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RESEARCH ARTICLE

Facile electrochemical preparation of overoxidizedpolypyrrole/RGO composite for ds-DNA immobilization: a novel signal amplified sensing platform for electrochemical determination of chlorpheniramine

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Abstract

Background Chlorpheniramine (CPA), thanks to its relatively lower side effects, is a widely prescribed medicine for alleviating allergic symptoms as well as some medical emergencies. Owning to this extensive use, many efforts have been directed to measure chlorpheniramine both in vivo and in vitro. High performance liquid chromatography (HPLC), both normal and reverse phase, as well as spectrochemical and electrochemical methods are analytical approaches which have been extensively exploited for determination of CPA. Among them, electrochemical techniques have found elegant place for analysis of CPA due to simplicity, sensitivity and ease of instrumentation.

Methods Herein, we have reported the preparation and characterization of a biosensor by immobilization of double-stranded DNA on the surface of overoxidizedpolypyrrole-reduced graphene oxide modified pencil graphite electrode (ds-DNA-PPyox/RGO/PGE) as well as its novel usability in measurement of chlorpheniramine (CPA). Scanning electron microscopy (SEM), electrochemical impedance spectroscopy (EIS), UV-Vis spectroscopy and differential pulse voltammetry (DPV) were exploited in order to characterize and evaluate the performance of the proposed biosensor.

Results Final results showed that proposed strategy for modification of PGE introduces an ultra-sensitive biosensor for CPA which offers the best detection limitamong all previously reported electrochemical sensors for CPA. Taking advantage of this biosensor for determination of CPA, a wide linear dynamic range from 0.05 to 200 μM, and a low limit of detection 0.023 μM were obtained by using DPV method. Usability of this biosensor was also confirmed by determination of CPA in tablet and spiked urine samples.

Conclusions Overoxidized polypyrrole-reduced graphene oxide offered a suitable substrate for immobilization of ds-DNA by which a new biosensor for determination of CPA was fabricated. Proposed biosensor can successfully be used for determination of CPA in urine samples taking advantage of electroanalytical methods.

Keywords RGO composite · Chlorpheniramine · DNA · Voltammetry

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Introduction

Chlorpheniramine (CPA), Fig. 1, with IUPAC name of 3-(4-Chlorophenyl)-N,N-dimethyl-3-pyridin-2-yl-propan-1-amine is a histamine H1 antagonist medicine which is widely administrated for treating allergic symptoms and asthma due to its relatively less side effects compared to other antihistamine drugs. It is also widely prescribed for anaphylaxis, which is a medical emergency caused by a severe allergic reaction [1, 2]. Due to this extensive application of CPA, researchers have been trying to develop efficient methods for determination of CPA. Sankalp et al. [3] introduced high performance liquid chromatography (HPLC) method in which phosphate buffer/methanol was used as mobile phase and UV detection of CPA was done at 230 nm. Limit of detection was calculated to be 11.3 ng mL^{-1}. To enhance the performance of HPLC, Daryakenary and Zeeb [4] used graphene oxide/Fe_3O_4/polythionine composite as sorbent in solid phase extraction prior to using HPLC with UV detector which resulted in detection limit of 0.4 ng mL^{-1}. Baviskar et al. [5] claimed that they have successfully used reversed-phase HPLC for determination of chlorpheniramine maleate where methanol and 0.1% triethylamine (55:45 *V*/V) with pH = 3 were used as mobile phase. Apart from chromatography, chemiluminescence [6, 7] and capillary electrophoresis [8] have also been used for determination of CPA. Though above-mentioned methods are popular among analytical chemists, they usually suffer from long pretreatment as well as analysis time for samples, using organic solvents that are usually expensive and harmful for environment and high cost instrumentation. Among the analytical techniques, electrochemical strategies have attracted a great deal of attention because of comparatively easier instrumentation, lower cost, being eco-friendly and having higher sensitivity and speed for analysis of samples. Regarding above-mentioned advantages, researchers have reported few electrochemical methods for evaluation of CPA [9–11].

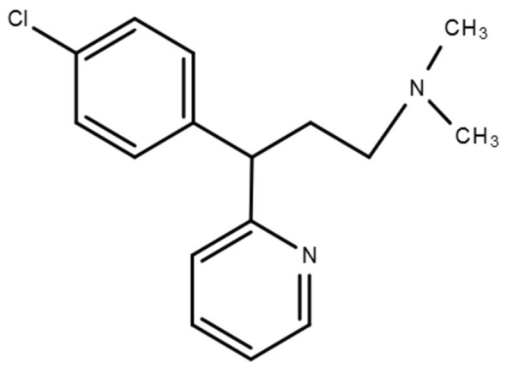

Fig. 1 Structure of chlorpheniramine

Double-stranded DNA (ds-DNA) film of nanometer thickness, which is immobilized on the surface of electrodes, can offer an active part for DNA biosensors. However, one of the most important stages in fabrication of DNA biosensors that should be carefully considered is using an appropriate strategy for immobilization of DNA, single or double stranded, as sensing element of sensor. Regarding this, either choosing a suitable immobilization surface or choosing a more effective strategy for immobilization of higher amount of well-aligned biorecognition element can bring about great impact on performance of DNA biosensor. The higher amount of well-organized biorecognition elements, e.g. ds-DNA, on the surface of electrode will result in higher sensitivity due to signal amplification. Conducting polymers such as polyaniline [12], polythiophene [13] and polypyrrole [14] have entered the field of electrochemical biosensors in order to enhance the performance. Among them, polypyrrole (PPy) is commonly exploited as immobilization substrate in which ds-DNA can be incorporated either into or onto the polypyrrole network. Acceptable conductivity, biocompatibility and stability as well as ease of preparation with both electrochemical and chemical methods have made polypyrrole an attractive option for researcher in this field. After all, since electropolymerization is simpler, more convenient, cost-effective and can provide better control over final structure compared to chemical methods, it is widely used to prepare polypyrrole. Overoxidation of as prepared PPy will result in formation of a film with conductivity of about 10–3 to 103 S cm^{-1} which provides an appropriate substrate for immobilization of negatively charged ds-DNA. Actually, the delocalized electronic structure of overoxidized PPy (PPyox) allows polyanionic ds-DNA to attach to mobile positively charged defect in PPyox network and, consequently, formation of a stable complex between ds-DNA and PPyox [15]. In order to take advantage of combination of PPy and carbon derivatives, reduced graphene oxide (RGO) was chosen owning to its high conductivity and biocompatibility. Prepared composite, PPyox/RGO, showed excellent capability to provide an appropriate substrate for ds-DNA immobilization and, subsequently, greatly enhanced the performance of biosensor towards determination of CPA [16].

Here, we aimed to develop a biosensor for evaluation of CPA using overoxidized polypyrrole/RGO composite as suitable matrix for immobilization of ds-DNA. Cyclic voltammetry was used for preparation of overoxidized polypyrrole/RGO composite onto the surface of inexpensive pencil graphite electrode (PGE). Due to ability of this proposed matrix for ds-DNA immobilization, proposed biosensor showed very good capability for determination of CPA by differential plus voltammetry (DPV) in both standard solutions and real sample (CPA tablet and urine samples). Proposed biosensor found to be time and cost-effective, simple and sensitive, for evaluation of chlorpheniramine.

Experimental

Materials

Double-stranded salmon sperm DNA was purchased from Fluka and suspended in 0.1 M acetate buffer solution pH = 4.8. Pyrrole monomer (Aldrich) was distilled prior to use in order to remove impurities. RGO was prepared using modified Hummers method according to our previous reported paper [17]. Pencil graphite (0.5 mm, Pentel, Japan) electrodes (PGEs) were bought from a local bookstore and were kept in nitric acid 0.1 M prior to use. Chlorpheniramine maleate, acetic acid (CH_3COOH), sodium hydroxide (NaOH), potassium sulfate (K_2SO_4) and other chemicals were bought from Sigma-Aldrich and used without any further treatment.

Instrumentation

Electrochemical analyses were carried out using a μAutolab type III in which saturated Ag/AgCl, and a platinum wire were acting as reference and auxiliary electrodes, respectively. PGE was used as working electrode to provide an appropriate substrate for further modifications. Scanning electron microscopy (SEM) analyses were performed with a Hitachi S4160 (Cold Field Emission). To evaluate the pH, a digital pH meter (Metrohm model 710) was used.

Fabrication of overoxidized polypyrrole-reduced graphene oxide modified pencil graphite electrode (PPyox/RGO/PGE)

PGEs were removed from nitric acid solution and washed carefully with distilled water prior to use. A solution containing 0.1 M pyrrole monomer and 0.05 M potassium sulfate was prepared. Afterwards, 0.1 g RGO was dispersed in above solution and let to be sonicated for 30 min till a homogeneous suspension acquired. Then, 5 mL of pyrrole/RGO suspension was transferred to an electrochemical cell and electropolymerization was performed onto the pencil graphite working electrode by using cyclic voltammetry (optimum conditions were found to be 5 cycles with scan rate 100 mV s^{-1} in potential range of 0.2 V to 1.2 V). This electrode, which was covered by a shiny black layer, was removed from above solution, washed with copious amount of distilled water and inserted in a solution containing 0.1 M sodium hydroxide to be overoxidized by applying 1.4 V for 120 s (PPyox/RGO/PGE).

Immobilization of ds-DNA

To immobilize ds-DNA onto the surface of electrode, PPyox/RGO/PGE was inserted into 0.1 M acetate buffer solution pH = 4.8 containing desired concentration of salmon sperm ds-DNA (optimum concentration found to be 1.5 mg mL^{-1}) and potential of 0.5 V was applied for desired time (optimum time found to be 120 s), see section 3.3. To evaluate the ability of proposed sensor towards determination of CPA, electrochemical oxidation signals of guanine and adenine were recorded in acetate buffer solution 0.1 M pH = 4.8 before and after interaction with CPA.

Preparation of real samples

After accurately weighting of tablets in a sheet, real sample of chlorpheniramine tablet was prepared in a clean mortar by finely powdering of 10 tablets which was labeled each contains 4 mg chlorpheniramine maleate. After that, an adequate amount of this powder was weighted (equal to average weigh of one tablet) and dissolved in Tris buffer solution using ultra sonication for 10 min and then was centrifuged. Appropriate amount of supernatant was used in order to prepare desired concentrations of CPA tablet sample by diluting this solution. Urine samples (drug free) was collected form two healthy men (authors of this paper) in the day of analysis. This urine samples were centrifuged at 5000 rpm for 15 min in order to separate unknown endogenous chemicals. Upper part of resulting urine sample was used after diluting and spiking with appropriate amount of CPA to check the recoveries. Unspiked aliquots of this urine sample were also used as blank. Each measurement was repeated three times. Human blood plasma sample was acquired from Iranian blood transfusion service (Tehran, Iran) and treated with nitric acid (1 mL, 0.1 mol L^{-1}) in order to precipitate the plasma proteins. Afterwards, the sample was vortexed and centrifuged for 10 min. at 12000 rpm. Subsequently, upper part of sample was collected in a clean vial for further analyses.

Results and discussion

Investigation on electrode surface modification

The surface morphology of proposed sensor was investigated by using SEM. Figure 2a, b, c and d illustrate the SEM images obtained upon changing the surface of bare pencil graphite electrode (PGE), RGO/PGE, PPy/PGE and PPy/RGO/PGE. Figure 2a shows smooth and layered surface of PGE before modification which is typical for a graphite-made material. Figure 2b demonstrates the electrode surface of PGE after modification with RGO which is harsh owning to formation of RGO on the surface of PGE. After treatment of PGE with electropolymerized PPy (Fig. 2c), surface roughness increases due to the presence of PPy agglomerations, which means more surface area will be available for electrochemical reactions. Finally, Fig. 2d shows the formation of composite of RGO/PPyox on the surface of PGE. As it will be mentioned

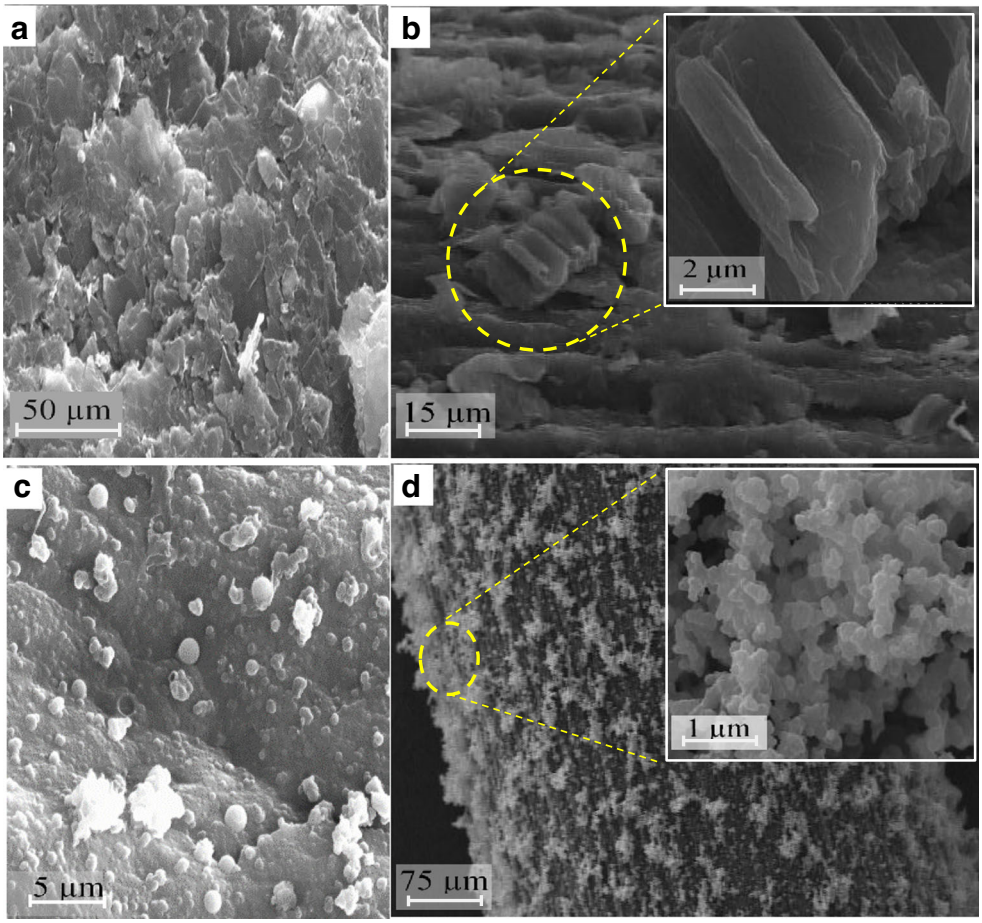

Fig. 2 SEM image of bare (**a**) PGE (**b**) RGO/PGE (**c**) PPy/PGE (d)PPy/RGO/PGE

later, in the next sections, homogeneity in distribution of PPy particles on the surface of electrode (see Fig. 2d) and, consequently, high surface area of this modified electrode will further provide an appropriate substrate for ds-DNA immobilization and thereby higher sensitivity for the sensor.

Electrochemical impedance spectroscopy (EIS) was also applied to evaluate the modification process of this bioelectrode. Figure 3 shows Nyquist diagrams of bare PGE, PPy/PGE, PPyox/RGO/PGE and ds-DNA-PPyox/RGO/PGE which were recorded in 1 mM $Fe[(CN)_6]^{3-/4-}$ redox couple (1:1) solution containing 0.1 M KCl. Z_{veiw} software was exploited to interpret the results using the shown equivalent circuit in the inset of Fig. 3. Here, Rct, Rs, CPEdl, and Zw stand for charge transfer resistance, solution resistance, constant phase element capacitance and Warburg element, respectively. The mean error for each element was less than 5% indicating validity of proposed model for EIS. As Fig. 3 shows, electron transfer resistance (R_{ct}), indicating by semicircle diameter, changes in the following order PPy/RGO/PGE << PGE < PPyox/RGO/PGE < ds-DNA- PPyox/RGO/PGE. These results confirm that PPy/RGO composite is much more conductive compare to bare PGE which can be known considering the nature of conducting polymers. However, overoxidation of PPy causes decreasing the conductivity, as expected, due to repelling doping ions from PPy network. Finally, ds-DNA-PPyox/RGO/PGE showed higher resistance in comparison to PPyox/RGO/PGE indicating successful immobilization of ds-DNA on the surface of PPyox/RGO/PGE.

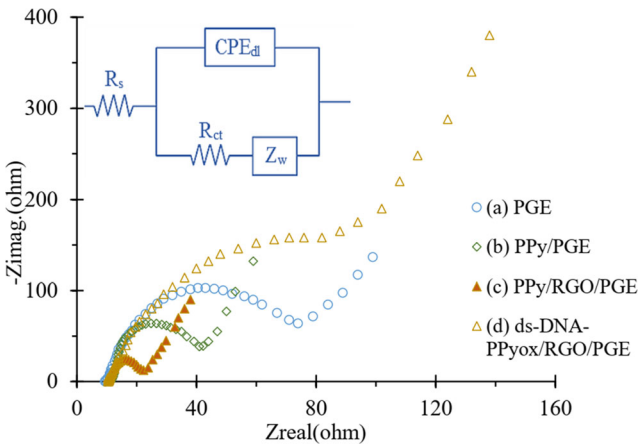

Fig. 3 Nyquist plots of bare PGE (a), PPy/PGE (b) PPy/RGO/PGE (c) and ds-DNA- RGO/PPyox/PGE (d) in the presence of 1.0 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) solution containing 0.1 M KCl

Electrochemical and UV-Vis investigation on PPyox/RGO/PGE interaction with CPA

Differential pulse voltammetry (DPV) is one of the most powerful electrochemical techniques by which we investigated the interaction between modified electrode and chlorpheniramine. Figure 4a clearly shows in the absence of CPA, two well-defined oxidation peaks for guanine and adenine were appeared at about 0.64 V and 0.91 V, respectively at ds-DNA-PPyox/RGO/PGE. However, after inserting modified electrode in a solution containing CPA and let it for a certain time to interact with CPA, these oxidation peaks of guanine and adenine deteriorate. This observation could be explained considering interaction (binding) of guanine and adenine bases in the immobilized ds-DNA on the surface of PPyox/RGO/PGE and CPA. This strong interaction between ds-DNA and CPA was further used to fabricate an electrochemical sensor for determination of CPA. In another parallel analysis, we tried to take advantage of UV-Vis spectroscopy to confirm the interaction of ds-DNA and CPA. Figure 4b illustrates that chlorpheniramine maleate has three absorbance peaks at 256, 262 and 271 nm in the range of 220 to 320 nm. As evident from Fig. 4b, the absorbance of CPA increased substantially upon addition of ds-DNA which is another proof to confirm the strong interaction between ds-DNA and CPA. Since intercalative binding mode characterized by noticeable bathochromy in the absorbance peak (≥ 15 nm), we suggest that electrostatic and/or groove binding mode is dominant interaction between CPA and ds-DNA due to smaller red shift in absorbance (less than 5 nm) [18].

Optimization of important parameters

Impact of ds-DNA concentration

In order to obtain the highest performance for proposed sensor some key parameters were selected to be optimized. Firstly, ds-DNA concentration, which is used for immobilization of ds-DNA on the surface of PPyox/RGO/PGE, was optimized by altering the concentration of ds-DNA from 0.3 to 3 mg mL^{-1} and following the oxidation peak currents (i_p) of guanine and adenine. As Fig. 5a shows peak currents increase along with increasing the concentration of ds-DNA up to 1.5 mg mL^{-1} and level off at higher concentrations. This shows adsorption of ds-DNA on the surface of PPyox/RGO/PGE increases gradually by increasing ds-DNA concentration and reaches to maximum when ds-DNA concentration is 1.5 mg mL^{-1}. Higher concentrations of ds-DNA cannot cause more adsorption of ds-DNA on the surface of PPyox/RGO/PGE since it has been already saturated at 1.5 mg mL^{-1}. Therefore, we chose 1.5 mg mL^{-1} as the optimum ds-concentration for fabrication of this sensor.

Impact of immobilization time

By applying different immobilization times ranging from 30 to 360 s, optimum immobilization time was evaluated using 1.5 mg mL^{-1} of ds-DNA. According to Fig. 5b increasing the accumulation time causes an enhancement in oxidation peak current of adenine and guanine up to 120 s and further increases in accumulation time don't show significant effect on peak currents. Then, 120 s was chosen as optimum immobilization time.

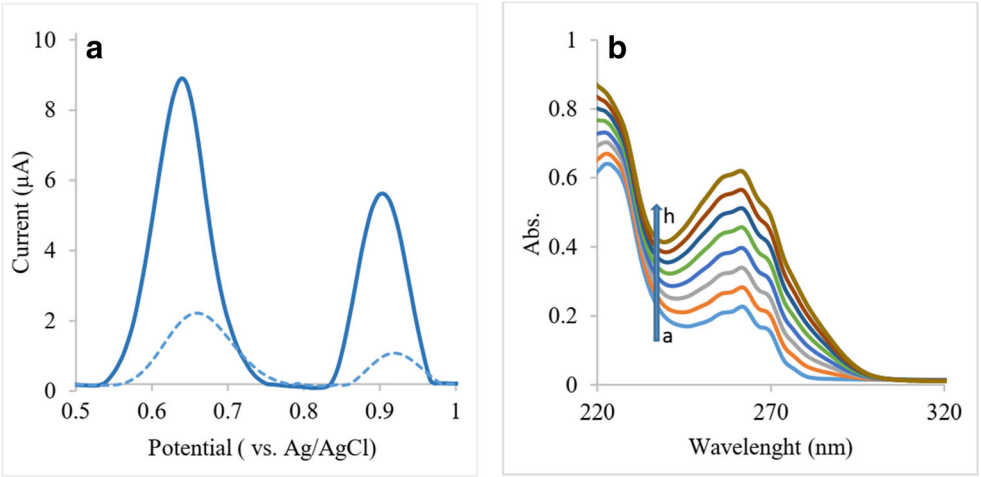

Fig. 4 **a** DPV of oxidation signals of guanine and adenine (a) before and (b) after interaction with 150 μM CPA. (Conditions: immobilization of ds-DNA on the surface of PPyox/RGO/PGE was performed with [ds-DNA] = 1.5 mg mL^{-1}, at +0.5 V for 120 s in acetate buffer pH = 4.8. Incubation of ds-DNA-PPyox/RGO/PGE with CPA at Open circuit potential (OCP) for 120 s in Tirs buffer solution pH = 7. DPV analyses were performed in acetate buffer solution pH = 4.8. **b** Absorption spectra of 50 μM CPA after addition of ds-DNA with different concentrations a) 0, b) 10, c)20, d) 30, e) 40, f) 50, g) 60 and h) 70 μM in Tris buffer solution (0.1 M)

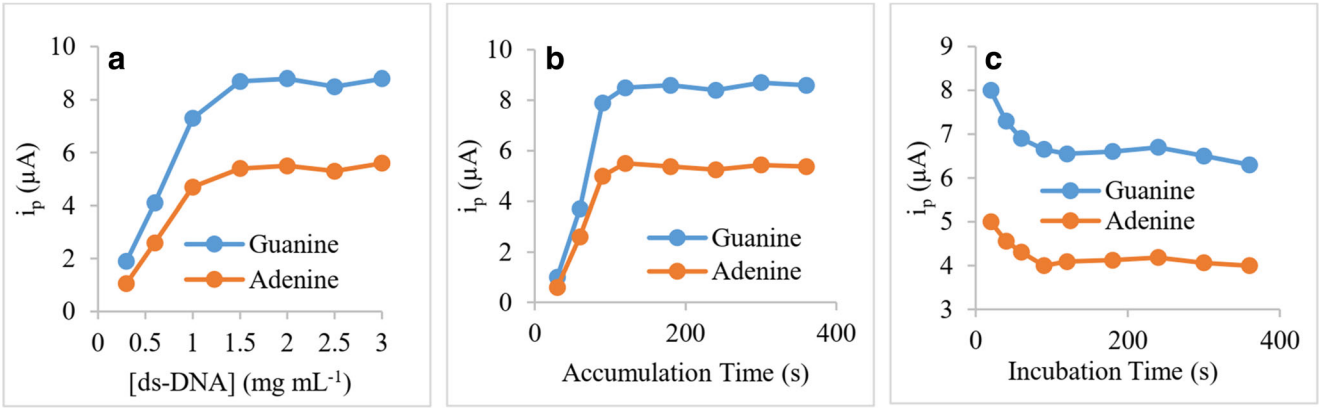

Fig. 5 Optimizations of: **a** effect of [ds-DNA] for immobilization at PPyox/RGO/PGE (conditions: immobilization potential 0.5 V for 240 s in acetate buffer solution pH = 4.8. **b** Effect of accumulation time for immobilization of ds-DNA on the surface of PPyox/RGO/PGE (conditions: [ds-DNA] = 1.5 mg mL^{-1}, at applied potential 0.5 V in acetate buffer solution pH = 4.8. **c** Incubation time of CPA with ds-DNA-PPyox/RGO/PGE in Tris buffer pH = 7. (Conditions: immobilization of ds-DNA at potential 0.5 V for 120 s in acetate buffer pH = 4.8 containing [ds-DNA] = 1.5 mg mL^{-1})

Impact of incubation time

Incubation time of ds-DNA-PPyox/RGO/PGE in the solution of CPA, was also investigated applying different times from 20 to 360 s. Figure 5c demonstrates that the higher the accumulation time, the higher decrease in oxidation peak currents of guanine and adenine up to 90 s. These results show that upon giving more time to the system, CPA can interact more with ds-DNA on the surface of electrode and causes more changes in the resulting signal, i.e. oxidation peak currents of guanine and adenine. However, accumulation time of 90 s is enough for completing the interaction of CPA and ds-DNA and further increasing in accumulation time is not effective for increasing the sensitivity of sensor. Accordingly, we chose 90 s as optimum value for further analyses.

Calibration curve

According to above-mentioned optimum conditions, we tried to investigate on possibility of using strong interaction of ds-DNA with CPA to fabricate an electrochemical sensor. To realize this goal, we exploited the changes in oxidation peak current of guanine and adenine upon incubation of proposed biosensor with different concentrations of CPA. Variations in

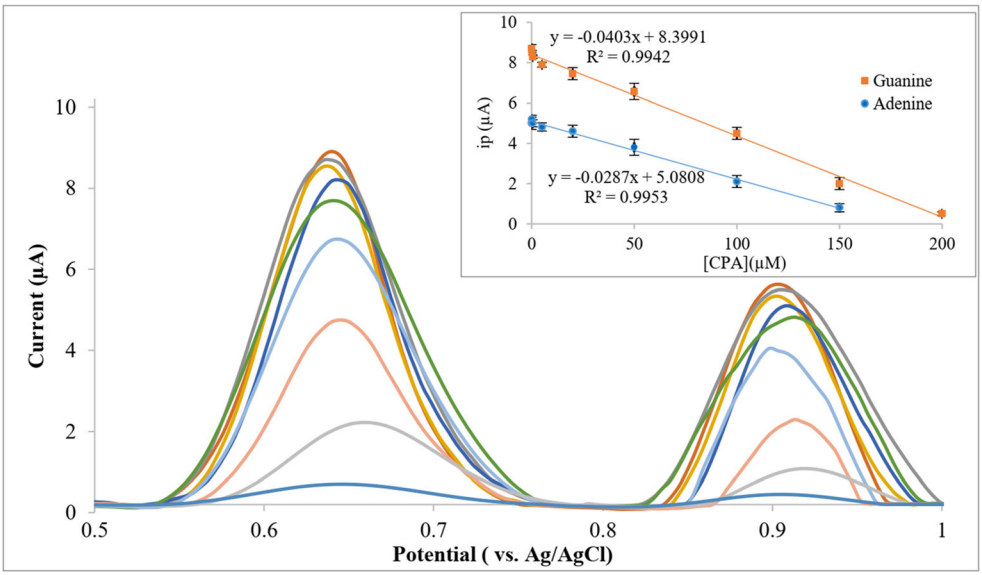

Fig. 6 Variation of DPV signals of oxidation of guanine and adenine after interaction with different concentrations of CPA from (up to down) 0, 0.05, 0.5, 5, 20, 50, 100, 150 and 200 µM. (conditions: immobilization of ds-DNA on PPyox/RGO/PGE were performed in acetate buffer pH = 4.8 by applying potential 0.5 V for 120 s. Incubation of CPA with ds-DNA-PPyox/RGO/PGE at open circuit potential for 90 s in Tris buffer pH = 7. DPV measurements were carried out in acetate buffer pH = 4.8). Inset: calibration curve for determination of CPA based on oxidation signals of guanine and adenine (each point represents 3 replicates)

Table 1 Comparative study of reported researches in literature for electrochemical determination of CPA

Modifier	Detection method	Linear range	Detection limit (μM)	Ref.
Nickel phosphate nanoparticle	DPV	0.05–10.0 mM	16	[6]
Sodium-dodecyl sulfate	Square wave voltammetry	1–100 μM	0.028	[1]
MWCNTs	DPV	5–500 μM	1.63	[10]
Polytyramine doped tris(2,2′-bipyridyl)Ru(II) complex	DPV	2–45 μM	0.338	[11]
Electrochemical pretreatment	Multiple pulse amperometric	16–120 μM	0.5	[19]
Flower-like cobalt	DPV	0.1–10 μM	0.08	[20]
Sodium-dodecyl sulfate	DPV	8–100 μM	1.7	[21]
ds-DNA-PPy_{ox}/RGO	DPV	0.05–200 μM	0.023	This work

oxidation peak currents were recorded by DPV. Figure 6 shows that changes in peak currents of guanine and adenine bases exhibit a linear trend in the range of 0.05 μM to 200 μM for guanine ($i_p = -0.0403[CPA] + 8.399$, $R^2 = 0.994$) and 0.05 to 150 μM for adenine ($i_p = -0.0287[CPA] + 5.080$, $R^2 = 0.995$). Detection limits were calculated, $S/N = 3$, to be 0.023 μM and 0.039 for guanine and adenine, respectively.

Table 1 shows a comparative study for determination of CPA using different analytical methods. As can be seen, proposed sensor offers the best detection limit among reported sensors in the literatures. This elegant feature can be attributed to strong interaction of CPA with ds-DNA as well as capability of PPyox/RGO/PGE in providing suitable substrate for immobilization of ds-DNA.

Interference study

Considering the importance of selectivity in applicability of sensors, we carried out interference study using some common substances which can be found in real samples. Here, we set 5% of change in oxidation peak current as acceptable threshold. Obtained results revealed that 500-fold excess for common simple sugars such as glucose, fructose and sucrose, as well as uric acid cannot interfere significantly with CPA determination using ds-DNA-PPyox/RGO/PGE. Moreover, even 1000-fold higher concentration of Na^+, K^+, NH^{4+}, Cl^- and Br^- ions didn't show any interference effect up to acceptable 5% error. Therefore, this good selectivity can offer another elegant feature for the proposed sensor. Noteworthy, Ca^{2+} ions showed more serious interfering effect as they can electrostatically attach to ds-DNA and weaken the interactions between ds-DNA and CPA.

Real sample analysis

Capability of proposed biosensor for determination of CPA in real samples was also tested. Chlorpheniramine maleate tablet, urine and human blood serum were evaluated as case study. Table 2 exhibits the results for analyses of CPA tablet and urine samples in which acceptable recoveries between 93.6%–104.3% can confirm the usability of ds-DNA-PPyox/RGO/PGE biosensor for measurement of CPA in real samples. Unfortunately, we couldn't use this biosensor to measure CPA in human blood serum even after diluting of serum sample (blood serum sample had the same source as urine sample, authors).

Conclusions

The in conclusion, herein, we reported on designing a novel, sensitive and inexpensive electrochemical sensor for determination of CPA. Proposed sensor can be used for determination of CPA in the range of 0.05 to 200 μM with detection limit of 0.023. SEM, EIS, UV-Vis and DPV were used to characterize and evaluate the proposed sensor. Results show, this novel

Table 2 Performance of DNA biosensors for determination of CPA in real samples

Sample	Spiked	Expected	Found	Recovery (%)	RSD (%)
CPA tablet	–	4 mg	3.8	–	2.6 ($n = 5$)
Urine 1	0.0	–	<limit of detection	–	–
	5 μM	–	4.7	94	3.8 ($n = 3$)
	100 μM	–	108	108	5.6 ($n = 3$)
Urine 2	0.0	–	<limit of detection	–	–
	5 μM	–	5.2	104	4.6 ($n = 3$)
	100 μM	–	94	94	5.1 ($n = 3$)

electrochemical biosensor is enough selective and sensitive to be used for determination of CPA in real samples such as CPA tablet and urine. Up to our best knowledge, proposed sensor offers the lowest limit of detection compare to all previous reported electrochemical sensor in the literature. This achievement can be assigned to, first, very effective role of overoxidized polypyrrole/RGO nanocomposite in providing a suitable substrate for immobilization of ds-DNA and, secondly, strong interaction of ds-DNA and CPA.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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