

Nano-biosensor based on reduced graphene oxide and gold nanoparticles, for detection of phenylketonuria-associated DNA mutation

ISSN 1751-8741

Received on 29th May 2017

Revised 28th November 2017

Accepted on 5th December 2017

E-First on 27th February 2018

doi: 10.1049/iet-nbt.2017.0128

www.ietdl.org

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Abstract: Phenylketonuria (PKU)-associated DNA mutation in newborn children can be harmful to his health and early detection is the best way to inhibit consequences. A novel electrochemical nano-biosensor was developed for PKU detection, based on signal amplification using nanomaterials, e.g. gold nanoparticles (AuNPs) decorated on the reduced graphene oxide sheet on the screen-printed carbon electrode. The fabrication steps were checked by field emission scanning electron microscope imaging as well as cyclic voltammetry analysis. The specific alkanethiol single-stranded DNA probes were attached by self-assembly methodology on the AuNPs surface and Oracet blue was used as an intercalating electrochemical label. The results showed the detection limit of 21.3 fM and the dynamic range of 80–1200 fM. Moreover, the selectivity results represented a great specificity of the nano-biosensor for its specific target DNA oligo versus other non-specific sequences. The real sample simulation was performed successfully with almost no difference than a synthetic buffer solution environment.

1 Introduction

Phenylketonuria (PKU) is a faulty metabolism of phenylalanine amino acid, caused by defective action of an enzyme phenylalanine hydroxylase (PAH) [1–3]. The phenylalanine is a necessary part of the human diet and is naturally present in all kinds of dietary protein [4, 5]. In a healthy person, the PAH enzyme breaks down any excess phenylalanine from these sources beyond what is needed by the body because high levels of phenylalanine could be toxic in blood and brain and consequently affecting development and also function of brain [3, 5, 6]. There are some mutations in the PAH gene which could lead to the PKU. There is no doubt that PKU should be diagnosed in early stages and treated from the very first days of life. Accordingly, it could be as a first inborn errors of metabolism to be screened [1, 3, 4, 7].

Among several PAH mutations, some seem more important, such as IVS10NT546 which has been explained for PKU was identified as a G to A transition at position 546 in intron 10 of the PAH gene is representing the presence of this disease in most of the countries including Bulgaria, Italy, Turkey and especially Iran suspected that this mutation may be one of the more frequent defects in the PAH gene causing classic PKU in southern Europe [4, 5, 8, 9]. There are several techniques used for its detection and screening, such as RT-PCR, PCR-RFLP, restriction enzymes, and sequencing [10–13].

Electrochemical biosensors are being used widely as important techniques for detection and quantification of the electroactive materials [14–16]. Detection of the important biological compounds such as DNAs and RNAs have been enabling us to detect, prognosis, and screening of a disease [15–17]. Electrochemical biosensors provide an attractive means to analyse the content of a biological sample due to the direct conversion of a biological event to an electronic signal and they are rapid, accurate, highly sensitive, and specific [18, 19]. In this way, nanobiosensors could be more sensitive due to taking advantage of application of the nanomaterials to enhance the sensitivity of the biosensors mostly by expanding the surface area and/or enhancing the electron transfer rate [20–22]. Plenty of electrochemical nanobiosensors

have been made so far based on various nanomaterials which is enabling us to detect oligonucleotides even down to atomolar level [23–26].

Electrochemical nanobiosensors have been used for detection of PKU so far, especially for the detection of mutations in the PAH gene. In addition, some electrochemical biosensors have been developed to detect the mentioned mutations. However, there are many developed electrochemical biosensors or nanobiosensors for the detection of DNA or RNA molecules as biomarkers [16, 27]. These biosensors are taking advantage of different techniques and methods to achieve better sensitivity, selectivity, and overall function [28, 29]. Most of them using nanomaterials for signal amplification and surface area increase [14, 23]. In addition, there are some other electrode modifications [30] and even other methods such as molecularly imprinted polymer [15, 31]. Electrochemical labels which are the electroactive materials to generate more sensitive signals and increase the sensitivity of the electrochemical biosensors [18, 19, 32]. In this study, we used an anthraquinone compound named Oracet blue (OB) to enhance the sensitivity of our biosensor.

Here, we developed an electrochemical nanobiosensor for the recognition of a PAH mutation to detect PKU disease. The target oligo of the nanobiosensor was designed to contain the flanking region of the mutation IVS10nt546 inside the PAH gene. The exact complementary oligo was designed to be single-stranded DNA probe (ssProbe) and it was thiolated at its 5' end to be self-assembly attached to the surface of gold nanoparticles (AuNPs). AuNPs were decorated on the reduced graphene oxide (rGO) sheet on the screen-printed carbon electrode (SPCE). We also applied OB as an electrochemical indicator which is relatively new among other electroactive labels so far, such as methylene blue [19, 33]. The application of carbon nanomaterials underneath of gold nanomaterials have been used in several combinations and forms so far for modification of the electrode [14, 16, 23, 29]; however, the combination of rGO and AuNPs is reasonably attended recently for different goals [34, 35], but based on our literature review, this combination has not been used yet for PKU detection. Once more, based on our literature review, there is no previous publication

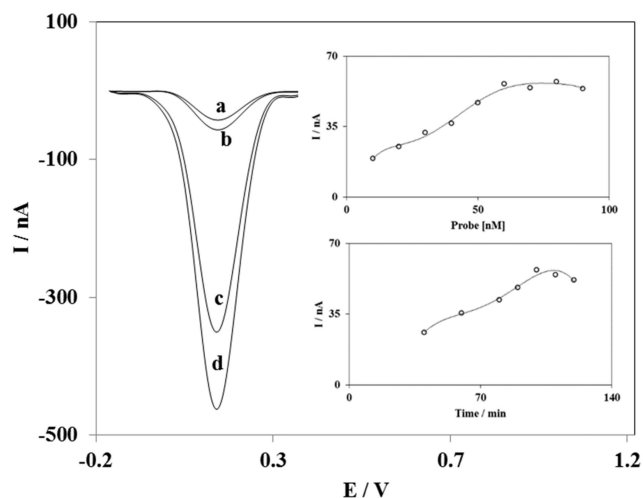


Fig. 1 Optimisation of the parameters about single-stranded alkanethiol probes
(a) Immobilisation methods, (b) Probe concentrations, (c) Probe immobilisation time periods

about a biosensor for recognition of the mutation IVS10nt546 inside the PAH gene. In this nanobiosensor, detection of the mentioned mutation would be based on the concept of typical sequence-specific DNA hybridisation electrochemical biosensors [36, 37] which is mostly able to detect a single nucleotide changes and/or damages as it was previously described in several publications before [38–42].

2 Materials and methods

2.1 Apparatuses and electrochemical methods

The electrochemical apparatus used in this study was an AutoLab potentiostat/galvanostat PGSTAT 101 (The Netherlands) which was connected to an SPCE from Dropsens Co. (DRP-C110) (Spain). The SPCE consisted of a carbon working electrode (with 4.0 mm diameter) and also the counter electrode and the reference electrode which were made from platinum ink and silver ink, respectively.

The electrochemical measurements were cyclic voltammetry (CV) for assessment of electrode modification and differential pulse voltammetry (DPV) for the final measurements. The DPV method was used with the following experimental parameters: an amplitude of 25 mV, a modulation time of 0.05 s, and a step potential of 50 mV which performed in a 0.1 M phosphate buffer solution (pH 7.0). However, the CV was performed in 1.0 mM $K_3[Fe(CN)_6]$ dissolved in PBS buffer and in the potential range from 0.025 to 0.33 V and a sweep rate of 0.02 V s^{-1} .

In addition to CV, the field emission scanning electron microscopy (FE-SEM) images were taken by the use of a TESCAN MIRA3 (Czech Republic) and a KYKY-EM3200 Digital Scanning Electron Microscope (China) to analyse the correct conformation of the modified electrode surface after modification with the rGO and AuNPs.

2.2 Reagents and solutions

All the chemicals used in this research were purchased from the Sigma-Aldrich Co. (USA), including the chemically rGO (prod. no. 777684), AuNPs (with 20 nm diameter) (prod. no. 741965), and OB (prod. no. 75355).

Moreover, the DNA oligonucleotides were provided from the Bioneer Company (South Korea). The target oligo sequence was designed to contain the flanking region of the mutation IVS10nt546 for the PAH gene. The sequences of all the used oligo were as follows:

Target oligonucleotide (from PAH gene):



ne-base mismatched target oligonucleotide:



Three-base mismatched target oligonucleotide:



Non-complementary oligonucleotide:



alkanethiol single-strand probe:



Furthermore, all the solutions which were used in this study were prepared based on the protocols from the previously published articles [18, 19].

2.3 Fabrication of the nanobiosensor

Before to use, solution of AuNPs (150.0 mg/ml in H_2O) and solution of rGO (1.25 mg/ml in H_2O) were dispersed separately in the sonication bath, for a short period of time. The working electrode of the SPCE, which was made of carbon, was firstly washed with doubly distilled water thoroughly. Next, a 4.0 μl drop of the rGO solution was applied on the surface of the SPCE working area and kept isolated until dried. Consequently, after a mild wash with water, 5.0 μl of AuNPs solution was applied to the rGO-modified electrode and also kept isolated in the container to be slowly dried to achieve the correct assembly of AuNPs on the rGO.

Once the modified electrode washed with double-distilled water, a 4.0 μl drop of the thiolated specific probes (80.0 nM) was applied on the modified electrode for 100 min and kept in the isolated container to be slowly dried for self-assembly attachment of the probe on the AuNPs surface. Next, a drop of 1.0 mM MCH solution was applied on the surface of the working electrode for 5 min and then washed up with the double-distilled water. Afterwards, based on our previously published protocol [17], a 3.0 μl of hybridisation buffer containing a proper concentration of the target DNA sequence was applied on the surface of the working electrode and kept for 120 min.

After hybridisation of target DNA with the probes, the modified electrode was washed with washing solution and consequently, a 4.0 μl drop containing 0.12 mM of OB (as electrochemical indicator) was dropped on the modified electrode for 100 min and then was rinsed with double-distilled water. Finally, the DPV measurement was performed in a 0.1 M phosphate buffer solution (pH 7.0) to measure the reduction curve of intercalated OB molecules. Besides, after every phase of electrode modifications, the CV analysis was performed; however, the FE-SEM imaging was done after modification of the working electrode with nanomaterials.

3 Results and discussion

3.1 Study of the alkanethiol probe parameters

Owing to its important role, the self-assembly process of the alkanethiol single-strand probes (ssProbe) on the surface of the decorated AuNPs was optimised through testing and evaluating different conditions. First of all, the method of ssProbe immobilisation was assessed by testing two different regular techniques; droplet self-assembly method and solution self-assembly method. In result, Fig. 1a is comparing these two methods which is demonstrating the advantage of the droplet self-assembly method. The DPVs obtained before (a, c) and after (b, d) the hybridisation of ssProbe with the target DNA.

In addition, the concentration and incubation time of the alkanethiol probe are really important. These parameters were tested in our optimisation experiments results and the results are shown in Figs. 1b and c for the concentration and incubation time of ssProbe, respectively. As it can be seen in Fig. 1b, different

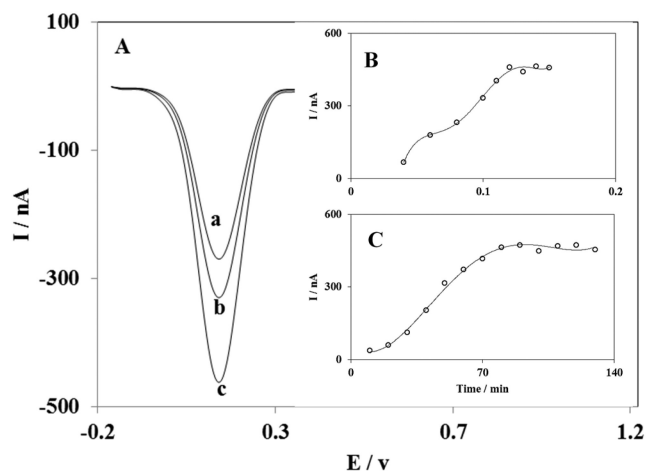


Fig. 2 Optimisation of the parameters about hybridisation and electrochemical label

(a) Hybridisation methods, (b) OB concentrations, (c) OB incubation time periods

concentrations of ssDNA were tested (from 10.0 to 90.0 nM) which the 80.0 nM had a higher current peak. However, seven different incubation time were tested (Fig. 1c), and 100 min was chosen as the best. Consequently, in the fabrication of biosensor, the ssProbe were used as in concentration of 80.0 nM for 100 min. It can be seen in Fig. 1b that the concentration of applied ssProbe on the electrode surface is very effective parameter, because it influences the resulted output current and is very different for various concentrations. The same explanation is also true about the incubation time which is shown in Fig. 1c and the influence of this parameter on the resulted output current is really important.

3.2 Study of the hybridisation parameters

Since the hybridisation method and its incubation time are very vital in the function of the biosensor, the evaluation of these parameters seem to be vital to develop new biosensor. For that reason, in this section, three different hybridisation methods were assessed and the comparison of their DPVs is shown in Fig. 2a. Among three methods, the solution hybridisation method was selected as the best method (curve c) and were choose to be the hybridisation strategy of the biosensor, while the hybrid drops (curve a) and hybrid boiling point (curve b) methods had a lower peak current.

3.3 Study of OB parameters

OB is an anthraquinone and were proved earlier for its intercalation interaction with the DNA [14]. This compound does have a reduction peak in low potential as well as intercalation mechanism which make it perfect electrochemical label to be used in biosensor. To find out its best concentration and incubation time, a series of experiments were performed and in results, it was discovered that from 0.04 to 0.15 mM OB concentration, the best one was 0.12 mM (Fig. 2b) and from 10 to 120 min incubation time, the 100 min was the best (Fig. 2c). Therefore, these selected parameters of OB were used to build the biosensor. As it can be concluded from the assessment of Fig. 2b, the concentration of the applied OB does really affect the output current of the final biosensor. In Fig. 2c, the incubation time of OB is also shown a various output currents for different time periods. Both Figs. 2b and c emphasise the importance of optimisation experiments for electrochemical label (OB) to be used in fabrication of the nanobiosensor.

3.4 Analysis of sensor fabrication phases

The SEM imaging is one of the most important characterisation methods in nanotechnology and has been used for different purposes, including but not limited to surface characterisation of a modified working electrode in electrochemical nanobiosensors. Hence, in this study, the SEM imaging of the nanomaterials-modified working area of the SPCEs was performed and it is

shown in Fig. 3. In this figure, the FE-SEM image of rGO (Fig. 3a) demonstrates the presence of the rGO on surface of the bare SPCE electrode. In another FE-SEM image, the distribution of the AuNPs on the rGO-modified SPCE is shown in Fig. 3b. In conclusion of this part of study, both SEM images were mainly demonstrated the assembly of the nanomaterials (rGO and AuNPs) modification steps on the SPCE electrode. Therefore, further modifications with ssProbes were carried on consequently. In addition, the energy-dispersive X-ray spectroscopy (EDS) analysis of the later modified SPCE electrode (AuNPs + rGO) was also performed by the SEM instrument which is reported in Fig. 3c. As it can be seen in the EDS spectrum, the chemical elements carbon (C) and gold (Au) are the main composition of the modified SPCE surface and are present on the modified SPCE electrode.

In addition to the FE-SEM imaging, the highly used electrochemical method, CV, was also performed to prove every modification phase of the electrode during biosensor fabrication. Fig. 4 represents the cyclic voltammograms of different modified electrodes in 1.0 mM $K_3[Fe(CN)_6]$ solution during biosensor fabrication. The CV curves in Fig. 3 recorded for the bare SPCE, rGO-modified SPCE (curve named rGO), SPCE/rGO/AuNPs electrode (curve named rGO + AuNPs), SPCE/rGO/AuNPs/ssProbe + MCH (curve named ssProbe + MCH), and finally, SPCE/rGO/AuNPs/ssProbe + target DNA (curve named hybridised).

As it can be seen, the application of the nanomaterials such as rGO (curve b) and AuNPs (curve a) significantly increased the currents, which is possibly because of the higher electron transfer rate [43–47] and/or expansion of the surface area [48–50] of nanomaterials. On the other hand, the probe immobilisation considerably decreased the obtained peak current (curve d), because the ssProbe does have negative charge and also when they are immobilised on the surface of the AuNPs, they block the electron transfer stream towards the electrode. Furthermore, this trend repeated after the hybridisation of the target DNA with ssDNA probes (curve f), which is expectable because the density of oligonucleotides after hybridisation (formation of double-stranded oligonucleotides) is more than just ssDNA-modified surface. As it is explained, the CV studies are representing the modification steps of the nanobiosensor fabrication process.

3.5 Selectivity of the sensor

The selectivity and specificity of the oligonucleotide-based biosensors basically originated from the highly selective nature of the two complementary oligonucleotides, which brings a feasible function of selectivity for the target oligos instead of non-specific oligos. For this reason, the developed electrochemical nanobiosensor was assessed for its selectivity by recording the DPV signals resulted from non-complementary and mismatched sequences instead of specific target DNA. Fig. 5 includes the final DPV graphs obtained from the bare SPCE electrode (a), ssProbe-modified SPCE/rGO/AuNPs (b), non-complementary oligo (c), three-base mismatched oligo (d), single-base mismatched oligo (e), and target DNA oligo (f).

With a simple comparison of the DPV currents of these curves, it can be seen that the target DNA (curve f) does have a very higher current than other non-specific oligos. In addition, the very high difference between the hybridised nanobiosensor (curve f) and the non-hybridised nanobiosensor (curve b) simply shows the appropriate function of the OB as an electrochemical label in this nanobiosensor, which could make a great contrast between single-stranded oligos versus double-strands and this phenomenon can be a reason for what previous studies prove that the OB–DNA interaction is intercalation [19].

As the result of specificity assessment, it can be stated that the more than sufficient function of the nanobiosensor as its selectivity is a supreme feature that fits to name of a biosensor which can be used in a complex solution of extracted DNA samples containing non-specific and partially specific oligos.

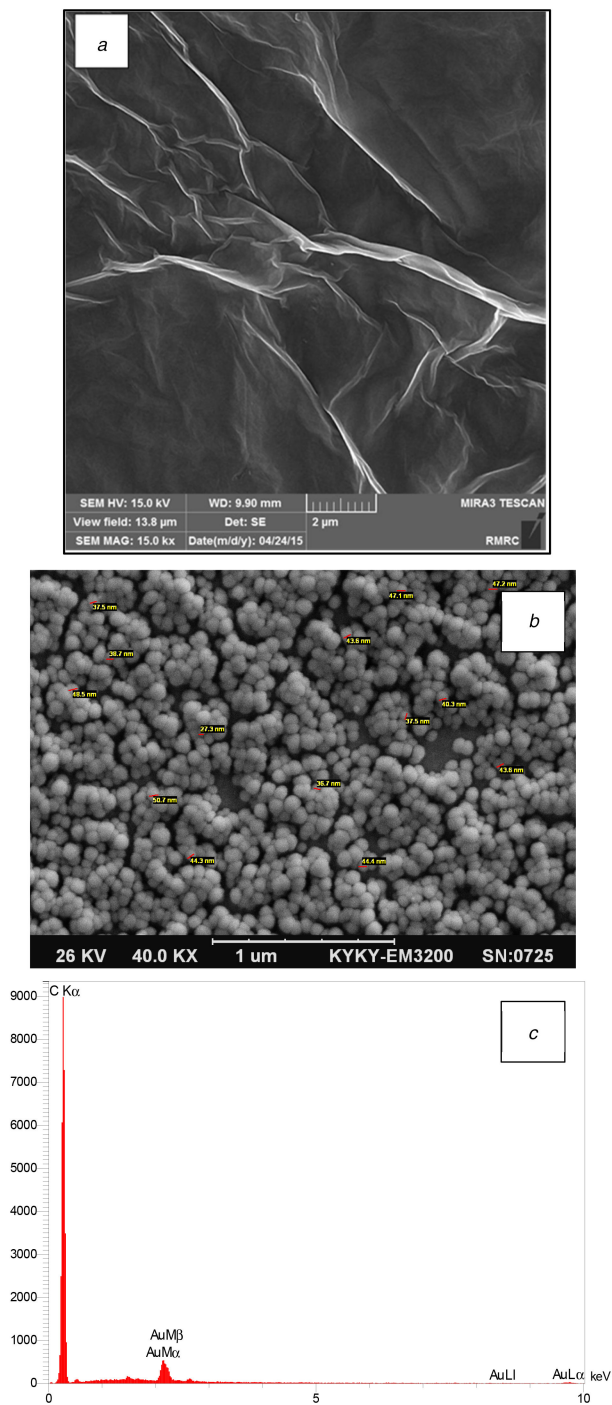


Fig. 3 SEM images of the modified SPCE surface (a) rGO on the SPCE, (b) AuNPs decorated on the rGO layer on the SPCE, (c) EDS analysis of the AuNP + rGO-modified SPCE

3.6 Detection of the target DNA concentrations

Undoubtedly, the most important specification of a DNA biosensor is the linear range and limit of detection which is representing its sensitivity and functionality for measuring different concentrations of the target DNA oligos. Therefore, the prepared nanobiosensor was applied to detect PAH DNA target concentrations where the peak current of electrochemical reduction of OB molecules considered as final output of the nanobiosensor.

In results, the differential pulse voltammograms recorded in PBS solution (0.1 M, pH 7.0) shown for the concentrations within linear range in Fig. 6. Furthermore, mean currents of DPVs for three replications of every concentration used for plotting the standard curve which was linear in concentrations from 80 to 1200 fM of target concentration (Fig. 6 inset). The limit of detection of this biosensor was calculated to be 21.3 fM using $C_m = 3s_{bl}/m$

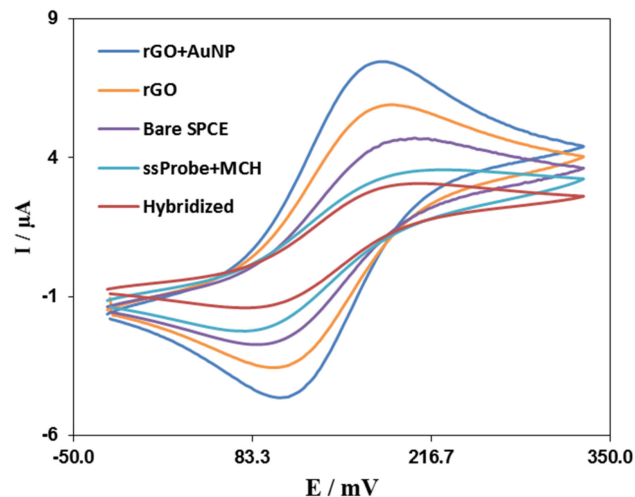


Fig. 4 Results of the CV analysis for different steps of SPCE modifications [The CVs performed in the 1.0 mM $K_3[Fe(CN)_6]$ solution in 0.1 M phosphate buffer solution (pH 7.0)]

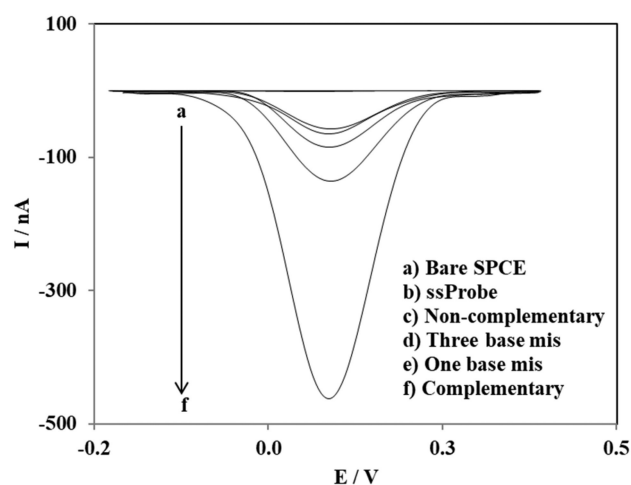


Fig. 5 Results of the selectivity assessment of the nanobiosensor for target DNA over the non-specific oligos [The DPVs performed in the 0.1 M phosphate buffer solution (pH 7.0)]

equation; where s_{bl} is the standard deviation of 16 repeated DPV of the blank sample (the signal of the OB on the non-hybridised probe), and m is the slope of the calibration plot. The mean current for the 14 repetitive measurements in a solution of 250.0 fM of target DNA was 105.2 ± 3.4 nA with the relative standard deviation (RSD) of 3.2%.

Additionally, the reproducibility of the nanobiosensor was also calculated from three replications of DPVs measured for two concentrations 200.0 fM with the RSD of 3.43% and 500.0 fM with the RSD of 2.89%. Such a low RSD values are clearly depicting the reliability of the fabrication method and representing its great repeatability for several fabrications which enable it to be reproduced with lowest possible error.

As it is concluded from the literatures of previously reported DNA nanobiosensors, the wide linear range and low detection limit of the proposed nanobiosensor are better than the most of them [16, 23, 28, 32, 51]. Moreover, the simple and cost-effective preparation of this nanobiosensor along with its remarkable sensitivity can be considered as its extra advantages.

The reasonable explanation for the high performance of the nanobiosensor may be became from application of rGO and AuNPs as very high conductive nanomaterials with fast electron transfer rate and also their effect in expansion of the electrode surface and consequently, the larger area of working electrode. Moreover, the application of an anthraquinone such as OB as electrochemical label with the mechanism of intercalation is taking the

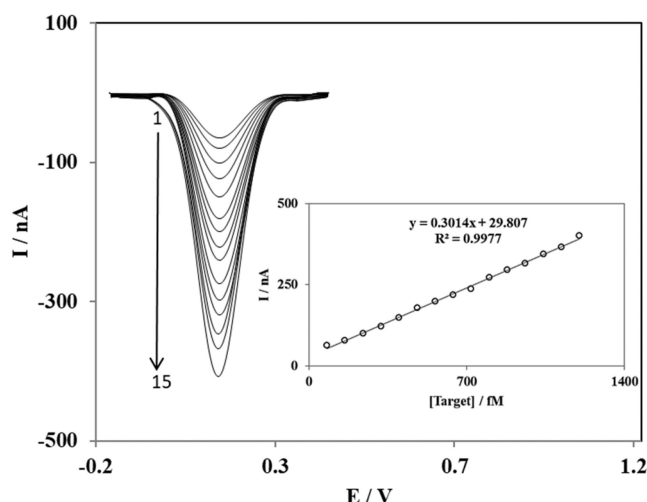


Fig. 6 Results of the analytical performance of the nanobiosensor: The DPVs of the OB reduction for the concentrations in the linear range. The inset is a plot of the mentioned DPVs currents versus their concentrations in the linear range (The DPVs performed in the 0.1 M phosphate buffer solution (pH 7.0))

Table 1 Real sample results of the nanobiosensor in the simulated extracted PAH DNA

Added DNA, fM	Detected DNA, fM	Recovery percentage, %	Relative standard deviation percentage, %
100.0	99.18	99.18	3.81
250.0	253.7	101.48	2.30
400.0	395.8	98.95	3.67

nanobiosensor to the higher level as its sensitivity and selectivity are improved surely.

Furthermore, the stability of the nanobiosensor was assessed within different time periods for the fabricated nanobiosensor which was stored in the 0.1 M PBS pH 7.0 solution inside the refrigerator (4°C) within 15 days. The results of DPV analysis showed almost 6.8% reduction in the performance of the nanobiosensor after 5 days of storage, 10.5% after 10 days, and 17.2% after 15 days, which is an acceptable result.

3.7 Real sample investigation

For application in clinical and/or real sample working environment, every developed analytical method such as nanobiosensors should be tested in these situations to be guaranteed for its functionality in such an environment. As the proposed nanobiosensor worked really well in PBS buffer as a working environment, its evaluation was also performed in the simulated extracted DNA solution as a real sample. For this, the combination of some potential interfering molecules, which could possibly found in the real extracted DNA, were added to the target DNA solution. The four interfering molecules were included ethanol (25%), glucose (25%), CTAB (Cetyl trimethylammonium bromide) (25%), and SDS (sodium dodecyl sulphate) (25%) which their mixture was added to the exact amount of target DNA solution. Then, the hybridisation of the prepared nanobiosensor with the target DNA was performed in the simulated mixture instead of the PBS buffer. The rest of experiments were the same as the previous conditions in this study. The resulted DPVs were recorded in four replications and the mean recovery percentages and their RSD values were calculated and the results are shown in Table 1. The recovery percentage was calculated as a ratio of detected DNA with biosensor over the added DNA concentration.

As it can be seen in Table 1, the recovery percentage of the 100.0 fM spiked concentration of DNA was $99.18 \pm 3.81\%$ in four replications. The recovery percentage for 250.0 fM spiked concentration of DNA was $101.48 \pm 2.30\%$ and for 400.0 fM

spiked concentration of DNA was $98.98 \pm 3.67\%$ in four replications. The recovery percentages lower and/or higher than 100% can be explained possibly with errors in repeatability of the biosensor (in four replications for each concentration), and possible human and instrumental errors. However, it can be said that the recovery percentages are in very common range and are near to 100% and also their respecting RSD percentage are very low. These results are a clear sign of good performance of the nanobiosensor in the simulated extracted DNA solution instead of the PBS buffer and it can be concluded that the four potential interferences agents could not interject the performance of the nanobiosensor. Such a performance indicates that the presented DNA nanobiosensor has promising potentials for future use in real clinical laboratory tests to detect/screen the mutation in PAH gene for PKU.

4 Conclusion

The electrochemical DNA became the most important area of research in analytical chemistry and biochemistry and their commercial applications in medicine, food science, and other fields are growing day after day. Most importantly in this case, nanomaterials are getting the most attention and application of nanomaterials in biosensors is a getting regular. For this reason, here we explained a novel electrochemical nanobiosensor based on AuNPs decorated rGO sheet on the SPCE and application of OB as an electrochemical indicator, for detection of a PAH mutation for screening or detection of PKU disease. The results of experimental tests of the proposed nanobiosensor revealed that the sensitivity of the method could be as low as 21.3 fM of target DNA concentration known as the limit of detection. The dynamic range of the biosensor was from 80 to 1200 fM, which is very wide and suitable range, especially for such a low-price and simple biosensing platform. Furthermore, the selectivity and specificity of the biosensor enable it to make clearly distinction between single-strand DNA and target hybridised double-strand DNA, as well as, the distinction between target DNA with mismatched and/or non-complementary oligonucleotides. Finally, the functionality of the designed and tested nanobiosensor was confirmed in the simulated real sample solution of extracted DNA which is promising its application in future medical tests for detection and/or screening of the PKU, to enhance the quality of life and even save the children from death.

5 Acknowledgment

The authors express their sincere appreciation to the Research Council of Islamic Azad University (Ashkezar branch) for their financial support of this research.

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