Studii şi Cercetări Ştiinţifice
Chimie şi Inginerie Chimică, Biotehnologii, Industrie Alimentară
2016, **17** (2), pp. 169 – 178

Scientific Study & Research
Chemistry & Chemical Engineering, Biotechnology, Food Industry
ISSN 1582-540X

ORIGINAL RESEARCH PAPER

NOVEL FREE LABEL BOTULINUM APTASENSOR BASED ON CAPACITANCE METHOD

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Received: April, 04, 2016
Accepted: June, 27, 2016

Abstract: A feasible and fast method to fabricate Botulinum neurotoxin serotype A (BoNT/A) biosensor was investigated by graphene-carbon paste nanocomposite electrode. In the present work, the graphene was synthesis by chemical method and characterized by SEM. The nanocomposite shows a high conductive and sensitivity for BoNT/A determination as shown by cyclic voltammetry and electrochemical impedance spectroscopy. Graphene-Carbone paste electrode was used for immobilization of aptamer in 0.1 M phosphate buffer solution at *p*H = 7. Aptamer was trapped in graphene modified carbon paste (Cp) electrodes. The electrodes were applied as indicator electrodes for capacitance determination of BoNT/A. Through this method, BoNT/A was detected in a concentration range from 0.16 to 0.68 ng·mL^{-1} with a correlation coefficient of 0.964 and detection limit of 0.09 ng·mL^{-1}. Also, the life time of sensor is in finite.

Keywords: *aptamer, Botulinum, biosensor, electrochemistry, graphene*

INTRODUCTION

In the recent years, graphene has great interest among in the fields of materials science, physics, chemistry and biology. This allotrope of carbon comprises layers of six-atom rings in a hexagonal configuration with atoms bonded by sp^2 bonds [1, 2]. As a basic building block of other carbon allotropes, graphite can be wrapped to generate 0D fullerenes, rolled up to form 1D carbon nanotubes, and stacked to produce 3D graphite [3].

Nowadays, Graphene can be produced relatively easily by mechanical exfoliation of graphite, heating SiC and reduction of graphene oxide [4]. Graphene has unique properties, such as high surface area, high electrical conductivity wide potential windows, fairly inert electrochemistry and good electrocatalytic activity for many redox reactions, low cost and strong mechanical strength. Also, in comparison with CNTs, graphene has many advantages, as follows: i) no metallic impurity, ii) cheap and easy production [5]. These properties have been caused that, the graphene to be ideal candidates for electrochemistry investigation and chemical sensors and biosensors fabrication. Recently, much attention was reported for protein immobilization on graphene [6, 7] and hybrid of graphene [8] was reported.

Recent years have witnessed the establishment of various synthetic routes for graphene, including chemically derived graphene from graphite oxide (graphene oxide, GO), chemical vapour deposition (CVD) of graphene on transition metal films, and epitaxial growth of graphene resulted from the high temperature reduction of silicon carbide. Nanocomposites have attracted great attention because of their unique and novel properties as structural or functional materials. With appropriate designs, nanocomposites can exploit the superlative properties of parent constituents, producing a desired material with improved performance. In this context, intensive efforts have been made to elaborating organic-inorganic nanocomposites for different implementations such as optoelectronics, sensors, biology. Catalysis, Organic components majorly include synthetic polymers and bio-macromolecules, while inorganic components typically include nanoparticles, nanotubes, and layer inorganic materials [9].

Botulinum neurotoxins (BoNT) are the most potent of all toxins, categorized into seven distinct types (A-G), immunologically. Among of the 7 serotype, Botulinum neurotoxin serotype A (BoNT/A) is the most frequent cause of cases humane botulism. BoNT/A is extremely toxic, showing an LD_{50} in humans of 1 $ng \cdot kg^{-1}$, making it the most poisonous naturally occurring substance known [10]. Apart from being a dangerous biohazard agent, BoNT/A has increasing therapeutic and clinical applications, and they are used for cosmetic purposes [11]. BoNT /A is a protein comprised of a light chain (LC) and a heavy chain (HC) that are held together through a single disulfide bond. While the HC is responsible for the transport of the toxin into the cell, it is the LC that is responsible to toxic effects [12]. Because of this and despite its widespread medicinal use, and important in nation define, the rapid or real time detection of BoNT/A becomes extremely important.

In this study, graphene synthesized in our laboratories, was used as a substrate modifier for aptamer immobilization. Graphene is very conductive for electron transferring and used for ratio/volume increasing. By this method, BoNT/A was detected by aptamer/graphene immobilized in label free procedure by LCR meter.

MATERIALS AND METHODS

Reagents

Graphite powder was purchased from Fisher. Sodium nitrate, potassium permanganate, sulfuric acid, Hydrogen peroxide, sodium bore hydride, paraffin oil, dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4) were purchased from Merck. A 0.1 M phosphate buffer solution *p*H - 7.4 was employed as supporting electrolyte. Ultrapure water from a Millipore-Milli Q system was used for preparing all solutions. All the reagents were used as received, without further purification and all experiments were carried out at room temperature (25 ± 2 °C).

Apparatus and measurements

Electrochemical experiments were performed with an Autolab potentiostat (PGSTAT 101). A working glassy carbon electrode with a diameter of 3 mm, a silver/silver chloride (Ag/AgCl) reference electrode, containing 3 M, KCl and a platinum rod auxiliary electrode were used from metrohm. Electrochemical studies were performed using a single-compartment conventional three-electrode cell (volume 300 µL). A carbon paste electrode was use as working electrode. Saturated silver/silver chloride (Ag/AgCl) reference electrode, containing 3 M KCl and a platinum rod auxiliary electrode were used. All potentials were measured and reported versus the Ag/AgCl reference electrode. Cyclic voltammetry (CVs) experiments were performed at 0.1 V/s. The amperometric experiments were carried out by applying the desired potential and allowing the transient current to reach the steady-state value prior to the addition of the analyte and the subsequent current monitoring.
Electrochemical impedance spectroscopy (EIS) measurements were performed using an Autolab potentiostat (PGSTAT 101). All capacitance measurements were performed with an LCR meter (GPS 3131B, Benchtop LCR Meter). Both modified carbon pastes were packed into a polyethylene tube (2.5 mm diameter), the tip of which had been cut off. Electrical contact to the paste was established via inserting a copper wire thorough flank.
The morphology of the synthesized nanocomposite was obtained using a SEM Model LEO 440i, UK. The electrochemical behavior of nanocomposite was carried out in an air-saturated solution for similarity of in vivo usage by Autolab potentiostat (PGSTAT 101).

Graphene synthesis

Graphene oxide (GrO) was synthesized from graphite using the Hummers method [13] and reduce graphene (RGr) was obtained by reduction of GrO with $NaBH_4$. Briefly, graphite, sodium nitrate and potassium permanganate were added to concentrated sulfuric acid. After heating at 35 °C for 30 min, the reaction mixture turned greenish and pasty. Then, the reaction was carefully quenched by the slow addition of water. The paste was kept at 100 °C for 15 min and turned brownish. After further dilution with water it was allowed to cool to 30 °C for 30 min, during which it turned yellow. Hydrogen peroxide was carefully added to form colourless soluble manganese sulphate.

The resulting GrO was isolated while still warm by filtration and the yellow-brown filter cake was washed with warm 5 % diluted hydrochloric acid and finally with water. The resulting stable and brownish GrO aqueous solution was reduced by 1:1 Gr/$NaBH_4$ mass ratio, at room temperature for overnight. The graphene black precipitate was filtrated, washed with water. The different steps of the synthesis were evaluated by FT-IR spectroscopy.

Botulinum neurotoxin type A/ Light chain as an analyte

The gene encoding the enzymatic light chain (LC) domain of BoNT/A with gene bank's Accession No:YP_001253342.1 has coloned in host (*E.coli*). In typical condition, a confirmed clone of recombinant *E.coli* harboring the synthetic gene encoding the LC of BoNT/A was grown at 39 °C to an OD 600 nm = 0.25 in a shake flask containing 10 mL of the LB media contain 20 μm of $ZnCl_2$ and Kanamycin (2×10^5 $g \cdot L^{-1}$) [14], then incubated the shake flask in 18 °C and lets to bacteria to growth continuously for 5 hours until an OD 600 = 0.7. Then we initiated the cells at this time by adding IPTG (final concentration, 0.2 mM). Induction continued for 15hr after adding isopropyl-β-D-thiogalactoseide (IPTG), the cells was harvested by centrifugation (Beckman, Palo Alto, CA) at 7000 rpm for 15 min at 4°C. Cells were washed with cold phosphate buffer saline (1×X) and centrifuged at 7000 rpm for 15 min then frozen at -20 °C. Wet cell yield was 45 $g \cdot L^{-1}$ [15]. Then *E.coli* cells was re-suspended in lysise buffer and were subjected to 5cycles of 1-min sonication (at 75 % power in a Fisher Model 300 Sonic Dismembrator) and 1.5 min cooling on ice. After centrifugation for 15 min at 14,000 × g. Purification of rBoNT/A-His from *E.coli* cell lysate was performed utilizing Ni-NTA agarose resin as recommended by the manufacturer. Shortly the column was equilibrated with equilibration buffer (50 mM NaH_2PO_4, 500 mM NaCl, *p*H = 8.0, and 20 mM Imidazole). After that, the supernatant was added to column and lets to recombinant BoNT/A LC (rBont A/LC) to attached to the resins, then flow was harvested and column was washed tow time by washing buffer I (50 mM NaH_2PO_4, 500 mM NaCl, *p*H 8.0, and 25 mM Imidazole) and wash buffer II (50 mM NaH_2PO_4, 500 mM NaCl, *p*H 8.0, and 40 mM Imidazole), then added one valium of elution buffer I (50 mM NaH_2PO_4, 500 mM NaCl, *p*H 8.0, and 250 mM Imidazole) and 1.5 valium of Elution buffer II (50 mM NaH_2PO_4, 500 mM NaCl, *p*H 8.0, and 350 mM Imidazole). The total product harvested from valiums of Elution buffer. I were purified using the Amicon filter (cot off = 37 KDa). The resulting rBoNT/A LC that appeared by SDS PAGE purified rBoNT/A LC was confirmed by ELISA technic [14] and Western blot (Figure 1). The purified recombinant enzyme was dialyzed overnight against 500 mL of PBS buffer containing 5 mM EDTA and 1 mM DTT. EDTA was removed by further dialysis for 36 hours against three changes of 500 mL of buffer containing 1 mM DTT. Result was showed near to 90 % of total cell protein was rBont A/LC that showed by Total cellular protein (T) in Figure 1.a, also the rBoNT A/LC was soluble in Bacteria (soluble supernatant (S) in Figure 1) against of insoluble pellet (P), and after purification the (I) the r BoNT A/ LC has minimal change as Figure 1.b. Western blot used affinity purified rabbit polyclonal antibodies against a 16-residue N-terminal sequence of the BoNT /A LC as the primary antibody and a peroxidase-coupled goat anti-rabbit IgG (H + L) as the secondary antibody (Figure 1.c). Bands were visualized by a chromogenic substrate (DAB).

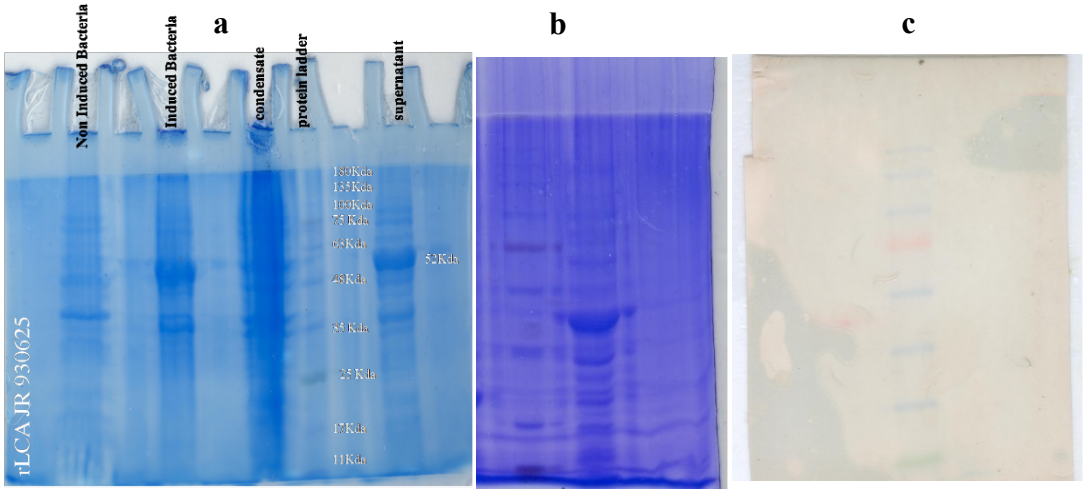


Figure 1. *SDS–PAGE followed by Coomassie stain (**a** and **b**) and Western blot (**c**) of crude and purified BoNT/A LC expressed in E. coli containing the synthetic gene for BoNT/A LC in a multicopy plasmid pET 28a*

Aptamer

One of the tree isolated DNA aptamers against BoNT/A-rLC, that was reported by Lou and his coworkers [16] selected and ordered to a Canadian Co. (Biomatik) forsynthesis. The synthesized DNA aptamer was aliquoted to favorite concentration and stored in 4 °C before use.

Electrode preparation

In the first step, 0.8 mg of graphite was mixed by 0.2 mg of graphene nanocomposite, and it was added to 10 µL of paraffin oil. The mixture of nanocomposite was fixed on syringe electrode and washed with the PBS three times. Finally, the prepared electrode was stored at 4 °C before use.

Sensing procedure

By making the serial concentration of r BoNT /A in optimum buffer condition and applying the electric current, the changes of capacitance of electrodes were measured. Then unknown concentrations of rBoNT /A has been detected according to the standard drown curve.

RESULTS AND DISCUSSION

Graphene characterization by SEM

Characterization of graphene is presented in Figure 2. It shows that, graphene was synthesis successfully.

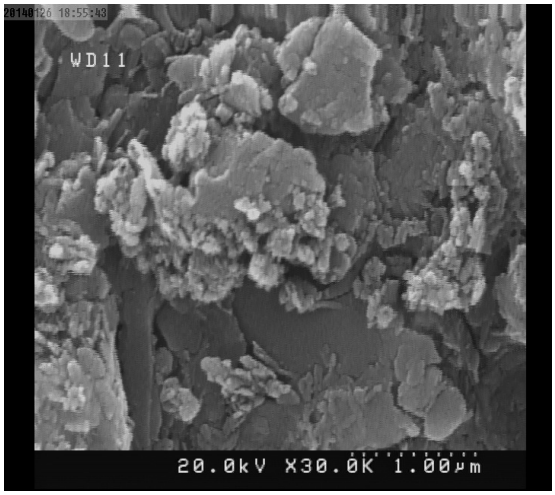


Figure 2. *SEM image of graphene*

FT-IR Spectroscopic characterization of Graphene

Figure 3 displays the spectroscopic FT-IR characterization of GrO and RGr. The IR spectrum of graphene oxide show bands attributed to oxygen containing groups, which confirmed the successful oxidation of graphite. These bands are assigned to (O-H) stretching vibration mode of intercalated water (3400 cm^{-1}); (C-O) stretching (1730 cm^{-1}); (CO epoxy) stretching (1170 cm^{-1}), and (CO alkoxy) stretching vibration (1014 cm^{-1}) [3]. It is obvious that, the intensity of the absorption peaks for reduce graphene were decreased. This figure proves that, the GrO was successfully reduced to RGr.

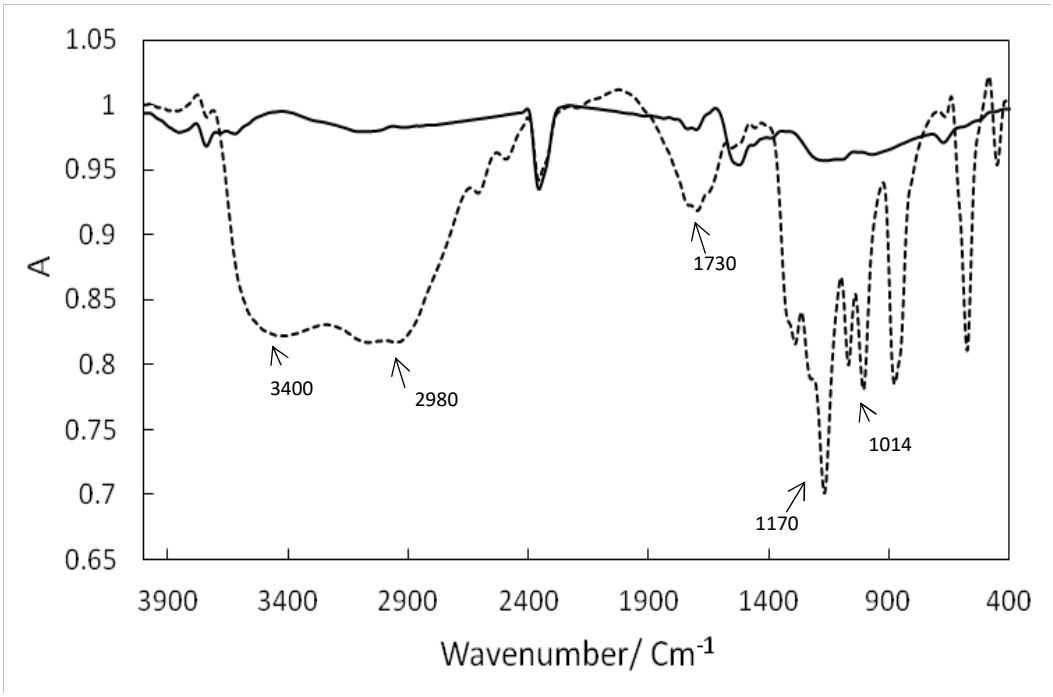


Figure 3. *FTIR spectra of graphene oxide (---) graphene (—)*

The investigation of conductivity by cyclic voltammetry

The electron conductivity of Cp and RGr + Cp nanocomposite electrodes were monitored by cyclic voltammograms (CVs) of $K_3[Fe(CN)_6]$ as redox marker (Figure 3a and 3b respectively). In this experiment, 1.0 mM hexacyanoferrate (II) / (III) (1:1 mixture) was dissolved in 0.1 M PBS as supporting electrolyte. As shown in Figure 3, the hexacyanoferrate (II) / (III) redox couple on Cp and RGr + Cp nanocomposite electrodes was appeared in potential range of - 0.5 to 0.9 V. The value of formal potential [$E^0 = (E_{pc} + E_{pa})/2$] for Cp and RGr + Cp nanocomposite electrodes were 0.2 and 0.15 V, respectively. This is obvious that, when RGr + Cp nanocomposite was fixed on the electrode surface, the intensity of hexacyanoferrate (II) / (III) current peaks was greatly increased. This is due to the high conductivity of RGr + Cp nanocomposite, which could promote electron transfer between the electrode surface and the electroactive species in the solution. This result was confirmed by electrochemical impedance spectroscopy, too.

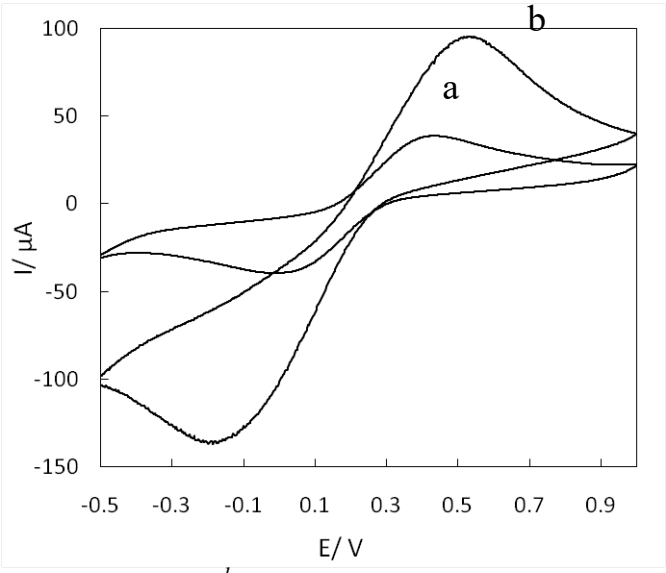
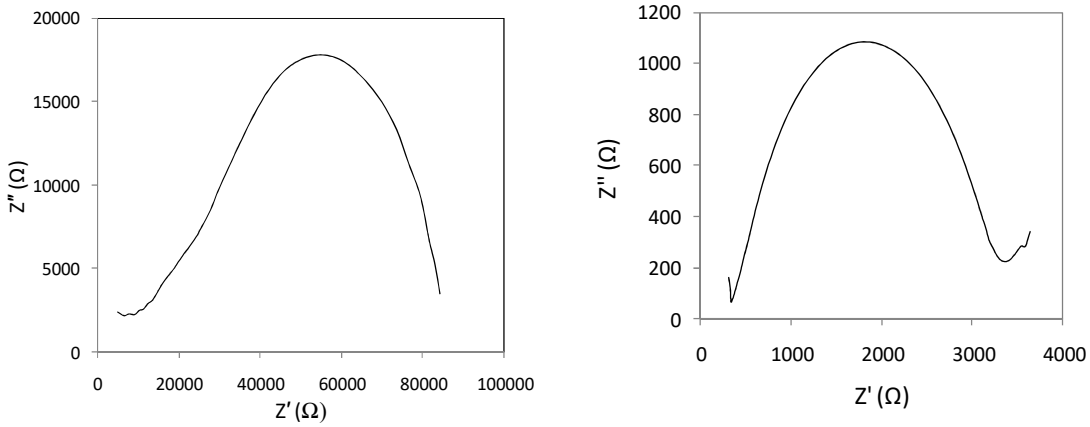


Figure 4. *CVs of 1.0 mmol· mL^{-1} hexacyanoferrate (II) / (III) (1:1 mixture) on Cp electrode* ***(a)*** *and RGr + Cp electrode* ***(b)*** *in 0.1 mol· $mL^{-1}KNO_3$ and 0.1 mol·mL^{-1} PBS solution (pH = 7.4) (the scan rate is 100 mV· s^{-1} at air saturated condition)*

The investigation of conductivity by electrochemical impedance spectroscopy (EIS)

The electrochemical study results are presented as Nyquist plots in Figure 5. According to the Figure 4 it is evident that all the Nyquist plots showed a simple semicircle that revealed a deposited layer on electrode surfaces. The nanocomposite plot showed the minimum radius related to low charge transfer resistance and high conductivity of the electrode surface layer referred to attend of graphene nanocomposition surface and concerned to its conductivity nature. The graphit plot showed the maximum semicircle radius related to maximum charge transfer resistance and minimum conductivity. Therefore, in presence of RGr, the Cp electrode is better capacitance sensor through high conductivity and high surface to area ratio.



a ***b***

Figure 5. *EIS of 1.0 mmol·mL^{-1} hexacyanoferrate (II) / (III) (1:1 mixture) on Cp electrode (a) and RGr + Cp electrode (b) in 0.1 mol·mL^{-1} KNO_3 and 0.1 mol·mL^{-1} PBS solution (pH = 7.4)*

Determination of BoNT/A by aptasensor

For measuring of BoNT/A the capacitance method was used. Figure 6 shows the relationship between the change of capacitance and BoNT/A concentration by graphene carbon paste modified electrode.

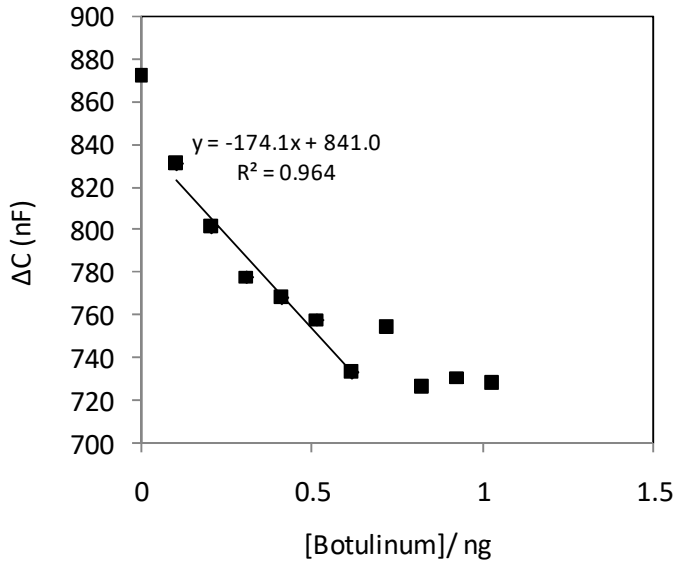


Figure 6. *Calibration curve of BoNT/A determination on aptamer/RGR modified Cp electrodes in 0.1 M PBS solution (pH = 7) by capacitance method*

In aptamer/graphene carbon paste modified electrode, BoNT/A was detected in a concentration range from 0.16 to 0.68 ng·mL^{-1} with a correlation coefficient of 0.964 and a detection limit (DL) at signal to noise ratio of 3 (S/N = 3) was calculated according to the Equation 1:

$$DL: 3.3\ \sigma/S \qquad (1)$$

where: σ is standard deviation of the response,

S is the slope of calibration curve [13]. The detection limit was calculated to be as low as 0.09 ng·mL^{-1} Based on 3 signal to noise.

Selectivity of the aptasensor

The selectivity of the clinical diagnostic methods is an important factor in analyzing biological samples that display a complex matrix. In the current study, the selectivity of the proposed aptasensor was evaluated in the presence of different species such as: high chain of recombinant Botulinum neurotoxin Type A (Bont/AHC), recombinant Botulinum neurotoxin Type E (rBont/E) and recombinant Botulinum neurotoxin Type B (rBont/B). It does not significant response to aptasensor, as showing to selectivity of system to Botulinum neurotoxin detection (Figure 7).

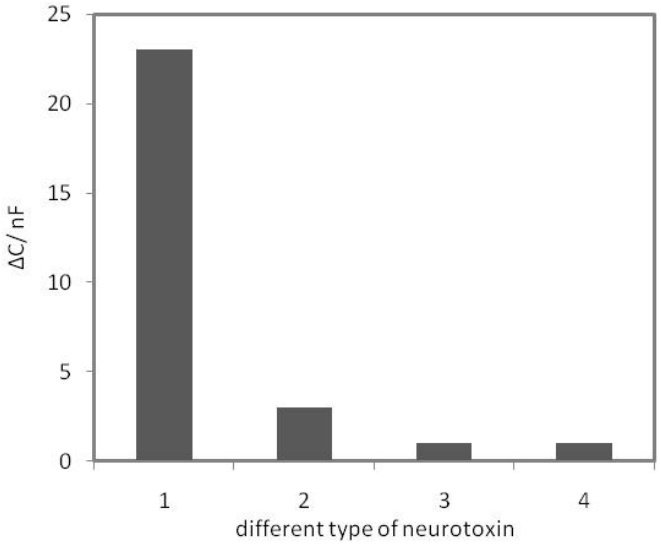


Figure 7. *Comparison of BoNT/A (1) response with rBont/B (2),* Bont/AHC *(3) and* rBont/E *(4) in concentration of 0.1 ng·mL^{-1} on aptamer/RGR modified Cp electrodes in 0.1 M PBS solution (pH = 7) by capacitance method*

CONCLUSIONS

The study and preparation of BoNT/A aptasensor is very important and required for food industry. Therefore, we propose label-free, inexpensive, cheap, rapid and renewable *Botulinum* apta-sensor by immobilizing of aptamer on RGr modified Cp electrode and this electrode was used in capacitance method. RGr was caused to high capacitance signal in apta-sensor trough of high conductivity and high surface area. The BoNT/A apta-sensor has good linear range, low detection limit and excellent selectivity. It seems that this system has the ability to be used as a platform for other pathogens and analyt, too.

ACKNOWLEDGMENTS

Financial supports provided by the Research Council of the Baqiyatallah University of Medical Science are gratefully appreciated.

REFERENCES

1. Jin, E., Lu, X., Cui, L., Chao, D., Wang, C.: Fabrication of graphene/prussian blue composite nanosheets and their electrocatalytic reduction of H_2O_2, *Electrochimica Acta*, **2010, 55** (24), 7230-7234;
2. Chen, L., Hernandez, Y., Feng, X., Müllen, K.: From nanographene and graphene nanoribbons to graphene sheets: chemical synthesis, *Angewandte Chemie International Edition*, **2012**, **51** (31), 7640-7654;
3. Wang, Y., Li Z, Wang J, Li J, Lin Y.: Graphene and graphene oxide: biofunctionalization and applications in biotechnology, *Trends in biotechnology*, **2011**, **29** (5), 205-212;
4. Gasnier, A., Pedano, M.L., Rubianes, M.D., Rivas, G.A.: Graphene paste electrode: electrochemical behavior and analytical applications for the quantification of NADH, *Sensors and Actuators B: Chemical*, **2013**, **176**, 921-926;
5. Ting, S.W., Periasamy, A.P., Chen, S.M., Saraswathi, R.: Direct electrochemistry of catalase immobilized at electrochemically reduced graphene oxide modified electrode for amperometric H_2O_2 biosensor, *International Journal of. Electrochemical. Science*, **2011**, **6**, 4438-4453;
6. Wu, J.F., Xu, M.Q. and Zhao, G.C.: Graphene-based modified electrode for the direct electron transfer of cytochrome c and biosensing, *Electrochemistry Communications*, **2010**, **12** (1), 175-177;
7. Wu, P., Shao, Q., Hu, Y., Jin, J., Yin, Y., Zhang, H., et. al.: Direct electrochemistry of glucose oxidase assembled on graphene and application to glucose detection, *Electrochimica Acta*, **2010**, **55** (28), 8606-8614;
8. Wu, H., Wang, J., Kang, X., Wang, C., Wang, D., Liu, J., et al.: Glucose biosensor based on immobilization of glucose oxidase in platinum nanoparticles/graphene/chitosan nanocomposite film, *Talanta*, **2009**, **80** (1), 403-406:
9. Kang, X., Wang, J., Wu, H., Aksay, IA., Liu, J., Lin, Y.: Glucose oxidase–graphene–chitosan modified electrode for direct electrochemistry and glucose sensing, *Biosensors and Bioelectronics*, **2009**, **25** (4), 901-905;
10. Stanker, L.H., Merrill, P., Scotcher, M.C., Cheng, L.W.: Development and partial characterization of high-affinity monoclonal antibodies for botulinum toxin type A and their use in analysis of milk by sandwich ELISA, *J Immunol Methods*, **2008**, **336** (1), 1-8;
11. Couesnon, A., Raffestin, S., Popoff, M.R., Popoff.: Expression of botulinum neurotoxins A and E, and associated non-toxin genes, during the transition phase and stability at high temperature: analysis by quantitative reverse transcription-PCR, *Microbiology*, **2006**, **152** (Pt 3), 759-70;
12. Moe, S.T., Thompson, A.B., Smith, G.M., Fredenburg, R.A., Stein, R.L., Jacobson, AR.: Botulinum neurotoxin serotype A inhibitors: Small-molecule mercaptoacetamide analogs, *Bioorganic & medicinal chemistry*, **2009**. **17** (8), 3072-3079;
13. Tang, L., Wang, Y., Li, Y., Feng, H., Lu, J., Li, J.: Preparation, structure, and electrochemical properties of reduced graphene sheet films, *Advanced Functional Materials*, **2009**, **19** (17), 2782-2789;
14. Ahmed, S.A., Smith, L.A.: Light chain of botulinum A neurotoxin expressed as an inclusion body from a synthetic gene is catalytically and functionally active, *Journal of protein chemistry*, **2000**, **19** (6), 475-487;
15. Cai, S., Singh, B.R.: Strategies to design inhibitors of Clostridium botulinum neurotoxins. *Infect Disord Drug Targets*, **2007**, **7** (1), 47-57;
16. Lou, X., Qian, J., Xiao, Y., Viel, L., Gerdon, AE., Lagally, ET., et al.: Micromagnetic selection of aptamers in microfluidic channels, *Proceedings of the National Academy of Sciences U S A*, **2009**, **106** (9), 2989-94.