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ORIGINAL ARTICLE

Nanowired dual-electrodes surface to monitor cerebral ischemia by current-volt measurements

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Abstract

The level of clotting protein 'factor IX' (FIX) is highly associated with cerebral ischemia, and this research work has developed a sensitive detection of FIX on dielectrode sensor by current-volt measurement. Sensing area was grown with zinc oxide nanowire to attach more probe for FIX interaction. Aptamer was utilized as the detection probe and attached on the sensing electrode surface through amine-aldehyde chemical linkage. In addition, biotin-streptavidin interaction was utilized to attach the higher number aptamers on the electrode surface connected with dual-probe station. FIX detection limit was found as 10 fM in the phosphate buffer saline spiked samples and 1:320 dilution of human serum. The linear ranges were as 10 fM to 100 pM and 1:320 to 1:80, respectively. With a good determination co-efficient [$y = 2.6813x - 3.8467$; $R^2 = 0.9479$] this biosensing strategy helps to quantify FIX and monitor the condition of cerebral ischemia.

Keywords Cerebral blood · Clotting protein · Aptamer · Biomarker · Biosensor

Introduction

Cerebral ischemia is also known as ischemic stroke/brain ischemia, a condition of blockage in blood vessel, causing restriction in oxygen-rich blood to the brain, resulting a damage to the tissues in the brain and leading a stroke. In worldwide, ischemic stroke is the fifth leading cause of death (Burden and Factors 2006). Cerebral ischemia has been categorized into two types, namely thrombotic and embolic. An embolic stroke occurs when bloods clots within the artery, while thrombotic occurs when the arteries in the cerebral areas were blocked by clotted blood in the brain (Albertyn 1987; Andrews and Berndt 2004; Gross et al. 2018; Beck et al. 2016; Sharpe and Pasternak 2017). In general, the embolism moves through the blood stream from the heart and settle down when it cannot move further, which cause the restriction of blood flow to the brain and leads to stroke. Various reasons are involving for the stroke occurrence, such as high-cholesterol, high-blood pressure, obese, atrial fibrillation and atherosclerosis.

Identification of cerebral ischemia with a suitable biomarker is essential to avoid the sudden stroke and improving the treatment and patient lifestyle. Various blood-based biomarkers are involving in the process of cerebral ischemia, such as thrombomodulin, calcium-binding protein, glial fibrillary acidic, clotting proteins, neuron-specific enolase, and insulin-like growth factor binding protein (Jickling and Sharp 2011, 2015). Since coagulation is the major cause for stroke, clotting proteins have received more attraction as biomarkers to identify the cerebral ischemia and the associated condition. In particular, research has proven that the coagulation factor IX (FIX) involves a major part in ischemic stroke. FIX is a vitamin K-dependent clotting protein playing a major action in the intrinsic pathway of the coagulation cascade. Research was proved that elevating level of FIX is highly associated with venous thromboembolism and cardiovascular disease (Appiah et al. 2017). Further studies have proven that activity levels of FIX are correlated with ischemic atherothromboembolic stroke (Yang et al. 2006; Heikal et al. 2013). In addition, coagulation and fibrinolysis are other major reasons for ischemic stroke (Bagoly et al.

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2019). So that, quantifying the clotting protein FIX by a suitable probe helps to monitor the stroke at its earlier stages. This research work was identified the clotting protein FIX at its lower level by its aptamer on dielectrode sensor to monitor the cerebral ischemia.

Aptamers are known as artificial antibodies, selected by in vitro process called Systematic Evaluation of Ligands by Exponential enrichment (Gopinath et al. 2008, 2012; Guo et al. 2020). Since aptamers have higher sensitivity and selectivity to the target, its applications are widely accepted in various medical fields, including imaging, drug-delivery and diagnosis. In particular aptamer are highly attractive in medical diagnosis due to its differentiation capability with the closely related molecule, which cannot do with antibody-based diagnosing. The specific RNA aptamer for FIX was developed by Rusconi et al. (2002) and various researches are going on for the utilization of FIX aptamer as the potential anticoagulant with its high stability and selective binding (Gopinath et al. 2006; Howard et al. 2007; Sullenger et al. 2012). Further, various FIX sensors have been developed by its aptamer to identify the thrombosis-related diseases (Lakshmipriya et al. 2014; Guo et al. 2019; Letchumanan et al. 2019; Yao et al. 2020). Utilizing this aptamer as the probe, aptasensor was developed to quantify the level of FIX in the human plasma. Biotinylated aptamer was attached to the streptavidin with sensing electrode and identified the FIX by measuring the current changes upon binding of aptamer. Further, to enhance the interaction of FIX more aptamer-probe was immobilized by growing zinc oxide nanowire as a wider sensing area.

Materials and methods

Reagents

Factor IX (FIX), Ethanolamine, Human serum, (3-Aminopropyl) triethoxysilane (APTES), zinc acetate dihydrate, monoethanolamine (MEA) were from Sigma Aldrich, USA. Glutaraldehyde was ordered from Wako chemicals, Japan. The oligonucleotide dT20 with biotin at its 5′ end and the other nucleotides for aptamer synthesis were commercially synthesized by the local supplier. Anti-FIX RNA aptamer was synthesized as described previously (Lakshmipriya et al. 2013, 2014). The DNA templates 5′-AGTAATACG ACTCACTATAGGGATGGGGACTATACCGCGTAATG CTG-3′ and 5′-AGTAATACGACTCACTATAGGGTACCC CTGATATGGCGCATTAC GAC-3′ were used to synthesize the aptamer and complementary aptamer sequences and the primers 5′-AGTAATACGACTCACTATAGG-3′ (forward) and 5′-(T_{24})ATGGGGAGGCAGCATTACGCGGTATA-3′ (reverse) was used for Polymerase chain reaction to synthesize aptamer and the same forward primer and different reverse primer 5′-(T_{24})TACCCCTCCGTCGTAATGCGC CATAT-3′ were used to synthesize the complementary aptamer (Cheen et al. 2017; Letchumanan et al. 2019).

Sensor surface fabrication for current-volt measurement

Dielectrode was fabricated by using silica substrate as a base. At first, the sensor was designed with AutoCAD software with a desired gap size. The silicon substrate was first cleaned with cleaning solutions, RCA1 and RCA2, followed by deionized water. Then SiO_2 layer was grew with 100 nm by oxidizing the substrate at 500 °C for 1 h. Next aluminium layer with a thickness of 200 nm was coated by using a thermal evaporator onto the SiO_2 layer. Further, Al layer was covered with 2000 nm of positive photoresist by using the process pf photolithography. Desired pattern was then further developed by UV-exposure on a photoresist and then the unnecessary area was removed by rinse the electrode by the developing and the Al-etching solutions. Measurements were performed by current-volt with ammeter at the range from 0 to 2 V with an interval of 0.1 V and operated at room temperature.

Modification of ZnO-nanowire on dielectrode

Following method was used to prepare the flower pattern of ZnO on the fabricated dielectrode. (i) $Zn(CH_3COO)_2.2H_2O$ (8 g) was dissolved in ethanol and mixed vigorously for 30 min at 60 °C; (ii) The stabilizer of MEA was introduced drop-by-drop to the mixture at continuous stirring for 2 h; (iii) The obtained homogenous and transparent solution was aged 1 day at room temperature; (iv) The next day ZnO gel was deposited on the sensing surface with the spin coating method for 20 s at 3000 rpm (3 times); (iv) The ZnO coated surface was annealed for 2 h at 300 °C; (v) Annealed substrate was dipped inside the growth solution (mixture of hexamethyltetramine and zinc nitrate hexahydrate) and keep in an oven for 5 h at 90 C; (vi) Final product was washed with isopropanol followed by distilled water and used for further surface modification.

Preparation of biotinylated aptamer modified electrode surface

ZNO nanowire modified sensing electrode as flower pattern was initially treated with 1% of KOH solution for 10 min and then washed thoroughly with HEPES buffer to remove the free KOH and then 1.5% of APTES in ethanol was dropped on the surface for 1.5 h. The free molecules were removed by ethanol washing and then aldehyde activated surface was created by treating the surface with 2% of aqueous glutaraldehyde for 2 h. After washing the surface with 10 mM

HEPES (pH 7.4), on this glutaraldehyde-activated electrode surface, 200 nM of streptavidin was attached through the covalent linking and the excess aldehyde groups on the electrode surface was covered by introducing the 1 mg/mL of PEG-COOH. Further, biotinylated FIX-aptamer (mixture of anti-FIX aptamer extended with dA_{24} at its 3′ end and biotin-dT_{20}) was allowed to interact with streptavidin modified electrode surface. This aptamer-modified surface was utilized to determine FIX.

Determination of FIX sensitivity on aptamer immobilized dielectrode surface by current-volt measurement

To determine the FIX, FIX with different concentrations were added individually and the current in voltage was measured to identify the FIX interaction with immobilized aptamer. The surface was washed with HEPES buffer before recording the current response with reduced biofouling. To maintain the aptamer-FIX stability, 2 mM $CaCl_2$ was mixed in the HEPES buffer as recommended previously (Rusconi et al. 2002). The interaction of factor IX and aptamer needs calcium ion to facilitate the high affinity, it might be due to the blood-based biomarker (FIX) used as the target. This has been proven by Gopinath et al. (2007) and they reported that at the minimal concentration of 1 mM calcium ion is necessary for this interaction. The difference in current for each FIX concentration (100 aM to 10 pM) was plotted in an excel to calculate the detection limit of FIX.

FIX detection in diluted serum by current-volt measurement

FIX was also identified from human serum by aptamer immobilized dielectrode surface. Instead of FIX, diluted human serum from 1:1280 to 1:40 were placed individually on aptamer immobilized electrode surface for 15 min. Then, the surface was washed with HEPES buffer, and current changes were registered for each dilution of human serum. The sensor was operated by a dual probe station connected to the ammeter. All operations were performed at room temperature and maintained the wet condition on the sensing surface. Both for making wet-condition and washing between each surface modification, 10 mM HEPES buffer (pH 7.2) was used. During the measurement 0–2 V was supplied with the interval changes of 0.1 V.

Results and discussion

The study has been launched to analyze the presence of human clotting FIX, which can monitor the cerebral ischemia and the associated issues. A laboratory fabricated dielectrode sensor surface was used with a proper surface modification (Fig. 1). This study uses ZnO nanowire with two major purposes, one is increment with the surface area for promoting surface-to-volume ratio. The second one is suitability of ZnO for the surface chemical modification to facilitate higher number of molecular immobilizations towards high-performance detection. The process of detecting FIX involved the following major steps. They are (i) Dielectrode with ZnO nanowire was treated with KOH; (ii) Amine modification on KOH treated electrode; (iii) Linker of glutaraldehyde on amine; (iv) Streptavidin was attached through covalent interaction; (v) Excess aldehyde surface was covered by PEG-COOH; (vi) Biotinylated aptamer was linked with streptavidin; (vii) FIX/Human serum was interacted. Since it has been proved that anti-FIX aptamer exhibits enough stability in human serum, it selectively binds FIX at higher sensitivity, expected to display the lower

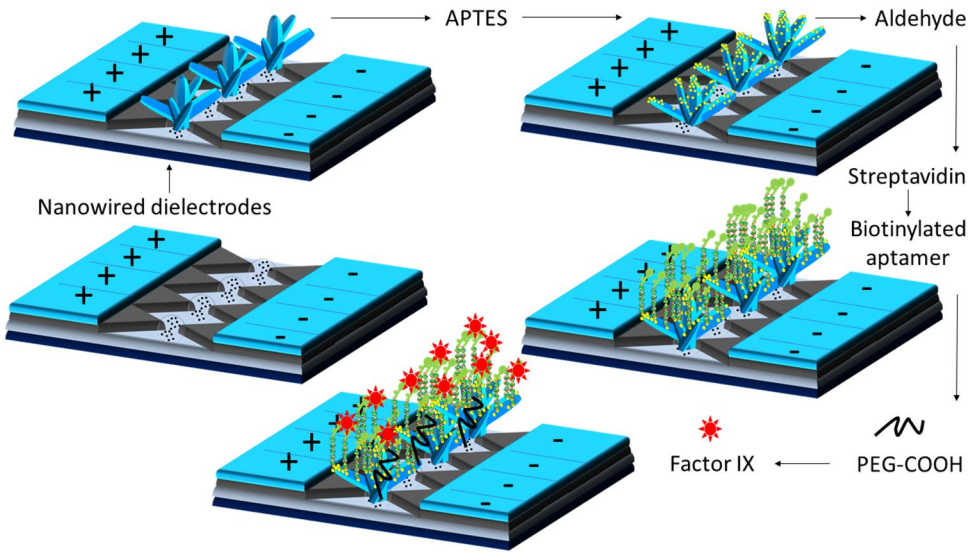

Fig. 1 Basic sensing set-up. Dielectrode sensing surface with the nanowire created is shown for determining cerebral ischemia. Scheme is for detecting FIX displayed. The aptamer as probe was attached to dielectrode surface through APTES-Glutaraldehyde interaction. PEG-COOH was used as a blocking agent

FIX detection. Research has proven that good limit of FIX detection by aptamer as a probe instead of anti-FIX antibody (Lakshmipriya et al. 2014). Apart from that, aptamer immobilization process is playing a vital role inr enhancing the detection limit, with a higher number of aptamers that can bind more FIX molecules. Herein, in the functionalization process, biotin-streptavidin interaction was followed to attach the aptamer on the sensing electrode surface. Since streptavidin has four binding sites for biotin, at least two to three biotinylated aptamer can bind with one streptavidin molecule, and gives a chance for a higher number of aptamer attachment on the electrode surface (Lakshmipriya et al. 2016). Further, PEG-COOH was used as the blocking molecule, this reduces biofouling, and yields a proper orientation of aptamer on the surface, which elevates the interaction of FIX (Nagasaki et al. 2007; Nagasaki 2011). Figure 2a displays the measuring system by a dual probed station to identify the FIX from human serum for monitoring cerebral ischemia. The intactness of originally fabricated sensing surface was confirmed by 3D-nanoprofiler imaging and SEM observation.

Functionalization of surface with aptamer

The aptamer functionalization process on sensing electrode surface was monitored and the current change recorded for each step to attest the proper immobilization of aptamer. As displayed in Fig. 3a, the silane-modified electrode displays the current response as 1.6 E-09 A, after adding glutaraldehyde (GLU), it increased to 5.85E-08 A. This result attests the aldehyde linker formation on the amine-modified surface. Further, when streptavidin was added, higher increment of current was recorded, the difference in current noticed as 1.356 E-07 A. This change of current was indicated the attachment of amine in the streptavidin with the aldehyde in the glutaraldehyde. When biotinylated aptamer was dropped, the current was enhanced to be 3.64 E-07 A. Higher changes in current difference were noted after adding the aptamer due to the interaction of higher number of biotinylated aptamers with the immobilized streptavidin (Fig. 3b). This current response confirms the aptamer immobilization on the sensor electrode. Finally, the remaining aldehyde tethered electrode surface was blocked with PEG-COOH, and then FIX was interacted.

Determination of FIX: probe-target interaction

FIX was identified on aptamer modified immobilized electrode surface. Figure 4a shows the current responses with different FIX concentrations with the constant immobilized aptamer. As displayed in Fig. 4a, after adding 100 aM of FIX, current was elevated from 6.58E-07 to 6.82E-07 A. Further increment with FIX concentrations to 1 fM, 10 fM, 100 fM, and 10 pM, the current response also promoted to 1.82 E-06, 3.24 E-06, 6.58 E-06, 9.52 E-06 and 1.42 E-05

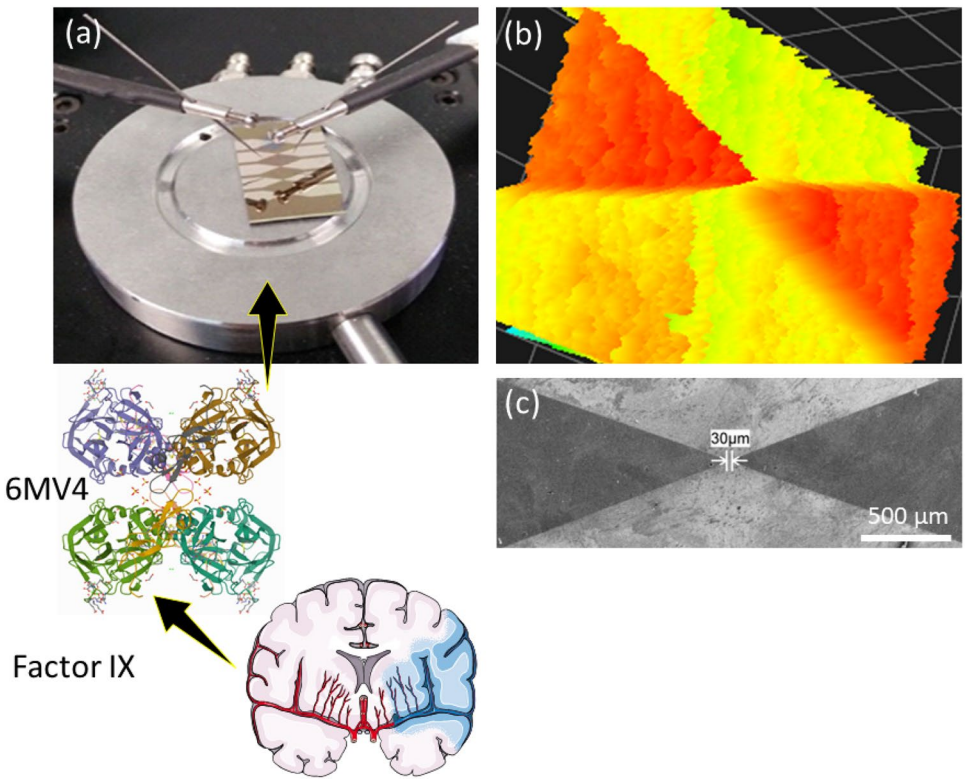

Fig. 2 Detection surface. **a** Original sensing surface with the dual-probe station. The probes supply positive and negative currents. The crystal structure of protein (Accession: 6MV4) is included. **b** 3D-nanoprofiler image at sensing region. **c** SEM image at sensing region. The gap region is measured

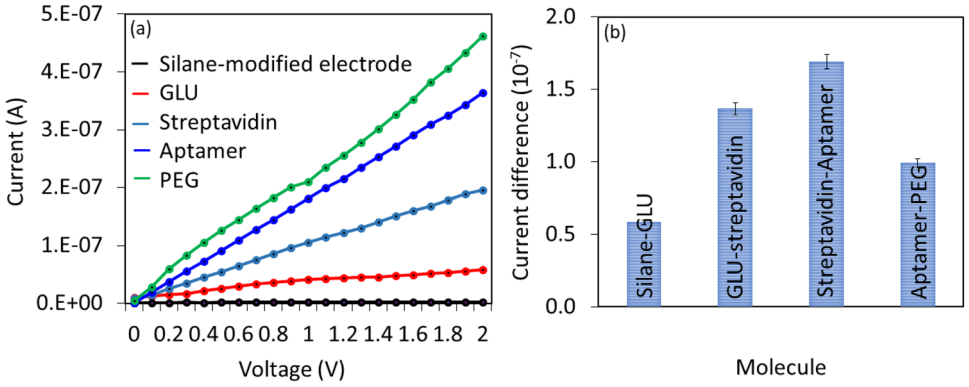

Fig. 3 **a** The aptamer functionalization process on sensing electrode surface. Current response was recorded after introducing each molecule. Clear changes in current were noticed for each modification step. **b** Current differences with aptamer functionalization process. Higher changes in current difference were noted after adding the aptamer due to the interaction of higher number of biotinylated aptamers with the immobilized streptavidin. Error values were calculated by averaging triplicate values

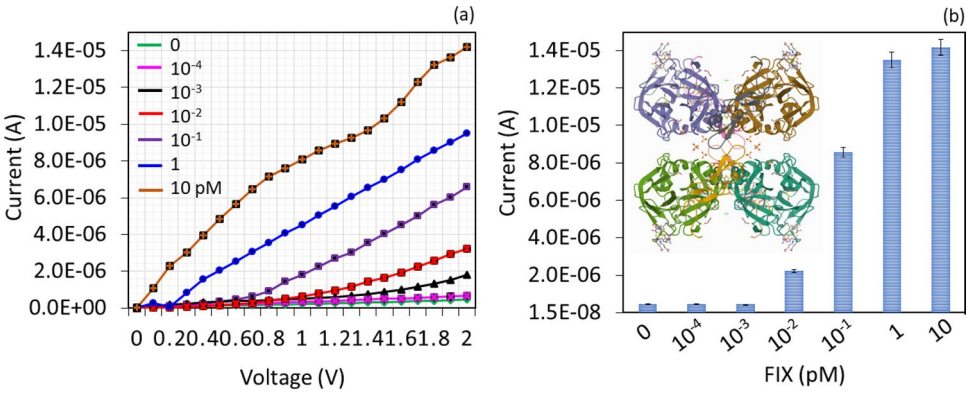

Fig. 4 **a** Identification on aptamer modified immobilized electrode. Different concentrations of FIX interaction with a constant immobilized aptamer. **b** Current responses for different concentrations of FIX interaction with the constant immobilized aptamer. With increasing FIX concentration, the current response also increased gradually. Error values were calculated by averaging triplicate values. The crystal structure of protein (Accession: 6MV4) is included as inset

A. It was found that with enhancing FIX concentration, the current response also increased gradually (Fig. 4b).

High-performance of sensor: linearity & determining FIX in human serum

The difference of current was plotted in an excel based on the above interactions and calculated the limit of detection of FIX as 10 fM by averaging the triplicate values (Fig. 5a). According to the earlier studies (Gopinath et al. 2006; Letchumanan et al. 2019), different sensing systems displayed the limit of detections/dissociation constants are from 1 to 418 pM. The current sensing strategy has improved a step further and attained the limit of detection to be 10 fM, with 100-fold higher sensitivity than other sensing systems. The normal level of FIX in the biological sample is around 87 nM (5 µg/mL). If the condition became abnormal, it causes clotting deficiency and leads to stroke. So that, it is necessary to quantify FIX in the human serum. Here, FIX in the human serum was identified by its aptamer immobilized surface. Human serum dilutions from 1: 1280 to 1:40 were placed independently on the aptamer-modified surfaces and the current responses were noted. From 1:320 dilution (which is equivalent to 250 pM) (Lakshmipriya et al. 2014), the current response was slowly increased when decrease the dilution of human serum (Fig. 5b). The higher stability of aptamer is also due to the 2′fluro modification performed during the synthesis. Generally, RNA molecule is expected to be unstable. To overcome this issue, this study uses 2′ fluoro incorporation at the end of 2′ ribose position (instead of a 2′-hydroxyl group, as recommended by Rusconi et al. (2002). The original SELEX process has been performed by using the 2′fluro molecules, which ended-up in the selection of stable aptamer. This result indicates the detection of FIX

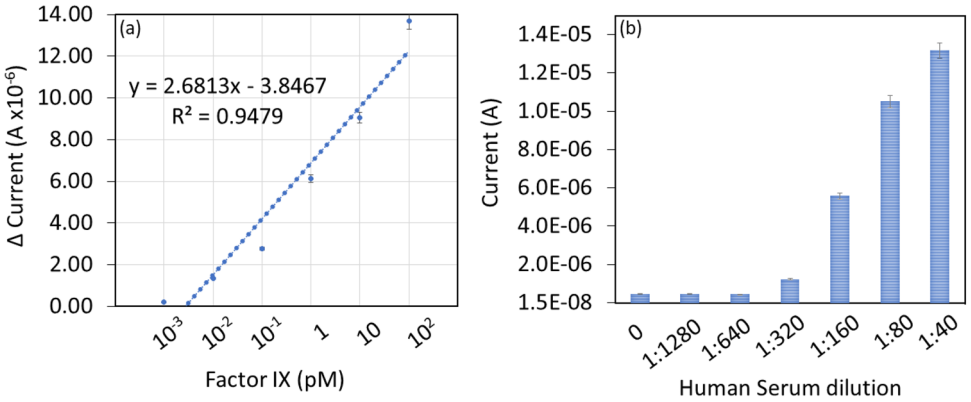

Fig. 5 **a** Limit of detection of FIX. A linear regression analysis with differences of current responses for the interaction of each FIX concentration with its aptamer. Plotted in an excel sheet and calculated the limit of detection of FIX as 10 fM. **b** FIX quantification in the human serum. FIX was identified by its aptamer immobilized surface from diluted human serum from (1:1280 to 1:40). Apparent detection was with 1:320 dilution. Current response was gradually increased when decrease the dilution of human serum

by its aptamer in the biological sample, which helps to monitor the level of FIX to identify the cerebral ischemia and its condition. Further, the selectivity of this aptamer to interact FIX was confirmed by interacting with the complementary aptamer, but there is no obvious interaction was noticed.

Conclusion

Cerebral ischemia is a stroke in the brain ischemia, caused due to the blockage of blood vessel and then restriction of oxygen rich blood flow to the brain. Identifying the ischemia and its condition with a suitable biomarker is mandatory to save the life. The clotting protein factor IX (FIX) is playing a crucial role in the clotting cascade, and also found to have a strong correlation to cerebral ischemia. This research was quantified the level of FIX in the human serum by its aptamer attached on the zinc oxide nanowire modified dielectrode surface. The detection limit was reached 10 fM with the diluted factor IX and 1:320 dilution in the human serum. This research work identifies FIX in human plasma with its lower level, which helps to identify the cerebral ischemia and its follow-up treatment.

Author contributions WG, MS and XL conceived and designed the study. XG, YL, HL and LX performed the literature search and data extraction. WX drafted the manuscript. The authors are contributed to the preparation of the manuscript and discussion. All authors read and approved the final manuscript.

Declarations

Conflict of interest Authors declare no conflict of interest.

Ethical statement None

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