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One-step detection for two serological biomarker species to improve the diagnostic accuracy of hepatocellular carcinoma

Tao Gao[a], Jun Zhi[a], Chaoli Mu[a], Shiyu Gu[a], Jing Xiao[a], Jie Yang[a], Zhaoxia Wang[b,*], Yang Xiang[a,*]

[a]State Key Laboratory of Pharmaceutical Biotechnology and Collaborative Innovation Center of Chemistry for Life Sciences, Department of Biochemistry, Nanjing University, Nanjing 210093, P. R. China.

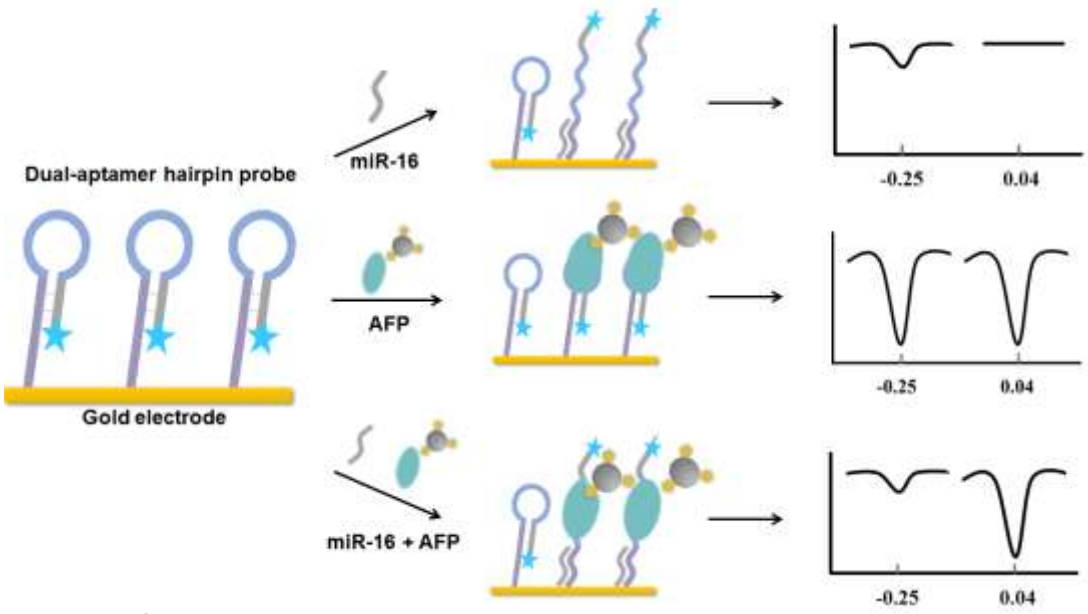
[b]Department of Oncology, The Second Affiliated Hospital of Nanjing Medical University, Nanjing 210011, P. R. China

At present, the accuracy of clinical hepatocellular carcinoma (HCC) diagnosis needs to be further improved. In this work, two kinds of serological biomarker species, microRNA and protein biomarker, have been detected simultaneously to identify HCC. Herein, a dual-aptamer hairpin DNA oligonucleotide is designed as the electrochemical sensing probe (ESP) to achieve this goal. The hairpin-structured DNA probe consists microRNA-16 (miR-16) complementary sequence and alpha fetoprotein (AFP) aptamer sequence, so it can both capture miR-16 and AFP. Once it hybridizes with miR-16, the hairpin structure is unlocked so that the terminal modified signal molecule (methylene blue, MB) would give a decreased electrochemical signal. Meanwhile, once it recognizes AFP, concanavalin A (ConA) modified silver nanoparticles (AgNPs) can bind to AFP at the sensing surface. An

*Corresponding authors. Fax: +86 25 58509994 (Z. Wang), +86 25 83592510 (Y. Xiang); E-mail addresses: wangzhaoxia@njmu.edu.cn (Z. Wang), xiangy@nju.edu.cn (Y. Xiang).

obvious electrochemical signal of AgNPs can thus be generated for AFP detection. In this way, one-step and simultaneous detection of miRNA-16 and AFP can easily be realized by collecting the two sensitive and non-interfering electrochemical signals. Compared with traditional single biomarker detection methods, this assay strategy can improve the accuracy of HCC by monitoring two kinds of serological biomarkers species. Besides, this novel electrochemical biosensor based on ESP is simple, low-cost and efficient, which make it promising to improve the accuracy and specificity for the diagnosis HCC in the future.

Graphical Abstracts



Keywords: electrochemical biosensor; hairpin-structured DNA probe; microRNA-16; alpha fetoprotein (AFP); hepatocellular carcinoma biomarkers

Introduction

Hepatocellular carcinoma (HCC), which seriously endangers human lives and

health, now is one of the major malignant diseases with high morbidity and mortality [1]. Accurate diagnosis of HCC can significantly improve the clinical curative effect and alleviate the suffering of patients [2, 3]. However, available techniques such as imaging and histology methods can only work at the late stage of HCC [4]. To extend time window for early detection and treatment, a great deal of efforts should be made for serological biomarker testing [5]. Thus, analysis of the commonly used serological protein biomarkers, such as alpha-fetoprotein (AFP) [6], cancer embryo antigen (CEA) and Golgi protein 73 (GP73) [7], has received great attention. Nevertheless, the reported methods are mainly towards the single-factor analysis, which may result in high rate of false positive and low accuracy [8]. Therefore, the analysis of multiple protein biomarkers has received much interests [9, 10].

In addition to the above mentioned protein biomarkers, some emerging nucleic acid biomarkers (circulating methylated DNA, miRNA, ect.) have been confirmed that their abnormal expressions are closely related to human cancer at the gene expression level [11-15]. Among them, microRNA-16 (miR-16) is suggested as a promising HCC biomarker due to its high specificity [16]. In the meantime, based on the serological analysis data of HCC patients, researchers have found a positive relationship between the amount of miR-16 and AFP [16]. So, a significantly improved accuracy of HCC diagnosis would be achieved when AFP and miR-16 are detected simultaneously [16].

So far, several methods can be used for AFP analysis, for instance enzyme-linked immunoassay, affinity chromatography, affinity imprinting [17-19]. At the same time,

available methods have also been reported for miR-16 detection, such as northern blotting, reverse transcription-polymerase chain reaction (RT-PCR), quantitative polymerase chain reaction (qPCR) and electrochemiluminescence [20, 21]. To be noted, however, there is no compatible approach for simultaneous detection of both the protein and miRNA biomarkers, which may provide more valuable information for HCC diagnosis. Therefore, there is a dire need of developing a simple, rapid, and easily operated method for the simultaneous detection of protein and nucleic acid biomarkers to improve the diagnostic accuracy.

So in this work, we have designed a dual-functional hairpin DNA probe, which consists miR-16 complementary sequence and AFP aptamer sequence [22, 23], as the electrochemical sensing probe (ESP) for simultaneous determination of miR-16 and AFP. Their corresponding quantitative signals can be obtained by coupling using two non-interfering electrochemical signals, which are generated from methylene blue (MB) and silver nanoparticles (AgNPs) (Scheme 1). Moreover, since MB and AgNPs have shown excellent electrochemical response, the sensitivity of this assay can also be guaranteed. As a result, a one-step and sensitive assay has been achieved in this ingenious design to monitor both miRNA-16 and AFP biomarkers. So, this assay strategy may have promising application in HCC diagnosis in the future.

Please insert Scheme 1 here.

Materials and methods

Materials and reagents

All the oligonucleotide sequences, including electrochemical sensing probe (ESP),

miR-16 and miR non-complementary sequence (miR-NC), were synthesized and purified by Sangon Biotechnology (Shanghai, China). They were listed in Table 1. AFP and GP73 proteins were purchased from Antu Biological Engineering Co., Ltd (Zhengzhou, China) and Hotgen Biological Technology Co., Ltd (Beijing, China), respectively. Concanavalin A (ConA), suberic acid bis(N-hydroxy-succinimide ester), glutathione (GSH), silver nitrate ($AgNO_3$) and other chemical reagents were obtained from Sigma-Aldrich. These chemicals here were all of analytical reagent grade without further purification. Human blood samples were obtained from the Affiliated Hospital of Nanjing University. Ultrafiltration filter (Amicon Ultra-4, 30 kDa NMWL) was purchased from Merck Millipore. UV-vis absorption spectra were acquired on a UV-1800 spectrometer (Japan). Electrochemical measurements were carried on a CHI660D electrochemical working station (CH Instruments, Austin, USA). All the solutions were prepared with deionized water, which was purified with a Millipore-Q purification system (Branstead, USA) to a specific resistance of 18.2 MΩ cm.

Table 1. Oligonucleotide sequences used in the experiments.

Oligonucleotide	Sequence (5'-3')
ESP	SH_TTTTTCGCCAATATTTACGTGCTGCTATCAGGTGCAGT TCTCGACTCGGTCTTGATGTGGGTTAGCAGC_MB
miR-16	UAGCAGCACGUAAAUAUUGGCG
miR-NC	UUGUACUACACAAAAGUACUGG

Preparation of ConA modified AgNPs (ConA-AgNPs)

In a solution containing 10 nM sodium hydroxide and 0.25 mM sodium citrate, 0.1 M silver nitrate solution were dropwise added, to a final concentration of 0.25 mM.

The mixed solution was continuously stirred at 200 rpm/min until the solution turned to bright yellow, GSH was then dropwise added with the final concentration of 1 μM. After further stirring for 20 mins, GSH modified AgNPs was obtained. Next, 5.0 μL of suberic acid bis (N-hydroxysuccinimide ester) was added into 1.0 mL of GSH-AgNPs solution. 3 mins later, 10 μL ConA (0.5 mg/mL) was added into the above solution. After incubation for 15 mins, ConA modified silver nanoparticle (ConA-AgNPs) was collected by ultrafiltration at 7500 g for 15 mins in a centrifugal filter (Amicon Ultra-4, 30 kDa NMWL, Millipore). Finally, the concentrated product was dispersed by half-and-half deionized water and PBS, then the ConA-AgNPs was stored at 4 °C before use.

Fabrication of the electrochemical biosensor

Modification steps of the gold electrode are based on our previous report with slight modification [24]. First, the gold electrode was polished and cleaned to be a mirror finish. Then the gold electrode surface was dried under pure nitrogen gas. The gold electrode was then brought to modification by ESP through Au-SH bond. Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) was used for the storage of ESP. 7 μL DNA hybridization solution was added into 3 μL ESP solution (10 μM). The mixture was incubated at 90 °C for 5 min, then cooled down to room temperature to let the DNA to be denatured. 10 μL DNA fixing solution (1.0 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and TCEP (1.0 mM, Tris (2-carboxyethyl) phosphine) were added into the above-mentioned solution, and then the mixture (20 μL, containing 1.5 μM ESP) was dropped onto the treated gold electrode surface and

incubated at 25 °C for 3 h. Afterward, the electrode was treated by 1 mM MCH (2-Mercaptoethanol) for 1 h at 25 °C so as to achieve a well-aligned ESP monolayer on the electrode surface and also avoid nonspecific adsorption. The modified electrode was finally ready for measurement.

Detection of miRNA-16 and AFP

Saline sodium citrate (SSC) buffer (0.15 M NaCl, 15 mM sodium citrate, pH 7.4) was used to dilute miR-16 and AFP. After incubating the miR-16 and AFP mixed solution (6 μL) on the electrode surface for 2 h at 37 °C, the electrode was rinsed in SSC buffer for three times. All the solution correlated with miR-16 was dealt with 1% DEPC (Diethy pyrocarbonate) in order to avoid RNase degradation. Above mentioned electrode was rinsed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) for three times, 10 μL prepared ConA-AgNPs was dropped onto electrode surface subsequently and incubated for 1 h at 37 °C. Finally, the electrode was washed in PBS and brought to measurement immediately.

Electrochemical measurements

All data was measured on a CHI660D electrochemical working station based on a three-electrode system by using the modified gold electrode as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum electrode as the counter electrode. 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 1 M KCl was used as the electrolyte solution for electrochemical impedance spectroscopy (EIS) test. EIS was measured with an amplitude of 5 mV, over the frequency ranging from 0.01 Hz to 10 kHz. Differential pulse voltammetry (DPV) were recorded in a mixture of 5 mL of

10 mM Tris-HNO_3 buffer (pH 7.4) and 100 mM KCl. The scan range of DPV was from -0.5 V to 0.3 V.

Please insert Scheme 2 here.

Results and discussion

Method design

In this work, we designed an electrochemical sensing probe (ESP) in order to simultaneously detect AFP and miR-16. This probe consists miR-16 complementary sequence and AFP aptamer sequence (Scheme 2), so that it can capture two biomarkers in one step at the electrode surface. Specifically, a thiol group is modified at 3'-terminal of the probe sequence, and MB moiety is modified on its 5'-terminal. Thus, the hairpin structured DNA can be immobilized on the gold electrode through the Au-SH bond. At this point, a relatively large electrical signal of MB can be detected as the modified MB on the hairpin-structured DNA is close to the surface of gold electrode. When miR-16 and AFP bind to ESP, the electrical signal of MB decreases obviously as the hairpin DNA structure is unlocked due to the hybridization with miR-16. At the same time, ConA-AgNPs would combine to the captured AFP through glycosidic bond, so the electrochemical signal of AgNPs can be detected. On the electrochemical workstation, two independent signals were recorded that do not interfere with each other. So we can achieve the purpose of simultaneous detection. In summary, through the design of dual-aptamer ESP and the combined use of two non-interfering electrochemical signals, one-step and simultaneous detection of the miRNA and protein biomarker targets can be achieved.

Please insert Fig. 1 here.

Characterizations of ConA-AgNPs

The modification of ConA on AgNPs is investigated by UV-vis spectrophotometry. The UV absorbance values of AgNPs, GSH-AgNPs and ConA-GSH-AgNPs were measured during the synthesized procedures. As shown in Fig. 1, all the three curves have strong absorption peaks at 400 nm, which is consistent with the specific absorbing peak of AgNPs [25, 26] and indicates that AgNPs has been synthesized successfully. At the same time, another strong shoulder peak at 260 nm with an obvious change in the shape, position and symmetry can be detected for ConA-AgNPs, which verifies the success modification of ConA to the surface of AgNPs as ConA protein has specific absorbing peak at 260 nm [27].

Characterizations of the electrochemical sensor

Please insert Fig. 2 here.

EIS is used to verify the modification steps on the electrode surface (Fig. 2). Fig. 2a shows that the Nyquist diagram of the bare electrode is in a straight line, which means that before surface modification of gold electrode, the resistance of charge transfer on the electrode surface is close to zero. When the gold electrode is modified with ESP, the resistance increases and an evident semicircle can be observed (Fig. 2b). The diameter of the semicircle further increases after miR-16 and AFP were captured, which was result from the increasing charge transfer resistance on the surface of the gold electrode. The result has indicated the successful fabrication of on the electrode sensing surface.

Please insert Fig. 3 here.

Performance of the biosensor

In this work, DPV is applied for the quantitative analysis of miR-16 and AFP. When only miR-16 is present, the MB signal detected by DPV decreases with the increment of miR-16 concentration. And a linear range can be obtained between 50 and 2000 nM (Fig. 3a). The linear regression equation is I = - 6.058-0.088lg$c$ (nM), R^2=0.983. The detection limit (DL) of the sensor for miR-16 was estimated to be 0.14 nM (DL = 3.3δ/S, where δ is the standard deviation of blank samples, S is detection sensitivity (slope of calibration curve)). When a constant concentration of 100 nM miR-16 is used in this system, the stripping peak current of AgNPs is observed to increase with the increasing concentration of AFP, and a linear range can be established between 50 pg/mL and 10 ng/mL (Fig. 3b), the linear regression equation is I = - 6.960-0.191lg$c$ (pg/mL), R^2=0.98. The detection limit (DL) of the sensor for miR-16 was estimated to be 8.76 pg/mL. Fig. 3c presents the dual electrochemical signals corresponding to the simultaneous capture of two targets after incubation with 500 nM miR-16 and 1.0 ng/mL. When AFP is present alone, ESP is also able to detect the electrochemical signal of AgNPs in response to AFP. As a result, the sensitivity of the detection method is proved high owing to this sensitive electrochemical method and the high affinity between the ligands. To assess the reproducibility of the sensor, the coefficient variations (CVs) of intra- and inter-assays were used. Both the intra- and inter-assay precisions of the sensor were examined with two samples containing 100 nM and 500 nM miR-16 respectively for three times. The valued CVs were 3.6% for 100 nM

miR-16 and 2.9% for 500 nM miR-16. The inter-assay was performed with three prepared electrochemical sensors. The CVs of inter-assay were 4.9% and 3.4%, respectively. The CVs of intra- and inter-assay have indicated good reproducibility of the fabricated electrochemical sensor.

Please insert Fig. 4 here.

Control experiments and specificity tests

To further explore the feasibility of the hairpin ESP, we choose miR-NC and GP73 as interfering components. The miR-16 and miR-NC are incubated with the gold electrode that have been modified with ESP. The signal responses of the gold electrode are characterized by DPV. Due to the structure characteristics of the designed hairpin DNA, MB modified on the 5'-terminal is close to the surface of the gold electrode, so a relative large electrical signal of MB can be detected. When the target miR-16 is incubated with the ESP modified gold electrode, miR-16 opens the DNA hairpin structure after the complementary base pairing. So a relatively low MB electrical signal was detected due to the DNA 5'-terminal being canted away from the surface of gold electrode. However, when miR-NC was incubated with the ESP modified gold electrode, the MB signal was changed little (Fig. 4a). This proves that the DNA hairpin structure was not opened as the nucleotide sequence of miR-NC is not complementary to ESP. In the absence of miR-16, the ESP modified gold electrode are incubated with AFP and GP73, followed by ConA-AgNPs. The signal of AgNPs could be detected, while no signal was detected for GP73 (Fig. 4b). Therefore, the ESP probe has been proven to be specific to miR-16 and AFP, and the assay

method is feasible.

Please insert Fig. 5 here.

In order to further explore the specificity of the ESP, we challenged the biosensor with other glycoproteins, such as GP73 and CEA. The AFP is mixed with GP73 and CEA in 1% serum, and then the mixture is incubated with the ESP probe modified gold electrode. The detected peak current is shown in Fig. 5, which shows the peak current can be detected only when AFP is present. The results have verified the good specificity of the biosensor.

Conclusions

In this work, a novel design for one-step and simultaneous detection of two kinds of biomarker species, nucleic acid and protein, to identify HCC with high specificity is firstly proposed. Based on the innovatively designed dual-aptamer hairpin DNA probe, serological miRNA-16 and AFP can be simultaneously detected to improve the diagnostic accuracy of HCC. Besides, collection of non-interfering quantitative signals from MB and AgNPs have enabled one detection for two electrochemical signals. Sensitive, time-effectiveness and low-cost of this electrochemical platform have also provided high sensitivity and usability. Therefore, compared with traditional single/multi-biomaker detection methods, this electrochemical biosensor may give great assistance to improve the accuracy and specificity for HCC and other clinical diagnosis in the future.

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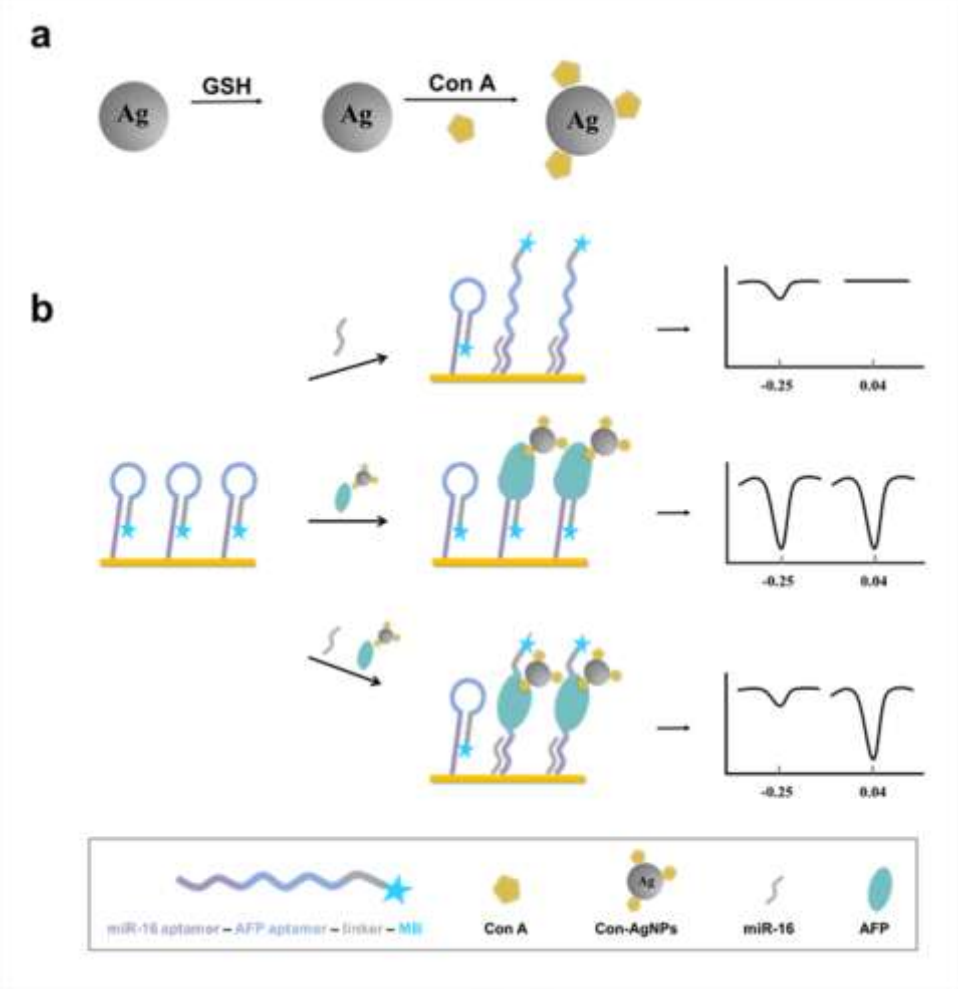
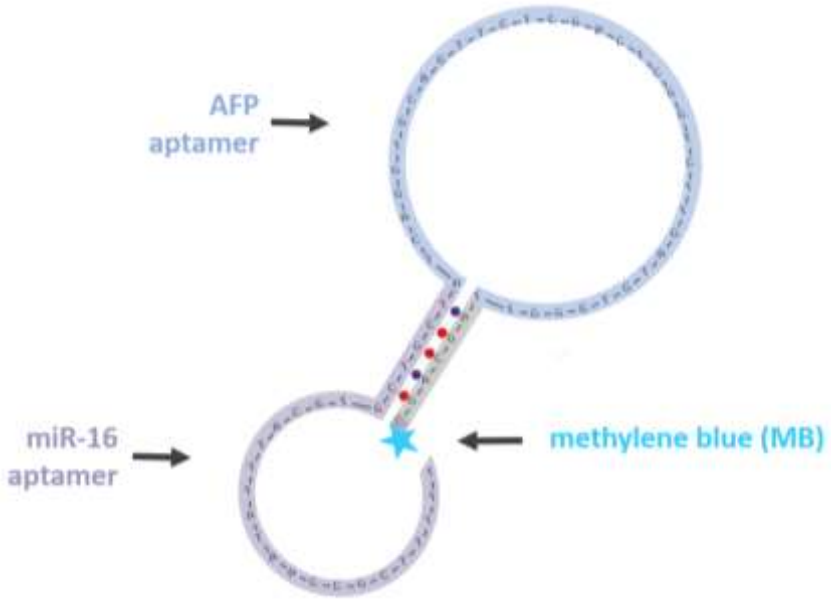


Scheme 1. Schematic illustration shows the fabrication process and performance of the electrochemical biosensor. (a) Preparation of ConA-AgNPs, and (b) electrochemical responses of the fabricated electrochemical biosensor in the presence of different biomarkers.



Scheme 2. The designed DNA hairpin (ESP) that has been used as the sensing probe, the structure of which is analyzed with OligoAnalyzer Tool from Integrated DNA Technologies (IDT). Specifically, the stem and single-stranded structure (purple) indicate miR-16 aptamer, and the loop structure (blue) indicates AFP aptamer.

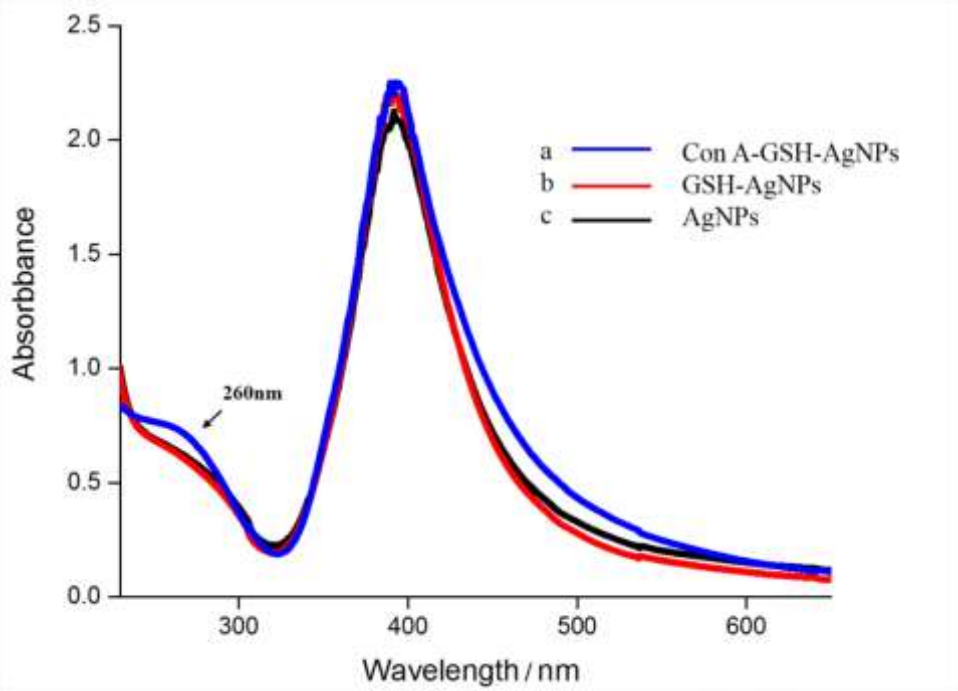


Fig. 1. UV–vis spectra of ConA-AgNPs (a), GSH-AgNPs (b), and AgNPs (c) in PBS buffer, pH 7.4.

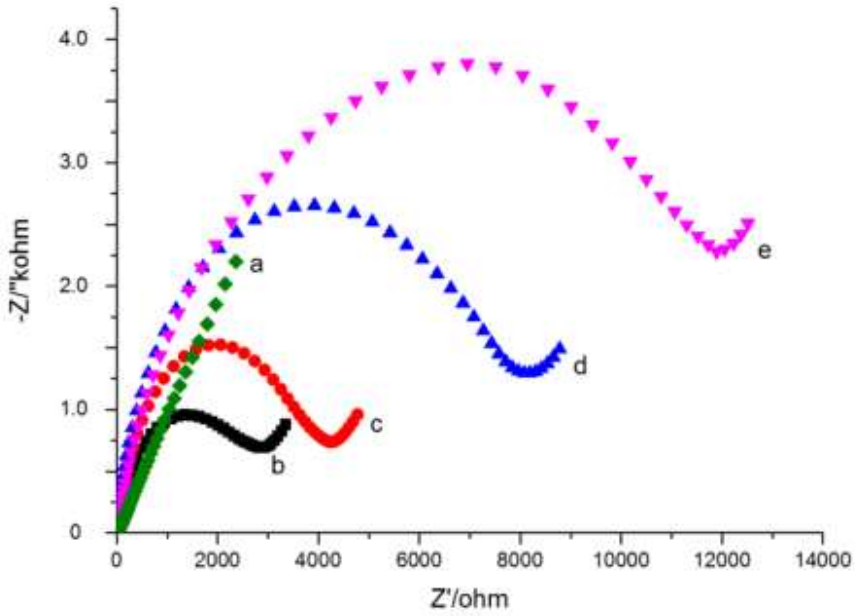


Fig. 2. EIS spectra corresponding to each step after treatments of the electrode: (a) bare electrode, (b) ESP/electrode, (c) miR-16/ESP/electrode, (d) AFP/miR-16/ESP/electrode, (e) ConA-AgNPs/ AFP/miR16/ESP/electrode.

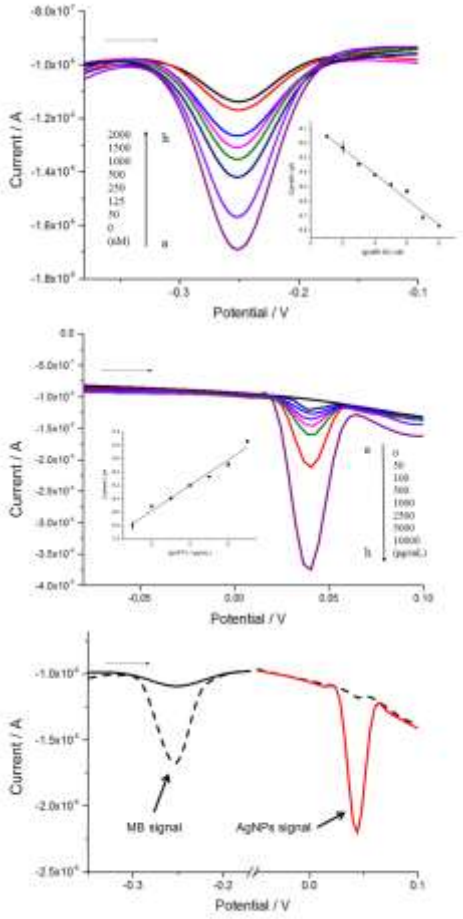


Fig. 3. (a) DPV obtained at the modified electrodes to quantify miR-16. Curves (a-h) corresponding to miR16 concentrations of 0, 50, 125, 250, 500, 1000, 1500, 2000 nM respectively. The inset is the calibration curve. Error bars indicate standard deviation of three independent experiments. (b) DPV obtained at the modified electrodes to quantify AFP. Curves (a-h) are corresponding to AFP concentrations of 0, 50, 100, 500, 1000, 2500, 5000, 10000 pg/mL respectively. The inset is the calibration curve. Error bars indicate standard deviation of three independent experiments. (c) DPV responses for simultaneous detection of miR-16 and AFP based on the ESP modified gold electrode using MB and AgNPs as indicators. The dashed lines indicate signal responses in the absence of target biomarkers.

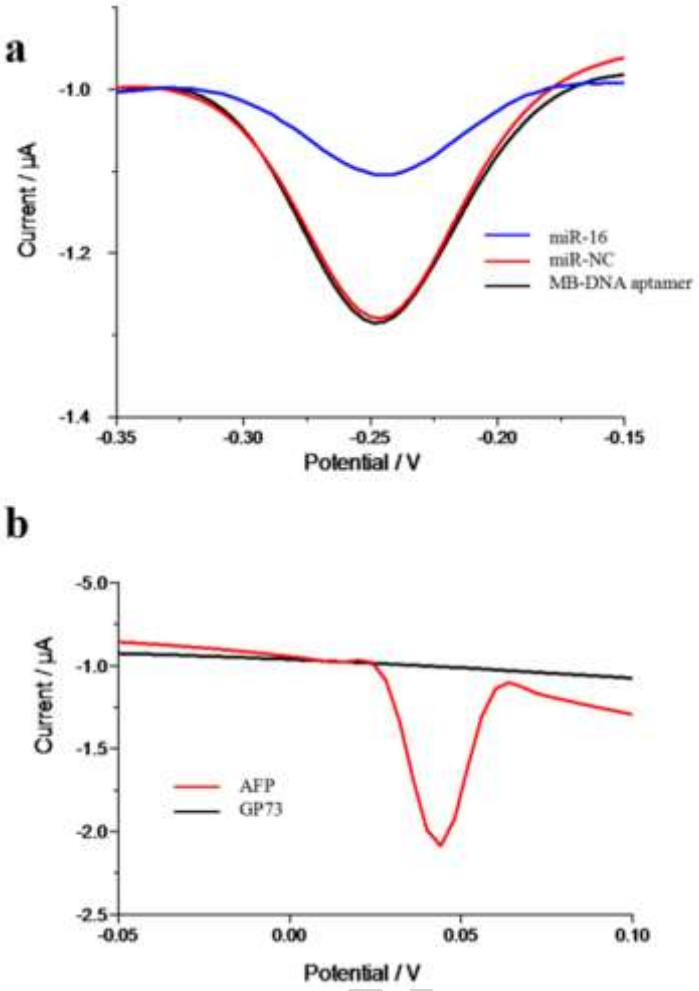


Fig. 4. (a) DPV of ESP modified gold electrodes after incubation with miR-16 and miR-NC. (b) DPV of ESP modified gold electrodes after incubation with AFP and GP73.

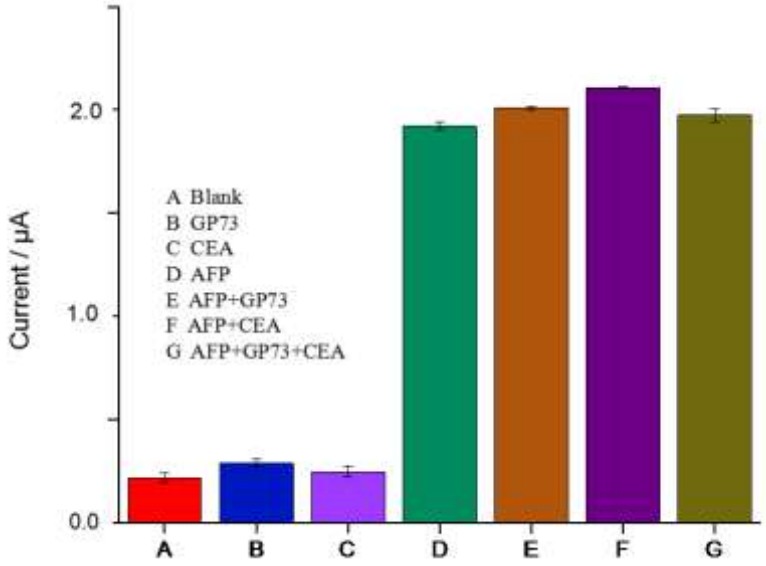


Fig. 5. Specificity of the proposed electrochemical biosensor. 5 ng/mL AFP and 5 ng/mL other interfering proteins were introduced in 1% serum. Error bars indicate standard deviations of three independent experiments.

Highlights

- One-step electrochemical detection of two serological biomarker species is achieved.
- A dual-functional DNA probe can both capture nucleic acid and protein biomarkers.
- Simultaneous detection of miR-16 and AFP improves the diagnostic accuracy of HCC.
- Two sensitive and non-interfering electrochemical signals are simultaneously given.