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Detection of plasma miR-223 by a novel label-free graphene oxide/gold nanocomposite immunosensor in colorectal cancer patients: An electrochemical biosensor approach

Abolfazl Akbari [a,1], Hadi Hashemzadeh [b,1], Zahra Shokati Eshkiki [c], Mohsen Masoodi [a], Seidamir Pasha Tabaeian [a,d,**], Hossein Naderi-Manesh [b,***], Ali Akbar Zare [e], Shahram Agah [a,*]

[a] Colorectal Research Center, Iran University of Medical Sciences, Tehran, Iran
[b] Department of Nanobiotechnology/Biophysics, Faculty of Bioscience, Tarbiat Modares University, Iran
[c] Alimentary Tract Research Center, Clinical Sciences Research Institute, Imam Khomeini Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[d] Department of Internal Medicine, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[e] Recombinant Proteins Department, Breast Cancer Research Center, Motamed Cancer Institute, ACECR, Tehran, Iran

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ABSTRACT

MicroRNAs (miRNAs) are one group of the pertinent molecular biomarkers in the early detection of cancers, especially colorectal cancer. A simple and label-free nanostructured electrochemical biosensor was fabricated to detect miR-223, a colorectal cancer miRNA, by a vertical arrangement of graphene oxide (GO) decorated with gold (Au) Nano-flower nanostructures (GO@Au-NS). The use of high surface-to-volume aspect ratio nanostructures with excellent electroactivity that contain thiolated DNA probes (Cap-223) could increase the accessibility, chance, and recognition performance of miR-223. The substrate characteristics are scanned by HRSEM, EDX, and electrochemical techniques. Furthermore, the successful immobilization of Cap-223 on GO@Au-NS and their hybridization with targeted miR-223 macro-molecules were investigated by electrochemical techniques (CV, EIS, and DPV). The electroactivity of the working electrode before and after GO@Au-NS fabrication showed a significant difference. A wide range of linear detection of miR-223 by a fabricated biosensor was from zM to nM, with a lower detection limit (LOD) of 0.012 aM. The selectivity of the nanostructured miR-223 biosensor was also investigated by studying the discrimination ability of mismatched miRNAs sequences, which resulted in high selectivity for the biosensor. In addition, its efficiency in the detection of miR-223 in a real sample (human serum) showed a remarkable ability of the biosensor to detect it with a low percent of relative standard deviation (RSD = 5.7%). The developed electrochemical biosensor exhibited great potential for miRNA-based early detection of colorectal cancer and had excellent sensitivity, and selectivity, making it helpful for use in translational medicine research and clinical applications.

1. Introduction

Currently, colorectal cancer (CRC), as the second deadliest cancer and the third most common cancer in the world, is one of the most important global public health challenges. By implementing screening programs, developed countries have been able to reduce more than 40% of deaths (Xi and Xu 2021). Colorectal and colon cancers can be significantly controlled with early-stage screening/diagnosis, allowing us to finally find the golden age of treatment. There are a number of available diagnostic approaches for early detection of the CRC, such as carcinoembryonic antigen (CEA), colonoscopy, fecal occult blood test (FOBT), carbohydrate antigen 19–9 (CA19-9) *etc*., with their advantages and disadvantages (specificity and sensitivity) (Inadomi 2017). The advancements in understanding and finding CRC diagnostic modalities have led to increased therapy options and inhibited its metastases (Xi and Xu 2021).

Most CRC diagnostic strategies are invasive, cost-effective, have low

* Corresponding author. Colorectal Research Center, Rasoul-e- Akram Hospital, Sattarkhan Ave, Niyayesh St, Tehran, Iran.
** Corresponding author. Colorectal Research Center, Rasoul-e- Akram Hospital, Sattarkhan Ave, Niyayesh St, Tehran, Iran.
*** Corresponding author.
E-mail addresses: aptabaiyan@gmail.com (S.P. Tabaeian), Naderman@modares.ac.ir (H. Naderi-Manesh), agah.sh@iums.ac.ir (S. Agah).
[1] These authors have contributed equally in this work as first authors.

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Abbreviation list

Graphene oxide nano-wall and gold nanostructure (GO@Au-NS)
MicroRNAs (miRNAs)
single strand DNA (ss-DNA)
bovine serum albumin (BSA)
beta mercapto ethanol (βME)
high resolution scanning electron microscopy (HRSEM)
energy dispersive X-Ray Analysis (EDX)
Fourier transform infrared (FT-IR)
capture DNA probe again miR- 223 (Cap-223)
colorectal cancer (CRC)
polymerase chain reaction (PCR)
gold nanoparticles (AuNPs)
graphene oxide (GO)
micro RNA (miRNA)
fluorine-doped tin oxide (FTO)
hydrochloric acid (HCl)
etheylenediaminetetraacetic acid (EDTA)
deuterium-depleted water (DDW)
ethyl (dimethylamino propyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS)
cyclic voltammetry (CV)
electrochemical impedance spectroscopy (EIS)
and differential pulse voltammetry (DPV)
Dithiothreitol (DTT)
Disodium phosphate (Na_2HPO_4)
grazing incidence X-ray diffraction (GIXRD)
limit of detection (LOD)
standard deviation (SD)
relative standard deviation (RSD)

accuracy, and are only able to find biomarkers in advanced or late stages of CRC. Hence, developing a reliable, rapid, and cost-effective CRC sensing strategy is an important challenge. Scientists have recently attempted to develop novel strategies for early detection of CRC using technologies such as microRNAs (miRNAs). The miRNAs play an important role in many metabolic and cellular regulations, especially in controlling cell proliferation, differentiation, and survival. Aberrant expression of miRNAs has a critical role in the pathology of many diseases, including cancer (Kashi and Einollahi 2022).

The expression of miRNAs can be measured by various molecular methods, such as PCR and microarray techniques. Due to the small size of miRNAs, these methods are all based on PCR, which has become very popular in recent years. According to the expression level of miRNAs, they can be used to diagnose the type of cancer, the clinical stage, and other clinical characteristics of tumors. The use of miRNAs in cancer classification is significantly more efficient than mRNAs, and they can be used as a tool to diagnose the type of cancer and its clinical stage (Kashi and Einollahi 2022; Vychytilova-Faltejskova et al., 2016). The miR-223 have been reported in different cancers (Mahmoud et al., 2022; Zhang et al., 2014) in which this non-coding RNA can be served as an important CRC biomarker and proposed to be valid and reliable for diagnosis as well as prognosis (Zhang et al., 2014). However, early detection of the cancer-related miRNAs by conventional techniques (*i.e.* PCR, and northern blotting), do not meet current demands due to limited sensitivity and also because they are complex or require special laboratory skills (Ye et al., 2019).

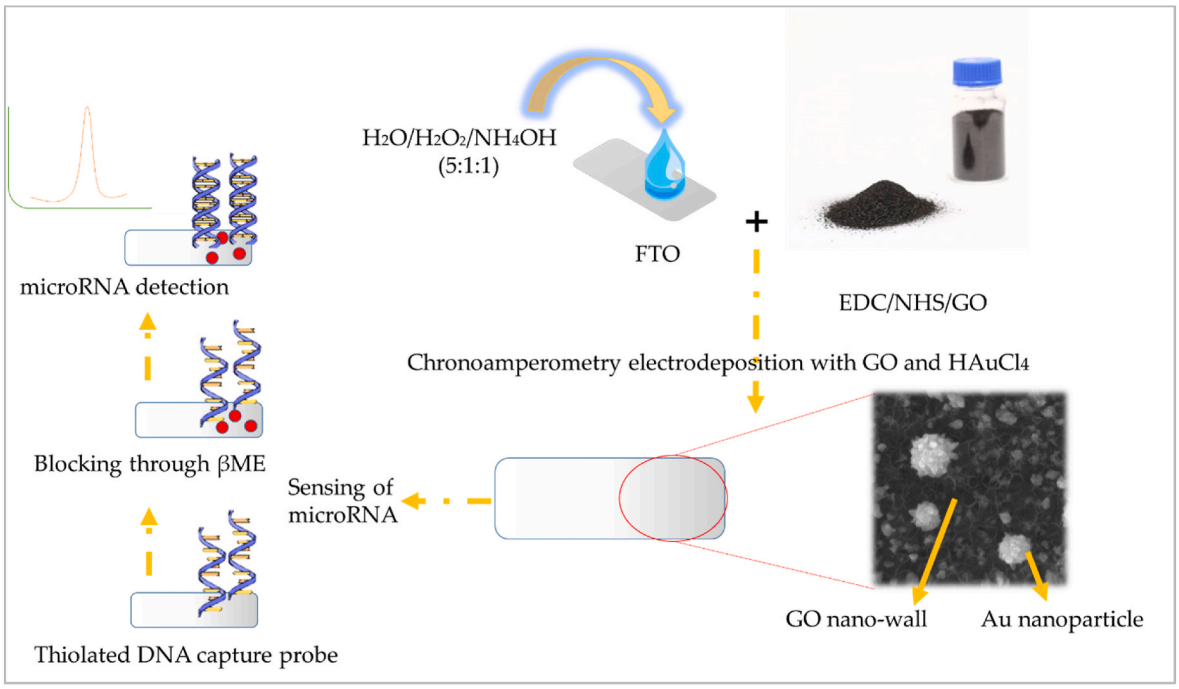
There are numerous nucleic acid molecule detection assays (signal readout techniques) such as electrochemiluminescence, electrochemistry, fluorescence, colorimetry, and so on (Ye et al., 2019). Developed biosensors integrated with nanomaterials have revolutionized sensing platforms for early detection of disease biomarkers. Nanomaterial-based biosensors as platforms for electrochemical miRNA detection are more sensitive, selective and can create better diagnostic signals. A large number of nanomaterials such as gold nanoparticles (AuNPs), graphene oxide nanosheets, magnetic nanoparticles and *etc.* have been applied within studies as biosensors for miRNAs detection. In the case of electrochemical sensing, the use of nanomaterials, due to their excellent electrical conductivity, high surface area, and ease of functionalization, makes them a good candidate and strong tool for improving performance and serving as an alternative to traditional detection techniques (Ye et al., 2019). Biocompatibility, strong surface-plasmon resonance absorptions, easy synthesis and functionalization, and a high surface area to volume aspect ratio make gold nanostructured platforms an excellent candidate for biosensing approaches (Borghei et al., 2018). The presence of Au-NPs in electrochemical biosensors can greatly enhance the sensitivity due to their high electrocatalytic activity and excellent electron-transfer characteristics (Yu et al., 2003). Fabricating robust electrochemical biosensing substrates with high sensitivity can be further tailored by synthesizing nanocomposites. The detection of cancer-related miRNAs by gold nanostructured platforms through target-triggered approaches can enhance the performance of electrochemical biosensors. Ultrasensitive miRNA detection via nanostructured electrochemical platforms is more accurate due to the quantification of miRNA. On the other hand, graphene oxide (GO) nanosheet owing to distinct properties such as thermal/chemical resistance, excellent electrical characteristics, and finally a high surface-to-volume aspect ratio, has attracted great attention as a potential nanostructure for a variety of uses in different *in vitro* and *in vivo* biomedical applications (Lehner et al., 2021). However, the poor intrinsic biocompatibility and difficult covalent biofunctionaliziation of GO-based nanostructures restrict their biological and biosensing applications. The use of GO-based nanostructures integrated with gold nanoparticles, as a platform for sensing approaches helps physicians and scientists detect diagnostic biomarkers early by creating a powerful and sensitive electrochemical biosensing platform. Electrochemical fabrication of nanostructured platforms has emerged as a novel method for the environment-friendly production of gold-graphene-based nanomaterials in a scalable way (Gao et al., 2010; Li et al., 2015).

In this study, as shown in schematic Fig. 1, we present a facile and robust nanostructured platform for miR-223 as a diagnostic biomarker for colorectal cancer. The GO-based nanosheet was chemically synthesized and electrochemically deposited on the FTO (fluorine-doped tin oxide) electrodes to fabricate the graphene oxide Nano-wall. The $HAuCl_4$ salt was used to fabricate and decorate gold Nano-flower like those on the graphene oxide Nano-wall. After that, the thiolated DNA capture probe was immobilized to the gold nanoparticles and finally miR-223 was detected electrochemically in a standard solution and real samples (human plasma). The presented biosensor for the ultralow concentration of miR-223 is a powerful tool for biomedical applications.

2. Materials and methods

2.1. Chemicals and materials

The $HAuCl_4.3H_2O$, Graphite powder (99.99% purity), H_2SO_4, $KMnO_4$, HCl, potassium ferricyanide [$K_3Fe(CN)_6$], potassium ferrocyanide [$K_4Fe(CN)_6$], potassium chloride (KCl) were acquired form Sigma-Aldrich, the FTO or Fluorine-doped tin oxide (2.2 mm thickness and 15 Ωsq^{-1} surface resistance) were bought from Nanogostar Sepahan (I.R.Iran).



Schematic Fig. 1. The graphical abstract of the immunosensor procedure to fabrication of biosensor from surface modification and GO treatment to microRNA detection.

2.2. Nanostructured platform fabrication

2.2.1. GO synthesis

At the first step, the graphene oxide nanosheet was prepared. The GO was synthesized by Hummer protocol with some modification (Ghorbani et al., 2021; Hashemzadeh et al., 2020; William et al., 1958). In this modified method, potassium permanganate was used instead of nitric acid, which has a stronger oxidizing effect. The $KMnO_4$ causes the graphite plates distance to be further apart and improved quality of obtained GO product. Briefly, first sulfuric acid (98%) and then graphite powder (2 g) were added in the Erlenmeyer flask on an ice bath with slow stirring. Then $KMnO_4$ (6 g) was slowly added to the solution (in several steps) and it was stirred for several hours. Finally, the product was concentrated with deuterium-depleted water (DDW) with H_2O_2 (30%) several times to remove the sulfuric acid. A high quality graphene oxide was obtained in an oven at 80 °C.

2.2.2. GO and FTO activation

At the second step, the graphene oxide nanosheet (100 mg) was immersed in solution of NHS (0.1 mM)@EDC (0.4 mM) for 1 h (1 mL volume). Then the 15 mL DDW was added to previous activated graphene oxide solution and applied for further electrochemical deposition on FTO surface. The FTO coated glass (20 × 8 mm^2) was cleaned with soap detergent solution, acetone, and DDW in ultrasonic bath and hot plate successively. Then, to form hydroxyl groups on FTO surface, its substrate was treated with a solution containing H_2O/H_2O_2 (30%)/NH_4OH (5:1:1 ratio, V/V) at RT for 90 min, followed by rinsing with DDW.

2.2.3. GO electrodeposition and gold nanoflower fabrication on the FTO

After washing the FTO coated glass and activated with mentioned solution, they were subjected to electrochemical deposition of activated GO to fabricate a thin film layer of graphene oxide Nano-wall. The electrochemical growth (Chronoamperometry) of synthesized GO was carried out in GO solution activated by EDC/NHS (15 min at 5 V, high voltage) on the activated FTO surface. A uniform GO vertically arrayed and furnished on the FTO was gently rinsed several times using DDW to remove un-attached GO and by-products. In the next step, further chronoamperometry applied for gold nanoflower decorating on the GO Nano-wall. To this end, the $HAuCl_4.3H_2O$ (0.5 mM) solution used for fabricating gold nanoflower (15 min at 0.1 V, low voltage) followed by rinsing with DDW.

Finally, the FT-IR, FESEM, and EDX of nanostructured electrodes were taken to characterize the surface. The functional group of GO and successfully synthesization of the GO was examined with FT-IR. The morphology of nanostructured surface was scanned by a field emission scanning electron microscopy (FESEM), MIRA3 TESCAN-XMU. Composition analysis of nanostructured platform was studied using grazing incidence X-ray diffraction (XRD) with Cu- Kα radiation.

2.3. Electrochemical characterization

The electrochemical techniques studies were performed by a potentiostat instrument (BioLogic Inc., SP-300 EC-Lab®). The cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and differential pulse voltammetry (DPV) techniques applied to characterization of nanostructured electrode performance. The experiments were done at RT using a three-electrode mode, including a modified (GO Nano-wall@ gold nanoflower) FTO as a working electrode, a gold sheet (1*2 cm^2) as a counter electrode, while an Ag/AgCl in saturated KCl used as reference electrode. All electrochemical studies performed in a standard redox couple solution containing KCl (0.1 M) and $(CN)_6]^{-4}$/$[Fe(CN)_6]^{-3}$ (2.5 mM).

2.4. Biosensing procedure

In this study, we have used the thiolated DNA capture probe against miR-223 (Cap-223) to detect colorectal cancer biomarker miR-223 as following sequences:

The 5′ thiolated Cap-223 (5′-TTTTTTTTTTTTGCACA-TAAACTGTTCGACTCAA-3′),

Hsa-miR-223 (5′-CGUGUAUUUGACAAGCUGAGUU-3′)

Hsa-miR-486 (5′-UCCUGUACUGAGCUGCCCCGAG-3′)

Hsa-miR-9 (5′-UCUUUGGUUAUCUAGCUGUAUGA-3′)

The BioRP purified oligonucleotide sequences received from Takapouzist, and Nedayefan Inc. (I.R.Iran).

The preparation of Cap-223 and miR-223 were followed by the manufacturer's protocol. To this end, a TE (Tris-HCl 10 mM pH 7.5, EDTA 1 mM) buffer was applied for dissolving of Cap-223 (freeze-dried powder) and stored at −20 °C before use. Then, an optimized concentration of 1.5 mM (based on the saturation peak) of Cap-223 was prepared and used to further processing. To *disulfide bond reducing (Cap-223 are 5′-thiolated) and preparing* final concentration, a mixing of the Cap-223 and 0.1 M of *Dithiothreitol (DTT) in a* 1: 1 ratio *(15*–30 min *at RT) followed by adding a developed solution* (NaCl 1 mM, citric acid 10 mM plus Na_2HPO_4 10 mM). In the next step, defined surface area of nanostructured platform was immersed in 1.5 mM Cap-223 for 2–3 h. Then, the blocking agent, βME 0.2 mM to cover uncoated surface area with Cap-223 applied for 15 min.

The miR-223 freeze dried powder prepared by dissolving it in same solution used for Cap-223 (TE buffer). A final solution containing trisodium citrate 30 mM, and NaCl 300 mM in various concentrations ranging from zM to nM of miR-223 applied for miR detection. The bare nanostructured platform efficiency, and all steps from Cap-223 immobilization to miR-223 detection changes was recorded by measuring the CV (potential of −300 to 600 mV, and 50 mV s^{-1} scan rate), EIS (frequency 100 kHz-100 mHz, AC current range of 100 mA, step duration of 6s, and AC sine wave amplitude of 5) and DPV (potential of −300 to 600 mV). All recorded raw data were analyzed by Prism GraphPad, MS Excel, and Origin Lab software and the data were subjected to error bare, relative standard deviation and mean comparison significancy analysis.

2.5. Ethical approval

To find fabricated biosensor efficacy, the real sample (human serum) was used. The ethical approval of sample preparing was according to the ethical principles of Tarbiat Modares University, I.R.Iran ethics committee.

3. Results and discussion

3.1. Nanostructured platform analysis using SEM-EDX and FT-IR

The development of robust platforms and substrate with a higher surface-area-to-volume aspect ratio and higher electroactivity gives more strength to electrochemical biosensors. Nanostructured platform, in comparison with their bulk, have excellent properties such as those mentioned. In this study, we have fabricated a robust substrate that has a high surface-area-to-volume aspect ratio and excellent electroactivity using a furnished GO-based Nano-wall decorated with gold Nano-flower.

We have synthesized the GO nanosheets based on the Hummer protocol. The FTIR spectrum of synthesized GO nanosheets (FT-IR spectrum not shown) exhibits numerous peaks at 3426 cm^{-1} in the high frequency area related to the stretching mode of the O–H bond, which can be attributed to the hydroxyl functional groups in GO. The narrow peak at 2926 cm^{-1} originates from symmetrical stretching of CH_2 groups, and the peak observed at 1691 cm^{-1} stretching vibrations of C═O (carboxyl groups). In addition, at 1630 cm^{-1} wavenumber a band is found that is assigned to the bond stretching vibration of the C═C functional group of GO. Transmission mode of FT-IR spectra reveals a sharp peak observed at 1383 cm^{-1} that can be assigned to the C–H bending vibration, and C–O stretch of the carboxylic acid group of GO functional group (Abbasi et al., 2020; Rashidian et al., 2021).

The surface characterization of substrate such as crystallographic properties, and morphology of GO@Au-NS were analyzed using grazing incidence X-ray diffraction (GIXRD), and field emission scanning electron microscopy (FESEM). A PANalytical X'Pert Pro MPDX-ray diffractometer with a CuKα source (λ = 1.5406 Å) applied to GIXRD crystallographic characterization of GO@Au-NS, in which the incidence angle was at 1° for 2θ values ranging from 0° to 10° (Siburian et al., 2018). The results of GIXRD patterns of the GO Nano-wall substrate are different from when it was subjected to electrodeposition of gold Nano-flower (Fig. 2). The nano-structure and energy spectrum analysis data on the intensity of GO Nano-wall and gold Nano-flower are shown in Fig. 2-D, and H, respectively. EDX spectra data of GO Nano-wall and gold Nano-flower were measured at a voltage of 15 kV. The GO Nano-wall EDX spectra data revealed that the oxygen atoms (O) carbon atoms (C) have the energy quantities captured by each detector at 0.525 and 0.28 keV, respectively. While the spectra data related to nanostructured substrate with gold Nano-flower showed different energy quantities measured by detector such as exhibited three distinct peaks at 2θ = 2.1, 2.2 keV (AuMβ and AuMα) and 9.73 keV for AuLα. The GO@Au-NS EDX pattern showed 3 peaks at 2θ = 2.1, 2.2, and 9.73, which related to different nanostructure based on Miller indices.

The morphology of nanostructured substrate fabricated on the FTO electrode was scanned by FESEM. As shown in Fig. 2 A-C and E-G, the morphology of the GO are Nano-wall shaped with a series of protrusions on it (may aggregated GO) and has a good nanoscale dimension. An increasing in surface-area-to-volume aspect ratio and roughness in the nanoscale dimension is shown in Fig. 2 FESEM images. The FESEM images shown in Fig. 2 revealed that the gold Nano-flower like nanoparticle fabricated and decorated on the surface of GO Nano-wall, resulting in an obvious landing area has been created for the proper placement of Cap-223. The FESEM scanned images and GIXRD patterns demonstrating that the electrochemical approaches of GO@Au-NS is well fabricated with an excellent surface area.

3.2. Electrochemical characterization of substrate

As an important concept for electrochemical biosensors, the electroactivity of the nanostructured platform is critical, which can enhance the performance of different electrochemical techniques such as CV, EIS, and DPV. The electroactivity of surface before and after nanostructured surface fabricated showed a significant difference (Fig. 3). Improved electroactivity of GO@Au-NS surface exhibited successful fabrication of the platform on the FTO coated glass surface. This in turn, leads to improvement in biosensing performance through record a small changes of resistance occurred on the surface.

Cyclic voltammetry techniques have been used as a technique to find changes in the potential of working nanostructured electrode. The results showed that there is a significant change before and after nanostructure fabrication on the working electrode and also for all step. In this work, a thiolated ss-DNA sequence (complementary of miR-223) was immobilized on the surface of GO@Au-NS platform as a capture probe (Fig. 3). The 5′-thiolated ss-DNA immobilized covalently to the GO@Au-NS surface by disulfide bonding, in which can react to miR-223 by hydrogen binding of nucleic acids. Not only the CV, but also the EIS, and DPV of bare GO@Au-NS electrode and functionalized with Cap-223 revealed a changes on the surface, resulting successful immobilization of Cap-223 on the surface (Fig. 3). In the next step, a blocking agent of uncoated surface (2-mercaptoethanol) was used which could occupy more free spaces without covering the Cap-223. We have used βME due to these fact that this blocking agent in comparison of bovine serum albumin (BSA) is not able to interact with Cap-223, and a smaller molecules than BSA can cause smaller changes in the EIS, CV, and DPV, in turns reliable data are achieved. Moreover, the DPV, and EIS electrochemical techniques applied to record changes occurred on the surface step-by-step from Cap-223 immobilization to final concentration of miR-223 (nano molar) detection. The results of EIS and DPV techniques are given in the Figs. 2 and 3. The results revealed that the Cap-223 is successfully immobilized on the surface followed by an increasing on the Z′ (Ω) of EIS compared to bare working electrode (Nanostructured platforms). Same results achieved by DPV techniques, where the peak height was decreased by increasing the resistance (Fig. 3).

By using the EIS different concentration data, the limit of detection

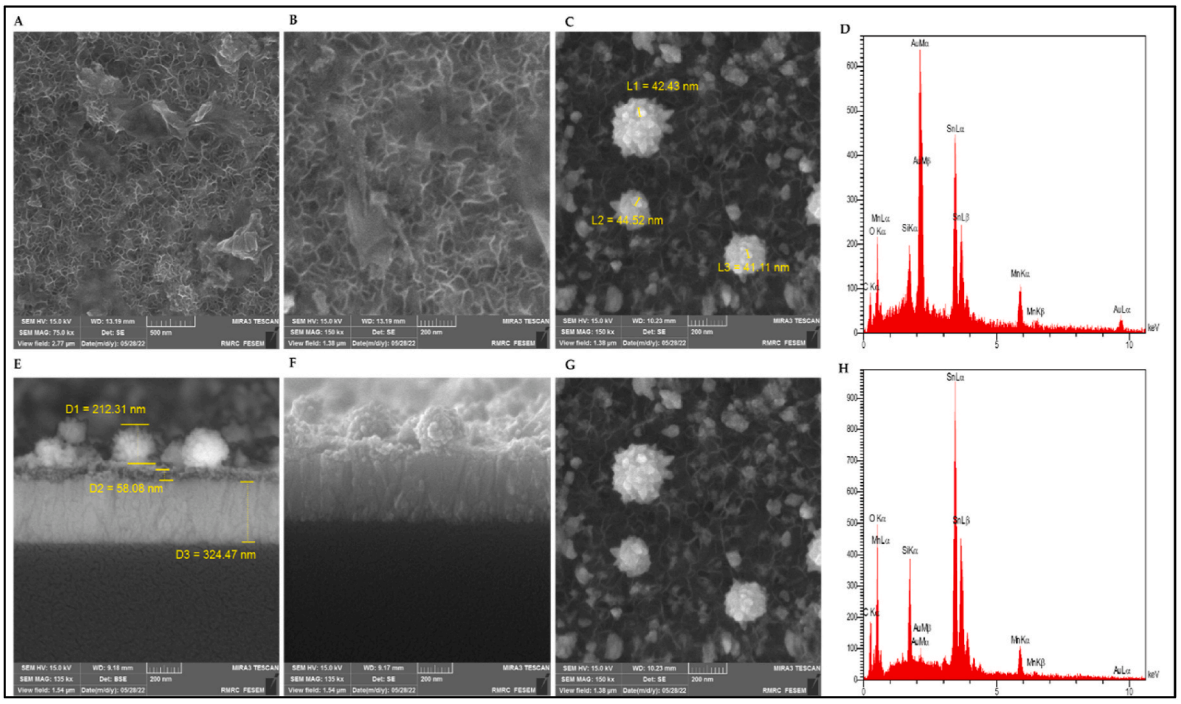


Fig. 2. The HRSEM, and EDX analysis of fabricated biosensor containing graphene oxide Nano-wall, and gold Nano-flower. A, and B are the HRSEM of GO Nano-wall shows a GO nanosheet that have a vertical arrangement and in shape of wall. C, is a GO Nano-wall@Au Nano-flower (also the G). D, is EDX analysis of substrate (also the H), and the E-F are the cross section of GO Nano-wall@Au Nano-flower fabricated on the FTO electrode. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(LOD) of biosensor was calculated according to the following equation:

$$LOD = 3.3\ (S_y/S)$$

Where, S_y: the standard deviation (SD) of the response (EISs) and S: the slope of the semi-log calibration curve. In the current study, a wide range of miR-223 (zM to nM) were tested to calculation of LOD of nanostructured biosensor and an ultra-low LOD estimated for this biosensor performance (0.012 aM). In comparison with previous biosensors developed by researchers, our fabricated nanostructured platform showed an excellent efficiency, which can be used as a good candidate diagnostic tools.

3.2.1. *Sensitivity and selectivity of biosensor*

To study the detectable performance of the proposed GO@Au-NS biosensor, a sensitivity study was carried out via both DPV and EIS measurements at various concentrations of the target miR-223. As illustrated in Fig. 4 A, the charge transfer resistance (R_{ct}) of the biosensor noticeably increased with an increase in the miR-223 concentrations. The result of logarithmic plot of miR-223 concentrations (Molarity, M) versus currents/resistance, as shown in Fig. 4-B, exhibits a good linear relationship over a range of zM to nM. The detection limit (LOD) of the proposed nanostructured biosensor was estimated to be as low as 0.012 aM, based on the mentioned equation. This relatively broad detection range and lower detection limit (LOD) showed that the fabricated miR-223 nanostructured platform, based on a label-free GO@Au-NS without any redox agents, can be prepared for miR-223 clinical assays. In this study we have applied EIS, CV, and DPV techniques to study the performance of fabricated biosensor. The EIS data were further proved by DPV technique analysis carried out in ([Fe $(CN)_6]^{3-/4-)}$ solution containing KCl. Both CV and DPV curves provided information regarding the electrochemical processes (in accordance EIS curve) occurred at the electrode/solution interface. The results of EIS, CV, and DPV indicated the behavior of the redox probe [Fe $(CN)_6]^{3-/4-}$ on the nanostructured platform electrode surface before and after each GO@Au-NS electrode modification step, in addition during the hybridization with the miR-223 target. When the GO@Au-NS was fabricated on the working electrode, an increase in peak current (CV), and in the peak height (DPV), resulting increase in the electroactivity of surface, while reducing the resistance of EIS were observed as compared with the bare FTO working electrode. This indicating improvement of the electron transfer. After the immobilization of Cap-223, blocking free surface using the 2-ME, hybridization of miR-223 with the DNA Cap-223 (with ascending increase in concentration), the CV, and DPV peaks decreased and the semi-circle diameter of EIS peaks increased. As a result of the spatial blockage of nanostructured platform between [Fe $(CN)_6]^{3-/4-}$ and the NS working electrode surface, in addition, decrease conductivity or increase the resistance of surface. The electrochemical techniques shows that the sensitivity of GO@Au-NS biosensor is excellent for biomedical applications. In this study, at least three replicates were applied to test the performance of the biosensor and results showed that a good reproducibility and repeatability (the error bar, relative standard deviation or RSD, and coefficient of variation). The stability of biosensor was tested after approximately one month storing the electrode and test the performance of its. The result showed that the stability of biosensor not changed and a significant changes was not found.

Furthermore, to test the specificity of the biosensor, different miRNAs have been used as mismatch target to find the biosensor selectivity performance. In this study the mismatch miR-486 sequences at same concentration applied instead of miR-223. The results of the electrochemical techniques such as DPV (EIS are not shown) of miR-486 are given in Fig. 5-A. As illustrated in the graphs Fig. 5-A, because the mismatch miRNAs could not hybridize with Cap-223, a significant decrease in peak height not observed in the DPV peak of miR-486. Resulting, no hydrogen bonding occurred between Cap-223 immobilized on the surface and miR-486, and dose not reduced conductivity of the surface resulted. The results of this step show us an excellent selectivity for our fabricated biosensor. Our results revealed that, the fabricated biosensor is able to differentiate the miR-223 from other miRNAs.

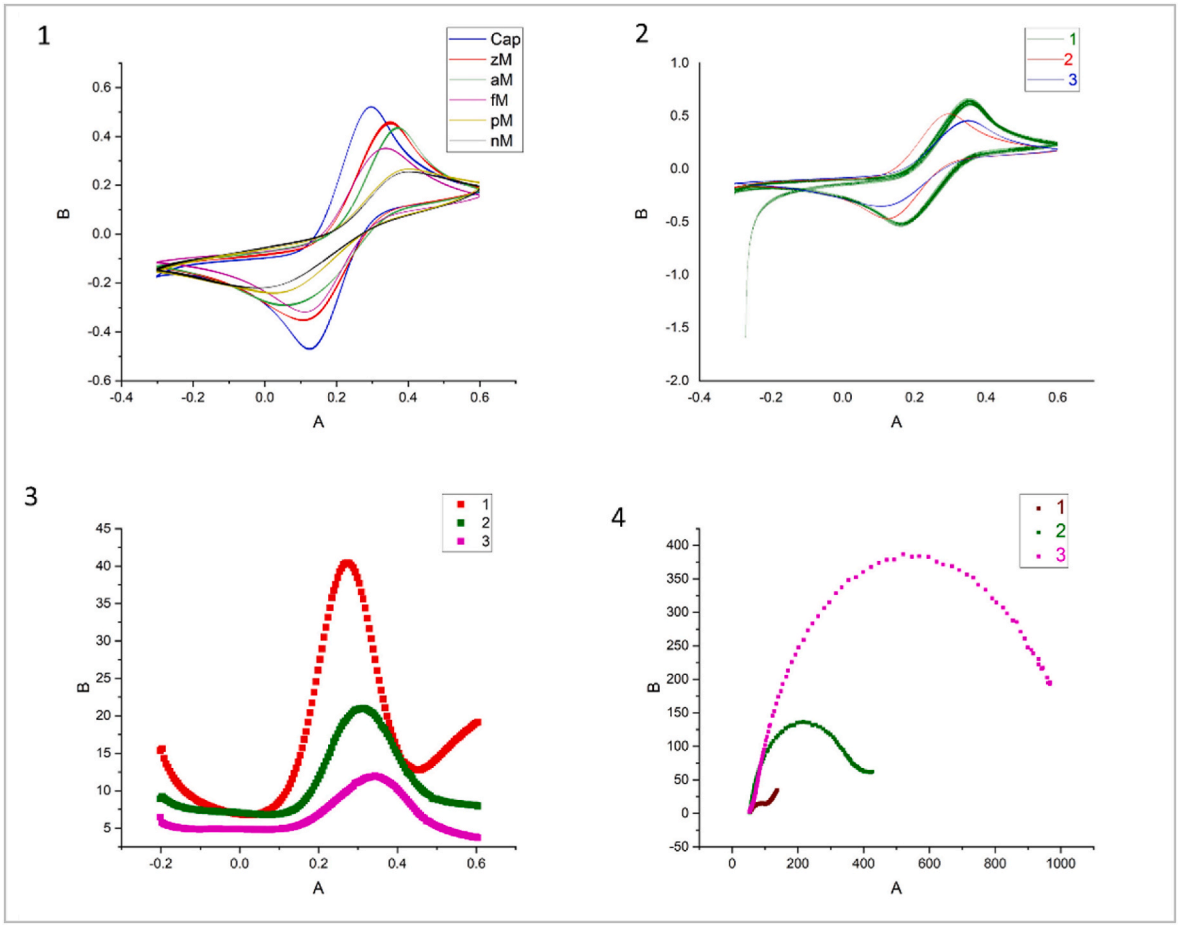


Fig. 3. Electrochemical measurements of surface modification. The section 1 is the CV peak from DNA capture probe immobilization to surface to nM concentration hybridization of miRNA, section 2 is CV peak of bare electrode (1), thiolated DNA capture probe (2), and miRNA hybridization with miRNA (3), the section 3 is DPV peak of bare electrode (1), thiolated DNA capture probe (2), and miRNA (3), and the section 4 is EIS peak of bare electrode (1), thiolated DNA capture probe (2), and miRNA (3).

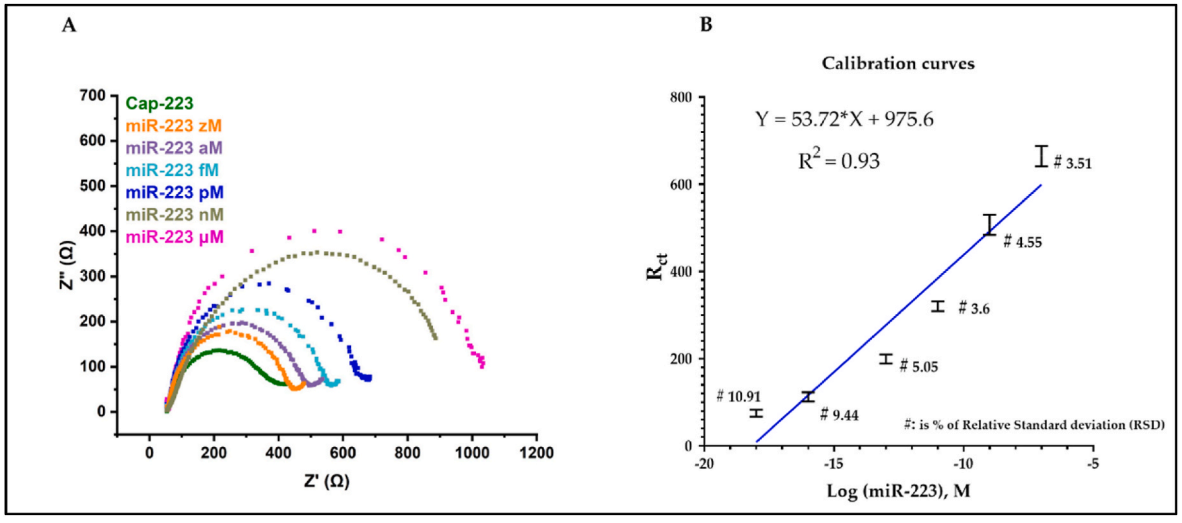


Fig. 4. The electrochemical analysis (4-A) and standard calibration curve (4-B) of different concentration of miR-223 tested by EIS, # in calibration curve is RSD or relative standard deviation, (n = 3).

3.2.2. The miR-223 analysis in real sample (human serum)

Human biological fluids, such as blood, plasma, saliva, mucus, and urine can contain many species of biomarkers (*i.e* miRNA), which are useful for disease analysis (Han et al., 2019). Hence, in the current study, a human real sample (serum) was utilized for target miR-223 detection assay. A concentrations of miR-223 diluted into 2% human serum (50:1 ratio with PBS) were analyzed by the fabricated biosensor. The results of DPV techniques of real samples containing miR-223 are given in

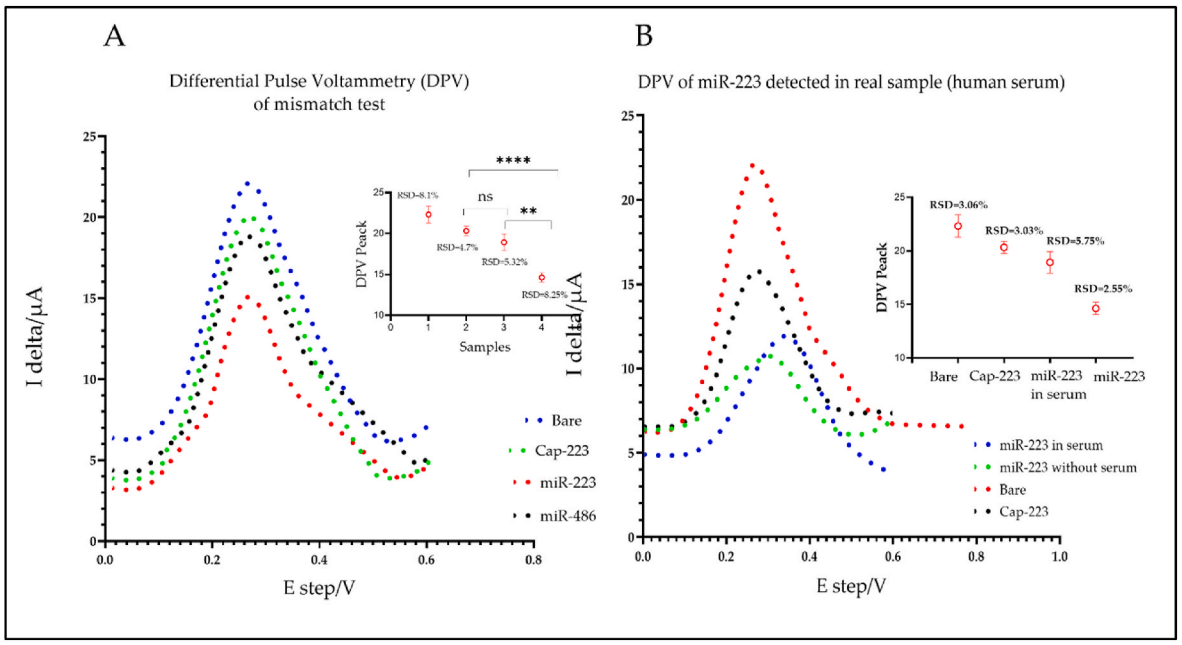


Fig. 5. The electrochemical analysis (DPV) of discrimination test by mismatch sequences and significancy analysis of it (ns, is non-significant, and ** is significant at p values of $P \leq 0.01$) (3-A), and 3-B is DPV plot of miR-223 detected in real sample or human serum with their RSD or relative standard deviation.

Fig. 5-B. The RSD or relative standard deviation (%) is estimated and listed above of each error bar and repeat of bare working electrode (NS platform), miR-223, miR-223 in serum and Cap-223. For every mentioned repeat especially miR-223 in human serum, RSD percentage was estimated with low RSD%, indicating that the GO@gold based biosensor is an excellent analytical tool in miR-223 assays even in real sample, which can be a good candidate for application in colorectal cancer clinics and diagnosis. Overall, the real-sample detection of miRNAs using nanostructured biosensor will be of high importance for early detection of disease. Finally, the efficacy of our developed electrochemical biosensor has been compared with recently strategies, in which they have utilized nanomaterials-based biosensor for detection of miRNAs. The biosensor fabricated for detection of miR-155 and 21 through paper-based voltammetric approaches by (Torul et al., 2021) and the result showed that the LOD is less than our biosensor. Although the limitation of their biosensor is LOD, however the portable of their sensor is an advantage, compared to our biosensor which needs readout platform. A published research work, carried out by Shahbazi-Derakhshi et al., (Khosravi et al., 2023) showed that when they have decorated AuNPs on the perovskite-graphene oxide surface, the performance of their biosensor enhanced. Khosravi et al., (2023), have developed an immunosensor to electrochemical miR-155 detection using nanocomposite biosensor fabricated by functionalized/conjugated graphene materials and gold nanostars, in addition modified with nickel-iron NPs. The LOD of their biosensor is less than our biosensor, in addition our biosensor is simple and not more process is needed to fabrication those complex systems. The results are inserted as a table in Table 1.

A major issue related to nanostructure-based working electrode is find the fabrication technique for biosensing approaches that is able to fabricate a stable and reproducible electrode. In addition, using surfactant and chemical agents to fabrication of working electrodes of electrochemical biosensor can hindered the electroactivity, finally the performance of the biosensors. In this case, we have used a surfactant-free approach to fabricate the biosensor, with high electroactivity. In addition, our biosensor is label-free immunosensor and have excellent performance to detect microRNA both in standard and human serum. Developing the label-free biosensors for electrochemical approaches is essential as the enzyme, chemical agents, and those agents used for labelling could affect the result (false-positive/negative).

Table 1
Comparison of the administration for detection of miRNA.

Strategy	Target	LOD	Year	Ref
GO Nano-wall@Au Nanoflower	miR-9	12.8 zM	Our work	
Paper-Based Electrochemical Biosensors by GO with $MoS_{2\ and}$ Au Nanoparticles	miR-155 and 21	12–59 nM	2021	(Torul et al., 2021)
decorated AuNPs on the perovskite-graphene oxide surface	miR-21	2.9 fM	2023	(Shahbazi-Derakhshi et al., 2023)
reduced graphene oxide modified with nickel-iron (Fe–Ni@rGO)	miR-155	0.05 fM-50.0 pM	2023	(Khosravi et al., 2023)
Surface modification of glassy carbon electrode surface (GCE) by ZnS quantum dots and L-cysteine - electrochemical approaches	miR-200a	8.4 fM	2021	(Moazampour et al., 2021)
Gold nanoparticle modified gold electrode biosensor - electrochemical approaches	miR-100	10 aM	2021	(Zhuang et al., 2021)
Liposome mixed with MoS_2/gold NPs on SPE-GNP	miR-21	10 aM	2020	(Yazdi et al., 2020)
Ferrocene-functionalized graphene oxide nanosheets (Fc-GO)	miR-200a	0.29 fM	2023	(Moazampour et al., 2023)
Thiolated dendritic gold nanoparticles with SWCNTs	miR-21	0.01 fM	2020	(Sabahi et al., 2020)

4. Conclusion

Nanostructured-based electrochemical biosensors with a high surface-to-volume aspect ratio have recently gained a lot of attention due to their enhanced sensitivity, and specificity. The fabrication of

electrochemical working electrodes as biosensors with excellent electroactivity is interesting for the early detection of disease biomarkers in clinical applications. Here, a label-free nanostructured platform as an electrochemical biosensor is fabricated, which is based on GO@gold NS, for miR-223 colorectal cancer biomarker detection. The GO was synthesized by Hummer's protocol and electrodeposited on the FTO surface to fabricate Nano-wall shapes, and decorated them with gold Nanoflowers. The thiolated ss-DNA, used as capture probe covalently immobilized on the nanostructured surface via thiol–Au interactions, was used to detect miR-223 as a colorectal cancer biomarker. The step-by-step procedures were confirmed by EIS, DPV, and CV techniques. The electrochemical signal of the miR-223 biosensor is caused by the different responses of the miR-223 macromolecules that were hybridized with Cap-223. The simple fabrication approaches of a nanostructured biosensor were utilized to this end, resulting in a highly sensitive LOD (0.012 aM) of miR-223. Furthermore, the fabricated nanostructure platform (biosensor) is effective to distinguish mismatched sequences from target miR223, and it also showed satisfactory performance in human serum, indicating its great potential for application in disease diagnosis and assessment. On the other hand, satisfying antifouling properties are significantly dependent on the hydrophilicity or hydrophobicity of the interface of the electrode, resulting in a higher hydrophilicity index that can promote more antifouling activities especially when a hydration layer is formed on the surface of the electrode, this can result in antifouling interface enhancement. In this study, we have applied numerous approaches to enhance the antifouling interface properties of our nanostructured surface. In the first step, the GO nanosheets were treated by EDC/NHS to more activate the GO, increasing its hydrophilicity (after the EDC/NHS treatment, the GO solubility increased significantly). In the next step, the FTO surface immersed in $H_2O/H_2O_2/NH_4OH$ was activated for better attachment of the functionalized GO. In turns, these FTO and GO functionalization procedures make our substrate more hydrophilic and, finally, enhance the antifouling property of the interface. In a research work, the researcher has developed an antifouling interface and claimed that the hydrophilicity of substrates is a result of antifouling properties, where the contact angles of substrates decrease (Chen et al., 2019). Our findings show that GO-NW@Au–NF biosensors with a significant hydrophilicity index can make our biosensor an excellent candidate with an excellent antifouling immunosensor for determining micro-RNA in biological fluids. Another potential of our work is that we fabricated nanostructure without surfactant, which it can hindered the electroactivity of electrochemical platform for sensing applications. Our future perspective can focus on the development and investigate biomarkers detection using portable bio-assay with excellent repeatability and reproducibility. In addition, it is necessary to plan microRNA detection bio-assay through a device (like our platform) with the reusable ability. As a limitation potential of our work, real-time sensing of more than one microRNA using a single platform is the most important challenges, which should be emphasized with other researchers.

CRediT authorship contribution statement

Abolfazl Akbari: Conceptualization, Methodology, Software, Data curation, Writing – original draft. **Hadi Hashemzadeh:** Conceptualization, Methodology, Software, Data curation, Writing – original draft. **Zahra Shokati Eshkiki:** Data curation, Software. **Mohsen Masoodi:** Editing, Visualization, Investigation. **Seidamir Pasha Tabaeian:** Supervision, Validation, Writing – review & editing. **Hossein Naderi-Manesh:** Supervision, Validation, Writing – review & editing. **Ali Akbar Zare:** Editing, Visualization, Investigation. **Shahram Agah:** Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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