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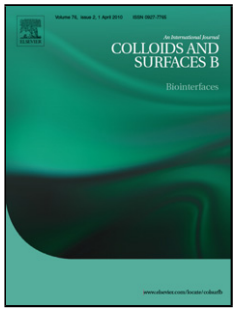

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Highly selective sensing and measurement of microRNA-541 based on its sequence-specific digestion by the restriction enzyme *Hinf1*

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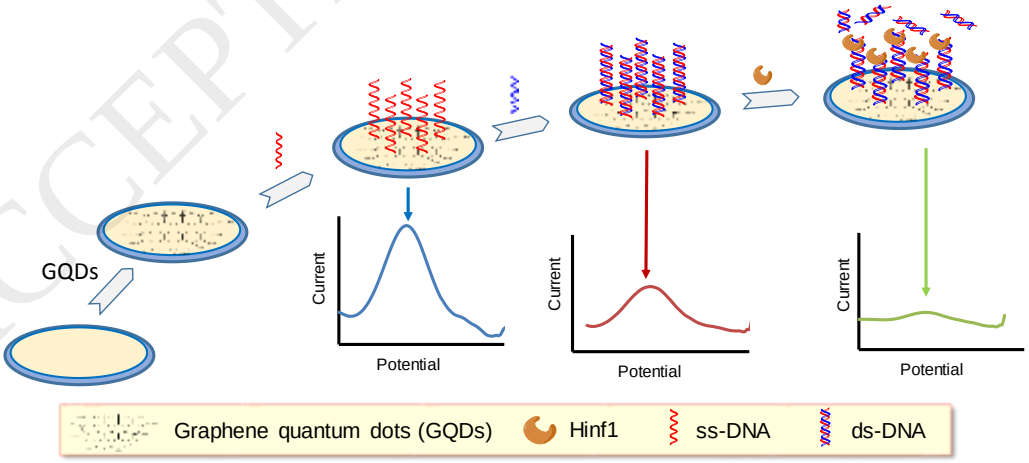
Graphical Abstract



Highlights:

- A genosensor is introduced for microRNA-541 detection based on an enzymatic method.
- Graphene quantum dots (GQDs) are used to modify a pencil graphite electrode (PGE).
- The hybridization event and the enzymatic reactions are studied through guanine signal tracing.
- *Hinf1* is applied as molecular scissors to recognize the specific sequences.
- A linear range of 1.0 fM to 1.0 nM and a detection limit of 0.7 fM were obtained for microRNA-541.

Abstract

In this study, a genosensor is introduced to detect microRNA-541 through an enzymatic digestion method and using a restriction enzyme (RE). *Hinf1* is a type of RE which can cut the double helix DNA at specific sequences. The hybridization event and the corresponding enzymatic reactions are studied through guanine signal tracing on a pencil graphite electrode modified with graphene quantum dots (GQDs/PGE). The stages of fabricating the electrode are monitored by atomic force microscopy, and its electrochemical behavior is studied by cyclic voltammetry. The results indicate that the guanine current response of a 25-mer oligonucleotide of 7-guanine immobilized on the electrode surface decreases after hybridization despite an increase in the number of the guanine bases. Also, after enzyme treatment, the current decreases further due to the separation of a number of guanine bases from ds-DNA. A comparison of the analytical parameters of the proposed method with those of the conventional guanine oxidation method indicates that the linear concentration range in the proposed method, i.e. 1.0 fM to 1.0 nM, is lower than that in the conventional method, i.e. 10.0 pM to 1.0 µM. On the basis of these findings, it is

concluded that the use of *Hinf1* enzyme makes it possible to measure microRNA at a femtomolar level. The selectivity of the designed biosensor has been proved using a non-complementary sequence with a one-base mismatch in the recognition site, rather than a complementary sequence. Finally, the proposed genosensor can be satisfactorily applied to measure microRNA-541 in human plasma samples.

Keywords: Electrochemical genosensor, Restriction enzyme, *Hinf1* enzyme, MicroRNA-541, Graphene quantum dots, Guanine signal

1. Introduction

Restriction enzymes (REs) are molecular scissors which can cut DNA at specific sequences into fragments at or near specific recognition sites known as restriction sites. Type IIP enzymes make up the most important subtype of restriction enzymes that typically have 250-350 amino acids in length and are usually used to selectively recognize ds-DNA sequences containing 4 to 8 base pairs [1]. *Hinf1* is a type IIP enzyme used to recognize ds-DNA sequences that contain a sequence of five base pairs. These pairs start with guanine-adenine (GA) and end with thymine–cytosine (TC). It can also have any base as the middle base pair, as shown in Scheme 1A [2, 3].

The presence of circulating microRNAs (microRNAs) has been proved in various diseases including tumor metastasis [4], viral infections [5], cancer [6, 7], diabetes [8, 9] and tissue injuries after strokes [10]. In such cases, microRNAs serve as potential clinical non-invasive biomarkers. MicroRNAs circulate in a stable form in body fluids (e.g, blood, urine and saliva) inside exosomes. Exosomes are nanoscale vesicles (40-100 nm) that protect numerous macromolecules from their parental cells including mRNA, microRNA, DNA and specific protein biomarkers. Thereafter,

they are taken up by target cells through various mechanisms and convey messages through the delivery of their content which can influence cell physiological pathways [11]. Recently, evidence has been found that the dysregulation of microRNA-541 (25 nt) expression contributes to a wide variety of human diseases such as lung cancer [12, 13], cardiac hypertrophy [14] and type 1 diabetes [15] by affecting both the stability and the translation of mRNAs.

Electrochemical biosensors based on hybridization of DNA have a good sensitivity and specificity in the diagnosis of genetic and infectious diseases. An electrical signal is produced when a target analyte is specifically complemented with short single-stranded DNA which is immobilized on the electrode surface [16]. In label-free detection, signal transduction is induced directly from the oxidation of guanine and adenine, as two important purine bases in DNA strands [17, 18]. However, due to low electron transfer, the response of the direct oxidation of guanine and adenine at the bare surface of electrodes is very poor [19].

So far, different strategies have been employed for signal amplification, such as using different nanomaterials to modify usual electrodes. Also, graphene quantum dots (GQDs), which possess graphene lattices inside the dots, circular or elliptical shapes and abundant edge sites, have been applied to modify electrodes [20]. Owing to their high mobility for charge carriers and large specific surface area, GQDs seem to have a great potential for the oxidation of guanine and adenine.

In this study, a label-free DNA assay was developed to detect microRNA-541 in highly diluted solutions. First, the guanine oxidation in ss-DNA was monitored by DPV. In the next step, ss-DNA was hybridized with microRNA-541 and treated with *Hinf1* enzyme as a restriction enzyme. Then, the guanine signal was recorded in the remaining ds-DNA. The use of the restriction enzyme,

i.e. *Hinf1*, made it possible to detect microRNA-541 in a lower concentration range than in the absence of the enzyme.

2. Experimental

2.1. Instruments, electrochemical measurements, and reagents

All the electrochemical experiments were performed by the AUTOLAB-PGSTAT 30 electrochemical analysis system supplied with the GPES software package (EcoChemic, Netherlands) and a conventional three-electrode system. A renewable pencil graphite electrode (PGE) was used as the working electrode. This electrode included a pencil graphite of HB type with the diameter of 0.9 mm vertically fixed inside a plastic tube with an epoxy adhesive so that only the cross section would be available. The electrical contact with the graphite was made through a copper wire. The reference electrode was a KCl-saturated calomel electrode (SCE), and a platinum wire served as a counter electrode. A Teflon-lined stainless autoclave (100 mL) was used for hydrothermal synthesis. Some atomic force microscope images were obtained using AFM from Nanosurf (Switzerland) and the SEM (scanning electron microscope) image was obtained with Phenom ProX. Differential pulse voltammetry (DPV) assays were conducted with the parameters of the initiative potential of 0.75 V, final potential of 1.2 V, amplitude of 0.025 V, modulation time of 0.05 seconds and a step potential of 0.005 V in a 0.1 M phosphate buffer solution (PBS) with pH 7.4.

The chemical materials required for the preparation of a 0.5 M acetate buffer solution (ABS) with pH 4.8 containing 20.0 mM NaCl, 0.1 M PBS (pH 7.4), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were bought from Sigma Aldrich Company. Certain reagents with analytical grades including K_2HPO_4, KH_2PO_4, NaCl, KCl, NaOH,

HCl, EDTA and citric acid (CA) were obtained from Merck Company and used without further purification. A 0.1 M PBS (pH 7.0) containing 5.0 mM $K_3[Fe(CN)_6]$, 5.0 mM $K_4[Fe(CN)_6]$ (1:1) and 0.1 M KCl was used for cyclic and differential pulse voltammetric experiments. Double distilled water was used to prepare all the experimental solutions, and the oligonucleotide solutions were prepared with ultra-pure and enzyme-free water. The restriction enzyme *Hinf1* 50 U μL^{-1} and a 10X RD blue buffer (1X: 10 mM Tris-HCl, pH 7.5 at 37°C, 10 mM $MgCl_2$, 0.1 mg mL^{-1} BSA) were obtained from Sinaclon Company (Iran). According to the supplier's instructions, *Hinf1* 5 U μL^{-1} was prepared in a blue buffer and kept frozen at -20°C. All the oligonucleotides were purchased as lyophilized powder from Bioneer Corporation (South Korea). The oligonucleotide sequences used for the fabrication of the nanogenosensor were as follows:

H_2N-probe sequence: 5'- H_2N-$(CH_2)_6$- AGT GGG ACC GAC AGC A**GA ATC** CTT T-3'.

Complementary (microRNA-541) sequence: 5'- AAA G**GA TTC** TGC TGT CGG TCC CAC T-3'.

Non-complementary (microRNA-105) sequence with one-base mismatch in the recognition site: 5'-TCA AAT GCT CA**G ACT C**CT GTG GT-3'.

The stock solutions of the oligonucleotides (100.0 µM) were prepared with a 10.0 mM Tris–HCl buffer (pH 8.0) containing 1.0 mM of EDTA. The immobilization solution was a 1.0 M PBS (pH 4.5), also the hybridization and washing solutions were a 0.05 M PBS (pH 7.0) containing 0.3 M of NaCl. The stock solutions of all the oligonucleotides were stored at -20°C until use.

2.2. Synthesis of graphene quantum dots, GQDs

GQDs were synthesized through the one-step hydrothermal method and with citric acid, as a source of carbon [21]. For this purpose, 1.26 g of citric acid and 0.72 g of NaOH were dissolved in 30 mL of water, and they were thoroughly stirred until a clear solution was obtained. The

solution was transferred to a 100 mL Teflon cup inside a stainless steel autoclave and heated at 160°C in an electric oven for six hours. After being cooled to room temperature, the resulting solution was stored in a dark and clean container away from the sun and at the temperature of 4°C. Under these conditions, the prepared GQDs were stable for two months.

2.3. Fabrication of the genosensor

Prior to use, a PGE was thoroughly polished with 0.05 μm alumina-water slurry on a smooth polishing cloth and then carefully rinsed. In all the cases, the PGE was electrochemically pretreated by applying +1.40 V in ABS (0.5 M, pH 4.8) for 30 seconds [22]. Schematic of the genosensor fabrication and its application are shown in Scheme 1B. The fabrication of the genosensor included the following steps:

a. To prepare a GQDs/PGE, GQDs were electrodeposited on the surface of a bare PGE by repeating the potential scans in the range of 0.0 to +1.0 V at the scan rate of 100.0 mV s^{-1} for 30 cycles.

b. The GQDs/PGE was inserted into a coupling reagent containing a 0.5 mM solution with equal volumes of EDC and NHS for 20 minutes to activate the electrode.

c. Immediately, 2.5 μL of an immobilization buffer solution containing a 10.0 μM H_2N-probe was dropped on the activated electrode, and the electrode was incubated in a humid container for one hour. During these step, an ss-DNA/GQDs/PGE was produced by a covalent bond between the 5'-amine group of the capture probe and the carboxyl group of the GQDs via the coupling reagent.

d. In this step, 2.5 μL of the target DNA (complementary or non-complementary) solution was dropped on the ss-DNA/GQDs/PGE surface, and a ds-DNA/GQDs/PGE genosensor was made during 90-min incubation. After both the immobilization and hybridization steps, the modified electrode was gently washed with a washing solution to remove any excess oligonucleotides.

e. Finally, the ds-DNA/GQDs/PGE was incubated with 5.0 µL of *Hinf1* (0.01 U μL^{-1}) at 37ºC for 20 minutes. Then, it was rinsed with a washing buffer. At this step, the prepared electrode was named H/ds-DNA/GQDs/PGE.

3. Results and discussion

3.1. Characterization of the genosensor

In order to confirm the different steps of PGE modification, the electrodeposition of GQDs on the PGE surface and the morphology changes of the electrode surface were investigated using scanning electron microscope (SEM) and atomic force microscopy (AFM), respectively. SEM image of the GQDs/PGE surface is presented in Fig. S1. The luminous points of this image indicate that GQDs have been effectively fixed on the activated PGE surface. Fig. S2 shows the AFM 3D-images and the distribution histogram of roughness at different stages of the genosensor preparation. The root mean square (rms) roughness was found notably higher in the GQDs/PGE than in the bare PGE. The topography of the ss-DNA modified surface suggested that the ss-DNA was strongly adsorbed on the GQDs/PGE surface, and the thickness of the assembled layers increased after hybridization. Finally, *Hinf1* shortened ds-DNA, and, as a result, the rms decreased. The variation in the roughness parameter appropriately proved the modification stages of the genosensor. Further details are presented in the supplementary file.

3.2. Genosensor electrochemical characterization

In order to follow the construction steps of the proposed genosensor, its cyclic voltammetric responses were recorded in a 0.1 M phosphate buffer solution (pH 7.0) containing 0.1 M KCl and 5.0 mM $[Fe(CN)_6]^{3-/4-}$, as a redox probe, during various stages of modification (Fig. 1A). Voltammograms (a) and (b) in Fig. 1A relate to the bare PGE and the GQDs/PGE respectively.

The electrode surface was densely covered with semiconducting GQDs. As a result, the peak current in plot (b) is evidently seen to be lower than that of the bare PGE. Also, the peak potentials separation (ΔE_p) increased at the GQDs/PGE surface. Fig. 1A, voltammogram (c), shows the cyclic voltammogram of the ss-DNA immobilized on the GQDs/PGE electrode surface (ss-DNA/GQDs/PGE). The ss-DNA bond at the GQDs/PGE surface induced electrostatic repulsion between the ss-DNA sugar-phosphate backbone and the redox probe. There were, therefore, a decrease in the peak currents and an increase in the value of ΔE_p, as observed in voltammogram (c). In the next step, the peak current of the ds-DNA decreased further as shown in voltammogram (d). This was due to the hybridization of the target microRNA-541 with the ss-DNA. Generally, in such conditions, the negative charge of ds-DNA has more electrostatic repulsion with the redox probe. After enzyme treatment, a piece of fixed ds-DNA was separated from the upper segment, exactly at the cutting site. Consequently, the peak current slightly increased, but the ΔE_p decreased in voltammogram (e).

As noted above, the electrochemical response of the genosensor was achieved through monitoring the intrinsic electroactivity of guanine. So, the performance of the designed genosensor is considered to be strongly dependent on the content [23, 24] and the availability [25] of guanine in oligonucleotides. To demonstrate this fact, the selectivity of the genosensor was evaluated using the differential pulse voltammetric method. Fig. 1B shows the differential pulse voltammograms of (a) ss-DNA/GQDs/PGE, (b) ds-DNA/GQDs/PGE, (c) ds-DNA/GQDs/PGE after *Hinf1* treatment (H/ds-DNA/GQDs/PGE) and (d) ss-DNA after hybridization with non-complementary oligonucleotide microRNA-105 and after enzyme treatment (H/NC/ss-DNA/GQDs/PGE) in a 0.1 M PBS (pH 7.4). As voltammogram (a) in this figure shows, the oxidation of guanine related to the ss-DNA/GQDs/PGE occurred at a potential of about +0.98 V in the PBS (pH 7.4), and it

produced the highest current. The double strand formation on the PGE surface (ds-DNA/GQDs/PGE) was confirmed when the magnitude of the guanine voltammetric peak current obviously decreased (voltammogram b). This decline in the peak current was because the redox active groups of guanine in the ds-DNA were only partly available for oxidation. It may be considered in comparison with ss-DNA; ds-DNA molecules are less flexible than ss-DNA molecules and, therefore, stand away from the electrode surface [26]. In addition, the DNA orientation is affected by the surface potential. When an attractive (positive) potential is applied, the upper segments of the flexible ss-DNA are pulled downward much more quickly than those of the ds-DNA [26]. In other words, the availability of guanine for electron transfer on the electrode surface is influenced by the differences in the upward and downward motions of DNA. As a result, in this study, the peak current related to the ds-DNA/GQDs/PGE decreased significantly. In order to improve the performance of the genosensor, its electrochemical response was also measured in enzymatic reactions. As illustrated in Scheme 1, *Hinf1* cleaved the DNA double helix at a well-defined point and decreased the guanine content. Therefore, the guanine current response on the H/ds-DNA/GQDs/PGE (voltammogram c) diminished more than that of the ds-DNA/GQDs/PGE (voltammogram b). This is why the difference between the current responses of the ss-DNA/GQDs/PGE and the H/ds-DNA/GQDs/PGE was clearly greater than that between the ss-DNA/GQDs/PGE and the ds-DNA/GQDs/PGE. This greater difference confirmed the greater sensitivity of the method in which the *Hinf1* enzyme was used.

In another experiment, a non-complementary oligonucleotide microRNA-105 (NC) sequence with a single-base mismatch in the recognition site was used to hybridize the probe (ss-DNA). The produced electrode (i.e. NC/ss-DNA/GQDs/PGE) was then reacted with *Hinf1* enzyme to obtain an H/NC/ss-DNA/GQDs/PGE. Fig. 1B, voltammogram (d), shows the voltammetric response of

ss-DNA after hybridization with non-complementary oligonucleotide microRNA-105, and voltammogram (e) presents the current response of the genosensor after enzyme treatment (i.e. in H/NC/ss-DNA/GQDs/PGE) in a 0.1 M phosphate buffer solution (pH 7.4). As it can be seen, the voltammetric response of the H/NC/ss-DNA/GQDs/PGE is obviously higher than that of the ds-DNA/GQDs/PGE. This is due to a couple of facts. First of all, as expected, the non-complementary oligonucleotide poorly paired to the probe, and, therefore, no full-match hybridization occurred between the two strands. Secondly, *Hinf1* did not recognize and cut the paired bases at the restriction site, and, consequently, the peak current decreased. However, the difference between the current responses of the ss-DNA/GQDs/PGE and the H/NC/ss-DNA/GQDs/PGE was clearly less than that between the ss-DNA/GQDs/PGE and the H/ds-DNA/GQDs/PGE. These findings suggest that the proposed method can be used to identify complementary oligonucleotide microRNA-541 sequences from non-complementary oligonucleotide microRNA-105 (NC) sequences with single-base mismatches at recognition sites.

3.3. Optimum conditions for the genosensor construction

In order to find the best conditions for preparing the genosensor, some of the experimental parameters that were used to make the genosensor and affected its response were investigated. Those parameters included the electrochemical modification conditions of PGE, EDC activation time, probe immobilization conditions, hybridization time and enzyme treatment conditions. The data in Fig. S3 (Supporting Information) indicate that an optimal response is obtained when certain values are implemented in the construction of a genosensor. These values are a) number of potential cycles: 30, b) EDC/NHS activation time: 20 minutes, c) concentration of ss-DNA: 10.0 μM, d) immobilization time: 60 minutes, e) hybridization time: 90 minutes, f) *Hinf1* concentration:

0.01 U μL^{-1}, and f) enzyme reaction time: 20 minutes. The details of the optimal conditions for the genosensor construction are reported in the Supporting Information file.

3.4. Quantitative measurement of microRNA-541

In this study, the analytical performance of the proposed genosensor was investigated by comparing the guanine signal of ss-DNA before and after hybridization with different concentrations of the target DNA using differential pulse voltammetry. The voltammograms were obtained under optimized experimental conditions, as shown in Fig. 2. They suggest that the current response of an ss-DNA/GQDs/PGE (I_p) is more than that of a ds-DNA/GQDs/PGE (I_t). It is also suggested that I_t decreases with an increase in the concentration of microRNA-541. The inset of Fig. 2 shows the variation of the difference between the current response (ΔI_H) of the ss-DNA/GQDs/PGE (I_p) and that of the ds-DNA/GQDs/PGE for different concentrations of microRNA-541, (I_t), ($\Delta I_H = I_p - I_t$) versus the logarithm concentration of microRNA-541. The calibration plot indicates that the variation of ΔI_H versus the concentrations of microRNA-541 is linear in the concentration range of 10.0 pM to 1.0 µM.

Furthermore, the effect of enzyme treatment on the efficiency and performance of the genosensor was evaluated. In this step, a similar procedure was used to measure microRNA-541, and the voltammograms of the ds-DNA/GQDs/PGE after treatment with *Hinf1* enzyme (H/ds-DNA/GQDs/PGE) were recorded (Fig. 3). Then, the difference between the current responses (ΔI_E) of the voltammograms of the ss-DNA/GQDs/PGE (I_p) and the H/ds-DNA/GQDs/PGE for different concentrations of microRNA-541 (I_e) against the logarithm of the microRNA-541 concentration were plotted and considered as the calibration plot (Fig. 3, inset). The results indicated that the difference of the current responses ($\Delta I_E = I_p - I_e$) of the proposed method is linear in the microRNA-541 concentration range of 1.0 fM to 1.0 nM.

By comparing the two calibrating plots in the insets of Figures 2 and 3, one can conclude that the sensitivity (represented by the slopes of the calibration plots) of both methods to measure microRNA-541 is almost the same. However, the concentration linear range for the second method, which is 1.0 fM to 1.0 nM, is lower than that for the first method, which is 10.0 pM to 1.0 μM. These findings suggest that, by using *Hinf1* enzyme, it is possible to measure microRNA in lower concentration ranges. The calibration plot in the inset of Fig. 3 and $3s_b/m$ equation were used to estimate the limit of detection (LOD) of microRNA-541 at the proposed genosensor. In that equation, s_b is the standard deviation of the blank measurements, and m is the slope of the calibration plot. Based on this description, the LOD of 0.7 fM was calculated to measure microRNA-541 quantitatively. The quantitative measurement results obtained on the basis of guanine oxidation signals are compared in Table 1. As the table shows, the proposed genosensor and the current response of the H/ds-DNA/GQDs/PGE can be used to measure microRNA-541 in a low concentration range. In addition, the detection limit of microRNA-541 is significantly decreased.

The reproducibility of the prepared genosensor was investigated by measuring different batches containing 1.0×10^{-12} M microRNA-541. The average value of ΔI_E and the relative standard deviation (RSD) of three independent genosensor were found to be 151.1 ± 0.8 nA and 9.69% (n = 3) respectively. This result indicates that the reproducibility of the genosensor is acceptable. It should be noted that the genosensor cannot be reused for microRNA-541 measurement because the oxidation of guanine is an irreversible process. It is, therefore, impossible to evaluate the stability and repeatability of the genosensor. The overall conclusion is that, by practicing the method developed in this study, the proposed genosensor can be of benefit in the clinical diagnosis and quantitative measurement of microRNA-541 in very diluted solutions.

3.5. Quantitative measurement of microRNA-541 in human plasma

Healthy human plasma was received from a local clinical laboratory (Sina Pathobiology Laboratory, Yazd, Iran) and used as a real sample. The original concentration of microRNA-541 in the freshly collected plasma samples is considered to be zero because of the very low abundance of this molecule in the plasma [37]. A portion of the sample diluted with PBS (at the ratio of 1:10) was used as a blank solution to which certain amounts of microRNA-541 were then added. The results of three repeated measurements using the proposed method are presented in Table 2. The recovery rates of 91.1% to 114.6% and relative standard deviations of 6.7% to 7.1% were obtained for microRNA-541 in the concentration range of 1.00×10^{-13} – 1.00×10^{-9} M. The results indicate that the proposed genosensor can satisfactorily be used to measure microRNA-541 in real samples.

A similar procedure was used to investigate the hybridization of ss-DNA with different concentrations of a non-complementary sequence (microRNA-105) in diluted plasma. The voltammetric responses (ΔI_E) of the ss-DNA/GQDs/PGE (I_p) and the H/ds-DNA/GQDs/PGE for different concentrations of microRNA-541 (as a complementary sequence) or microRNA-105 (as a non-complementary sequence), (I_e), are compared in Fig. S4. As it can be seen, the ΔI_E value is enhanced by an increase in the microRNA-541 concentration, but the response of the genosensor to microRNA-105 is negligible. These findings clearly confirm that the proposed genosensor is capable of both detecting the presence of microRNA-541 and distinguishing it from non-complementary sequences in human serum samples.

4. Conclusion

One of the main objectives of this study was to introduce the use of a restriction enzyme as a powerful tool for sequence-specific digestion that this property made it possible to detect microRNA-541 in a low concentration range than in the absence of the enzyme. Here a label-free

DNA assay was developed to detect microRNA-541 in very diluted solutions. First, guanine oxidation in ss-DNA was monitored by DPV. In the next step, ss-DNA was hybridized with microRNA-541 and treated with *Hinf1* as a restriction enzyme. Then, the guanine signal was recorded in the remaining ds-DNA. The findings of the study make it safe to claim that the proposed method can be used to distinguish the complementary oligonucleotide microRNA-541 sequence from the non-complementary oligonucleotide microRNA-105 sequence with the single-base mismatch at recognition sites.

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Legends of figures and tables

Scheme 1. (A) Schematic display of ds-DNA cutting mechanism by *Hinf1* enzyme (a) before cleavage, (b) after exposure to enzyme where N = A, C, G, T and (c) cutting at the restriction site. (B) Schematic representation of the genosensor fabrication and its application for detection of microRNA based on enzymatic digestion method and use of the restriction enzyme of *Hinf1*.

Fig. 1. (A) Cyclic voltammograms of (a) bare PGE, (b) GQDs-modified PGE (GQDs/PGE), (c) ss-DNA immobilized on the GQDs/PGE surface (ss-DNA/GQDs/PGE), (d) ss-DNA after hybridization with the complementary DNA (ds-DNA/GQDs/PGE) and (e) ds-DNA/GQDs/PGE after the *Hinf1* treatment step (H/ds-DNA/GQDs/PGE) in a 0.1 M phosphate buffer solution (pH 7.0) containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl. (B) Differential pulse voltammograms of (a) ss-DNA immobilized on the GQDs-modified PGE surface (ss-DNA/GQDs/PGE), (b) ss-DNA after hybridization with the complementary oligonucleotide microRNA-541 (ds-DNA/GQDs/PGE), (c) ds-DNA/GQDs/PGE after the *Hinf1* treatment step (H/ds-DNA/GQDs/PGE), (d) ss-DNA after hybridization with the non-complementary oligonucleotide microRNA-105 (NC/ss-DNA/GQDs/PGE), and (e) after enzyme treatment (H/NC/ss-DNA/GQDs/PGE) in a 0.1 M phosphate buffer solution (pH 7.4)

Fig. 2. Differential pulse voltammograms of (a) ss-DNA immobilized on the GQDs-modified PGE surface (ss-DNA/GQDs/PGE) and ss-DNA after hybridization with the complementary oligonucleotide microRNA-541 (ds-DNA/GQDs/PGE) in different concentrations: (b) 1.0×10^{-11} M, (c) 1.0×10^{-10} M, (d) 1.0×10^{-9} M, (e) 1.0×10^{-8} M, (f) 1.0×10^{-7} M, (g) 1.0×10^{-6} M. The inset shows the calibration plots of the changes in the ΔI_H value ($\Delta I_H = I_p - I_t$) *vs.* the logarithm concentration of microRNA-541. I_p and I_t refer to the peak current of DPV corresponding to

guanine oxidation at the ss-DNA/GQDs/PGE and the ds-DNA/GQDs/PGE. The error bars are estimated from the relative standard deviations of three experiments.

Fig. 3. The DPVs obtained for different concentrations of the target: (a) 1.0×10^{-15} M, (b) 1.0×10^{-14} M, (c) 1.0×10^{-13} M, (d) 1.0×10^{-12} M, (e) 1.0×10^{-11} M, (f) 1.0×10^{-10} M, (g) 1.0×10^{-9} M. The inset shows the calibration plots of the changes in the ΔI_E value ($\Delta I_E = I_p - I_e$) *vs.* the logarithm concentration of microRNA-541. I_p and I_e refer to the peak current of DPV corresponding to the guanine oxidation at the ss-DNA/GQDs/PGE and the ds-DNA/GQDs/PGE after enzyme treatment (H/ds-DNA/GQDs/PGE). The error bars are estimated from the relative standard deviations of three experiments.

Table 1. Comparison of the analytical parameters obtained from the guanine oxidation signals corresponding to different targets reported in the literature with those of the proposed genosensor

Table 2. Quantitative determination and recovery results (n = 3) of various amounts of the complementary microRNA-541 spiked in diluted human plasma

Scheme 1.

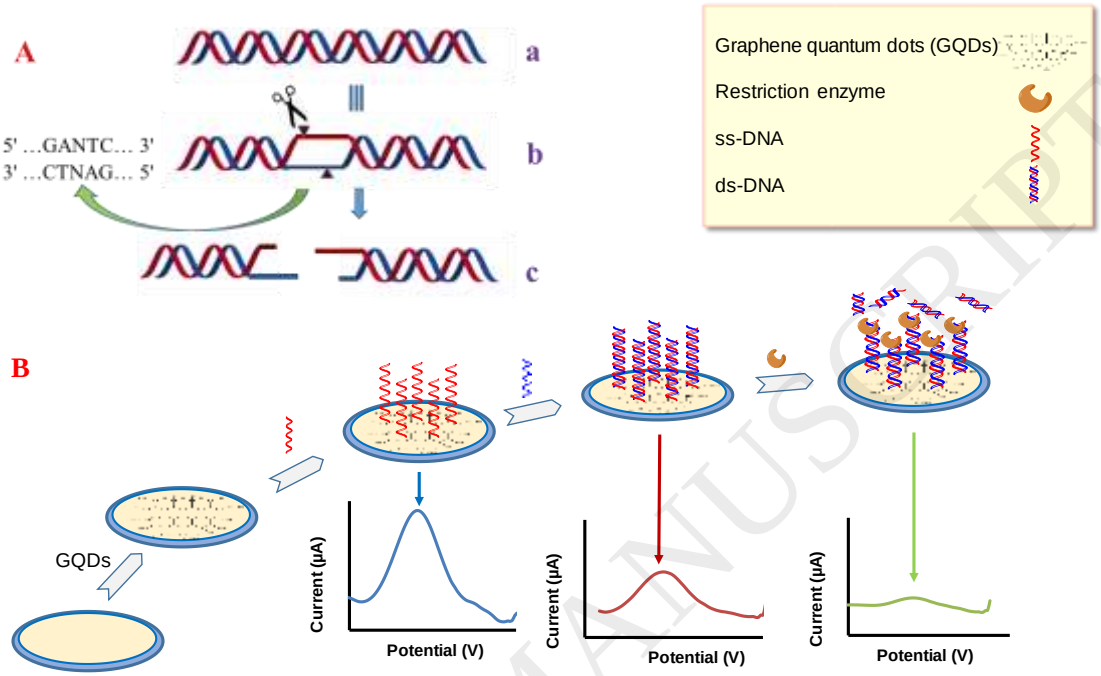

Fig. 1

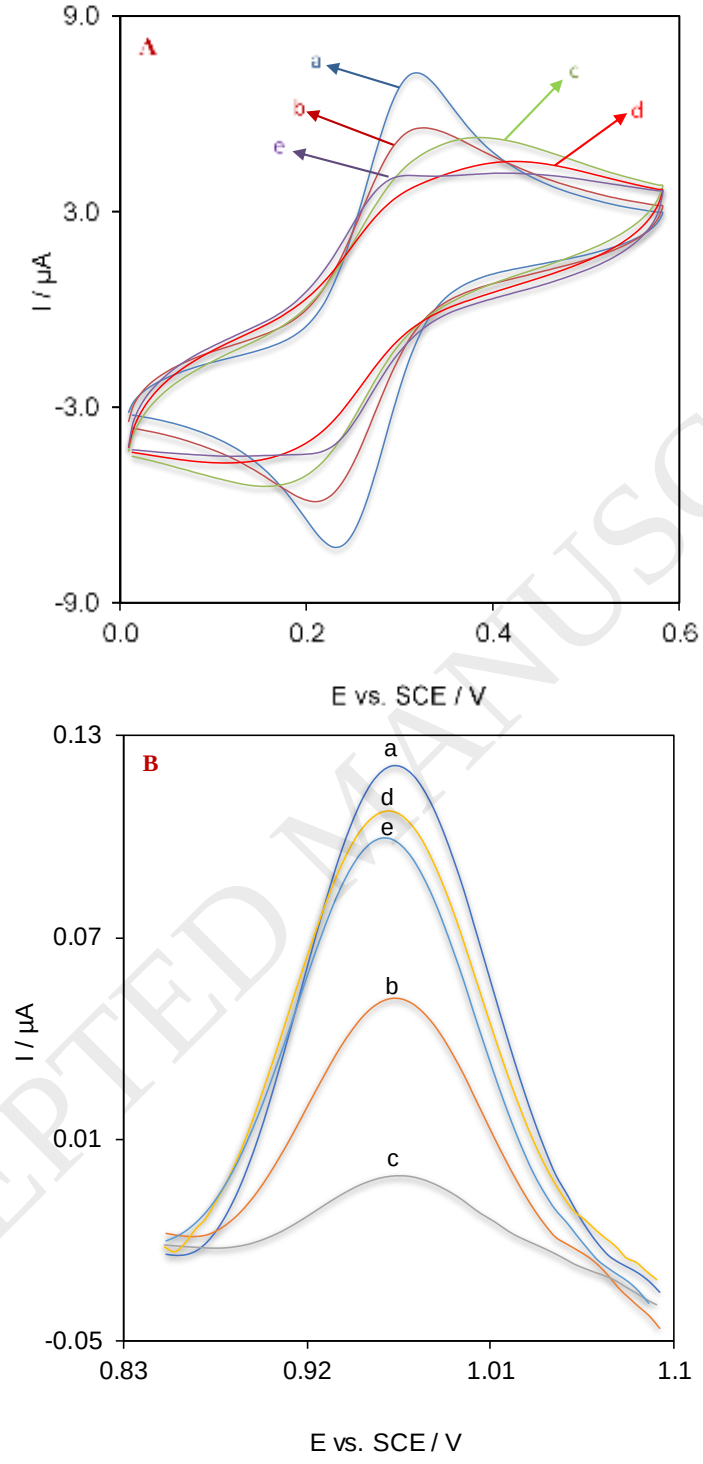

Fig. 2

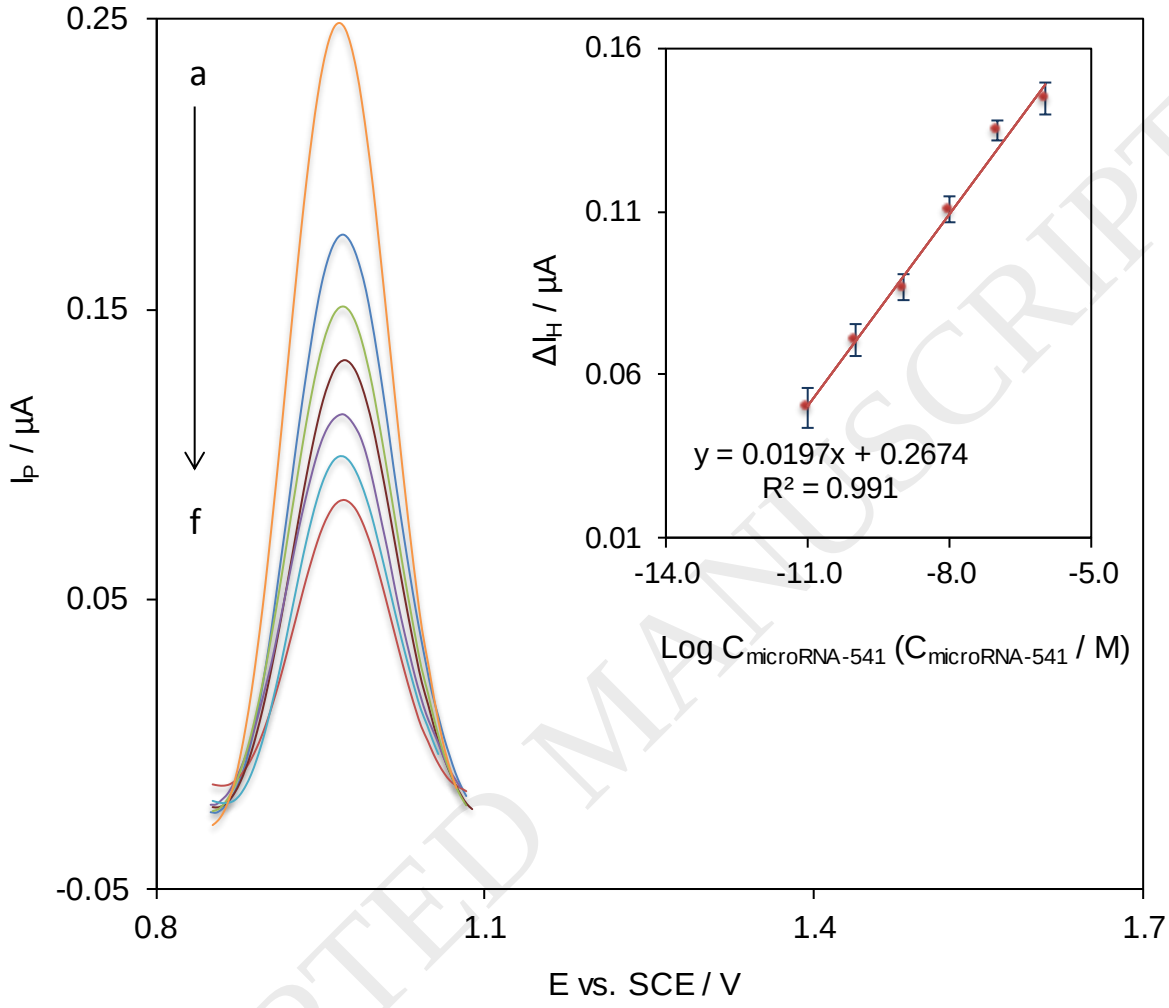

Fig. 3

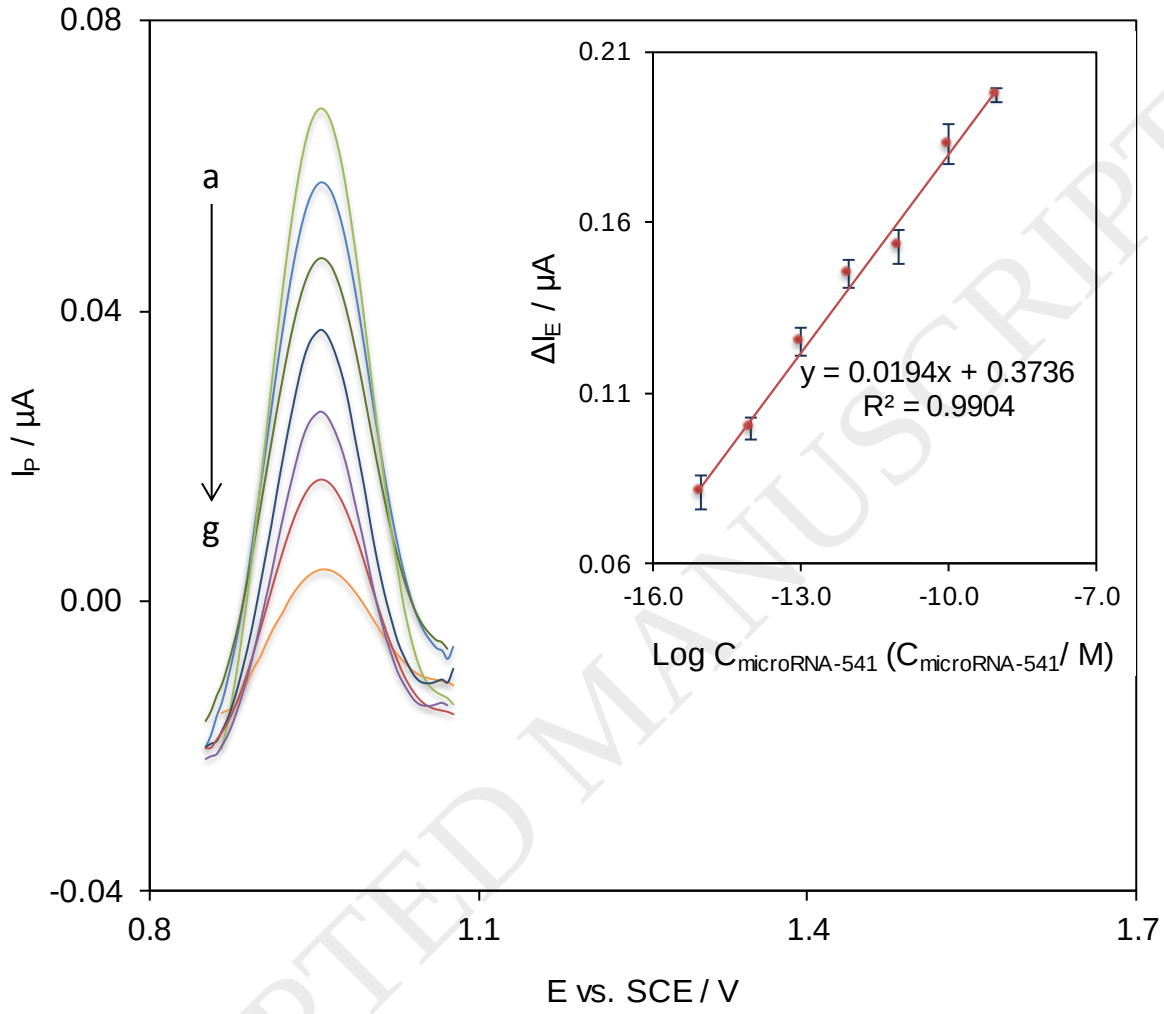

Table 1

Electrode	Method	Target	Linear range	LOD	Ref.
GOB/PGE	DPV	fs-DNA	5-20 μg mL^{-1}	1.11 μg mL^{-1}	[27]
G2-PS/PGE	DPV	DNA	20-80 μg mL^{-1}	4.20 μg mL^{-1}	[28]
PGE	DPV	DENV-3	10-100 nM	3.09 nM	[29]
PGE	DPV	IL-2 DNA	1000-2300 pM	69 pM	[30]
PGE	DPV	HPV16	40–5000 pg μL^{-1}	16 pg μL^{-1}	[31]
CB/PGE	EIS	microRNA-125a	1-2000 nM	10 pM	[32]
PGA/PGE	SWV	HCV1a	50-1000 nM	40.60 nM	[33]
GO/PGE	DPV	HCV1a	0.1-500 nM	43 pM	[34]
MWCNT/GCE	DPV	microRNA-24	1-1000 pM	1 pM	[35]
SPEs	DPV	microRNA-122	---	0.005 μM	[36]
GQDs/PGE	DPV	microRNA-541	1.0 fM -1.0 nM	0.7 fM	This work

GOB: Benzoic acid-functionalized graphene oxide; G2-PS: Succinamic acid functionalized second generation poly(amidoamine) dendrimer; PGE: Pencil graphite electrode; CB: Carbon black; PGA: Poly(l-glutamic acid); GO: Graphene oxide; MWCNT: Multi-walled carbon nanotube; GCE: Glassy carbon electrode; SPEs: Screen-printed graphite electrodes; GQDs: Graphene quantum dots; fs-DNA: Fish sperm DNA; DENV-3: Dengue virus serotype-3; IL-2: Interleukin-2; HPV16: Human papillomavirus 16 type; HCV1a: Hepatitis C virus 1a; DPV: Differential pulse voltammetry; EIS: Electrochemical Impedance Spectroscopy; SWV: Square wave voltammetry.

Table 2

Sample	Added / M	Detected / M	Recovery (%)	RSD (%)
Plasma	1.00×10^{-9}	9.11×10^{-10}	91.1	7.1
	1.00×10^{-11}	1.15×10^{-11}	114.6	7.0
	1.00×10^{-13}	1.03×10^{-13}	103.4	6.7