RESEARCH PAPER

The potentiality of graphene quantum dots functionalized by nitrogen and thiol-doped (GQDs-N-S) to stabilize the antibodies in designing of human chorionic gonadotropin immunosensor

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ABSTRACT

In this study, for the first time, a simple immunosensor for ultrasensitive recognition of human chorionic gonadotropin (HCG) in serum samples was fabricated using a simple approach. In this method, a low-cost, sensitive immunosensor was made based on QDs-N-S/Au nanoparticles (NPs) modified screen-printed carbon electrode (SPCE). It seems that QDs-N-S/AuNPs/ antibody as a biocomposite can be a good choice for development as an impressive immunosensor. In modifying the proposed immunosensor, the length of the process was reduced and the use of other substrates was eliminated. The pH and incubation time were optimized as two important parameters. Under the optimal conditions at modified SPCE, a linear communication in the range of 0.1 to 125 pg mL^{-1} and the detection limit of 12.5 fg mL^{-1} were obtained. The present procedure was utilized to the detection of HCG in real samples with the desirable results.

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INTRODUCTION

Human chorionic gonadotropin (HCG) is a momentous diagnostic marker of pregnancy and tumour marker for certain cancers. So, accurate and sensitive recognition of HCG in blood specimen is important. Chemiluminescence and enzyme-linked immunosorbent assay (ELISA) are conventional recognition methods to identify antigens. Electrochemical method has displayed several advantages including feasibility of miniaturization, simplicity of instrument, low cost and subsequent portability which distinguishes it from other methods [1]. In order to increase the sensitivity of electrochemical immunosensors different species of nanomaterial, such as quantum dots (QDs) [2], nanoparticles (NPs) [3] and carbon nanotubes have been employed. QDs are well-known for their unique features including biocompatibility, low cytotoxicity, controllable size, and possibility to be functionalized easily [4, 5]. Several studies (listed in Table S1) have disclosed sensitive and accurate detection of HCG in blood samples, however, to the best of our knowledge, there is no report about the use of QDs to modify the HCG electrochemical immunosensor. QDs are similar to proteins, nucleic and antibodies and they form conjugates with these compounds because of their small size [6, 7]. QDs can increase the effective surface of immunosensor with absorbing more antibodies because of their large surface area. Graphene quantum dots (GQDs) have been applied for designing electrochemical immunosensors and sensors [8, 9] due to the advantages of

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biocompatibility, low toxicity, smaller volumes, and large specific surface area.

Thio-GQDs (SH-GQDs) can form stable colloidal suspensions in different solvents containing dimethylformamide and ethanol. Also, SH-GQD has lower solubility compared to ox-GQD [10]. In this study, we proposed a simple green method to utilize the QDs-N-S as the nanomaterial for accurate and selective determination of HCG. Screen-printed carbon (SPC) has been used for designing this sensor as other studies [11-13]. In order to explain the choice of material in this study, it can be said that screen-printed carbon electrodes (SPCE) are carbon electrodes which are well-known for their unique features, such as good reproducibility and wide potential window [12-14]. Also, gold NPs (AuNPs) have been employed for modeling the sensor because of their well biocompatibility, excellent electrocatalytic activity, and high electrode conductivity. In this method, AuNPs have been attached on the SPCE surface modified by QDs-N-S. Thus, an antibody containing NH_2 group can attach to the AuNPs. Based on our knowledge, the usage of bovine serum albumin (BSA)/ antibody (Ab)/AuNPs/ QDs-N- S/ SPCE has not been employed to identify HCG yet.

EXPERIMENT

Materials and instrumentation

HCG (37 kDa), antibody (1 mg.mL^{-1}), sodium citrate ($Na_3C_6H_5O_7.2H_2O$), $HAuCl_4$, hepatitis virus core antigen (HCV), BSA, and progesterone were provided from LLC (USA) and Sigma-Aldrich Co. Ascorbic acid, glucose, and NaOH were purchased from Merck. All experiments were carried out at 25°C. 10 mol.L^{-1} $K_3Fe(CN)_6/K_4Fe(CN)_6$ and 0.1 mol.L^{-1} KCl were used for the electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) experiments. Phosphate buffer (0.1 mmol L^{-1}, pH = 7.5) containing 5 mmol L^{-1} [$Fe(CN)_6$]$^{3-/4-}$ and 0.1 mmol L^{-1} KCl was applied as a working solution to determine HCG. The blood serums as real samples were used and diluted with phosphate buffer (0.1 mmol L^{-1}) for 50 times, and then were analysed. Scanning electron microscope (MIRA3TESCAN-XMU) was utilized to take field-emission scanning electron microscopy (FE-SEM) images. Philips X'pert diffractometer with an X-Ray tube anode Co was used to obtain the X-ray diffraction (XRD) patterns. All electrochemical measurements were carried out with a μ-AUTOLAB electrochemical system (Eco-Chemie, Switzerland) driven with NOVA software. EIS analysis was performed with a frequency range between 0.1 Hz and 100 kHz with signal amplitude of 5 mV and with a bias potential of 0.2 V. The DPV measurements were carried out by scanning the potential of −0.1 to 0.5 V.

Formation of graphene quantum dots (GQDs)

GQDs were synthesized according to reference [11]. To do so, citric acid (2g) was added to a 5 mL beaker in a mantle and the temperature was raised up to 200 °C. After 5 min, the beaker content became liquid and turned pale yellow from colorless. After half an hour, the beaker content became orange which was added immediately to the aqueous solution of NaOH (10 mg mL^{-1}, 100 mL) using a dropper while stirring on the magnetic stirrer. When the pH was neutralized to 7.0, the GQDs were prepared.

Preparation of the GQDs functionalized with amine and thiol (GQDs-N-S)

The GQDs solution prepared in the previous step was heated up evaporation all the solvents. This solid (172 mg) was dissolved in a dry toluene (30 mL) and sonicated for 20 min followed by the addition of (3-aminopropyl)triethoxysilane (1.79 mL) and (3-mercaptopropyl)triethoxysilane (1.79 mL). This reaction was progressed under nitrogen atmosphere and refluxed for 12 h. The pale yellow precipitate was removed by centrifugation and washed with dry toluene for three times and dried in vacuo.

As is obvious in the FE-SEM image of GQDSs-N-S (Fig.1A), the mean diameter of this product is between 400 nm to 2.5 μm. The presence of C, N, O, Si and S elements in GQDs-N-S was confirmed by energy dispersive

analysis of X-ray (EDAX) and elemental mapping analysis, shown in (Fig.1B and Fig.1C). Furthermore, the characteristic peaks of GQDs-N-S are apparent in XRD (Fig.1D) confirming the formation of GQDs-N-S.

Designing the sensor

The construction of the sensor is realised in some steps (Scheme 1). First, in order to cleaning the bare SPCE, it was electrochemically activated in the presence of 2 mol L^{-1} H_2SO_4 by applying a voltage of +1.6 V during 150 s and then washed with Milli-Q water [3]. 10 μL of GQD-N-S solution (the synthesis of GQDs are shown in Electronic Supporting

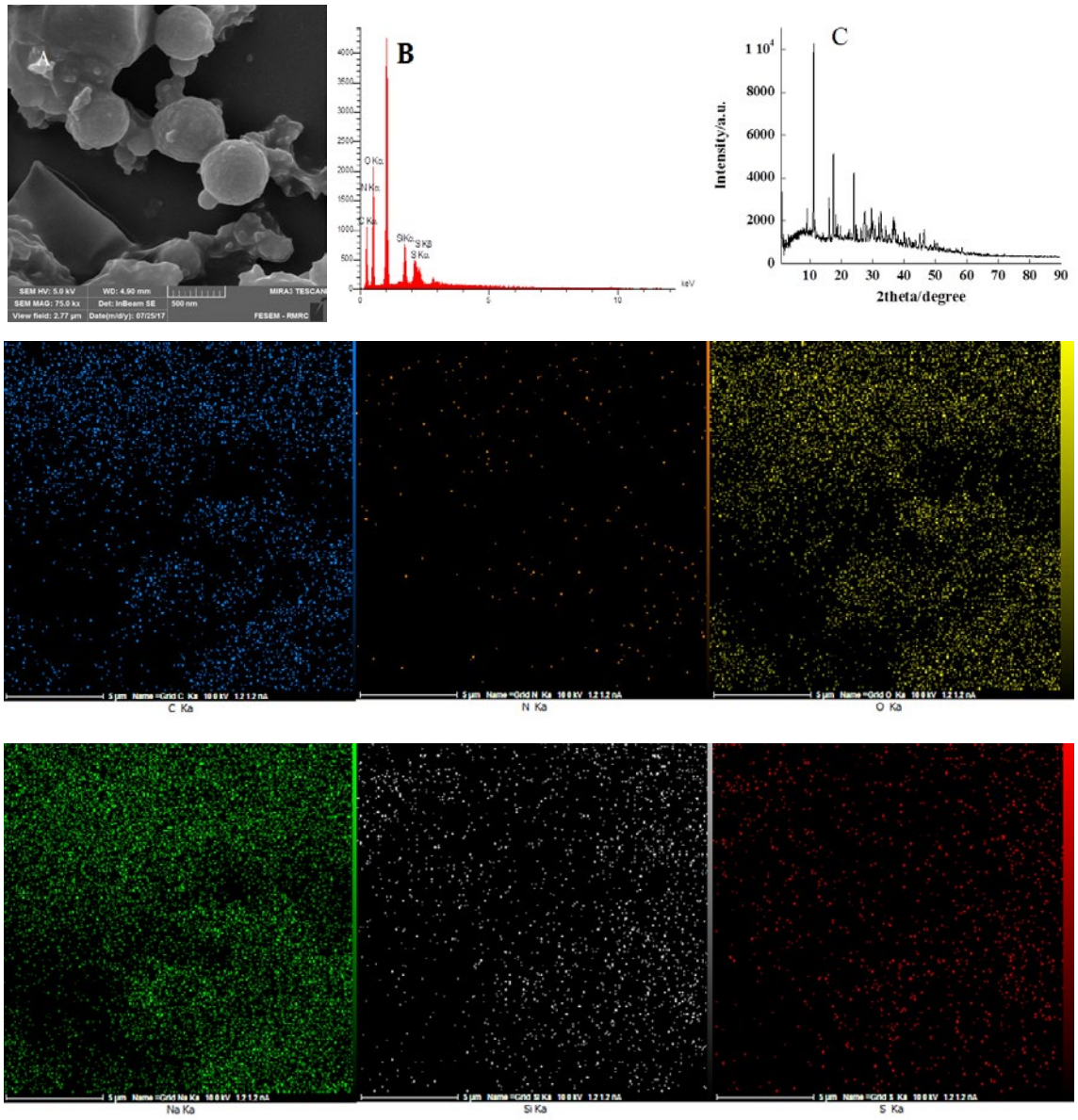
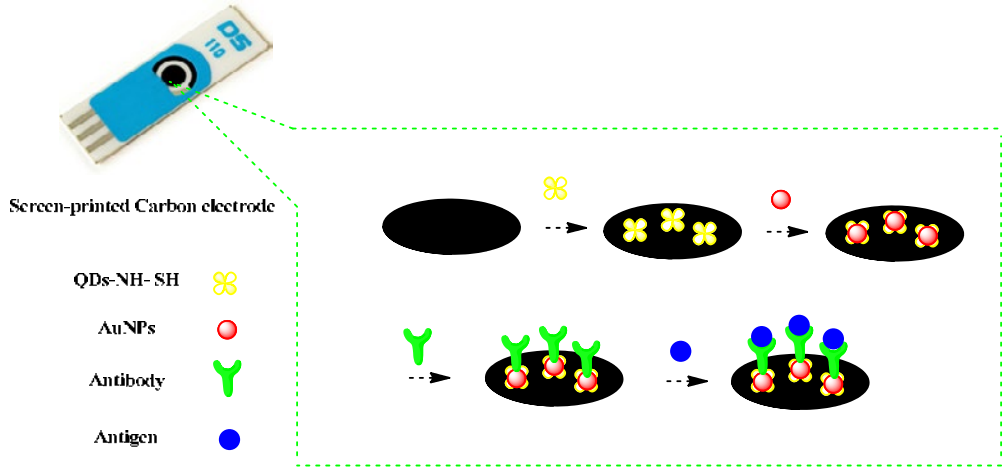


Fig. 1. (A) FE-SEM images of GQDs-N-S, (B) EDAX spectrum, (C) XRD spectra of GQDs-N-S, and (D) elemental mapping of GQDs-N-S.

Material, Fig. S1) was casted on the surface of electrode and dried in the room temperature. In the next stage, 10 µL of AuNPs solution was casted on the surface of the modified SPCE. AuNPs are attached on thiol and amine groups of the GQD-N-S/ SPCE surface after three hours. The AuNPs /GQD-N-S/ SPCE were incubated in anti-HCG (1 mg/mL) solution for about 1 h for interaction of NH_2 groups of anti-HCG molecules with tiol groups present on its surface. Finally, in order to avoid non-specific adsorption, 10 µL of BSA (10%) was dropped on the modified SPCE surface.

Electrochemical detection

A three-electrode system was used to perform measurements. Ag/AgCl (satd. 3.0 M KCl), platinum electrode and SPCE were applied as the reference, counter and working electrodes, respectively. In order to electrochemically characterize the modifications, the electrode was submitted to CV and ESI techniques. EIS measurements were carried out under a bias potential of 0.2 V and a frequency range between 0.1 Hz and 100 kHz with signal amplitude of 5 Mv. The CV analysis was carried out at 50 mV s^{-1} scan rate in a potential



Scheme 1. Schematic illustration of the proposed electrochemical immunosensor for HCG detection.

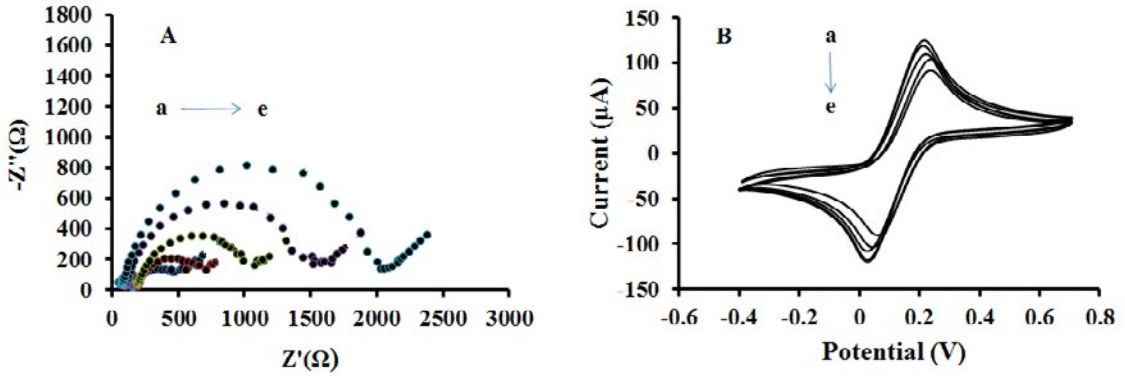


Fig. 2. (A) Recorded EIS for the different steps of the modified electrode: (5 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$, KCl 0.1 mmol L^{-1}), in 0.1 mmol L^{-1} PBS (pH=7.5) at (a) SPCE, (b) GQDs-N-S / SPCE, (c) AuNPs/GQDs-N-S / SPCE, (d) Ab/AuNPs/GQDs-N-S, (e) BSA/Ab/AuNPs/GQDs-N-S. (B) Typical CV studies of $[Fe(CN)_6]^{3-/4-}$ (a) to (e) are the same as (A).

between -0.4- 0.7 using 10 mmol.L^{-1} of $K_3Fe(CN)_6$/ $K_4Fe(CN)_6$ prepared in 0.1 mol.L^{-1} KCl as a redox probe. DPV technique was applied to record the analytical responses. This technique was carried out by scanning the potential of -0.1 to 0.5 V with modulation time of 50 ms and modulation amplitude of 25 mV. A solution containing 5 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ and 0.1 mmol L^{-1} KCl was prepared with phosphate buffer (0.1 mmol L^{-1}, pH = 7.5) and applied as a working solution for determination of HCV.

DISCUSSION

Potassium ferricyanide, as an electrochemical probe, was used to investigate the electrochemical performance of the bare SPCE and modified SPCE. After each immobilization step, the impedance spectroscopy of different modified SPCEs was recorded in 5 mmol mL^{-1} of $[Fe(CN)_6]^{3-/4-}$ solution. As shown in Fig. 2, R_{ct} value was increased from 288.04 Ω for the bare SPCE (a) to 445.35 Ω for the GQD-N-S/ SPCE (b) after casting some of GQD-N-S on the surface of SPCE. Subsequently, solution of AuNPs was casted on the surface of the modified SPCE. After 3 hours, AuNPs were attached on thiol groups of the modified SPCE because of interaction between AuNPs and thiol groups. AuNPs were attached on thiol groups of the GQD-N-S / SPCE surface. In Fig. 2 A (c), after coating with the Au NPs, the electron-transfer resistance (744.58 Ω) of the electrode in phosphate buffer containing $K_3[Fe(CN)_6]$ was apparently larger than that of the GQD-N-S / SPCE. In the next step, by attaching antibody on the electrode with the interaction between amine group of antibody and AuNPs, R_{ct} was increased to 1179.89. After casting of BSA on

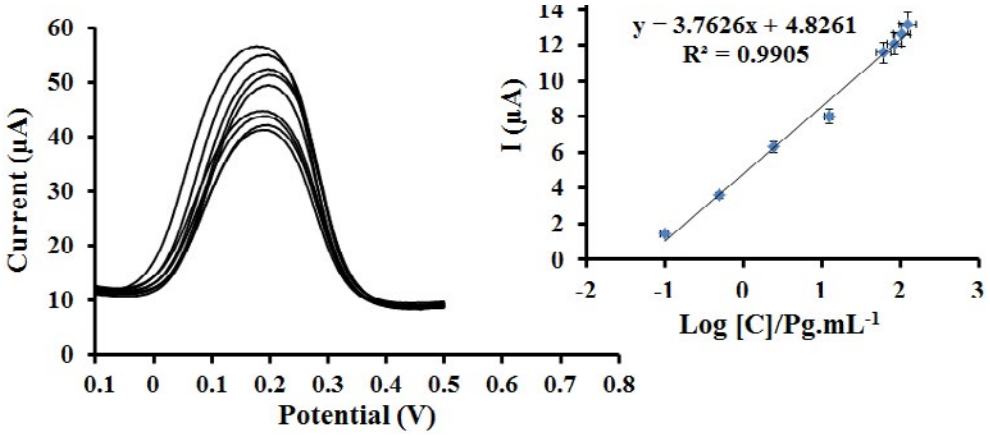


Fig. 3. (A) DPV responses of the immunosensor with increasing concentrations of HCG (from up to down: 0, 0.1, 0.5, 2.5, 12.5, 62.5, 85, 105 and 125 pg mL^{-1}). (B) Calibration curve of the peak current response *versus* the logarithm of HCG concentration. The parameters of DPV are as follows: initial potential, −0.1 V; final potential, −0.5 V; amplitude, 25 mV; modulation time of 50 ms.

the immunosensor, R_{ct} increased in the same way (curve d, R_{ct}= 2253.342 Ω).

CV experiments further confirmed that the QDs-N-S, AuNPs and antibody were successfully attached on the surface of SPCE.The cyclic voltammograms of the probe at different electrodes containing bare SPCE (a), QDs-N-S -modified electrode (b), AuNPs/ QDs-N-S -modified electrode (c) and antibody-immobilized electrodes (d) are shown in Fig. 2B. On treatment with QDs-N-S, the value of peak current decreased after making QDs-N-S film on SPCE. The presence of negative charge caused by thiol groups on the surface of modified SPCE led to $[Fe(CN)_6]^{3-/4-}$ repulsion. AuNPs with negative charge were casted on the surface of electrode and attached on the S and N groups of QDs-N-S. The result showed that the response of redox probe was reduced because of repelling the negatively charged $[Fe(CN)_6]^{3-/4-}$ anions with negative charge of the electrode surface. As shown in Fig. 2 B, the assembly of antibody molecules on AuNPs/ QDs-N-S -modified electrode leads to decrease the cyclic voltammetric response of the electrode. After casting the BSA solution on the surface of immunosensor in order to decrease available active sites, the cyclic voltammetric response decreased even more.

Optimization of method

In order to reach a proper performance of the immunosensor, some important parameters such as pH and incubation time were optimized. The reaction of the antibodies and antigens and amperometric signal were affected by the pH and incubation time. Different phosphate buffer solutions were prepared with pH values from 5.5 to 8.0. As shown in Fig. S1 A, the highest value of electrochemical signal was achieved at pH: 7.5 among different values ranging from 5.5 to 8.0. The incubation time was investigated due to the detection of the sufficient recognition time of target. Due to the formation of antigen–antibody complexes, the amperometric signal increased from 0 to 50 min, and then tended to level off. So, an incubation time of 50 min was selected for the recognition of HCG (Fig. S1 B).

Analytical detection

The immunosensor was used to measure a range of different concentrations containing 0, 0.1, 0.5, 2.5, 12.5, 62.5, 85, 105 and 125 pg mL^{-1} of HCG under optimal conditions. DPV measurements for which one of the concentrations was recorded after incubation of immunosensor in the HCG solution for 50 min. The measurements were carried out in 0.1 mol L^{-1} phosphate buffere solution (pH 7.5) containing 10 mmol L^{-1} $K_3[Fe(CN)_6]$ at 25 °C. Difference between the peaks current related to which one of the concentrations and zero concentration were calculated. The amount of this difference decreased with increasing the concentration of HCG because of the increased number of antigen/antibody complexes. A linear relationship was obtained between difference of current and the logarithm of HCG concentrations (Fig. 3). The regression equation was found to be ΔI (μA) = 3.7626 Log C + 4.8261 and the correlation coefficient R was 0.9905. The limit of detection was

Table 1. Detection of HCG in real samples.

Added (Pg mL^{-1})	Founded (Pg mL^{-1} + RSD%)	Recovery (%)
0.10	0.095±0.20	95
2.5	2.5±0.15	102
60.0	60.2±0.15	100.33
100.0	99.3±0.10	99.30

calculated as 12.5 fg mL^{-1}. The performance of the sensor was contrasted with other sensors in Table S1. Short stages of approach and simple fabrication process are major advantages of the designed immunosensor in comparison with other HCG immunosensors.

Interference study

In the presence and absence of HCG, some biomolecules such as HCV, progesterone, glucose and ascorbic acid that could be present in the physiological samples along with HCG were monitored from amperometric response of the modified electrode. When 3 pg mL^{-1} HCG with 500 times of interfering material was casted on the surface of immunosensor, no considerable differences were observed which is in contrast with the response of the immunosensor related to 3 pg mL^{-1} HCG on the calibration curve. However, a considerable change was found in the response for extra HCG concentration. Variation of the current response caused by the interference was less than 5%. These data showed that the selectivity of the designed immunosensor was good. As shown in Fig. S2, these observations suggest a good specificity of the designed immunosensor.

Reproducibility and stability

The reproducibility of the immunosensor is a key factor in the practical application. Inter-electrode and intra-electrode coefficients of variation were applied to study the reproducibility. The intra-assay precision was investigated by analyzing 100 pg mL^{-1} HCG for five replicate measurements. The relative standard deviation (RSDs) was 3.5%. Similarly, the inter-assay precision was investigated by analysing the same HCG level with five immunosensors and the RSD was 2.9%. The results obtained were good.

Real sample

Standard addition method in serum sample was applied to investigate the feasibility of the immunoassay. Human serum samples were put in a centrifugal filtration tube at 1008 rcf (30 min). Phosphate buffer (0.1 $mol.L^{-1}$) was used to dilution the serum samples (50 times) and different concentrations of HCG were spiked to the samples. As shown in Table 1, the recoveries ranging from 95% to 102% and the RSD ranging from 0.1% to 0.2% were found for different concentrations of HCG in human serum samples. These data indicated that the accuracy of this method was acceptable.

CONCLUSION

The use of nitrogen and thiol -doped GQDs (GQDs-N- S) that increases the loaded antibody and enhances the electrochemical response signal for protein analysis effectively improved the sensitivity of the electrochemical immunosensor. The modified electrode provides a new method for HCG antigen determination and expands the application of GQDs-N-S to modelling other sensors. The developed method provides a promising NP for different analytes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://nanochemres.org/.

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