



An amperometric nanobiosensor for the selective detection of K^+ -induced dopamine released from living cells

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ABSTRACT

A highly sensitive amperometric sensor has been studied for selective monitoring of K^+ -induced dopamine released from dopaminergic cells (PC12) which is based on an EDTA immobilized-poly(1,5-diaminonaphthalene) (poly-DAN) layer comprising graphene oxide (GO) and gold nanoparticles (GO/AuNPs). The integration of a negatively charged probe molecule on the poly-DAN/GO/AuNPs nanohybrid attained the signal enhancement to discriminate dopamine (DA) molecules from foreign species by catalytic effect and surface charge, and hydrogen bonding-based interactions with a probe molecule. The sensor performance and morphology were investigated using voltammetry, impedance spectrometry, SEM, and XPS. Experimental variables affecting the analytical performance of the sensor probe were optimized, and linear response was observed in the range of 10 nM–1 μ M with a detection limit of 5.0 nM (± 0.01) for DA. Then, the sensor was applied to monitor dopamine released from PC12 cells upon extracellular stimulation of K^+ ions. It was also confirmed that K^+ -induced dopamine release was inhibited by a calcium channel inhibitor (Nifedipine). The results demonstrated that the presented biosensor could be used as an excellent tool for monitoring the effect of exogenous agents on living cells and drug efficacy tests.

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1. Introduction

Label free monitoring of nerve cell excitation and release of neurochemicals in living state has fundamental significance in the better understanding of various processes implicated in neuronal functions. Among biologically interesting neurochemicals released from neuronal cells, dopamine is a vital signaling biomolecule, which plays significant role in the regulation of various physiological activities of the peripheral and central nervous systems (Hefco et al., 2003). Thus, precise monitoring of dopamine has become a critical research issue due to its low concentration in biological fluids and high clinical research significances. Previous studies have shown that an excessive stimulation of membrane bound receptors or ion channels in the dopaminergic cells trigger an abnormal neurotransmission of dopamine, which can result in the development of several psychiatric and neurodegenerative disorders such as Alzheimer's, Parkinson's, and Schizophrenia (Montague et al., 2004; Tobler et al., 2005). It is believed that exogenous substances such as drug molecules, environmental

contaminants, and toxins largely influence on the activation of dopaminergic cells as well as exocytosis of dopamine. Development of fast and label-free biosensing strategies for the observation of neuronal cell excitation and the determination of neurotransmitters dynamics using exogenous activators and inhibitors are obviously of much importance for neurobiology research.

To date, the dopamine determination from dopaminergic cell populations as well as from striatal brain slices has been carried out using conventional analytical techniques including fluorescence microscopy (Wachman et al., 2004), high-performance liquid chromatography (Cheng et al., 2000), capillary electrophoresis (Zhang et al., 2003) and spectrophotometry (Lapainis et al., 2007); however, most of these existing techniques are expensive, require sophisticated instruments to maintain and run, and the procedures for preparation of biosensors are rather time consuming. Thus, the development of rapid, facile and inexpensive analytical methods for sensitive and selective detection of trace dopamine release from living cells is very essential for routine analysis, precise clinical diagnosis of neuronal disorders and disease prevention. Thus, a PC12 cell line was used for DA release because they possess similar characteristics to that of mature sympathetic neurons (Westerink and Ewing 2008).

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Electrochemical methods have appeared to be suitable and more often employed in the clinical analysis to determine the concentration of DA owing to easy operation, cost effectiveness, and providing enough sensitivity to real time monitoring of the analytes in submicromolar concentrations (Chandra et al., 2013). Nonetheless, electrochemical observation of DA under physiological conditions is a challenging issue because its presence in the biological fluids is extremely low compared to ascorbic acid (AA) and uric acid (UA) which usually coexist with DA. Hence, the elimination of the interference caused by these species is very crucial as their oxidation potentials are almost similar, which results in overlapped voltammetric signal and the electrode often suffer from fouling effects. To overcome the influence of these factors, a variety of surface polymer modification approaches (Won et al., 2005; Abdelwahab et al., 2009; Lee et al., 2010; Chandra et al., 2013) have been exploited to enhance the sensitivity and selectivity of electrochemical techniques for dopamine detection. Despite of great performances of above mentioned techniques, improving the electrocatalytic properties of substrates for highly sensitive and target selective sensing is still considered as a challenge for bioanalytical research and medical diagnosis.

Recently, graphene oxide has attracted significant attention due to its electrical, optical, chemical and mechanical properties and many potential applications in bionanotechnology. The abundant reactive surface oxygen-containing functional groups and aromatic domains on the basal planes and edges of graphene oxide (GO) makes it to be an excellent material for biomolecular interactions (Zheng et al., 2013). In addition, Au nanoparticles (AuNPs) were often used to enhance the sensitivity of electrochemical sensors substrate (Noh, et al., 2012; Zhu et al., 2013). Thus, it is expected that the more enhanced performance can be attained when introducing of GO and AuNPs mixed composites to the conducting polymers. Since the incorporation of AuNPs and GO in the conductive polymer layer not only enhance the electronic interactions with surface polymer matrices but could also provide sufficient conductivity and large surface area, the immobilization of adequate probe molecule on the composite polymer could be readily applied for the detection of trace target species. Hence, immobilization of probe EDTA on the GO/AuNPs and polydiaminonaphthalene (poly-DAN) composite was studied for the dopamine detection through the interaction between EDTA and dopamine by hydrophilic and charge interaction. To the best of our knowledge, there is no previous report on the design of GO/AuNPs/pDAN-EDTA composite film for the determination of dopamine.

In the present work, a simple, sensitive, and selective electrochemical sensor platform for the observation of dopamine released from living cells was constructed. The surface of the modified electrode was characterized by X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy (SEM). The interaction between EDTA and dopamine was also simulated using energy minimized diagram. All the analytical parameters of the sensor probe were optimized and performance was evaluated for the accurate electrochemical detection of low concentration of dopamine (DA) in the presence of high concentration of interfering agents (AA and UA). Finally, the proposed biosensor was applied for the label free monitoring of K^+ -induced DA exocytosis from living PC12 cells, where the influence of different concentration of extracellular K^+ ions on the membrane depolarization via the opening of voltage-dependent Ca^{2+} channels was investigated.

2. Experimental

2.1. Materials and apparatus

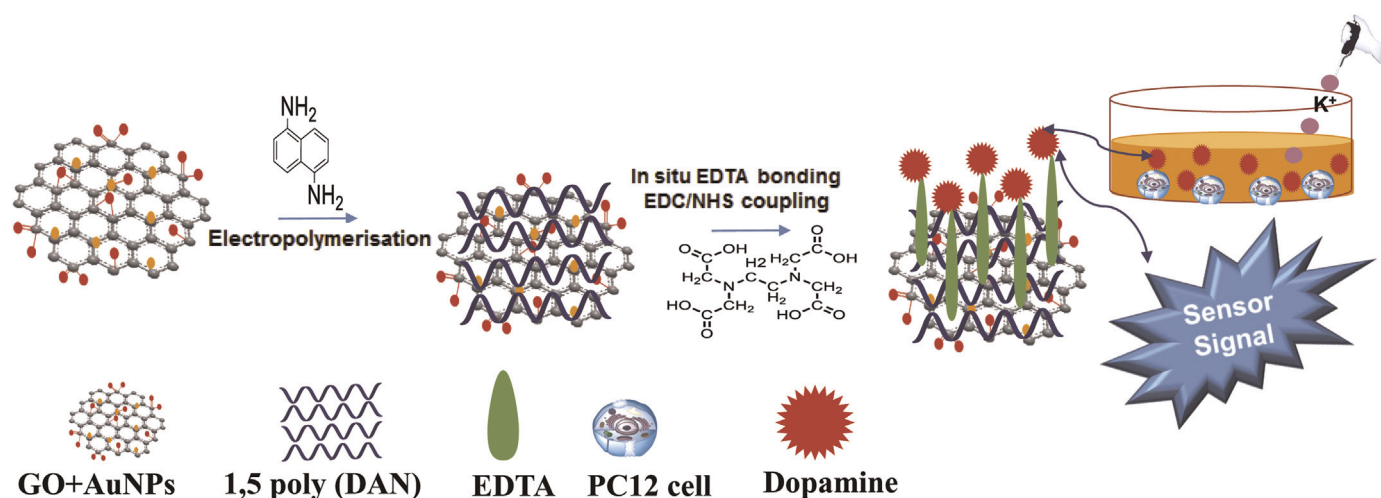
PC12 cells (KCLB 21721) were obtained Korean Cell Bank. Dulbecco's modified Eagle (DMEM) medium, fetal bovine serum (FBS), horse

serum, trypsin EDTA, penicillin/streptomycin, 1,5-diaminonaphthalene (DAN), dopamine (DA), ascorbic acid (AA), uric acid (UA), glucose, Graphite, sulfuric acid (98%), aluminum chloride and Hydrogen peroxide (H_2O_2), Ethylenediaminetetraacetic acid (EDTA), Potassium permanganate Phosphate dibasic, sodium phosphate monobasic, Nifedipine, Sodium nitrate ($NaNO_3$), potassium chloride (KCl), $K_3[Fe(CN)_6]^{4-}$, $K_3[Fe(CN)_6]^{3-}$, $[Ru(NH_3)_6]Cl_3$, and Phosphate buffer saline solutions (PBS) were obtained from Sigma-Aldrich (USA). All aqueous solutions were prepared in ultra-pure water obtained from a Milli-Q water purification system (18 M Ω cm). All electrochemical measurements were carried out at room temperature using conventional three-electrode cell system. A modified glassy carbon with geometric area of 0.07 cm², Ag/AgCl (in saturated KCl), and a platinum (Pt) wire were used as the working, reference, and counter electrodes, respectively. Cyclic voltammograms (CVs) were recorded using a potentiostat/galvanostat Kosentech, model PT-1 and EG & G PAR model PAR 273A. The impedance spectra were measured with the EG&G Princeton Applied Research PARSTAT. Scanning electron microscopy (SEM) images were obtained with a Cambridge Stereoscan 240, and X-ray photoelectron spectroscopy (XPS) experiments were performed using a VG Scientific ESCA Lab 250 XPS spectrometer coupled with a monochromatic Al K α source with charge compensation.

2.2. Preparation of sensor probe materials

Graphene oxide (GO) was synthesized from graphite powder according to modified Hummer's method (Zheng et al., 2013). Typically, graphite powder (5.0 g) and sodium nitrate (2.5 g) were blended in 120.0 ml of concentrated sulfuric acid (95%) and stirred for 30 min in an ice bath ($\leq 0^\circ C$). Then (15.0 g) of potassium permanganate was gently added in the prepared mixture solution, and the reactants were stirred out for a whole night at temperature $< 20^\circ C$. Afterwards, double-distilled water (150.0 ml) was added and the color of the mixture solution was transformed from dark greenish to brownish. The suspension was heated at $98^\circ C$ for one day with stirring to fully oxidize graphite. After cooling, hydrogen peroxide (30%) was added to the mixture, and the mixture was washed out several times with diluted HCl (5%) solution followed by washing with water. Finally, the product was filtered and dried under vacuum. In addition, AuNPs were prepared separately according to the previously reported protocol (Chandra et al., 2011). Briefly, 50 mL of 0.01 wt % HAuCl₄ in double-distilled H₂O was mixed with 1 ml of 38.8 mM trisodium citrate. After 1 min, 0.5 ml of a freshly prepared NaBH₄ solution was slowly added to the mixture. During the addition of NaBH₄, the color of the resulting solution changed from yellow to pink-violet, indicating the formation of AuNPs. Thereafter, Graphene oxide 0.5 mg/ml and AuNPs (1:1) were dispersed by ultrasonic agitation for 1 hr to give a homogeneous suspension.

Prior to modifications, the bare GC electrode was sequentially hand polished with alumina powder (0.3, and 0.05 μm) on a wet soft polishing cloth, and then washed ultrasonically in ethanol followed by ultra-pure water for a few minutes to remove the adsorbed residual alumina particles. The cleaned electrode was dried and a mirror-like surface was obtained. To deposit a nanocomposite film on the electrode surface, a certain amount (5 μl) of the GO/AuNPs was deposited by casting the suspension onto a cleaned GCE surface and dried at room temperature. Further modification was carried out by the electropolymerization reaction in phosphate buffer solution (pH=7.4) containing 1.0 mM DAN. The polymer films were formed by the potential cycles five times between 0.0 and +0.8 V at the scan rate of 100 mV/s. Then, the prepared electrode was rinsed with distilled water to remove loosely adsorbed GO/AuNPs/pDAN. Subsequently, the modified electrode was dipped in the EDC/NHS solution containing EDTA (10 mM) for 12 h for activation of carboxylic acid groups of EDTA



Scheme 1. Schematic representation of the fabrication steps of GO/AuNPs/pDAN-EDTA modified electrode and dopamine detection process.

and it was covalently bonded on poly-DAN. GO/AuNPs/pDAN-EDTA sensor probe was thus constructed, optimized, and finally applied for dopamine detection released from living PC12 cells (Scheme 1).

2.3. Preparation of cell sample and DA monitoring

To analyze DA using the GO/AuNPs/pDAN-EDTA sensor probe, CV measurements were carried out by cycling the potential between 0.0 V and 0.8 V at a scan rate of 50 mV/s. The real sample analyses of DA were carried out at optimized experimental conditions using living mammalian cells as a biological sample. *In vitro* experiments were carried out on a rat pheochromocytoma cell line, because of their wide use as a model for studying neurobiological functions and regulation of neurotransmitter release (Mir et al., 2011). A stock PC12 cell line was cultivated and maintained in Petri dishes using Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 10% heat-inactivated horse serum, and 1% penicillin-streptomycin at 37 °C, placed in a humidified atmosphere of 95% air and 5% carbon dioxide. Cells were passaged every five days and the medium was changed two–three times a week throughout the lifetime of all cultures. Prior to the electrochemical experiments, PC12 cells were removed from the bottom of cell culture flasks by standard trypsinization followed by centrifugation and suspended in 5 ml of culture medium. The cell suspension was then diluted to a desired concentration, and a volume of 1 ml of cell suspension was poured into wells of 24 well plate and allowed to incubate for 24 h. On removal from the incubator, the culture medium was discarded, and the cells were washed thrice with PBS solution (pH 7.4). The dopamine exocytosis was triggered by K^+ stimulation. For inhibitory test, cells were pretreated with 10 μ M nifedipine solution for 30 min prior to K^+ stimulation. Cell sample without treating with K^+ solution and only PBS solution were used for control experiments. All experiments were conducted under ambient laboratory conditions.

3. Results and discussion

3.1. FE-SEM and XPS characterization of the GO/AuNPs/pDAN-EDTA probe

The surface morphologies of each layer of the sensor probe were examined by FE-SEM as shown in Fig. 1. Fig. 1(a–c) displays

the images of (a) GO/AuNPs, (b) GO/AuNPs/pDAN and (c) GO/AuNPs/pDAN-EDTA modified layers. As displayed, the layer of drop casted GO/AuNPs mixture on the GCE shows a smooth GO film incorporated with small AuNPs (approx. 10 nm), whereas after electropolymerization of pDAN on the layer, a relatively rough morphological structure is observed. However, the FE-SEM image obtained for the final probe surface (GO/AuNPs/pDAN-EDTA) exhibits rough clusters due to aggregation of surface molecules, indicating the successful fabrication of the sensor probe.

XPS spectra were studied to elucidate the deposition of key elements on the GC surface for conducting polymer-nanocomposite film formation. Fig. 1(d) shows the XPS survey spectra obtained for (i) GO/AuNPs, (ii) GO/AuNPs/pDAN, and (iii) GO/AuNPs/pDAN-EDTA modified electrode surfaces. In the core-level high-resolution XPS spectra of (i) GO/AuNPs layer, Au, O and C elements were observed. However, after the electropolymerization of 1, 5-diaminonaphthalene onto the GO/AuNPs layer and fixation of EDTA onto poly-DAN layer, the existence of N along with Au, C, and O was also observed. Fig. 1(e) shows the deconvoluted peaks for Au 4f in (i) GO/AuNPs, (ii) GO/AuNPs/pDAN and (iii) GO/AuNPs/pDAN-EDTA layers. As shown, peaks for Au 4f_{7/2} were observed at binding energies of 83.98, 83.62 and 83.75 eV, respectively. While, peaks for Au 4f_{5/2} were observed at higher binding energies such as 87.48, 87.39 and 87.57 eV, indicating the clear existence of Au at each step of sensor fabrication. Fig. 1(f) shows the deconvoluted peak for C1s at each step of sensor probe fabrication. At the first step (i), four peaks were observed at binding energies of 284.59, 286.58, 287.8 and 289.3 eV which corresponds to C–C/C=C, C–O/C–O–C, C=O and HO–C=O (carboxylic group). Similarly, at step (ii) and (iii) C–C/C=C peaks were observed at binding energies of 284.63 and 284.61 eV, and carbonyl peaks were also observed at binding energies of 287.92 and 287.41 eV, respectively. Moreover, additional peaks for C–N bond were also observed at step (ii) and (iii) with binding energies of 285.88 and 285.69 eV. The presence of C–N bond indicates the successful polymerization of DAN and formation of GO/AuNPs/pDAN layer. The deconvoluted peaks for N1s at the steps (ii) and (iii) are shown in Fig. 1(g). As can be seen, at the second step (ii) (GO/AuNPs/pDAN) two peaks were observed at binding energies of 399.12 and 400.72 eV which corresponds to C–N and amide bond formation (Prasad et al., 2013), while at the step (iii) (GO/AuNPs/pDAN-EDTA) due to amide (–NH₂⁺–) bond, additional three peaks were observed at binding energies of 399.49, 400.87 and 402.51 eV, which indicates that the polymerization of DAN and subsequent attachment of EDTA through the amide bond formation.

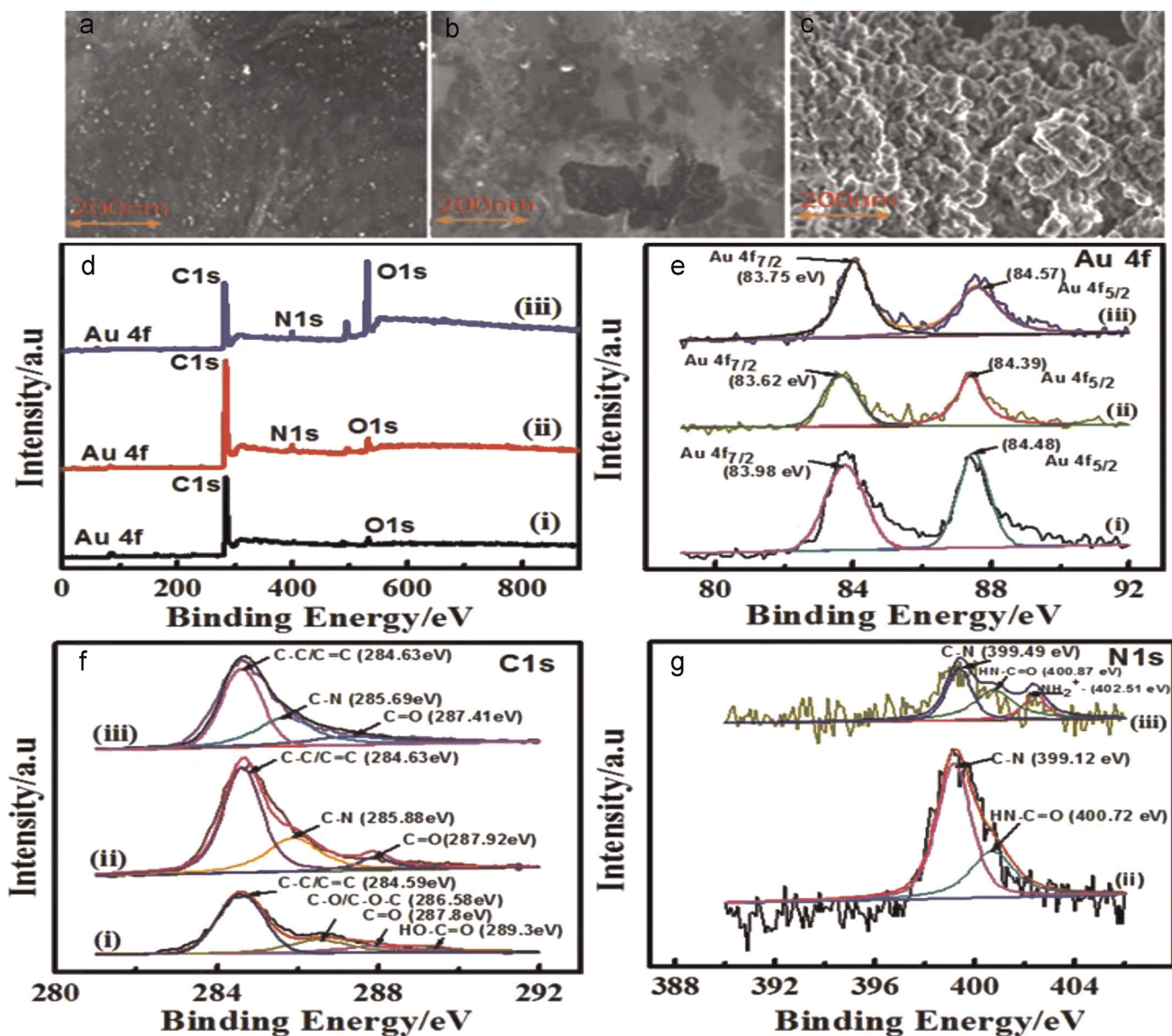


Fig. 1. FE-SEM images obtained at (a) GO/AuNPs, (b) GO/AuNPs/pDAN and (c) GO/AuNPs/pDAN-EDTA surfaces, (d) XPS survey spectra of (i) GO/AuNPs, (ii) GO/AuNPs/pDAN and (iii) GO/AuNPs/pDAN-EDTA, (e) deconvoluted peaks of Au 4f at step (i), (ii) and (iii), (f) deconvoluted peaks of C 1s at step (i), (ii) and (iii), and (g) deconvoluted peaks of N 1s at step (i), (ii) and (iii) respectively.

3.2. Electrochemical behavior of the GO/AuNPs/pDAN-EDTA sensor probe

To elucidate the electrochemical characteristics of the prepared GO/AuNPs/pDAN-EDTA sensor probe, CVs were carried out using negatively charged $[\text{Fe}(\text{CN})_6]^{3-}$ (4 mM) and positively charged $[\text{Ru}(\text{NH}_3)_6]^{3+}$ (4 mM) redox indicators, and compared with the bare GCE. It is well known that EDTA contains four $-\text{COOH}$ groups that offer anionic characteristic at the neutral pH, therefore it was assumed that the electron diffusion of negatively charged $[\text{Fe}(\text{CN})_6]^{3-}$ system will be blocked by modifying the electrode surface with the nanocomposite-conducting polymer film containing negatively charged EDTA groups and thus, the modified electrode should repel anionic species through the electrostatic repulsion phenomenon. Fig. 2(a) shows CVs recorded for bare GC (i) and modified electrodes (ii). As shown, a pair of well-defined redox peak of the $[\text{Fe}(\text{CN})_6]^{4-/3-}$ couple was observed at the bare electrode, while no detectable redox peak current was observed in

CVs recorded for GO/AuNPs/pDAN-EDTA modified surface. The results supported our assumption. In contrary, it was expected that GO/AuNPs/pDAN-EDTA modified electrode could attract cationic species through electrostatic phenomenon. Thus, additional experiments were carried out in the solution containing $[\text{Ru}(\text{NH}_3)_6]^{3+}$ ions to confirm the surface charge as shown in Fig. 2 (b). As depicted, after exposing the modified electrode to the $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$ solution, a well-defined current response was observed obviously (ii), while no noticeable current response was observed at the bare electrode (i), ascribing to the electrostatic attraction between the positively charged $[\text{Ru}(\text{NH}_3)_6]^{3+}$ ions in the measuring solution and the negatively charged EDTA modified GO nanocomposite film.

Additionally, electrochemical impedance spectroscopy was performed at an open circuit voltage to monitor the impedance variation of the electrode interfaces. Fig. 2(c and d) shows EIS spectra in the Nyquist plots corresponding to the bare and modified electrodes in the solutions containing cationic and anionic

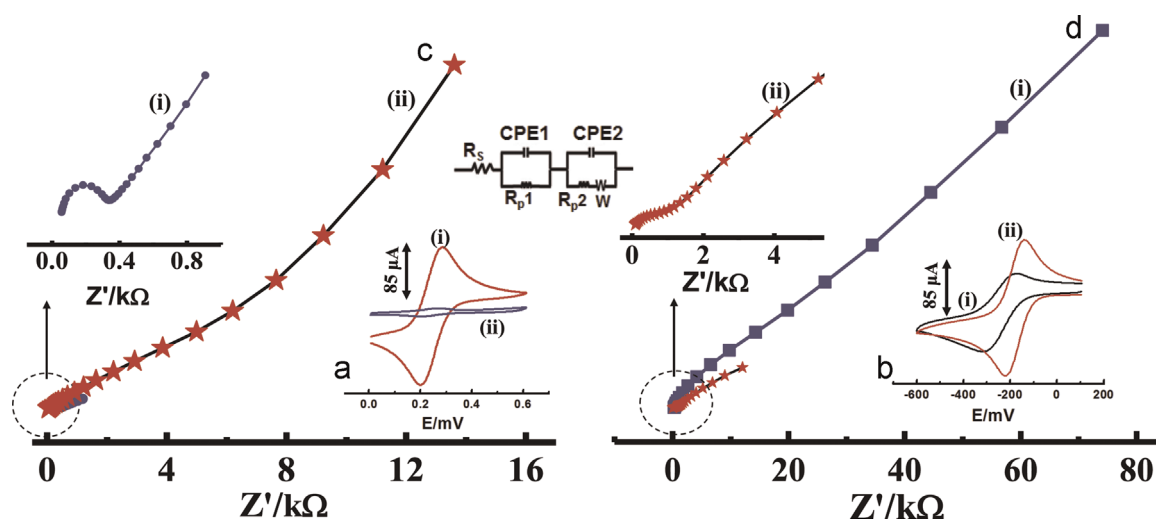


Fig. 2. Cyclic voltammograms obtained in solutions containing (a) 4.0 mM $[\text{Fe}(\text{CN})_6]^{4-}$ and (b) 4.0 mM $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$ using bare (i) and GO/AuNPs/pDAN-EDTA nanocomposite modified (ii) GC electrodes. EIS spectra obtained in solutions containing 4.0 mM $[\text{Fe}(\text{CN})_6]^{4-}$ ions (c), and 4.0 mM $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$ ions (d), using bare (i) and GO/AuNPs/pDAN-EDTA nanocomposite modified (ii) GC electrodes.

redox indicators. Impedance results were evaluated by employing Randle equivalent circuit which included the solution resistance (R_s), the polarization resistances (R_{p1} , R_{p2}), Warburg impedance (Z_W), and CPE constant phase elements. R_{p1} , R_{p2} , CPE1, and CPE2 parameters were acquired by adjusting the experimental values to the equivalent circuit using a Zview2 software. Fig. 2(c) shows the

R_p values obtained for (i) bare (104.4 Ω), and (ii) GO/AuNPs/pDAN-EDTA modified electrodes (9.8 K Ω) after exposing them to the solution containing $[\text{Fe}(\text{CN})_6]^{3-}$ ions. As shown, a remarkable increase in the interfacial charge resistance is estimated at the GO/AuNPs/pDAN-EDTA modified electrode, while very less charge resistance value is estimated for the bare electrode, which clearly

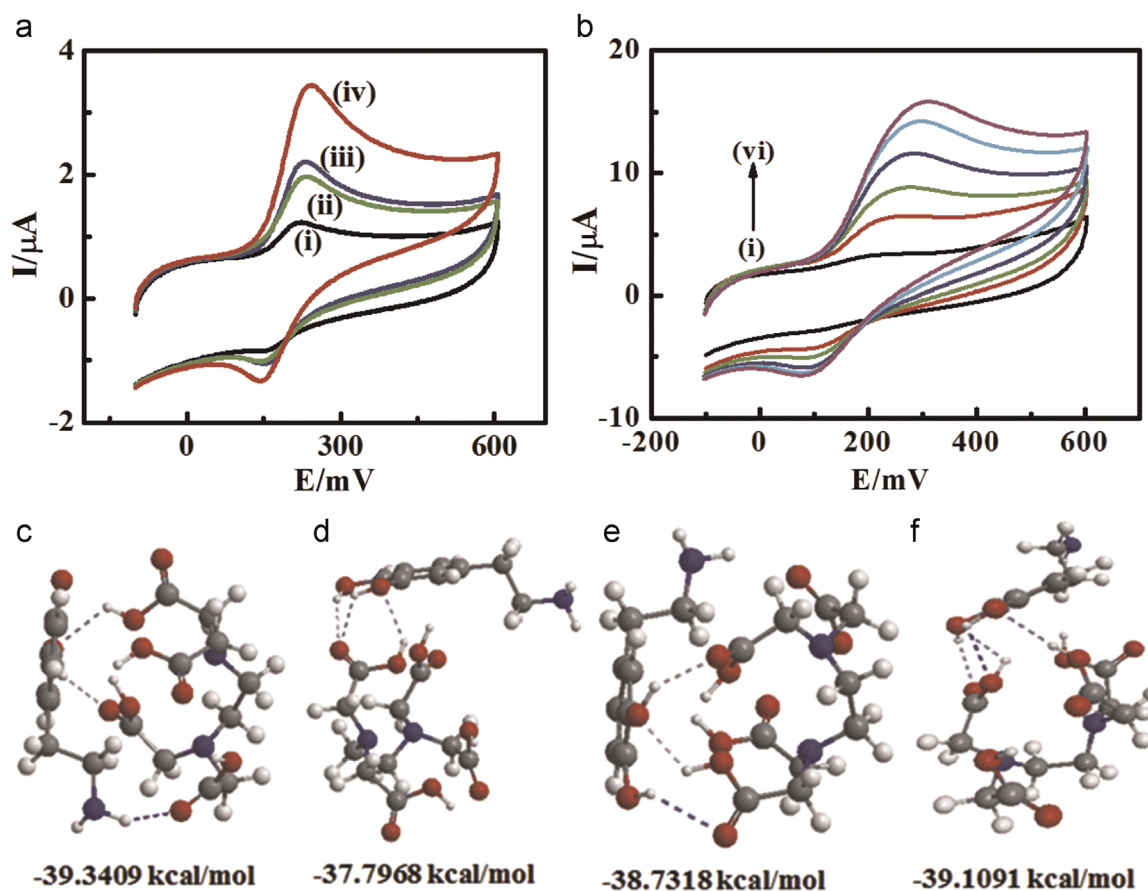


Fig. 3. (a) Cyclic voltammograms obtained in 100 μM DA solution at (i) bare, (ii) GO/AuNPs, (iii) GO/AuNPs/pDAN, and (iv) GO/AuNPs/pDAN-EDTA modified GCEs. (b) Cyclic voltammograms obtained at GO/AuNPs/pDAN-EDTA nanocomposite film modified GCE in 0.1 M PBS (pH 7.4) for various concentrations of DA: (i) 0, (ii) 12.5, (iii) 50, (iv) 100, (v) 250, and (vi) 500 μM . (c–f) Simulated structures of the dopamine-EDTA conjugate.

indicates that negatively charged sensor probe has been formed. The result could be attributed to the aspect that the diffusion of negatively charged $[\text{Fe}(\text{CN})_6]^{3-}$ ion is blocked by the negatively charged sensor surface through electrostatic repulsive forces. Since the conducting polymer nanocomposite containing GO and EDTA groups makes the electrode surface negatively charged and highly conductive, it is therefore expected that an easy electrostatic interaction could be facilitated between the negatively charged sensor probe and positively charged redox ions in the solution. Fig. 2(d) shows the R_p values for (i) bare ($30.8 \text{ k}\Omega$), and (ii) GO/AuNPs/pDAN-EDTA ($2.3 \text{ k}\Omega$) modified electrodes obtained after exposing the sensor to the solution containing $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$. As shown, a remarkable decrease in the charge resistance of GO/AuNPs/pDAN-EDTA modified probe compared to the bare electrode was observed. The impedance results were coincident to that obtained by voltammetric experiments. The variation in CV response and the impedance interfacial properties of the bare and modified electrodes clearly demonstrate that the negatively charged nanosensor probe has been successfully fabricated for dopamine analysis.

To observe the behavior of sensor probe towards the detection of dopamine, CV experiments were carried out for DA ($100 \mu\text{M}$) monitoring using bare, GO/AuNPs, GO/AuNPs/pDAN and GO/AuNPs/pDAN-EDTA modified electrodes. The comparative graph showed that when AuNPs/pDAN-EDTA modified electrode was used (Fig. 3(a)), the response for the dopamine oxidation was remarkably enhanced due to the electrostatic attraction between negatively charged sensor surfaces and positively charged DA molecules, indicating that the conductive polymer nanocomposite probe is very effective for enhancing the electrochemical response toward DA (Fig. 3(b)).

Finally, it was assumed that the complex formation for the EDTA-DA interaction could be expected through the calculation of the minimized energy of formed complex using Chem3D pro 12.0. Fig. 3(c–f) shows simulated structures of dopamine (DA) interacted on the EDTA molecule. The structure of the dopamine-EDTA complex was obtained by the calculation in four different possible ways occurred between DA and EDTA molecules. Fig. 3(c) shows that one of two hydroxyl groups of DA interacts with two carboxylic acid groups of EDTA and an amine group of DA interacts with a carboxylic acid group through three hydrogen bonding with a minimized energy value of $-39.3409 \text{ kcal/mol}$. In Fig. 3(d), it is found that a carboxylic acid group of EDTA interacts with two hydroxyl groups of DA and the calculated minimized energy value was $-39.3409 \text{ kcal/mol}$. Fig. 3(e) shows two hydroxy groups of DA interact with two carboxylic acid groups of EDTA through four hydrogen bonding with a minimized energy value of $-38.7318 \text{ kcal/mol}$. Whereas, Fig. 3(f) shows that two hydroxyl groups of DA interact with two carboxylic acid groups through three hydrogen bonding and the obtained minimized energy value is $-39.1091 \text{ kcal/mol}$. The simulated minimized-energy values shown in Fig. 3(c–f) confirm that DA and EDTA molecules interact with each other through the hydrogen bond formation in four possible ways. The simulated data showed that four different possible interactions could occur during DA and EDTA complex formation. Among them, the complex formed at minimized energy value of $-39.3409 \text{ kcal/mol}$ (Fig. 3(c)) is more favorable compared to the others as shown in (Fig. 3(c–f)), which may be due to less steric hindrance. The minimized energy values for DA and EDTA interaction indicate that EDTA augmented with conducting polymers or other conductive biocompatible materials could be applied as a potential substrate interface for robust bisensing applications.

3.3. Optimization of analytical parameters

The experimental conditions strongly affect the sensitivity and performance of the sensor. In order to achieve maximum sensitivity, analytical parameters for the detection of DA with the proposed biosensor were investigated and optimized in terms of EDTA concentration, pH, temperature, and applied potential. First, the effect of EDTA concentration on the dopamine detection was investigated over the range of 2.0 to 12 mM (Fig. S1a). The response enhanced gradually as the concentration of EDTA increased from 2.0 to 12 mM, and it reached the constant state over 10 mM EDTA. Amounts of EDTA greater than 10 mM did not steeply increase in the signal response. Hence, 10 mM was selected as the optimum concentration of EDTA and used for subsequent experiments. The effect of media pH on dopamine was investigated between pH 3.0 and 9.0 (Fig. S1b). The response peak current of the sensor gradually increased with increased pH values from 5.0 to 7.4, but it decreased with further increase in pH values. The maximum response was obtained at pH 7.4; thus, the optimum pH value was determined to be 7.4 at subsequent experiments. The effect of temperature dependency on the response of DA was then studied over the range from 10 to 55°C and optimized (Fig. S1c). For practical application, all experiments were performed at 37°C . The influence of applied potential on the response of DA was investigated at the potential range from 0.2 to 0.34 V (Fig. S1d). The maximum response was obtained at 0.3 V. Therefore, the potential of 0.3 V was applied for the final measurements.

3.4. Calibration plot and interfering study

Chronoamperometry was employed for the dopamine analysis due to rather higher sensitivity than voltammetry. Fig. 4(a) depicts a typical current-time response curve for successive injections of different concentrations of DA in PBS at steady intervals of 50 s. The applied potential of the electrode was adjusted at 300 mV. As shown, a well defined amperometric response was observed upon each addition of DA solution, and the calibration plot was linearly proportional to the dopamine amount over the range of 10 nM to $1 \mu\text{M}$. The linear regression equation was expressed as: $I_p (\mu\text{A}) = 0.0103 (\pm 0.001) + 0.0016 (\pm 0.001)$, with a correlation coefficient of 0.9993. The relative standard deviation (RSD) was 2.5%, and the detection limit was found to be $5 \text{ nM} (\pm 0.01)$, which was lower than the other DA sensors. The analytical performance of the proposed sensor for the detection of dopamine and some other sensors are summarized in Table 1.

DA, UA and AA usually make their oxidation potentials overlap mostly, because they are structurally similar. In addition, they usually coexist in the extracellular fluid of the central nervous system/body fluids. Thus, specificity and selectivity is one of the most critical issues for dopamine biosensor to be used in biological samples or cellular investigation. The initial results in this work have shown that the negatively charged ions can be eliminated by the negatively charged surface of GO/AuNPs/pDAN-EDTA nanocomposite film, whereas the electrochemical behavior of positively charged ions (DA) can be significantly promoted. Based on this feature, the GO/AuNPs/pDAN-EDTA nanocomposite film was applied to observe the oxidation process of DA in the presence of negatively charged foreign species commonly existing in complex biological samples. As shown in Fig. 4(b), the sensor exhibited a clear amperometric response towards DA ($100 \mu\text{M}$), while no noticeable signals were observed for AA (1 mM) and UA (1 mM) due to the electrostatic repulsion between the negatively charged sensor probe and the anions of AA and UA. The results indicated that electrochemically active positively charged molecules can interact significantly with the surface of GO/AuNPs/pDAN-EDTA sensor. Thus, the sensor is highly selective and can be effectively

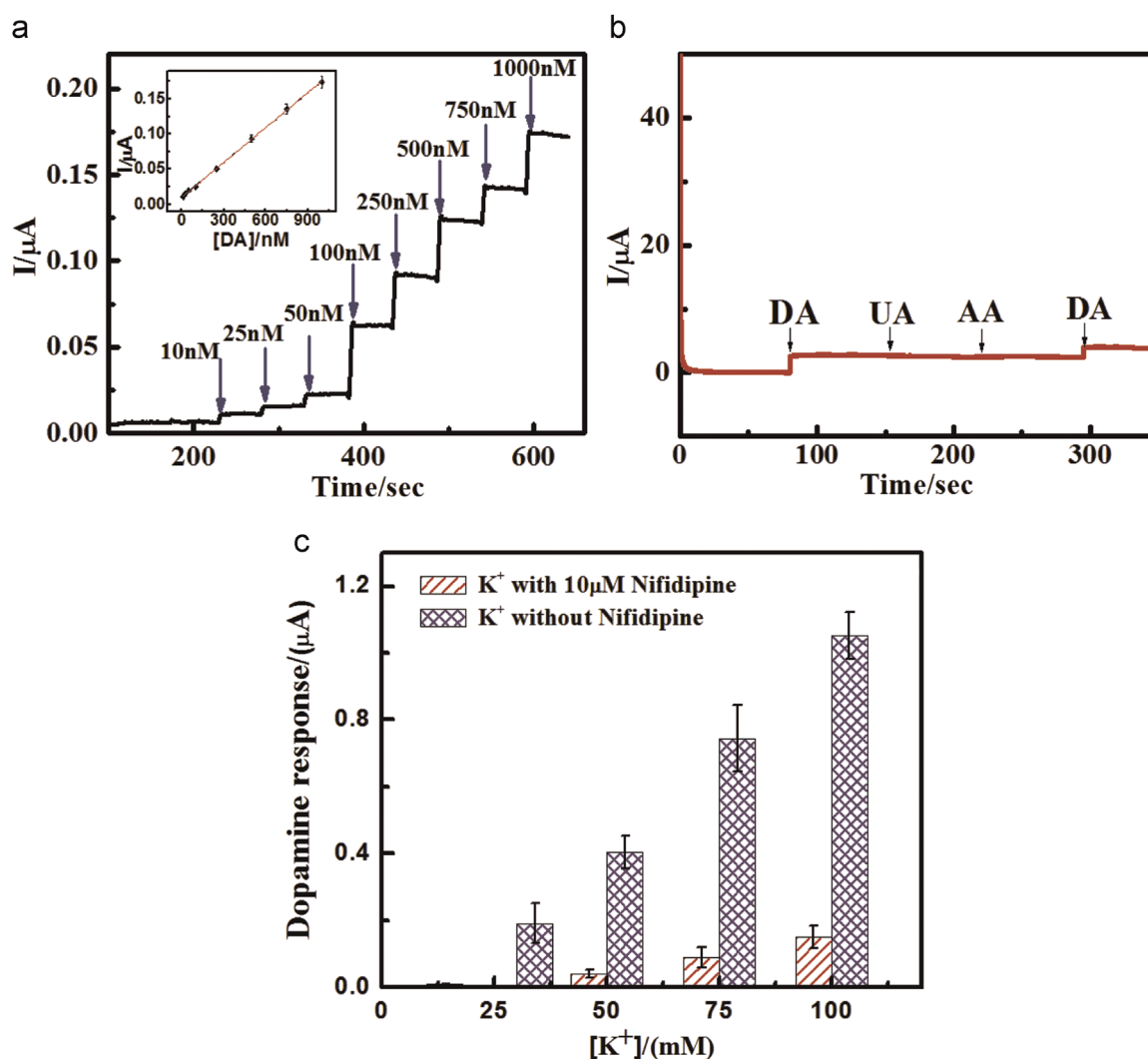


Fig. 4. (a) Amperometric response obtained at GO/AuNPs/pDAN-EDTA nanocomposite film modified GCE upon successive additions of DA into stirring PBS. Inset is the linear calibration plot of response current versus DA. (b) Chronoamperometric response for dopamine in the presence of potentially interfering bio-molecules (1 mM AA and UA). (c) The column indicate the chronoamperometric response for dopamine released from PC12 cells upon stimulating with different concentrations of K^+ (0–100 mM) in the absence (▨) or in the presence (▧) of nifedipine (10 μ M).

applied for the label free determination of dopamine release from dopaminergic cells or tissues.

3.5. Reproducibility and stability studies

To evaluate the reproducibility of the proposed sensor, 6 parallel measurements were carried out in 0.1 M PBS (pH 7.4) containing 100 μ M of DA. The relative standard deviation (R.S.D.) of 2.5% was observed which clearly indicated that the proposed sensor exhibited good reproducibility. The electrode-to-electrode

variation in the current signal of 100 μ M DA was recorded for five different GC electrodes with same area modified with GO/AuNPs/pDAN-EDTA. The variation in current was 3.7%, indicating good sensor-to-sensor reproducibility. The long term storage stability of the sensor was also monitored by recording the CVs of 100 μ M DA at regular intervals (two weeks) for a period of two months. When the sensor was stored at 4 $^{\circ}$ C for a period of two months, the sensor retained more than 90% of its initial response. The accuracy of the sensor was also verified through the standard addition method (S-Table 1). The excellent stability of the sensor may be

Table 1

Comparison of GO/AuNPs/pDAN-EDTA dopamine sensor with other reported electrochemical sensors.

Electrode material	Method	Dynamic range (μ M)	Detection limit (μ M)	Ref.
Nafion/graphene/Fc-NH ₂	DPV	0.5–200	0.02	Liu et al. (2012)
rGO/TiO ₂	DPV	2–60	6	How et al. (2014)
MWCNT/GONR	Amperometry	0.5–50	0.77	Sun et al. (2011)
TCPP/CCG	DPV	0.01–70	0.01	Wu et al. (2012)
MBIP	DPV	0.02–7	6	Rezaei et al. (2015)
LaFeO ₃	Amperometry	0.02–1.6	59	Thirumalairajan et al. (2014)
EDTA/AuGO	Amperometry	0.01–1	0.005	This work

ascribed to the stable conductive polymer nanocomposite formation and EDTA layer. The results demonstrated that the proposed sensor possess an acceptable stability, accuracy and reusability.

3.6. Determination of K^+ -induced DA released from living PC12 Cells

The potential applicability of the proposed sensor in real sample analysis was investigated by direct analysis of DA released from PC12 cells in living state. Briefly, PC12 cells were stimulated with different concentrations of K^+ solution to depolarize the cells (Fig. S2). Fig. 4(c) shows a typical dopamine release experimental results which were recorded by chronoamperometry versus stimulation of different concentrations of K^+ solution (0–100 mM) (Fig. 4c). It has been reported that stimulation of PC12 cells by elevated concentration of extracellular K^+ leads to depolarization of cell membrane which induces an influx of Ca^{2+} and Na^{2+} through opening of voltage-sensitive Na^{2+} channels and subsequent opening of voltage-sensitive Ca^{2+} channels (Shinohara et al., 2013). Elevation in intracellular Ca^{2+} level evokes the release of dopamine from large dense-core vesicles of the cells. The opening of voltage-sensitive Ca^{2+} channels allows an increase in the intracellular Ca^{2+} to a threshold sufficient to trigger the exocytosis. K^+ -induced cell membrane depolarization cause the fusion of dopamine containing vesicles with cell membrane and release the dopamine to the extracellular region. To determine whether the observed signal was not due to a response of the proposed sensor to K^+ stimulation, two different solutions were prepared to perform control experiments. One was only the PBS solution and did not contain the PC12 cells, and the other was a control PC12 cells solution sample that was not stimulated with the K^+ solution, no detectable change of response was obtained in the control experiments.

It is well known that PC 12 cell line possess voltage-dependent calcium channels that bind to calcium inhibitors and suppress depolarization-mediated calcium uptake (Morad et al., 1988). Thus, in order to validate whether the DA current response enhancement observed by our biosensor with the stimulation of K^+ solutions indeed involved exocytosis of dopamine, we observed the effect of treating PC12 cells with a calcium channel antagonist (nifedipine). To assess the ability of nifedipine to interfere with the response elicited by K^+ stimulation, first we treated cells with nifedipine (10 μ M) for 30 min, and then cells were stimulated with different concentrations of K^+ solutions (Fig. S3). The comparative graph for the dependence of current response induced by stimulation with various concentrations of K^+ in the presence or absence of nifedipine can be seen in Fig. 5(c) (Fig. 5c). The normalized current response for dopamine released from nifedipine-treated PC 12 cells induced by K^+ stimulation decayed and response decreased toward the basal level. The results indicate that the proposed biosensor is feasible for rapid and label free determination of various biological exocytotic events and monitoring the harmful effect of drugs or toxins on human nervous system.

4. Conclusions

In this work, a simple, sensitive and selective amperometric nanobiosensor was successfully developed for the determination of K^+ -induced dopamine released from dopaminergic cells (PC12) using a novel conducting polymer nanocomposite film. The GO/AuNPs/pDAN-EDTA nanocomposite sensor probe was characterized by SEM, XPS, and electrochemical methods. The interaction

between the DA and EDTA molecules was confirmed by estimating the minimized energy for possible interactions and complex formation using Chem3D pro 12.0. A calibration plot for dopamine oxidation revealed the linear dynamic range between 10 nM and 1 μ M with the detection limit of 5 nM (± 0.01), under the optimized experimental conditions. The overall results for monitoring the effect of nerve cell activator (K^+) or inhibitor (nifedipine) on dopamine released from PC12 cells indicated that the proposed biosensor is an excellent tool for label free detection of dopamine exocytosis and it can be applied for the observation of dopamine related intracellular signal transduction events, cell-based assessment of neuroprotective drug screenings and clinical diagnostics.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2015.01.024>.

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