Analytical Methods

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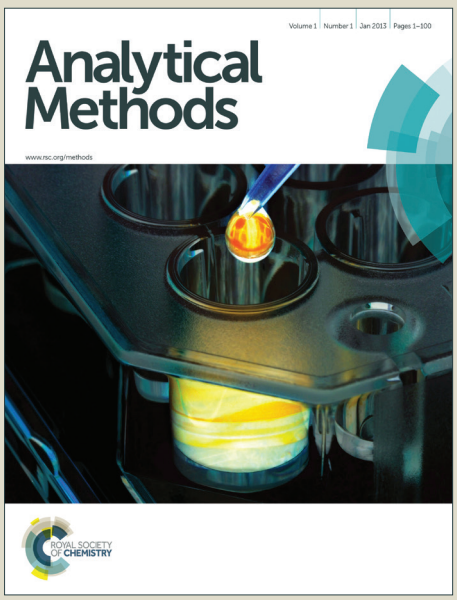

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Paper

Application of Oracet Blue in a novel and sensitive electrochemical biosensor for detection of microRNA

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MicroRNAs in serum/plasma, have been introduced as a novel, reliable and specific biomarkers for detection, status monitoring and population screening of a disease. Along with the molecular biology techniques, electrochemical biosensors have become the major biosensing methods for miRNA quantification. Here we have tried to develop an electrochemical biosensing system which is simple, easy to prepare, highly sensitive and selective, cost-effective and no need for sample preparation and/or amplification. We have evaluated an anthraquinone compound Oracet Blue (OB), as an intercalative electroactive label for miRNA electrochemical biosensing. The proposed electrochemical biosensor was made of a thiolated single strand capture probe (SH-modified SS-Probe) on an Au electrode (AuE). The role of OB was electrochemical signal transducing upon hybridization of the SS-Probe to the target miRNA. Under optimized conditions, target miRNAs can be detected from 50 pM to 15 nM with a detection limit of 13.5 pM. The biosensor clearly discriminated the target miRNA from a single base mismatch and non-complementary target oligonucleotides, which could guarantee a high selectivity and specificity. Moreover, the results of real sample assay of the proposed biosensor in human serum showed a good recovery percentage as well as high reproducibility which is promising its future potential use in clinical applications.

Introduction

MicroRNAs (miRNAs) have become one of the most accurate and reliable biomarkers which are used widely in detection of the cancer and other diseases. [1-3] miRNAs are short single-stranded ribonucleic acid (RNA) molecules of about 18–25 nucleotides in length (average ~22 nucleotides) that could play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression. The circulating miRNAs are present in the blood stream which made them easy to access for non-invasive sampling. [4, 5] Although they are already high-impact biomarkers, but easy and low-cost miRNA quantification method is still a real challenge. The current conventional miRNA detection strategies are majorly expensive, time-consuming and need a complex instruments and perhaps a qualified and trained operator. [5-7]

Along with the conventional miRNA quantification methods, different biosensors which the majority of them are Electrochemical have developed so far. Electrochemical biosensing methods are combining the high selectivity of the biochemical recognition with the high sensitivity of the electrochemical detection. Also, they are un-expensive, capable of being used by semi-skilled operators, their measurement is rapid and need a small volume of sample. [8-10] It is already reported that almost all of the electrochemical miRNA detection methods relying on hybridization; and the translation of hybridization into a measurable signal is mostly done with electroactive labels. [11, 12] These labels, which bind to DNA or RNA, are redox active molecules that preferably are either intercalated between two adjacent base pairs, or just interact with the DNA major or minor groove. [13, 14] Anthraquinones are redox-active molecules which can be utilized for electrochemical labelling of biomolecules and some of their derivatives are proven as remarkable intercalators. [13, 15, 16] Oracet Blue (OB) or 1-Amino-4-anilinoanthraquinone is an anthraquinone that we examined its function as a miRNA

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intercalator in electrochemical biosensing application for the first time. In Previous reports, Zare and his colleagues developed an OB modified glassy carbon electrode for the simultaneous detection of dopamine, ascorbic acid and uric acid. In another report, hydroxylamine sensor was built based on an OB-Multi-Walled Carbon Nanotubes film deposited on an electrode surface. In both cases, OB showed a great promise and exhibited a potent and persistent electrocatalytic behaviour for using as an electroactive compound. [17, 18] The chemical structures of the OB and its proposed electroactive reaction are shown in Fig. 1.

Fig. 1

So far, in many biosensors, the sulfhydryl (SH) modified single strand oligonucleotide probes (SH modified SS-probes) is attached and stabilized to the gold surface due to self-assembled and strong covalent bond between gold and sulphur which is transmitting electronic interactions between gold and sulphur-containing organic molecules. [19, 20]

Human miR-155 evaluated in this research as a target miRNA model for electrochemical studies, which is an oncogenic miRNA and up-regulated in some specific cancers and it is considered as a great diagnostic and prognostic biomarker in the serum. [6, 21, 22]

The electrochemical biosensors for miRNA detection have been applied widely, since the Gao and Yu developed an electrochemical miRNA assay for the first time at 2007. [23] The electrochemical sensing platform using biotin-streptavidin system have been developed with some modifications for detection of miR-222 [24]; let-7a [25, 26] miR-16, miR-15a and miR-660 [27]; miR-21 [28]; miR-200a [29]. In addition, application of enzymes have been reported with high-impact results with great sensitivity and detection limits for quantification of let-7b [30]; miR-720, let-7b, miR-1248 [31]; miR-21, miR-126 [32].

An innovation in using new components as signal transduction had reasonable results; such as detection of miR-21 using the oxidation signal of protein 19 (p19) as a molecular caliper [33]; detection of miR-141, miR-29b-1 and miRNA-103 using a conjugated copolymer [34], detection of let 7a using an electroactive ferroceneboronic acid (FBA) [35]. Also, the combination of some signal amplification methods could always led to great achievement and performance, despite the complexity of the platform. In this case, expert researchers have been shown their superb vision in electrochemical biosensors which could produce excellent results, for example the biosensors developed for let 7c [36] and miRNA-21 [37].

In this work, we evaluated for the first time, the application of the OB as an electroactive label for miRNA intercalation in hybridization-based biosensor with thiolated single strand capture probe (SS-probe) on a gold electrode (AuE). Furthermore, the proposed biosensor was tested in a real sample assay with the human serum.

2. Experimental

2.1. Instrumentations

The work electrode was the Gold electrode (AuE) with 2 mm diameter (geometric surface area of 0.0314 cm^2, and real surface area of 0.0208 cm^2). Also, a Saturated Calomel Electrode (SCE) was the reference electrode and a platinum electrode used as an auxiliary electrode. The Electrochemical experiments including Cyclic Voltammetry (CV), Differential Pulse Voltammetry (DPV) and Electrochemical Impedance Spectroscopy (EIS), were carried out using an Autolab potentiostat/galvanostat coupled with a frequency response analyzer (FRA), model PGSTAT 30 (EcoChemic, Utrecht, Netherlands) and GPES 4.9/FRA software at laboratory temperature (25 ± 1 °C). Moving average baseline correction with a peak width of 0.01 was used for treatment of DPV raw data. All the measured potentials were reported compared to the reference electrode.

2.2. Chemicals and solutions

All chemicals used in this study were of analytical grade and were used as received without further purification unless otherwise noted. 6-Mercapto-1-hexanol (MCH) was purchased from Sigma-Aldrich Company, USA, and the Oracet blue (OB) together with the other chemicals were purchased from Merck, Germany. All solutions, glassware and centrifuge tubes were autoclaved and kept isolated in room temperature. The buffer solutions used in this study as the following were prepared with ultra-pure water. TE buffer (pH 8.0), for dissolution and storage of nucleic acids, was prepared by adding 1.0 mL 1.0 M Tris (pH 8.0) and 20.0 µL 0.5 M Na_2EDTA (pH 8.0) to 800.0 mL deionized water and volume adjusted to 100.0 mL. Phosphate buffer solutions (PBS 0.1 M, pH 7.0), was prepared by adding 0.1 M H_3PO_4–NaH_2PO_4 to deionized water and the pH was adjusted with 2.0 M NaOH. The immobilization buffer was a 1.0 M phosphate buffer (pH 4.5) where the Washing and hybridization solutions were a 0.05 M phosphate buffer (pH 7.0) containing 0.3 M of NaCl. Oracet blue solution (0.15 mM) was prepared by adding Oracet blue powder to 42.0 mL of methanol in order to dissolve and 8.0 mL of phosphate buffer solution (pH 7.0) was added. The solution of 0.1 M H_2SO_4 was an electrochemical washing solution of the bare AuE, and, MCH solution was as 1.0 mM of MCH in deionized water. PBS was used for electrochemical measurements; except for the EIS experiments which were measured in a solution of 5.0 mM $K_3[Fe(CN)_6]$ containing 1.0 M KCl; and CV experiments which were measured in a solution of in 1.0 mM $K_3[Fe(CN)_6]$ in PBS buffer. In addition, the $[Ru(NH_3)_6]Cl_3$

solution, containing total concentration of 0.25 mM $[Ru(NH_3)_6]^{3+}$, was used for CV experiments.

2.3. Oligonucleotides

All the oligonucleotides were obtained HPLC purified in the form of lyophilized powder from Macrogen Corporation (South Korea) and used without further purification. The stock solutions of these oligonucleotides, were prepared with 0.1 M TE buffer solution for final concentration of 100.0 µM and kept frozen at -20 °C. The sequence of mature miR-155 (5'-TTAATGCTAATCGTGATAGGGGT-3') which have used as miRNA Target, and also miR-21 (5'-TAGCTTATCAGACTGATGTTGA-3') as non-complementary target, given from the miRBase database (http://www.mirbase.org). Furthermore, the substitution in single-based mismatch target, which is represented as an underlined letter in the regarding sequences, was based on replacing Purines for Pyrimidines or vice versa. The sequences of the other oligonucleotides are given below:
Single strand probe (SS-Probe): 5'-HS-(CH2)6-ACCCCTATCACGATTAGCATTAA-3'; Single-base mismatched Target: 5'-TTAATGCTAATAGTGATAGGGGT-3'

2.4. Pre-Treatment of the bare gold electrode

The bare Gold electrode was thoroughly polished with a 1.0 µm and 0.05 µm alumina–water slurry on a smooth polishing cloth until a mirror-like surface was obtained. Successively, the electrode was sonicated for 5 min in distilled deionized water to remove the alumina residual particles, and rinsed thoroughly with distilled water. Finally, the electrode was electrochemically cleaned by electrochemical cycling between -0.3 V and +1.5 V in a 0.1 M H_2SO_4 solution at a scan rate of 100 mVs^{-1} until reproducible cyclic voltammograms were obtained. Then it was rinsed with distilled water and dried under a nitrogen stream.

2.5. Biosensor fabrication

An aliquot of 2.0 µL of immobilization buffer solution containing 8.0 µM thiolated probes applied to the gold electrode surface and incubated in a high-humidity chamber for 120 min at room temperature for attachment of probes onto gold surface. Afterwards, the electrode was washed with a washing solution and was incubated in distilled water containing 1.0 mM of MCH for 5 min. At the end; the functionalized electrode was gently rinsed with distilled water to remove the loose adsorbed materials. The incubation time, concentration and immobilization method of single strand probe, the incubation time and concentration of the MCH, and also hybridization method and time were mainly based on our previous work. [38] To ensure the correct modification and assembly of the biosensor, CV performed after every preparation step in 1.0 mM $K_3[Fe(CN)_6]$ in PBS buffer at the potential range of -0.025 to 0.33 V and sweep rate of 0.02 Vs^{-1}; and the resulted cyclic voltammograms were compared to ensure the validity of the every modification steps. Additionally, the Electrochemical impedance spectroscopy (EIS) experiments were done for biosensor preparation assessment in a solution of 5.0 mM $K_3[Fe(CN)_6]$ containing 1.0 M KCl, from 100 KHz to 0.01 Hz with amplitude 5 mV and potential 0.27 V. Additionally, different CV experiment have performed in a solution of 0.25 mM $[Ru(NH_3)_6]^{3+}$ in PBS buffer to reconfirm the results of CV and EIS experiments in $K_3[Fe(CN)_6]$ solution.

2.6. miRNA measurements

The resulted modified electrode was subjected to desired concentration of target miRNA solution for 120 min and then gently rinsed with washing solution. Afterwards, it was immersed in OB solution for 60 min on a stirrer with very slow rotation for better accumulation on the resulted hybrid of probe-miRNA assumed via intercalation. The reduction signals of OB were measured by DPV at 25 mV modulation amplitude scanning from +0.23 to 0.0 V, with step potential 0.0049 in PBS buffer (pH 7.0). To provide a control, as soon as the biosensor prepared, it was directly immersed in the OB solution without hybridization by target miRNA, and the resulted DPV voltammogram (as a control) compared with the voltammogram of the target-hybridized biosensor.

2.7. Real sample assay

For the real sample evaluation of the biosensor, the EDTA-treated serum was used instead of hybridization buffer and final desired concentrations were made by adding synthesized miRNA into an EDTA-treated blood serum. Three different final concentrations (50.0, 500.0, 5000.0 pM) of miRNA in serum with five replications were analysed as same as the previous analytical studies of the biosensor.

For isolating the serum, the blood was taken from a healthy non-cancerous man (age 30) and collected in the simple container without any additive and allowed to clot at room temperature for 60 min; then it was kept in the refrigerator (4 °C) overnight. The samples were centrifuged twice at 150 g for 5 min and then 350 g for 15 min and the expressed serum was collected in a tube with 0.2 M EDTA in order to inactivate enzymes and also disrupt the miRNA containing exosomes. After all, the tubes were gently inverted several times and divided into aliquots and kept frozen at -20 °C. All real sample assays performed only with fresh or short-term stored serum (maximum 3 days) and avoided any freeze-thaw cycles.

3. Results and Discussion

In the presented biosensor, the concentration of miRNA target was detected by redox signals DPV of the intercalated OB and comparing the signals of the probe with hybridized miRNA-probe. We used a very efficient, easy and convenient combination of methods and techniques to build a simple and effective biosensor where the OB provides the high resolution and sensitive discrimination. We used miR-155 as a model for this research to prove the functionality and performance of the biosensor. Furthermore, the assay in human serum was performed to assess the usability of the biosensor directly in medical samples. The Scheme 1 provides a clear schematic of the proposed biosensor work steps, material and short comments of concentration and time period of applied materials.

Scheme. 1

3.1. Assessment of the fabrication procedure

To ensure the accuracy of the biosensor preparation procedure, The cyclic voltammograms of biosensor (measured in 1.0 mM $K_3[Fe(CN)_6]$ solution after every step of the assembling), were compared (Fig. 2A). As it can be seen, high peak current voltammogram obtained for the bare AuE (curve a), while for the SS-probe/AuE, the current dramatically decreased due to the unordered immobilization of the thiolated SS-probe to the electrode surface which blocks and reduces the electron transfer of the $K_3[Fe(CN)_6]$ to the AuE (curve b). After MCH treatment, the probes decorates more organized and the wrong orientated oligos were removed, thus the peak current increased a little (curve c). Finally, after hybridization of the target miRNA/SS-Probe, the current decreased significantly (curve d). The possible explanation is the accumulation of the hybridized oligonucleotides on the AuE surface which are negatively charged and also making extra blocking layer for the electron transfer of the $K_3[Fe(CN)_6]$ on the AuE surface. Beside the changes in the current which were explained, for the curve b and curve d, a slight positive shift occurred in the anodic peak potential, and also a negative shift in the cathodic peak potential was observed. Again this could be explained by the fact that after immobilization of the SS-probe, the oxidation and reduction of the $[Fe(CN)_6]^{3-/4-}$ ions on the modified AuE is occurred harder and even much harder when the target miRNA is hybridized with the SS-probe on the AuE surface. This might be originating from the negative charge and also the spatial blockage of the oligonucleotides for $[Fe(CN)_6]^{3-/4-}$ ions to reach the AuE surface.

Additionally, the results of CV experiments of the modified electrodes in the $[Ru(NH_3)_6]^{3+}$ have shown in Fig 2B. As it is presented, the bare AuE had a high peak current voltammogram (curve a), but after immobilization of the SS-Probe and MCH treatment on the AuE surface, the current decreased due to block up the surface of the electrode by SS-probes and MCh molecules (curve b). With hybridization of the target miRNA with the immobilized SS-probes, the electrode surface is further blocked; therefore the current decreased more than before (curve c). These observations also support the results of CV experiments in $[Fe(CN)_6]^{3-/4-}$ solution and is a valid method to evaluate the electrode surface in every modification step.

Fig 2

The results of the EIS study in the solution of 5.0 mM $K_3[Fe(CN)_6]$ containing 1.0 M KCl are shown as a Nyquist plot in Fig. 3. The plot represents changes in the charge transfer resistance (R_{ct}) of the electrode surface during the biosensor preparation after every electrode modification. In comparison with the bare AuE (curve a), the probe modified AuE showed a much higher charge transfer resistance (3764 Ω) (curve b), but at the probe/MCH modified AuE, the charge transfer resistance slightly decreased (3071 Ω) (curve c). Finally, the target hybridized/probe modified AuE showed significantly higher charge transfer resistance, which is a proof for the accuracy of the hybridization process (8036 Ω) (curve d). These results also confirm the results of CV experiment, and validate the successful self-assembly of SS-probe on the AuE and also hybridization of target miRNA with SS-probe.

Fig 3

3.2. Optimization of the fabrication procedure

In order to utilize the fabricated biosensor for actual medical laboratory detection, the whole procedure of the biosensor preparation was performed in 25 °C Temperature and pH 7.0. Such biosensor does not need any pre-treatment of serum/plasma samples, RNA extraction or amplifications. In addition, as it is mentioned earlier in the biosensor fabrication section, the incubation time, concentration and immobilization method of single strand probe, and also the incubation time and concentration of the MCH, and also hybridization method and time were mainly based on our previous work. [38]

To define the optimized condition, OB concentration and incubation time were optimized separately by different experiments on the biosensors which were hybridized with the target miRNA. The OB was tested in 10 different concentrations from 0.005 mM to 0.3 mM, and 11 different incubation time ranges from 5 to 120 minutes (Fig. 4). The results showed that the highest current of the OB concentrations (369.1 ± 4.8 nA) belonged to the 0.15 mM OB, while for the OB incubation time, the highest current (415.9 ± 6.2 nA) belonged to the 60 min incubation time. In conclusion, for all analytical experiments of the biosensor, the OB solution was used as 0.15 mM concentration for 60 minutes. All these experiments have performed in three replications and

respected error bars of RSD percentages have reported in the Fig 4.

Fig 4

3.3. General assessment of the biosensor

To assess the performance of OB as an electroactive label in the presented biosensor, the DPV voltammograms of the control, target-hybridized biosensor and the MCH modified bare Au electrode which were treated in OB solution for 60 min were compared on a single graph (Fig. 5A). The large difference between current of the probe and target in this voltammograms presented the perfect resolution of the OB to distinct a single strand from double strand oligonucleotide which proves an intercalating method as its functional method. Also, a very low difference between bare and Probe-modified Au electrode showed a little interaction of the OB with single strand oligonucleotide, as well. Furthermore, a non-significant difference between bare and MCH modified Au electrode presented that the OB does not interact with neither of them.

3.4. Selectivity evaluation

The specificity of the method for the presented miRNA assay was evaluated using the comparison of a target miRNA (human miR-155), a one-base mismatch target, and the non-complementary target (human miR-21). The resulting graph (Fig. 5B) showing clear discrimination between the Probe modified electrode, the non-complementary target (miR-21), single base-mismatched miRNA target and the complementary target (miR-155). The significant results of the selectivity tests might be derived from the intercalating method of the OB interacting with oligonucleotides which can make a difference from single strand oligo, partly hybridized targets and fully hybridized targets. Therefore, just a complementary oligonucleotide, specific to target miRNA-155, could cause a significant increase in OB attachments and its reduction signal current.

Fig 5

3.5. Analytical performance of the biosensor

The differential pulse voltammograms of intercalation of the OB in the different concentrations of the target miRNA for 120 min in 0.1 M phosphate buffer solution is shown in Fig. 6. As presented, the OB intercalation signals are increased with the increase of the target concentration and levelled off at 15.0 nM. As revealed in the calibration plot, log Concentrations of miRNA versus current, for the wide concentration range from 50.0 pM to 15.0 nM of the target miRNA is linear. The Detection limit estimated 13.5 pM from calculation of the standard deviation of the 12 replications of blank response and the slope of the calibration plot, with signal/noise ratio of 3.

The results were satisfying considering the rapid response, fast preparation, uncomplicated and low-cost preparation of the biosensor, and no need of any sample preparation and amplification of the target miRNA. Moreover, the linear range and detection limit of the biosensor is better than the average reported by previous studies. Furthermore, the conventional miRNA quantification methods such as RT-qPCR, Microarray, Sequencing, etc., in comparison to this simple electrochemical biosensor are more expensive, complex and time-consuming procedure with low throughput, less sensitivity and selectivity, and require a skilled operator and advance technologies. [39-43]

Fig 6

Reproducibility of the biosensor was also evaluated by repeating the optimized fabrication process for four times in 2 nM target miRNA and the peak currents of the output DPV voltammograms were statistically analyzed. The average of the highest resulted currents in four distinct replications was 304.1 nA with RSD of 2.34%. The low RSD percentage represents the ability of the fabrication procedure to be reproducible and can perform easily without reasonable error or bias.

Table 1, is comparing some specifications of the proposed biosensor to previously published electrochemical miRNA biosensors. As it can be seen, the responses of the fabricated biosensor are superior in some cases, especially linear dynamic range and detection limit comparing to other reported biosensors, and also to most of the conventional miRNA quantification methods. In addition, it is the most simple and cost-effective sensor among all of them, without the need of RNA extraction or amplification. [39-43] This biosensor is superior in linear range and detection limit compare to our previous DNA biosensor which were based on thiolated SS-probe on the AuE with measurable signals of hematoxylin with a linear range of 12.5-350.0 nM and detection limit of 3.8 nM. Therefore, based on the similarity of the present OB-based biosensor and this hematoxylin-based biosensor, it can be concluded that the OB is relatively better for sensitivity reasons [38]. Additionally, with comparison of OB-based biosensor with the Methylene blue-based biosensors from previously published DNA biosensors made it clear that OB, as an anthraquinone, is equally acceptable in high detection limit and repeatability while, it is superior in time consumption and stability. [44-45]

Table 1

3.6. Real sample analysis

As the Table 2 shows, the results of the real sample analyses which were done by the proposed biosensor in three different concentrations of the target miRNA added to the human serum. The resulted high recovery and low RSD percentages were excessive and acceptable for such a simple and fast prepared biosensor. Despite the high electrochemical activity of the serum components, the OB showed reduction peaks without any background noises. Furthermore, the reproducibility of the biosensor in the real sample was substantial according to the low RSD values.

To confirm the results of serum assay, we have quantified the miRNA-155 in two different sample types, blood and breast tissue samples from breast cancer patients, with 50.0 pM of initial extracted microRNA. The results obtained from 9 different biological samples (with 3 replications for each), the RSD of 4.17 % for blood samples, and 7.5 % for tissue samples were obtained. Therefore, based on this finding, the RSD of the presented biosensors in real samples are superior in comparison to the usual RT-PCR technique (SYBR-green). As it is clear, the lower RSD represents the outstanding accuracy and reliability of the biosensor.

Table 2

4. Conclusions

For the first time, we have developed an electrochemical biosensor for miRNA detection based on an anthraquinone component named Oracet Blue (OB). The simple mechanism and fabrication of the biosensor was based on the thiol modified SS-probe immobilized on the AuE surface, but the sensitivity of the biosensor was very considerable due to high resolution and low-noise reduction signals of the OB. With the assumption of the OB intercalation with hybridized oligonucleotides, it was able to discriminate and distinguish very well between target miRNA, single-base mismatch target, and non-complementary target. Moreover, the biosensor was tested in the human serum containing synthetic target miRNAs and the results were excessive. Considering the analytical performance of the proposed biosensor in synthetic and real sample environments, with advantages of simple, low cost and reproducibility of the preparation method, the proposed biosensor has several potential for clinical applications in circulating miRNAs quantification as biomarkers. It also could be used for screening of the patients or early detection of diseases such as cancer, and also for evaluating the efficiency of therapies.

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References

1 W. Zheng, T. E. Chase and L. He, Anal. Methods, 2014, 6, 2399-2405. (DOI: 10.1039/c3ay41752b)
2 M. D. Jansson and A. H. Lund, Mol. Oncol., 2012, 6, 590-610. (DOI: 10.1016/j.molonc.2012.09.006)
3 S. Srivastava, A. Bhardwaj, S. Leavesley, W. Grizzle, S. Singh and A. Singh, Biotechnic. Histochem., 2013, 88, 373-387. (DOI: 10.3109/10520295.2012.730153)
4 D. P. Bartel, Cell, 2004, 116, 281-297 (DOI: 10.1016/S0092-8674(04)00045-5)
5 H. Dong, J. Lei, L. Ding, Y. Wen, H. Ju and X. Zhang, , Chem. Rev., 2013, 113, 6207-6233. (DOI: 10.1021/cr300362f)
6 G. Di Leva and C. M. Croce, Clin. Biochem., 2013, 46, 840-841. (DOI: 10.1016/j.clinbiochem.2013.05.060)
7 J. Li, S. Tan, R. Kooger, C. Zhang and Y. Zhang, Chem. Soc. Rev., 2014, 43, 506-517. (DOI: 10.1039/c3cs60312a)
8 T. Tian, J. Wang and X. Zhou, Org. Biomol. Chem., 2015, 13, 2226-2238. (DOI: 10.1039/c4ob02104e)
9 B. N. Johnson and R. Mutharasan, Analyst, 2014, 139, 1576-1588. (DOI: 10.1039/c3an01677c)
10 M. Labib and M. V. Berezovski, Biosens. Bioelectron., 2015, 68, 83-94. (DOI: 10.1016/j.bios.2014.12.026)
11 E. Hamidi-Asl, I. Palchetti, E. Hasheminejad and M. Mascini, Talanta, 2013, 115, 74-83. (DOI: 10.1016/j.talanta.2013.03.061)
12 S. Campuzano, M. Pedrero and J. M. Pingarrón, Anal. Bioanal. Chem., 2014, 406, 27-33. (DOI: 10.1007/s00216-013-7459-z)
13 M. B. Gholivand, S. Kashanian and H. Peyman, Spectrochimica Acta Part A, 2012, 87, 232–240. (DOI: 10.1016/j.saa.2011.11.045)
14 C. Batchelor-McAuley, Q. Li, S. Dapin and R. Compton, J. Phys. Chem. B, 2010, 114, 4094-4100. (DOI: 10.1021/jp1008187)
15 J. Balintova, R. Pohl, P. Horakova, P. Vidlakova, L. Havran, M. Fojta and M., Hocek, Chem. Eur. J., 2011, 17, 14063-14073. (DOI: 10.1002/chem.201101883)
16 M. B. Gholivand, S. Kashanian, H. Peyman and H. Roshanfekr, Eur. J. Med. Chem., 2011, 46, 2630-2638. (DOI: 10.1016/j.ejmech.2011.03.034)
17 H. R. Zare and N. Nasirizadeh, J. Braz. Chem. Soc., 2012, 23, 1070-1077. (10.1590/S0103-50532012000600011)
18 H. R. Zare, N. Rajabzadeh, N. Nasirizadeh and M. Mazloum Ardakani, J. Electro. Chem., 2006, 589, 60-69. (DOI:10.1016/j.jelechem.2006.01.011)
19 H. Häkkinen, Nat. Chem., 2012, 4, 443-455. (DOI: 10.1038/nchem.1352)

20 B. N. Johnson and R. Mutharasan, Anal. Chem., 2014, 84, 10426-36. (DOI: 10.1021/ac303055c)
21 W. Zhu, W. Qin, U. Atasoy and E. R. Sauter, BMC. Res. Notes, 2009, 2, 89. (DOI: 10.1186/1756-0500-2-89)
22 I. Berindan-Neagoe, P. C. Monroig, Pasculli, and G. A. Calin, CA Cancer J. Clin., 2014, 64, 311-336. (DOI: 10.3322/caac.21244)
23 Z. Gao and Y. H. Yu, Biosens. Bioelectron., 2007, 22, 933-940. (DOI: 10.1016/j.bios.2006.04.020)
24 F. Bettazzi, E. Hamid-Asl, C. L. Esposito, C. Quintavalle, N. Formisano, S. Laschi, S. Catuogno, M. Iaboni, G. Marrazza, M. Mascini, L. Cerchia, V. De Franciscis, G. Condorelli and I. Palchetti, Anal. Bioanal. Chem., 2013, 405, 2-3: 1025-1034. (DOI 10.1007/s00216-012-6476-7)
25 Z. Cai, Y. Song, Y. Wu, Z. Zhu, C. J. Yang and X. Chen, Biosens. Bioelectron., 2013, 41, 783-788. (DOI: 10.1016/j.bios.2012.10.002)
26 Y. Peng, J. Jiang and R. Yu, Anal. Methods, 2014, 6, 2889-2893. (DOI: 10.1039/c4ay00033a)
27 A. Erdem and G. Congur, Talanta, 2014, 118, 7-13. (DOI: 10.1016/j.talanta.2013.09.041)
28 T. Kilic, S. N. Topkaya, D. Ozkan Ariksoysal, M. Ozsoz, P. Ballar, Y. Erac and O. Gozen, Biosens. Bioelectron., 2012, 38, 195-201. (DOI: 10.1016/j.bios.2012.05.031)
29 Z. Wang, J, Zhang, Y, Guo, X. Wu, W. Yang, L. Xu, J. Chen and F. Fu, Biosens. Bioelectron., 2013, 45, 108-113. (DOI: 10.1016/j.bios.2013.02.007)
30 Y. Ren, H. Deng, W. Shen and Z. Gao, Anal. Chem., 2013, 85, 4784-4789. (DOI: 10.1021/ac400583e)
31 Z. Gao and Y. Peng, Biosens. Bioelectron., 2011, 26, 3768-3773. (DOI: 10.1016/j.bios.2011.02.029)
32 Y. Yu, Z. Chen, L. Shi, F. Yang, J. Pan, B. Zhang and D. Sun, Anal. Chem, 2014, 86, 8200-8205. (DOI: 10.1021/ac501505a)
33 T. Kilic, S. N. Topkaya and M. Ozsoz, Biosens. Bioelectron., 2013, 48, 165-171. (DOI: 10.1016/j.bios.2013.04.011)
34 H. V. Tran, B. Piro, S. Reisberg, G. Anquetin, H. T. Duc and M.C. Pham, Anal. Bioanal. Chem., 2014, 406, 1241-1244. (DOI: 10.1007/s00216-013-7292-4)
35 N. Xia, X. Wang, D. Deng, G. Wang, H. Zhai and S. J. Li, Int. J. Electrochem. Sci., 2013, 8, 9714-9722. (DOI: Not Available)
36 Z. Gao, H. Deng, W. Shen and Y. Ren, Anal. Chem., 2013, 85, 1624-1630. (DOI: 10.1021/ac302883c)
37 C. Y. Hong, X. Chen, T. Liu, J. Li, H. H. Yang, J. H. Chen and G. N. Chen, Biosens. Bioelectron., 2013, 50, 132-136. (DOI: 10.1016/j.bios.2013.06.040)
38 N. Nasirizadeh, H. R. Zare, M. H. Pournaghi-Azar and M. S. Hejazi, Biosens. Bioelectron., 2011, 26, 2638-2644. (DOI: 10.1016/j.bios.2010.11.026)
39 Z. Jiang, H. Wang, X. Zhang, C. Liu and Z. Li, Anal. Methods, 2014, 6, 9477-9482. (DOI: 10.1039/c4ay02142h)
40 S. Guo, F. Yang, Y. Zhang, Y. Ning, Q. Yao and G. Zhang, Anal. Methods, 2014, 6, 3598-3603. (DOI: 10.1039/c4ay00345d)
41 H. L. Chen, M. M. Guo, H. Tang, Z. Wu, L. J. Tang, R. Q. Yu and J. H. Jiang, Anal. Methods, 2015, 7, 2258-2263. (DOI: 10.1039/c4ay02938k)
42 M. Keshavarz, M. Behpour, H.A. Rafiee-pour, RSC Adv., 2015, 5, 35651–35660. (DOI: 10.1039/c5ra01726b)
43 A.L.K. Teo, C.L. Lim, Z. Gao. Electrochimica Acta, 2014, 126, 19–30. (DOI: 10.1016/j.electacta.2013.06.113)
44 E. L. Wong, P. Erohkin, J.J. Gooding, Electrochemistry Communications, 2004, 6, 648–654. (10.1016/j.elecom.2004.05.002)
45 E. L. Wong, J.J. Gooding, Anal. Chem, 2003, 75, 3845-3852. (DOI: 10.1021/ac034129d)

Analytical Methods

Paper

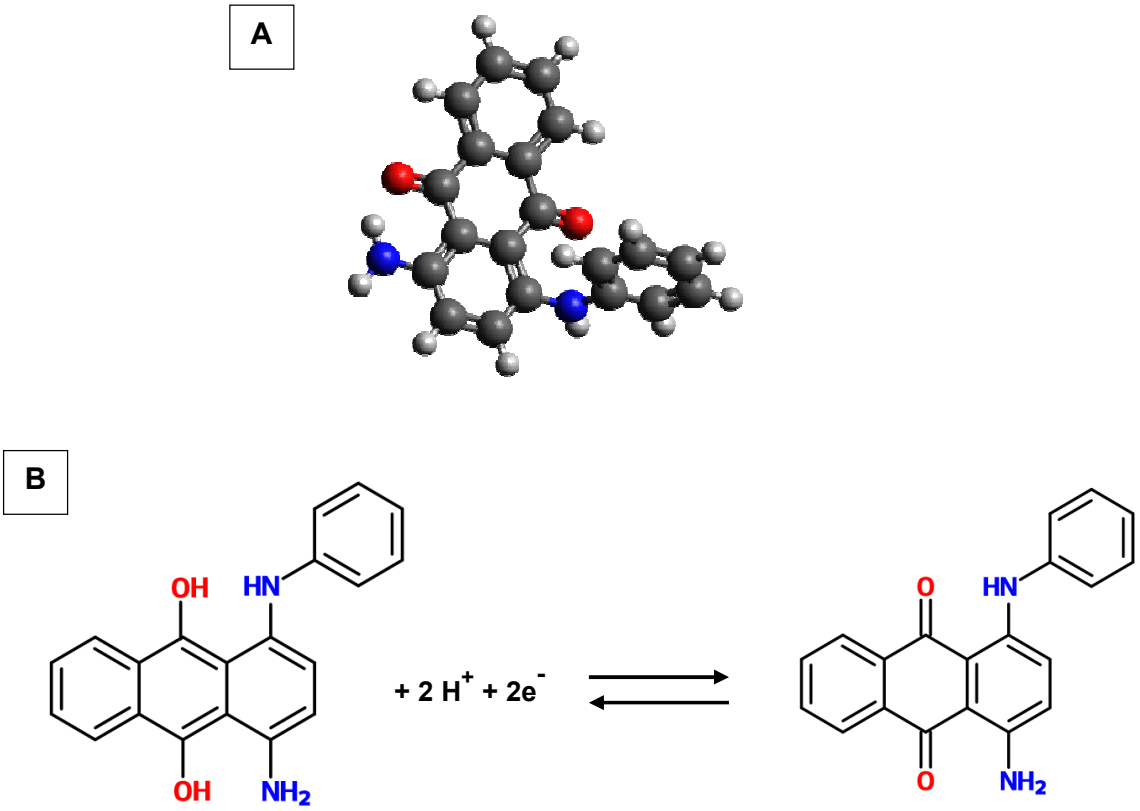
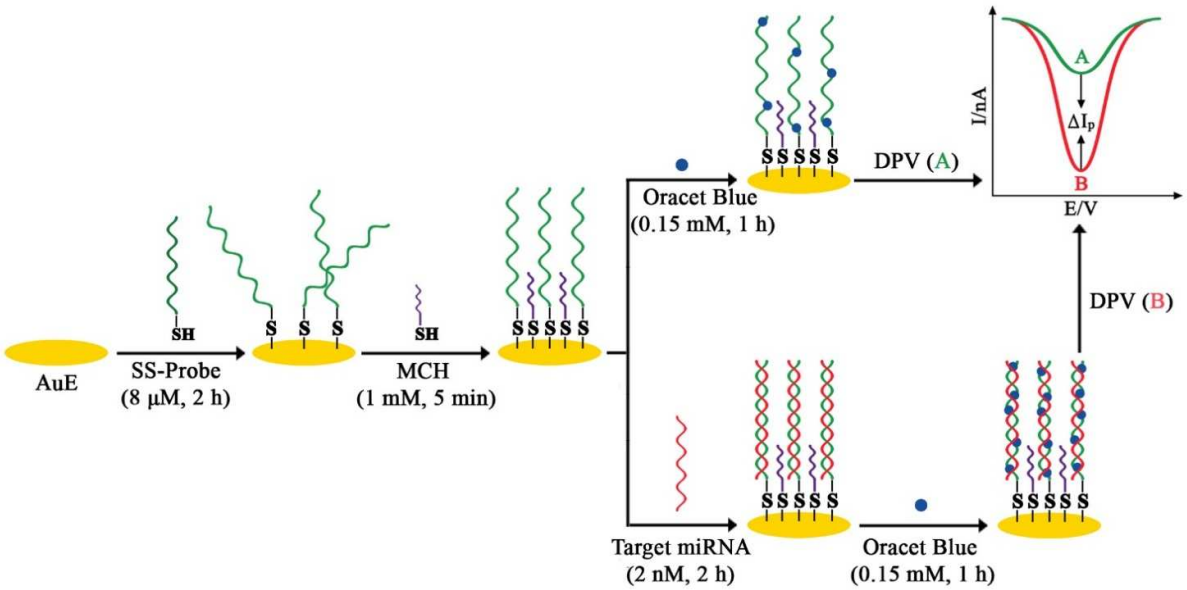


Fig.1. The structure (A) and the proposed electroactive reaction (B) of the Oracet Blue molecule.



Scheme. 1. Schematic illustration of the biosensor preparation for miRNA detection.

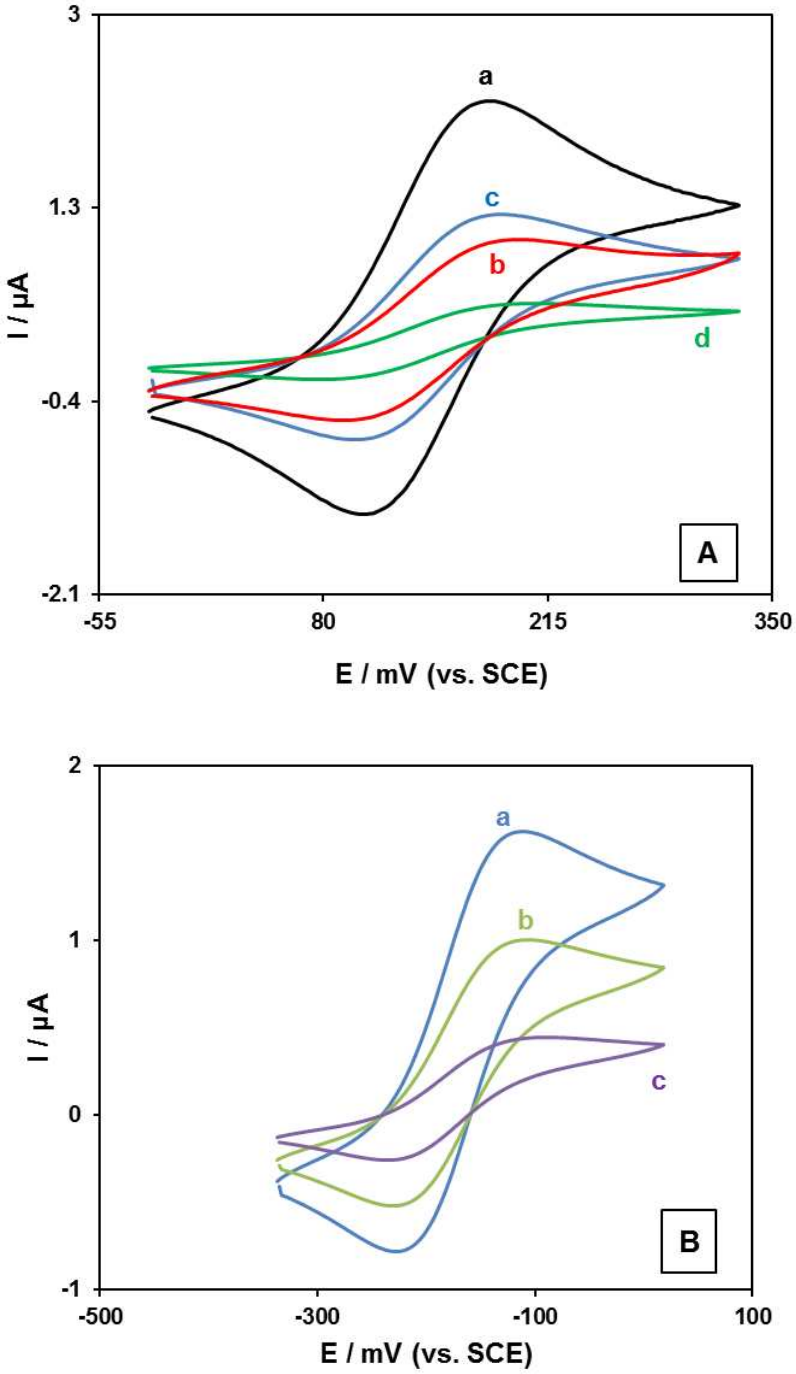


Fig 2. (A)The Cyclic Voltammograms of the modified electrode in the $K_3[Fe(CN)_6]$ solution after every step of the biosensor preparation. (a) Bare AuE, (b) SS-probe modified, (c) SS-probe/MCH modified, (d) Target miRNA hybridized with SS-probe/MCH/ modified AuE. (B) The Cyclic Voltammograms of the modified electrode in the $[Ru(NH3)_6]Cl_3$ solution after every step of the biosensor preparation. (a) Bare AuE, (b) SS-probe/MCH modified, (d) Target miRNA hybridized with SS-probe/MCH/ modified AuE.

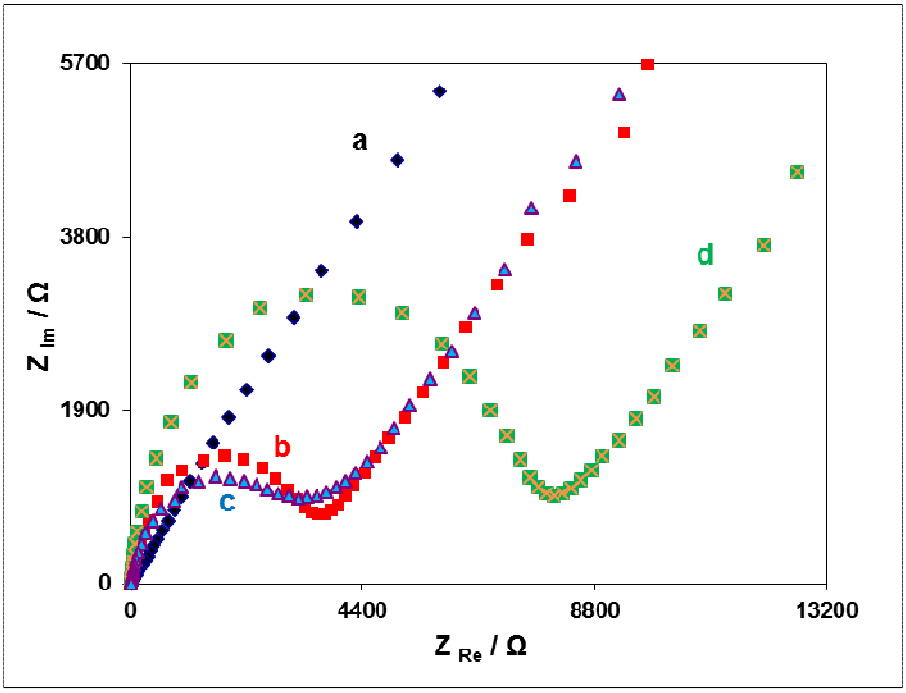


Fig. 3. The Nyquist plot of different electrodes in the solution of 5.0 mM $K_3[Fe(CN)_6]$ containing 1.0 M KCl. (a) bare AuE, (b) SS-probe modified, (c) SS-probe/MCH modified, (d) Target miRNA hybridized with SS-probe/MCH/ modified AuE.

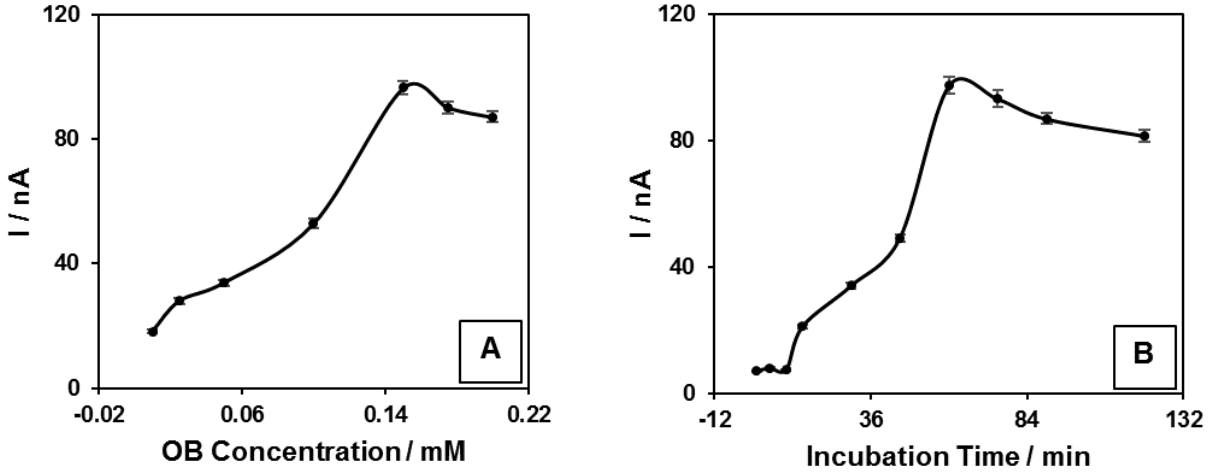


Fig. 4. The optimization results of the (A) concentration and (B) incubation time of Oracet Blue for the biosensor preparation.

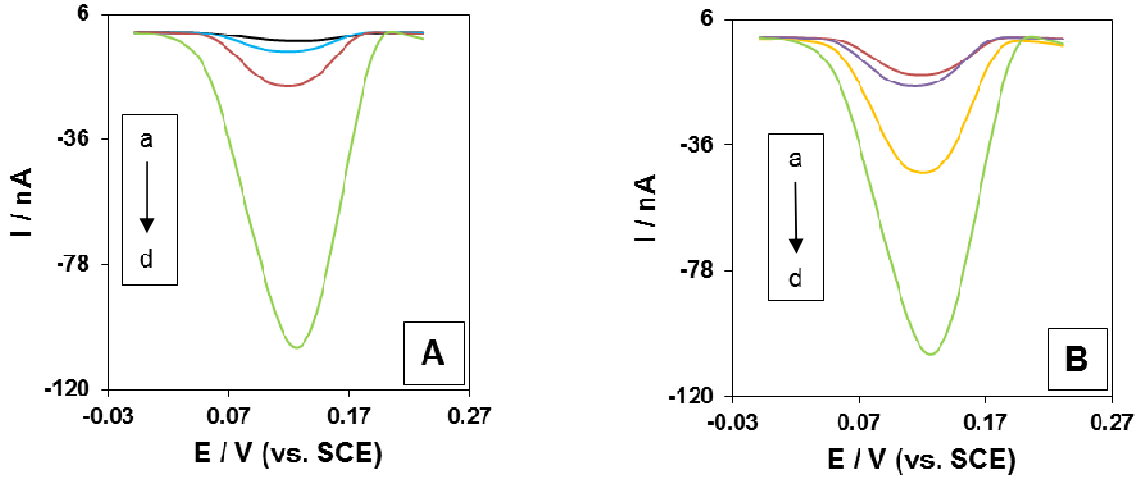


Fig. 5. DPVs of the different modified electrodes performed in the PBS (pH=7.0) which the electrodes were incubated in the OB solution for 120 min, for representing performance of the OB (A) and Selectivity of the biosensor (B). In the graph A, the letters correspond to the (a) bare AuE, (b) MCH modified, (c) SS-Probe modified, and (d) 2 nM complementary miRNA modified AuE. In the graph B, the letters correspond to the (a) SS-Probe modified AuE, (b) non-complementary miRNA, (c) single-base mismatch target miRNA, and (d) complementary target miRNA.

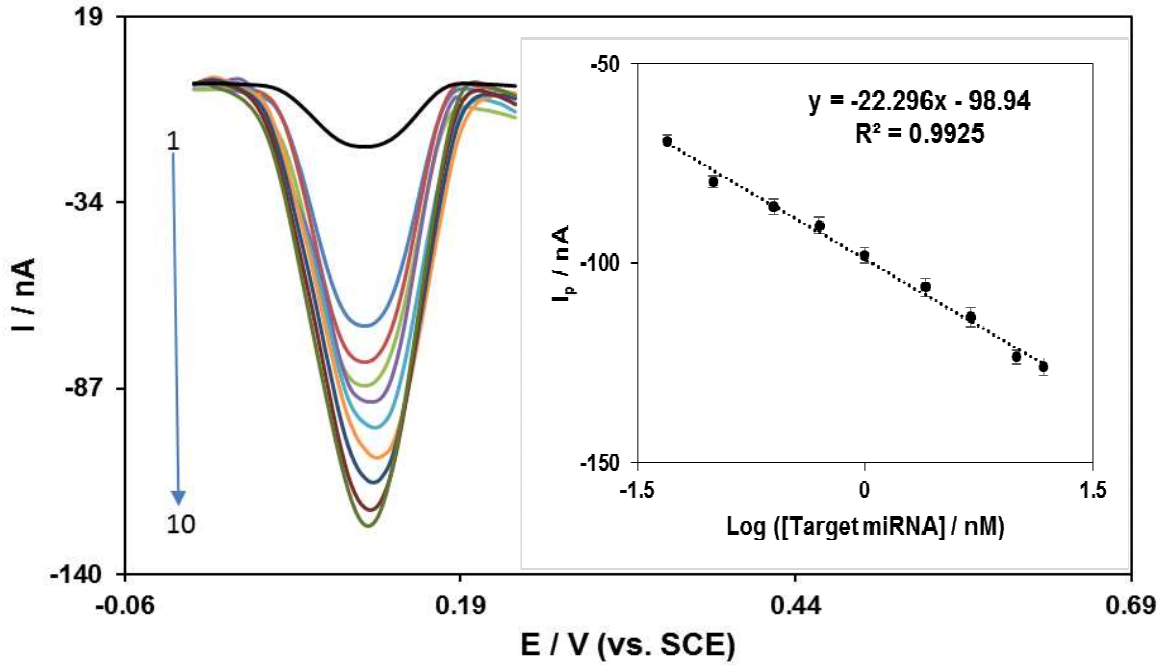


Fig. 6. DPVs of OB intercalation on the hybridized probes with different concentrations of miRNA for 120 min. The number 1 correspond to 0 pM, and numbers 2 - 10 correspond to 50 pM - 15 nM target miRNA, respectively. Inset shows the plot of log concentration of miRNA versus current response of the OB, and the error bars are RSD from 4 replications.

Table 1. The comparison of our biosensor specification with previously published electrochemical miRNA biosensors.

Base Electrode + Biosensor Components/Modifications	Electrochemical Method	Linear Range	Detection Limit	Ref.
ITO coated glass slides+CP/$Ru(PD)_2Cl_2$/hydrazine	Amp	0.50 pM -400.0 pM	0.20 pM	23
CSP+Biotinylated CP/streptavidin/magnetic beads/enzymatic reaction	DPV	0.01 nM -1.0 nM	12.0 pM	24
AuE+Allosteric MB/streptavidin peroxidase polymer/TMB	CV	20.0 pM – 100.0 nM	3.4 pM	25
SP arrays+Streptavidin/Magnetic beads/biotinalted CP/G oxidation	DPV	1.3 µM-5.5 µM	2.1 µM	27
PGE+Probe/EDC/NHS/Extravidin labeled alkaline phosphatase	DPV	NA	100.0 nM	28
AuE+CP/nuclease/isothermal amplification/ $K_3Fe(CN)_6/K_4Fe(CN)_6$	EIS	2.0 fM-2.0 pM	1.0 fM	30
AuE+Long probe/enzyme digestion/GO-tagged PNA CP	Amp	20.0 fM-10.0 pM	10.0 fM	31
AuE+Arched CP/isothermal amplification/hemin/polymerase/nickase	DPV	20.0 fM-50.0 pM	5.36 fM	32
PGE+Protein 19/Glucose oxidase	DPV	NA	160.0 nM	33
AuE+Electrochemically active FA/conformational change aptamer	DPV	5.0 nM-5.0 µM	1.0 nM	35
ITO coated glass slides+Poly(3,3 ’-dimethoxybenzidine) film/HRP	EIS	5.0 fM-2.0 pM	2.0 fM	36
AuE+Thiolated CP/ Oracet Blue	DPV	50.0 pM- 15.0 nM	13.5 pM	This work

Abbreviations: ITO: Indium tin oxide, CP: Capture probe, AuE: Au Electrode, CSP: Carbon Screen Printed, SP: Screen Printed, MB: Molecular Beacons, PGE: Pencil graphite electrode, GCE: Glassy Carbon Electrode, TMB: (3,3',5,5'-tetramethylbenzidine), EDC: N-(dimethylamino)propyl-N’-ethylcarbodiimide hydrochloride, NHS: N-hydroxysulfosuccinimide, PNA: peptide nucleic acid, GO: glucose oxidase, FA: ferroceneboronic acid, HRP: horseradish peroxidase, CV: Cyclic Voltammetry, DPV: Differential Pulse Voltammetry, SWV: Squarewave Voltammetry, EIS: Electrochemical Impedance Spectroscopy, Amp: Amperometry.

Table 2. The Real sample analysis of the biosensor with three different added miRNA concentrations in five replications.

Sample Number	Added miRNA/pM	Detected/pM	Recovery (%) (n=5)	RSD (%)
1	50.0	50.7	101.5	4.0
2	500.0	491.6	98.3	2.4
3	5000.0	4899.6	98.0	3.1

Graphical Abstract