the 15% human serum exhibited the same phenomena as those in ordinary PBS buffer but less sensitive. The results definitely illuminated the potential application of our aptasensor in samples.

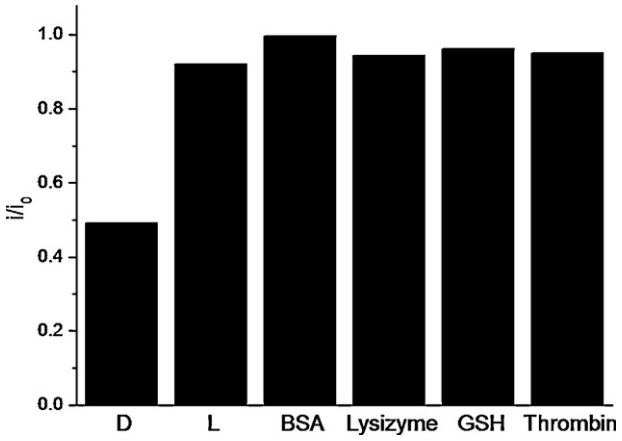


Fig. 5. Control experiments for 465 ng mL^{-1} D-VP, 1 μg mL^{-1} L-VP, 1 mg mL^{-1} BSA, 1 mg mL^{-1} lysozyme, 1 mg mL^{-1} GSH, and 600 μg mL^{-1} α-thrombin.

3.3. Selectivity of the sensing interface

An excellent electrochemical aptasensor has to illustrate good selectivity. One very important indicator is whether our sensing platform meet this requirement is that it has to selectively discriminate L-VP from D-VP. According to the previous literature, L-VP binds D-VP-specific aptamer (VBA) with an affinity at least 1000-fold lower than D-VP, which is the fundamental basis used to detect D-VP. In the same time, other proteins such as BSA, lysozyme, GSH and α-thrombin, were also chosen to test the selectivity of this electrochemical aptasensor. In Fig. 5, there were no remarkable changes of DPV signals for detection of 1 μg mL^{-1} L-VP, 1 mg mL^{-1} BSA, 1 mg mL^{-1} lysozyme, 1 mg mL^{-1} GSH and 600 μg mL^{-1} α-thrombin solutions, respectively. However, the peak current was reduced significantly with 465 ng mL^{-1} of D-VP. It was indicated that other proteins or peptides, especially L-VP, could not interact with the VBA and would not interfere with the detection of D-VP.

4. Conclusions

Herein, a sensitive label-free electrochemical aptasensor with redox MB-confined graphene multilayer as sensing interface for detecting chiral peptide is developed, which provides a detection limit of 1 ng mL^{-1}. The as-prepared aptasensor has several advantages. (1) The utilization of MB as an electron transfer probe avoided DNA tagging process, which made the sensor convenient and economical. Furthermore, these LBL multilayers with three-dimensional structure could bring in more redox probes and more molecular recognition elements which could improve the sensitivity of the biosensor. (2) Graphene nanosheet with extraordinary electric property and large surface-to-volume area, which facilitates accumulation of more redox probe between multilayers, is demonstrated as a new way to be applied in electrochemical aptasensors. The introduction of grapheme nanosheets highly increased the sensitivity. (3) A new method for chiral peptide detection was developed, which could distinguish the D-vasopressin from its enantiomer as well as other proteins such as BSA, lysozyme, GSH and α-thrombin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2011.10.044.

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