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RESEARCH ARTICLE

ANTI-miRNA IMMOBILIZATION OPTIMIZATION ON THE SCREEN PRINTED ELECTRODES FOR ELECTROCHEMICAL miRNA BIOSENSORS

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ABSTRACT

Synthetically produced miRNA molecules plays an important role as biomarker to examine and investigate the diagnosis of some diseases including cancer. In order to develop a sensitive electrochemical biosensor system for the detection of miRNA molecules, the anti-miRNA molecules are synthesized and immobilized on the biosensor surfaces and observe the signal changes via a proper measurement. Immobilization time and temperature along with the anti-miRNA concentration are critically important for an appropriate observation of the miRNA detection sensitivity of the prepared biosensor system. In this regard, synthetically produced anti-miRNA **(**anti-miR451(G)**)** was purchased and diluted into different concentration by using phosphate buffer solution. Then, the solutions were immobilized on the screen printed electrodes (SPEs) and the guanine oxidation signal of the anti-miRNA molecules were observed via differential pulse voltammetry method (DPV). An appropriate concentration of the solution was selected and dropped on the SPEs and held on at different temperatures (-18, +5 and +25 ºC) for 1, 3, 14 and 21 days and DPV measurements were conducted to investigate the optimum immobilization time and temperature. The result shown that guanine oxidation signal was increased by increasing the concentration of the genetic molecules in the immobilization solution and increased less after that point when the concentration increased more because the surface reached to a certain saturation value . The guanine oxidation signal revealed that the best suitable storing temperature after the immobilization was +5 ºC determined.

Keywords: miRNA detection, Screen printed electrode, DPV measurement, Guanine oxidation

1. INTRODUCTION

Early diagnosis of diseases is important for determining an effective treatment program. Diagnosis of many diseases can be achieved by detecting and examining genetic molecules. For this, the genetic molecule can be obtained directly from the living creature that has caught the disease, or can be obtained and examined from the microorganism that causes the disease. For example, some diseases such as breast cancer[1], haemophilia A [2] and cystic fibrosis [3] are related with the human genome and could be diagnosed via genetic methods. Covid-19 [4] and influenza [5] are virus related diseases and could be diagnosed by extracting the genetic molecules from the infecting virus and investigating them by using genetic characterization methods.

The proper detection of genetics base sequences in human, viral and bacterial nucleic acids is very important in a wide range of fields, it include the detection of diseases-causing, food-contamination and a large type of pathogenic diseases. A convenient and efficient diagnostic system should be developed with caring the same level of sensitivity, precision with the ability of detecting different targets, in view of the most used diagnostic methods are laborious, time consuming and complicated. The properties of selectivity, compatibility with micro-scale technologies and the label-free characteristic make electrochemical biosensors a wide opted tool for the large applications [7]. A variety of immobilization strategies and transducing materials have been developed [6].

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Screen printing microfabrication technology is continuously in development and have been combined with the electrochemical DNA biosensors research to fabricate analyte specific sensors [8][9], the screen printed electrodes SPE proved a ductility and flexibility to be designed with a wide range of materials and able to be modified with a different biological elements such enzymes, antibodies, DNA and other recognition elements [10]. Nanomaterials have also accelerated the performance of the electrochemical application by a high bio-compatibility and enhancing the electron transfer [11], and the combination with the SPE properties could lead to a synergistic effect eliciting unforeseen advantages. As presented in the literature studies, there is a wide range of conventional electrodes, the carbon based screen printed electrodes have shown a good extent in comparison to other metals based electrodes, it was also demonstrated that there is a function between the surface morphology and the electrochemical activity of the electrodes which makes the choice of the working electrode depending upon the electroanalytical application and technique used [8].

MicroRNAs (miRNAs) are single stranded, non-coding RNA molecules established of a class of 18-22 endogenous nucleotides in length that affect the mRNA stability and translation, combining with the 3′ end of the untranslated regions (3′UTR) of target mRNAs, resulting in target gene degradation or inhibition of its translation [12]. MicroRNA play a crucial role in tumor proliferation, tumor development is associated to the glucose metabolism [13] and are predicted to regulate the expression of approximately the one-third of the human genes [14]. The literature present a wide scale of studies and have shown that some miRNA regulate cellular differentiation, proliferation and apoptotic processes, that are proved with a higher importance in cancer aggravation, the miRNA profiling across different human cancers has proved that miRNAs were an efficient indicators of the developmental origin of cancers [15].various technologies for detecting miRNA have been developed such template repairing-PCR [16], microarrays [17] and northern blotting [18], the disadvantages of these processes are the high cost and production time which is long, this make the research in continuous development to conceive a low cost, flexible and easy techniques for miRNA analysis without losing the efficiency and the level of sensitivity. miRNA-451 exists in the human genome and has been considered as biomarker for some types of cancers [19] [20]. The recent studies in this field concentrate the effort on the properties of the miRNA-451 and shown that it can modulate process of tumorigenesis and the behaviour of cancers cells by deleting a series of oncogenes [21].

Electrochemical genetic sensors are considered as an alternative way for the detection of miRNA biomarkers and have promising feature in the miRNA biosensor field because of their fast, repeatable, feasible and easy applying properties. Various electrodes such as glassy carbon electrodes[22], pencil graphite electrodes[23], screen printed electrodes[24] and carbon paste electrodes[25] are used in electrochemical genetic biosensor measurements. During the sample preparation and the measurement, first the genetic molecules were attached on the electrode surfaces and the electrochemical measurements were carried out by using a proper buffer solution. The determination of the minimum amount of the genetic molecules that are attached on the electrode surface is important to obtain an interpretable signal with a proper sensitivity. Additionally, the time between sample preparation and measurement is also another critical parameter for a correct measurement. SPEs have been widely used for the detection of miRNA molecules [24, 26].

In this study, different concentrations of the miRNA molecules were immobilized on the SPEs and the guanine oxidation signals were observed from these electrodes via differential pulse voltammetry method. Synthetic anti-miRNA molecules **(**antimiR451 (G)**)** were purchased and diluted with phosphate buffer solution. Effect of the time and temperatures were also investigated by keeping the samples at -18, +5 and +25 ^{o}C for 1, 3, 14 and 21 days and conducting measurement with these samples.

2. Material and Methods

2.1. Chemicals

All the buffer solutions used in the electrochemical measurements were prepared by using ultra-*pure water* (DNase and RNase free). The prepared solutions were kept in the sterilized glass bottles at +4 ºC until they are used. *Potassium phosphate monobasic* (KH_2PO_4), *Sodium hydroxide* (NaOH), Hydrochloric acid (HCl) and glacial acetic acid were purchased from Sigma Aldrich. Potassium phosphate dibasic (HK_2O_4P) was purchased from Scharlau. Anti-miRNA (**polyT-antimiR451(G),** 28mer DNA, Scale M inclusive HPLC) molecules used in this study were received from Genaxxon Bioscience (Germany). The sequence of the oligonucleotide is given as following: *5'-TTT TTT AAC TCA GTA ATG GTA ACG GTT T-3'*

2.2. Preparation of Phosphate Buffer Solution (PBS)

0.05 M PBS solution was prepared with 1.36 gr (0.01 mol) KH_2PO_4 and 6.96 gr (0.04 mol) HK_2O_4P by dissolving them in 1 lt ultra-*pure* water. In order to bring the solution to pH 7.4 NaOH or HCl was added into the solution. Finally, 1.168 gr NaCl was put into the prepared solution to adjust the NaCl as 0.02 M in the solution.

2.3. Preparation of Acetate Buffer Solution (ABS)

0.5 M ABS solution was prepared by setting 0.5 M glacial acetic acid in 500 ml ultra-pure water. NaOH or HCl was added into the solution in order to bring the solution to pH 4.8. Finally, 1.168 gr NaCl was put into the prepared solution to adjust the NaCl as 0.02 M in the solution.

2.4. Preparation of Anti-miRNA Stock and Measurement Solutions

Anti-miRNA stock solution was prepared as 1000 ppm. The purchased anti-miRNA was firstly dissolved in ultra-pure water for stock solution preparation. The stock solution was poured in the PCR tubes as 50 µl and stored at -18 ºC until they are going to be used. The measurement anti-miRNA solutions were prepared by diluting the stock solutions. Stock solutions were shared into empty PCR tubes as 10 µl, and then 50 µl of PBS was added into the PCR tubes for the dilution. A vortex mixture was used for the stock and measurement solution preparations.

2.5. Attachment of Anti-miRNA Molecules on SPEs Surfaces

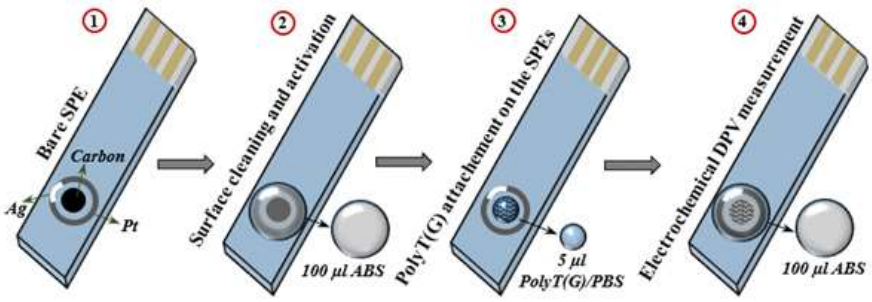

Figure 1. Surface cleaning and activation, anti-miRNA attachment and measurement process.

Surface cleaning and activation of the SPEs were carried out electrochemically with DPV method before the immobilization of anti-miRNA molecules. All the process steps were schematically illustrated in Figure 1. This process was conducted by dropping 100 µl of ABS on the electrode surface and applying

1.4 deposition potential for 60 s with 600 estimated number of the points and flowingly apply potential between 0.7-1.4 V with 10 mV/s scanning rate. After the cleaning and activation process, the surface of the SPEs were washed by dropping 300 µl PBS with a micropipette and left them to dry until they are going to be used. The attachment of the anti-miRNA molecules on the SPEs were carried out by dropping the measurement solution on the surfaces. 5 µl of anti-miRNA measurement solution was dropped on the previously cleaned and activated SPEs and waited for 1 hr. Then, electrode parts of the SPEs surfaces were washed with 300 µl of PBS before the measurement in order to remove unattached anti-miRNA molecules on SPEs. After the PBS washing, the sample surfaces were dried for 1 hr before the measurement.

2.6. Electrochemical Measurements of the Biosensory System via DPV Measurements

Electrochemical measurements were performed by using Potansiyostat - AUTOLAB AUT204 (Eco Chemie, Nederland) and NOVA 1.11 software. Screen-printed electrodes (SPEs) were purchased from *Metrohm* with DS 150 product code which has carbon working electrode, platinum auxiliary electrode (PAE) and silver reference electrode. DS 110 product code model which differently has carbon auxiliary electrode (CAE) was also tried in the initial measurements. Photograph images of the SPEs during anti-miRNA immobilization and DPV measurement were shown in Figure 2. The measurements were carried out between 0.75-1.3 V with 10 mV/s scanning rate by dropping 100 µl ABS on the electrode surface.

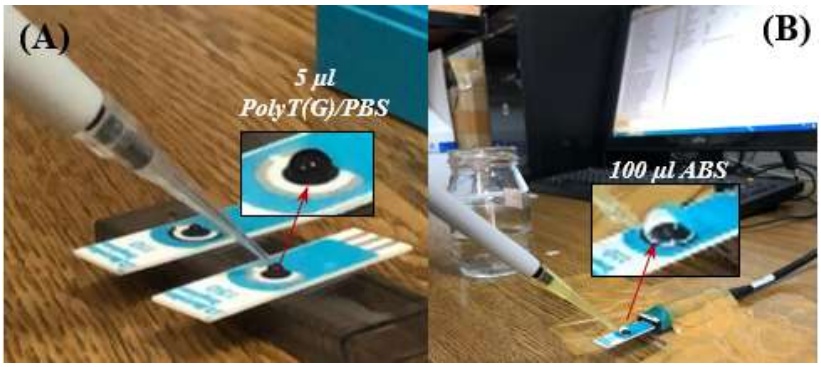

Figure 2. (A) Measurement anti-miRNA solution drop on the working electrode of SPE and (B) ABS solution drop on SPE during the measurement.

3. RESULTS AND DISCUSSION

3.1. DPV Measurement by using PBS Buffer Solution

PBS and ABS as two different buffer solutions were prepared in order to observe which buffer solution is better for an appropriate DPV measurement. It was begun with PBS solution at the measurements. In this regard, two different ways have been tried. First, the prepared measurement solution (anti-miRNA/PBS) was directly dropped on the SPE and the DPV measurement was conducted (Figure 3a and 3c). In the second way, the measurement solution was dropped on the SPE, waited for 60 min and then washed with just PBS the remove unattached molecules and DPV measurement was carried out (Figure 3b and 3d). These two methods were examined by using two different SPE types which has either platinum (Figure 3a and 3b) or carbon (Figure 3c and 3d) auxiliary electrode. Both guanine and adenine oxidation peaks were appeared at all the measurements when the plots were observed as given in Figure 3. When the results were compared with the literature, these peaks are generally appeared around 1 and 1.25 V correspond to guanine and adenine oxidations [27,28]. These peaks seems shifted to lower voltage values when the measurements were conducted by using PBS solution. In order to

observe the effect clearly with PBS measurement, ABS cleaning and activation was not applied to the SPEs in this group of the measurement before the anti-miRNA attachment and the measurement.

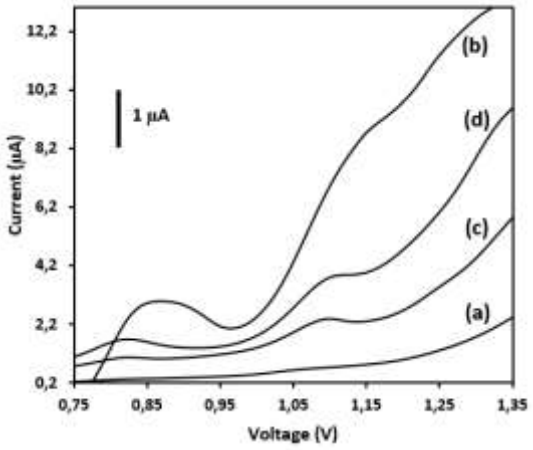

Figure 3. DPV measurement with PBS: (a) anti-miRNA/PBS (with PAE), (b) anti-miRNA/PBS wait 60 min-wash –measure (with PAE), (c) anti-miRNA/PBS (with CAE) and (d) anti-miRNA/PBS wait 60 min-wash – measure (with CAE).

3.2. DPV Measurement by using ABS Buffer Solution

ABS solution was also used during the DPV measurement, since when PBS solution was used the guanine and oxidation peak positions were not matched as in the studies published literature. Metrohm with DS 150 product code SPEs were used at all the measurements when ABS was used. In the measurement, 5 µl of anti-miRNA measurement solution was dropped on the previously cleaned and activated SPEs and waited for 1 hr. Then the samples were washed with PBS, dried and the measurement was carried out by putting 100 µl of ABS solution during the measurement. When the measurement plot was observed (obtained) as in Figure 4, it was observed that guanine and adenine oxidation peaks positions were matching as in the literature [27,28]. After the comparison of the results of PBS and ABS measurements with the literature, since the results were compatible when ABS was used as the buffer solution for the measurement, all the measurements were continued by using ABS solution.

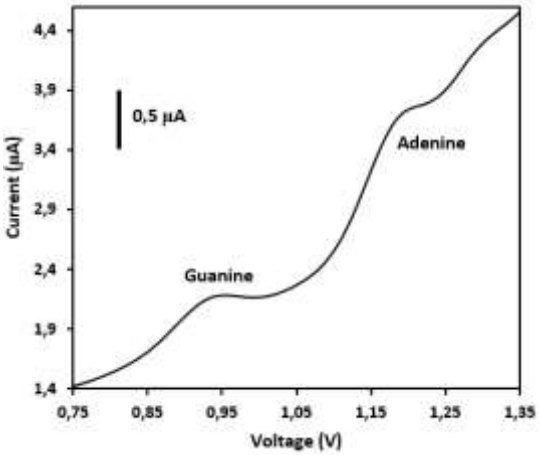

Figure 4. DPV measurement with ABS buffer solution.

3.3. DPV Measurement with Different Rate of Anti-miRNA Attached SPEs

The effect of the anti-miRNA concentration in the measurement solutions was studied by preparing five different diluting concentrations. The dilution was carried out by adjusting the anti-miRNA measurement solution/PBS (v/v) rate a 1/50, 2/50, 5/50, 10/50 and 20/50. Then, the prepared diluted solutions were dropped on the previously surface cleaned and activated SPEs, waited 1 hr, washed with PBS, dried for 1 hr and then the measurements were conducted. As seen from Figure 5, guanine and adenine oxidation peaks were clearly detected around 1 and 1.25 V sequentially [27,28]. The intensity of the guanine oxidation peak dramatically increased when the ratio of the anti-miRNA molecules were increased in the measurement solution up to 10/50 anti-miRNA measurement solution/PBS rate, and was not increased dramatically while increasing anti-miRNA ratio in the solution. On the other hand, there was a prominent enhancement at the adenine oxidation peak when the anti-miRNA measurement solution/PBS rate increased from 2/50 to 5/50, and not significant changes were observed at other concentrations. The results revealed that guanine and adenine oxidation peaks are detectable at every concentration, so the minimum concentration can be used in the following studies.

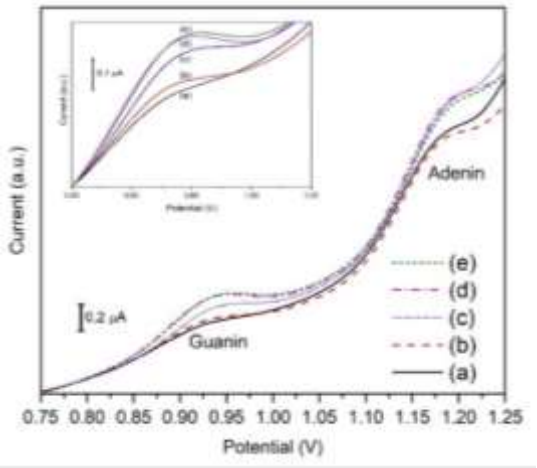

Figure 5. DPV measurement with different rate of anti-miRNA attached SPEs: anti-miRNA measurement solution/PBS (v/v) rates are (a) 1/50, (b) 2/50, (c) 5/50, (d) 10/50 and (e) 20/50.

3.4. Storage Time and Temperature Study of anti-miRNA Attached SPEs

In order to investigate the appropriate time and temperature of the anti-miRNA immobilized SPEs, DPV measurements were carried out after the samples stored at -18, +5 and +25 ^{o}C for 1, 3, 14 and 21 days. The DPV measurements plots were demonstrated in Figure 6. When it was focused on the guanine oxidation signal around 0.9-1.0 V, there was a dramatic decrease at the -18 ^{o}C stored sample when the measurement was conducted after one day stored samples. On the other hand, no significant change was observed at +5 ve +25 ^{o}C stored samples. The measured oxidation signal was always lower at -18 and +25 ^{o}C stored sample than +5 ^{o}C stored sample. Overall results revealed that the most suitable storage temperature after anti-miRNA attached samples until the measurement was +5 ^{o}C.

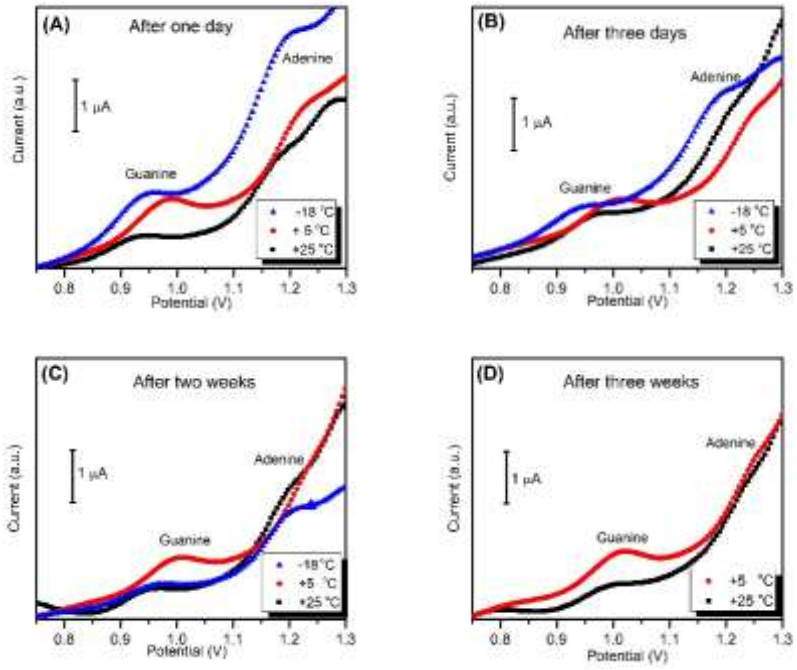

Figure 6. DPV measurements of anti-miRNA attached SPEs at different storage times at -18, +5 and +25 ºC stored samples: (A) 1 day, (B) 3 days, (C) 14 days and (D) 21 days.

4. CONCLUSION

Anti-miRNA immobilization optimization on the screen-printed electrodes for the sensitive detection of miRNA molecules were carried out. In this regard, different amounts of anti-miRNA molecules were attached on SPE surfaces and guanine oxidation signals were electrochemically measured and analysed in order to determine the best adequate immobilization concentration. Additionally, time and temperature after the anti-miRNA attachments on the SPEs were also investigated. The same amount of anti-miRNA molecules was immobilized on the SPEs and stored in -18, +5 and +25 ºC for 1, 3, 14 and 21 days. Then, guanine oxidation signals were observed again to determine the best time and temperature. The results demonstrated that even though the guanine oxidation signal dramatically increased by increasing anti-miRNA content in the immobilization solution, the signal was not increased more when it was continued to increase anti-miRNA content. Time and temperature result revealed that the best suitable condition was +5 ºC since the guanine oxidation signal intensity decrease less with time and was more stable comparing the other sample groups.

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CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

REFERENCES

[1] Wooster R, Weber BL. Breast and ovarian cancer. N Engl J Med, 2003; 348: 2339-2347.

[2] Rezaei H, Motovali-bashi M, Radfar S. An enzyme-free electrochemical biosensor for simultaneous detection of two hemophilia A biomarkers: Combining target recycling with quantum dots-encapsulated metal-organic frameworks for signal amplification. Anal Chim Acta, 2019; 1092: 66-74.

[3] Dequeker E, Stuhrmann M, Morris M.A., Casals T., Castellani C, Claustres M, Cuppens H, des Georges M, Ferec C., Macek M, Pignatti PF., Scheffer H, Schwartz M., Witt M, Schwarz M, Girodon, E. Best practice guidelines for molecular genetic diagnosis of cystic fibrosis and CFTR-related disorders - Updated European recommendations. Eur J Hum Genet, 2009; 17: 51–65.

[4] Chan JF, Yip CC, To KK, Tang TH, Wong SC, Leung KH, Fung AY, Ng AC, Zou, Z, Tsoi HW, Choi GK, Tam, AR, Cheng VC, Chan KH, Tsang OT. Yuen KY. Improved molecular diagnosis of COVID-19 by the novel, highly sensitive and specific COVID-19-RdRp/Hel real-time reverse transcription-polymerase chain reaction assay validated in vitro and with clinical specimens . J Clin Microbiol, 2020; 58: e00310-20.

[5] Van TT, Miller J, Warshauer DM, Reisdorf E, Jernigan D, Humes R, Shult PA. Pooling nasopharyngeal/throat swab specimens to increase testing capacity for influenza viruses by PCR. J Clin Microbiol, 2012; 50: 891-896.

[6] Carpini G, Lucarelli F, Marrazza G, Mascini M. Oligonucleotide-modified screen-printed gold electrodes for enzyme-amplified sensing of nucleic acids. Biosens Bioelectron, 2004; 20: 167-175.

[7] Malecka K, Stachyra A, Góra-Sochacka A, Sirko A, Zagórski-Ostoja W, Radecka H, Radecki J. Electrochemical genosensor based on disc and screen printed gold electrodes for detection of specific DNA and RNA sequences derived from Avian Influenza Virus H5N1. Sensors Actuators B Chem, 2016; 224: 290-297.

[8] Fanjul-Bolado P, Hernández-Santos D, Lamas-Ardisana PJ, Martín-Pernía A, Costa-García A. Electrochemical characterization of screen-printed and conventional carbon paste electrodes. Electrochim Acta, 2008; 53: 3635–3642.

[9] Kondo T, Sakamoto H, Kato T, Horitani M, Shitanda I, Itagaki M, Yuasa M. Screen-printed diamond electrode: A disposable sensitive electrochemical electrode. Electrochem Commun, 2011; 13: 1546-1549.

[10] Arduini F, Micheli L, Moscone D, Palleschi G, Piermarini S, Ricci F, Volpe G. Electrochemical biosensors based on nanomodified screen-printed electrodes: Recent applications in clinical analysis. Trends Anal Chem, 2016; 79: 114-126.

[11] Das R, Sharma MK, Rao VK, Bhattacharya BK, Garg I, Venkatesh V, Upadhyay S. An electrochemical genosensor for Salmonella typhi on gold nanoparticles-mercaptosilane modified screen printed electrode. J Biotechnol, 2014; 188, 9-16.

[12] Kloosterman WP, Plasterk RH. The Diverse Functions of MicroRNAs in Animal Development and Disease. Dev Cell, 2006; 11: 441-450.

[13] Guo H, Nan Y, Zhen Y, Zhang Y, Guo L, Yu K, Huang Q, Zhong Y. miRNA-451 inhibits glioma cell proliferation and invasion by downregulating glucose transporter 1. Tumor Biol, 2016; 37: 13751–13761.

[14] Gartel AL, Kandel ES. miRNAs: Little known mediators of oncogenesis. Semin Cancer Biol, 2008; 18: 103-110.

[15] Wang XC, Tian LL, Jiang XY, Wang YY, Li DG, She Y, Chang JH., Meng AM. The expression and function of miRNA-451 in non-small cell lung cancer. Cancer Lett, 2011; 311: 203–209.

[16] Zhou D, Lin X, Gao W, Piao J, Li S, He N, Qian Z, Zhao M, Gong X. A novel template repairing-PCR (TR-PCR) reaction platform for microRNA detection using translesional synthesis on DNA templates containing abasic sites. Chem Commun, 2019; 55: 2932-2935.

[17] Dong R, Shen Z, Zheng C, Chen G, Zheng S. Serum microRNA microarray analysis identifies miR-4429 and miR-4689 are potential diagnostic biomarkers for biliary atresia. Sci Rep, 2016; 6: 21084.

[18] Shabaninejad Z, Yousefi F, Movahedpour A, Ghasemi Y, Dokanehiifard S, Rezaei S, Aryan R, Savardashtaki A, Mirzaei H. Electrochemical-based biosensors for microRNA detection: Nanotechnology comes into view. Anal Biochem, 2019; 581: 113349.

[19] Wang H, Zhang G, Wu Z, Lu B, Yuan D, Li X, Lu Z. MicoRNA-451 is a novel tumor suppressor via targeting c-myc in head and neck squamous cell carcinomas. J Cancer Res Ther, 2015; 11: 216-221.

[20] Kovalchuk O, Filkowski J, Meservy J, Ilnytskyy Y, Tryndyak VP, Chekhun VF, Pogribny IP. Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. Mol Cancer Ther, 2008; 7: 2152–2159.

[21] Yuan J, Lang J, Liu C, Zhou K, Chen L, Liu Y. The expression and function of miRNA-451 in osteosarcoma. Med Oncol, 2015; 32, 1-7.

[22] Ehzari H, Amiri M, Safari M. Enzyme-free sandwich-type electrochemical immunosensor for highly sensitive prostate specific antigen based on conjugation of quantum dots and antibody on surface of modified glassy carbon electrode with core–shell magnetic metal-organic frameworks. Talanta, 2020; 210: 120641.

[23] Yammouri G, Mandli J, Mohammadi H, Amine A. Development of an electrochemical label-free biosensor for microRNA-125a detection using pencil graphite electrode modified with different carbon nanomaterials. J Electroanal Chem, 2017; 806: 75-81.

[24] Erdem A, Eksin E, Congur G. Indicator-free electrochemical biosensor for microRNA detection based on carbon nanofibers modified screen printed electrodes. J Electroanal Chem, 2015; 755: 167-173.

[25] Azab SM, Elhakim HA, Fekry A. The strategy of nanoparticles and the flavone chrysin to quantify miRNA-let 7a in zepto-molar level: Its application as tumor marker. J Mol Struct, 2019; 1196: 647-652.

[26] Ganguly A, Benson J, Papakonstantinou P. Sensitive chronocoulometric detection of miRNA at screen-printed electrodes modified by gold-decorated MoS2 nanosheets. ACS Appl Bio Mater,

2018; 1: 1184-1194.

[27] Bagni G, Hernandez S, Mascini M, Sturchio E, Boccia P, Marconi S. DNA biosensor for rapid detection of genotoxic compounds in soil samples. Sensors, 2005; 5: 394-410.

[28] Lin X, Ni Y, Pei X, Kokot S. Electrochemical detection of DNA damage induced by clenbuterol at a reduced graphene oxide-Nafion modified glassy carbon electrode. Anal Methods, 2017; 9: 1105-1111.