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Dual-probe sandwich for Lewy body detection on nano-composite modified dielectric surface to determine Parkinson's disease

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ABSTRACT

Parkinson's disorder (PD) is a chronic and central nervous system disorder that occurs when neurons in the area of the brain impairs or dies, in particular, it affects the dopamine-producing neurons. People having PD also loose chemical messenger norepinephrine, which involves in regulating the main function of physiological systems, such as heart rate and blood pressure. Researchers are working on to identify PD with a suitable biomarker and alpha-synuclein is the presynaptic neuronal protein, in the neurological lesions (Lewy bodies). This research was focused to develop an alpha-synuclein biosensor on an iron oxide (IO) nanomaterial-modified interdigitated electrode surface. Anti-alpha-synuclein aptamer was attached on IO through the amine-linker and aggregated alpha-synuclein was sandwiched between aptamer and antibody. To enhance the analytical performance, antibody was modified with gold nanoparticles and reached the detection limit of 10 aM, determined on a linear range between 10^1 and 10^7 aM [$y = 2.5812\times$-0.0081; $R^2 = 0.9729$]. Further, control molecules failed to increase the current responses indicating the specific detection of alpha-synuclein and the CSF-spiked alpha-synuclein increases the current responses, confirming the selective detection.

1. Introduction

Parkinson's disorder (PD) is a second common neurodegenerative disorder, known as 'the movement disorder', and was first identified in 1817 by Doctor James Parkinson, and PD occurs when the central nervous system dies or breaks down [1]. Particularly, when substantia nigra is getting affected, which controls the amount of dopamine production, and the condition with lesser dopamine leads to PD [2–4]. Since dopamine plays a major role in the movement of our body, it affects the central controlling system of our body. After the dopamine started to reduce, the symptoms such as stiff muscles, tremors, trembling, and loose balance will occur. Apart from these, people with PD is also likely to have other complaints, including eating and sewing problem, depression, difficulty in thinking, sleeping problem, and constipation.

The primary causes of PD are still unknown, but researchers believe that genes and environmental factors are the main reasons and several genes are found to be associated with PD [5]. Until now five genes, such as SNCA, PARK2, PARK7, PINK1, and LRRK2 are highly related to PD. The gene SNCA synthesizes the alpha-synuclein protein, and the aggregation (the clumps of aggregation called 'Lewy bodies') of this protein was found in people with PD. Generally, the misfolding of alpha-nuclein causes clumps together and forms the oligomers/protofibrils. And then, the oligomers accumulated into a form called 'fiber'; the fiber of alpha-synuclein has the possibility of damaging the neurons and causing PD (Fig. 1a). It has been found that a mutation in the SNCA gene at the early stages of PD. The gene PARK7 creates the protein DJ-1, which helps to protect the cell from oxidative stress, this gene is also found during the early stages of PD. The gene PARK2 synthesizes the protein parkin, which helps to recycle and breakdown of the cells. The protein synthesized by the gene PINK1 may help to produce the cells from the stress. LRRK2 makes the dardarin protein, mutations of this gene were found in early PD-affected people. Apart from gene

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mutations, environmental factors mainly toxic elements influence the occurrence of PD. Exposure to chemical agents found in herbicides and pesticides are increasing the risk of PD [6]. In addition, industrial toxins that have been used for the production of household and commercial products cause PD [7]. Carbon disulfide, organo-halogen, polychlorinated biphenyls, and polybrominated diphenyl ethers are the toxic elements used in industries that enhance the risk of PD. Not only that, toxic metals such as copper, mercury, lead, and manganese, are also potential reasons for PD [8]. Exposure of other neurotoxins namely, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahdropyridine), paraquat, rotenone, and 6-hydroxydopamine leads to the PD [9].

Neuroinflammation plays a major role in the progression of various neurological disorders, such as Alzheimer's Multiple Sclerosis, and Parkinson's (PD) diseases [10,11]. The main progress of PD is known to have neuroinflammation, playing a major role in affecting the degeneration of dopaminergic in PD. Alpha-synuclein in cerebrospinal fluid refers to the measurement of this protein levels in the clear, colourless fluid that surrounds the brain and spinal cord. Cerebrospinal fluid analysis is useful in the investigation and diagnosis of a variety of neurological illnesses, including those related to alpha-synuclein pathology, such as Parkinson's disease. Alpha-synuclein is prevalent in the cerebrospinal fluid of healthy people, however its amounts vary. Changes in the concentration of alpha-synuclein in cerebrospinal fluid may occur in persons with alpha-synucleinopathies, generally indicating protein aggregation in the brain. Alpha-synuclein is the presynaptic neuronal protein, that plays a key role in a number of ways to cause PD, it is an important protein contained in the defining neurological lesions (Lewy bodies) of PD [12,13]. Moreover, alpha-synuclein is the primary structure of Lewy bodies, suggesting the aggregation of these proteins plays a major role in PD. The above confirmation believed by researches, alpha-synuclein is one of the efficient biomarkers for PD. Current study determines the presence of aggregated alpha-synculein, a amyloid fibril formed in the lewy bodies, on the nanocomposites created on the dielectric surface and detected by dual probed (aptamer and antibody) sandwich.

The current study explores the potential use of nanomaterial on interdigitated dielectrode (IDE) surfaces as it has been one of the well-established sensing systems [14–16]. IDE is a sort of electrode configuration that is employed in a wide range of electrochemical and electrical applications. IDE is made up of a sequence of alternating metal fingers or electrodes that are closely spaced. This design maximises the available surface area for interactions while also providing a controlled and well-defined environment for various electrochemical/electrical processes. The tightly spaced electrodes produce a high surface area-to-volume ratio, which is especially beneficial in applications requiring a large surface area, such as biosensors and supercapacitors. Electrochemical sensing and biosensing applications frequently employ IDEs. When a target analyte interacts with the surface of the electrodes, it can cause changes in electrical properties such as impedance or current to be monitored in order to identify and quantify the analyte. Depending on the purpose, these electrodes can be built of a number of conductive materials such as gold, platinum, or other metals. The current study utilizes iron oxide nanoparticles as this material gained a significant attention, because of their unique magnetic, optical, and chemical features, biosensors have received a lot of attention in the development process. Because of these characteristics, they are appropriate for a wide range of biosensing applications, such as medical diagnostics, environmental monitoring, and biotechnology [17,18]. With biocompatibility, versatility, and potential to improve sensitivity and selectivity in detection, iron oxide nanoparticles have shown promise in biosensing applications. Researchers are still looking for novel ways to use the characteristics of these nanoparticles in a variety of biosensor applications.

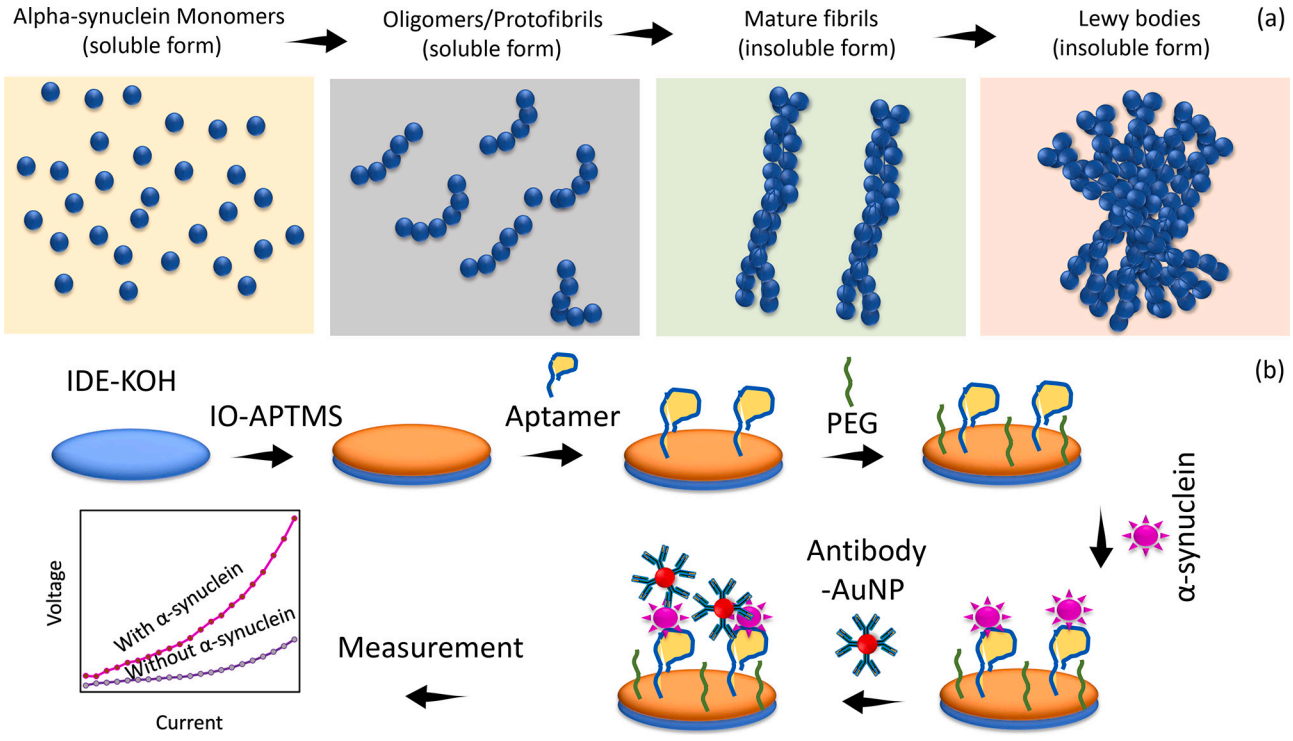

Fig. 1. (a) Process with alpha-synuclein aggregation. Misfolding of alpha-synuclein causes clumps and forms oligomers/protofibrils. And then, the oligomers accumulated into a form called, 'fiber'. (b) Schematic representation of alpha-synuclein biosensor. IDE was modified by KOH and then amine functionalized IO was introduced. Further, COOH-ended anti-alpha-synuclein aptamer was added to the surface, and the uncovered surface was blocked with PEG-COOH. And then, alpha-synuclein was allowed to interact the immobilized aptamer surface and sandwiched by AuNP modified anti-alpha-synuclein antibody.

2. Materials and methods

2.1. Materials and biomolecules

Alpha-synuclein, Anti-alpha-synuclein antibody, Gold nanoparticle (AuNP), (3-Aminopropyl)triethoxysilane (APTMS), PEG-COOH, Phosphate buffer saline (PBS), and 16-mercaptoundecanoic acid (16-MDA) were ordered from Sigma-Aldrich, USA. To generate the fibril formation, alpha-synuclein was mixed with Glycine-NaOH buffer and kept at 37 °C for 5 days. The aptamer sequence (5′-ATCGAGTGTGTACGGGGTCCGGTAGGGTGGCGAGGTCTTCCTGTCGTAGCAGG ATCCA-3′) was synthesized commercially by the local supplier [19]. Interdigitated electrode (IDE) sensing surface was purchased from Metrohm, Malaysia. For the measurements, Keithley picoammeter was used at the operating range of 0–2 V. The voltage at 2 V was read to calculate the experimental output. The prepared nanomaterials were characterized by high-resolution microscopic assessments.

2.2. Preparing plant extract

Papaya plant extract was used to synthesize the iron oxide nanomaterial. For this process, initially, the fresh papaya leaves were collected and washed thoroughly with distilled water and then dried in the oven and grind to a powder. Ten grams of the papaya powder was diluted in 500 μL of water and boiled for 30 min at 100 °C. And then the filtrate was collected through a filter paper, concentrated with a rotary evaporator at 50 °C for further experiment.

2.3. Synthesis of iron oxide nanomaterial

Ferric chloride hexahydrate was utilized as a precursor for synthesizing iron oxide (IO) nanomaterial. For this process, 100 mL of papaya extract was added into 0.1 M (100 mL) of ferric chloride hexahydrate dropwise and then sodium hydroxide with 1 M was added to the solution until it reaches the pH 11. The obtained solution was continuously stirred for 30 min to obtain a black-colored solution. The resulting nanomaterial was centrifuged at a speed of 10,000 rpm for 15 min, washed three times by ethanol followed by water, and then separated. The IO material was gathered, dried in a 100 °C oven for three hours, and then stored in an airtight container.

2.4. IO-APTMS complex preparation

IO nanomaterial was modified with APTMS for the propose of surface functionalization. For that, APTMS with 2% was mixed with 1 mg/mL of IO and placed in a hot stirring plate, and stirred for 3 h. Then the nanoparticle was rinsed with 25% ethanol and collected by centrifuging the mixtures at 5000 rpm/g for 10 min. The prepared nanoparticle was diluted in 25% ethanol and stored at 4 °C.

2.5. Preparation of Gold nanoparticle anti-alpha-synuclein antibody conjugation

Anti-alpha-synuclein antibody was immobilized on the gold nanoparticle (AuNP) to enhance the detection of alpha-synuclein. For this process, first, AuNP was linked with 5 mM of 16-MDA for 15 min. After that, the free 16-MDA was removed by centrifugation. And then, 16-MDA was activated by EDC (100 mM) and NHS (50 mM) for 10 min. Further, 250 nM of anti-alpha-synuclein antibody was added and rested for 1 h. The AuNP-antibody conjugates were collected by centrifugation.

2.6. Surface functionalization of aptamer on IO nanomaterial modified IDE

Anti-alpha-synuclein aptamer was attached to IDE through IO nanomaterial. Following steps are involved in this process. (i) Electrode was dipped in 1% of KOH and kept for 10 min and washed with distilled water; (ii) IO-APTMS complex formation; (iii) IO-APTMS was introduced on the surface and rested for 3 h. Washed the surface by 25% ethanol; (iv) Different concentrations of COOH-ended anti-alpha-synuclein aptamer (200 to 1200 nM) was added on the surface; (v) PEG-COOH (1 mg/mL) was placed on the aptamer-modified IDE and rested for 1 h. Washed the surface with PBS to remove the unbound PEG-COOH. The current-volt measurements for each surface functionalization step were performed to confirm the surface was modified with anti-alpha-synuclein aptamer properly.

2.7. Detection of α-synuclein by aptamer-antibody sandwich assay

Using sandwich assay with aptamer and antibody, alpha-synuclein was detected on IO-modified IDE. For this detection, various concentrations of alpha -synuclein (10 aM to 10 nM) were applied to the optimized aptamer attached surfaces, and the current-volt measurements were taken. The differences between the current responses were calculated and plotted in an Excel sheet to estimate the detection limit of alpha-synuclein. The limit of detection (LOD) was considered the lowest concentration of an analyte on the calibration line against the background signal (S/N = 3:1; LOD = standard deviation of the baseline+3σ).

2.8. Selective and specific detection of alpha-synuclein

To identify the selective detection, different concentrations of alpha-synuclein was diluted in an artificial cerebrospinal fluid (CSF), and detected by sandwich pattern on IO-immobilized IDE. Further, to identify the specific detection of alpha-synuclein, four control performances such as (i) complementary aptamer, (ii) non-immune antibody, and with parkinsonism-related proteins namely, (iii) PINK 1 [20], and (iv) DJ-1 [21]. The current-volt measurements were recorded and compared with the specific experiments.

3. Results and discussion

The protein alpha-synuclein is found largely in neurons, and its aggregation is linked to various neurodegenerative illnesses, but predominantly PD. The use of alpha-synuclein as a potential biomarker for PD disorders has been investigated by researchers. A biomarker is a quantifiable one that can detect the existence and progression of PD. Aggregation of alpha-synuclein is linked to the clinical features of the disease. It is crucial to emphasise, however, that while alpha-synuclein is a hallmark of PD, it is not totally unique to the disorder. Alpha-synuclein accumulates form structures known as Lewy bodies, which are observed in affected people's brain cells. These are a degenerative features of PD, but they can also be found in other alpha-synucleinopathies, indicating the relevance of PD to other neurogenerative diseases. The early detection of Parkinson's is a paramount and mandatory to give a proper treatment to the patients with PD and improve their lifestyle [22–24]. The biosensor is used to detect the PD with suitable probe and biomarker at an earlier stage. Probes such as an antibody, aptamer, and nucleic acids have been used to identify PD. Various sensors, such as Surface Plasmon Resonance (SPR), Enzyme-Linked Immunoassay (ELISA), Waveguide mode sensor (WMS), RAMAN spectroscopy, and Electrochemical sensor were used to identify the targets [25–29]. Analyte and target binding efficiency play a major role to improve the limit of detection. Apart from that proper surface functionalization also plays an important role, it was proved that the higher immobilization of the target or analyte molecule on the sensing surface improved the limit of detection. In addition, nanomaterials are effectively utilized in the medical field including diagnosis, treatment, and imaging [30–35]. In this research, iron oxide nanomaterial (IO) and gold nanoparticle (AuNP) were utilized to enhance the detection of PD biomarker, alpha-synuclein. Fig. 1b shows the surface functionalization of alpha-synuclein

identification on IO-modified interdigitated electrode. Initially, KOH was used to modify the surface of IDE, and then amine-functionalized IO was added. The surface was then treated with a COOH-ending anti-alpha-synuclein aptamer, and the exposed portion was coated with PEG-COOH. After that, alpha-synuclein was sandwiched between an AuNP-modified anti-alpha-synuclein antibody and allowed to interact with the immobilized aptamer. The capture probe in this experiment was an aptamer, and the detection probe was an antibody. Various research proved that aptamers specifically bind the target molecule with higher sensitivity and help to detect the target molecule at its lower level [36,37]. Further, AuNP modified-antibodies enhance the current-volt measurement, which helps for the accurate identification of alpha-synuclein. This study uses the current voltage range at 0–2 V, measuring at 2 V is ideal for achieving a good signal-to-noise ratio and sensitivity. This is especially significant when detecting analytes with low concentrations. When compared to lower voltages, a higher measurement voltage (2 V), can typically result in a larger signal response. This bigger signal can help identify the signal of the target analyte from background noise, resulting in more accuracy.

3.1. Surface characterization of IO nanomaterial

Fig. 2a-d describes the surface imaging of prepared IO nanomaterial and its elemental abundance. Based on FESEM analysis at different magnifications (1 μm and 200 nm), it was clear that the prepared nanomaterial is intact and uniform in size and slightly higher from the nanoscale range (Fig. 2a & b). Further, it was confirmed by FETEM observation at the magnifications (1 μm and 100 nm) (Fig. 2c). The EDX analysis of IO apparently stated the presence of major elements as Fe and O (Fig. 2d).

3.2. Surface functionalization of aptamer on IO-modified electrode

Through the amine-modified IO, an anti-alpha-synuclein aptamer was coupled to the IDE. The current-voltage measurement of the aptamer immobilization process on the IDE is shown in Fig. 3a. In which, KOH-treated IDE exhibits a current response of 1.31×10^{-9} A. After IO-APTMS surface modification, the current level was significantly raised to 1.87×10^{-9} A. The changes on the IDE with IO-APTMS is confirmed by the increase in current. On these IO-modified surfaces, 200 nM of aptamer shows the current level as 2.65×10^{-9} A, which confirms the binding of aptamer with IO-APTMS. Further, increasing the aptamer concentrations to 400, 600, 800,1000 and 1200 nM, the current levels were increased to 3×10^{-9}, 3.31×10^{-9}, 3.9×10^{-9}, 4.6×10^{-9}, and 4.81×10^{-9} A (Fig. 3b), which confirms the interaction of COOH in the aptamer with the amine on IO. Further, it was found that aptamer with 1000 and 1200 nM shows the closest current responses, indicating the saturation of aptamer immobilization on IDE. So that, for the alpha-synuclein sandwich assay, 1000 nM of aptamer was used to interact alpha-synuclein.

3.3. Detection of alpha-synuclein by a sandwich assay

A sandwich assay with aptamer and antibody was performed for the detection of alpha-synuclein. On the optimized aptamer immobilized surface, different concentrations of alpha-synuclein and then sandwiched by the constant level of AuNP-antibody. Fig. 4a shows the graph of current-potential for the detection of different concentrations of alpha-synuclein interaction with its aptamer and antibody. Before the detection, to minimize the background level, the IDE surface was blocked with PEG-COOH. The PEG-COOH blocked surface shows the current response as 8.29×10^{-9} A, after adding 10 aM of alpha-synuclein, the current level was increased to 2.02×10^{-8} A, which confirms the binding of alpha-synuclein with its aptamer and antibody. Further, increasing the concentration to 10^2,10^3,10^4,10^5,10^6,10^7 aM, the current responses were increased gradually to 6.01×10^{-9}, 9.66×10^{-8}, 1.21×10^{-7}, 1.42×10^{-7}, 1.61×10^{-7}, and 1.79×10^{-7} A (Fig. 4b). This increment of current confirms the detection of alpha-synuclein by sandwich assay with aptamer and antibody. The difference in current responses from the background for

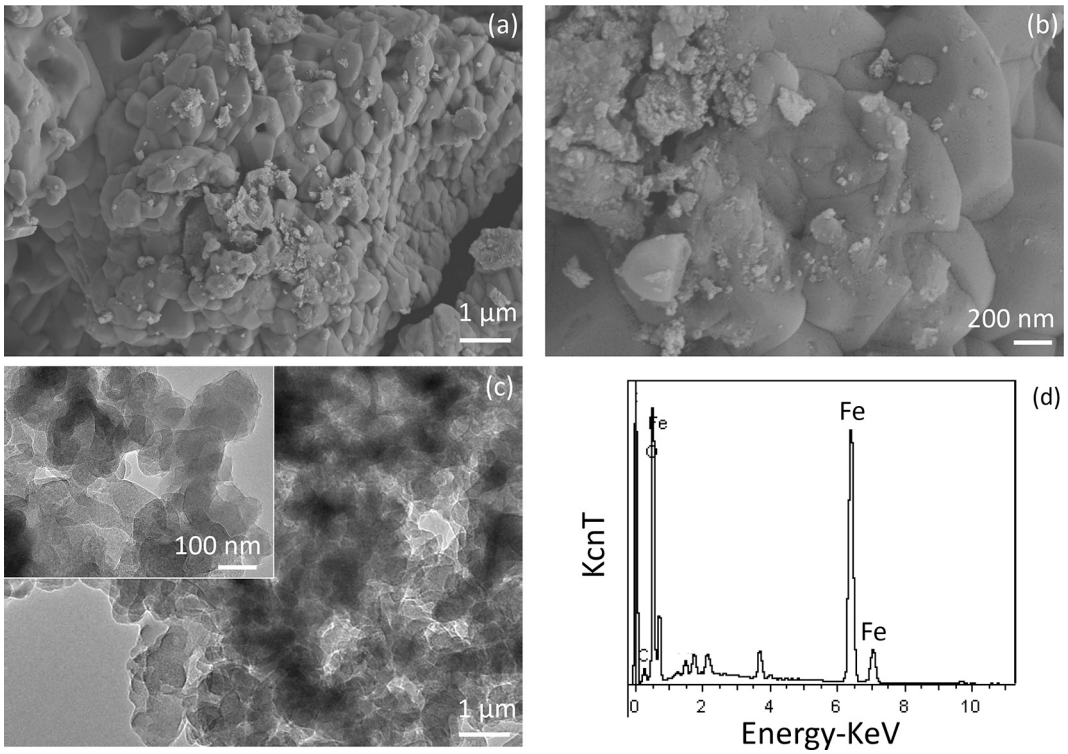

Fig. 2. Imaging of IO nanomaterial. (a) FESEM with the magnification at 1 μm. (b) FESEM with the magnification of 200 nm; (c) FETEM with the magnification of 1 μm (inset figure is with100 nm); (d) EDX analysis of IO. The presence of major elements of Fe and O was confirmed.

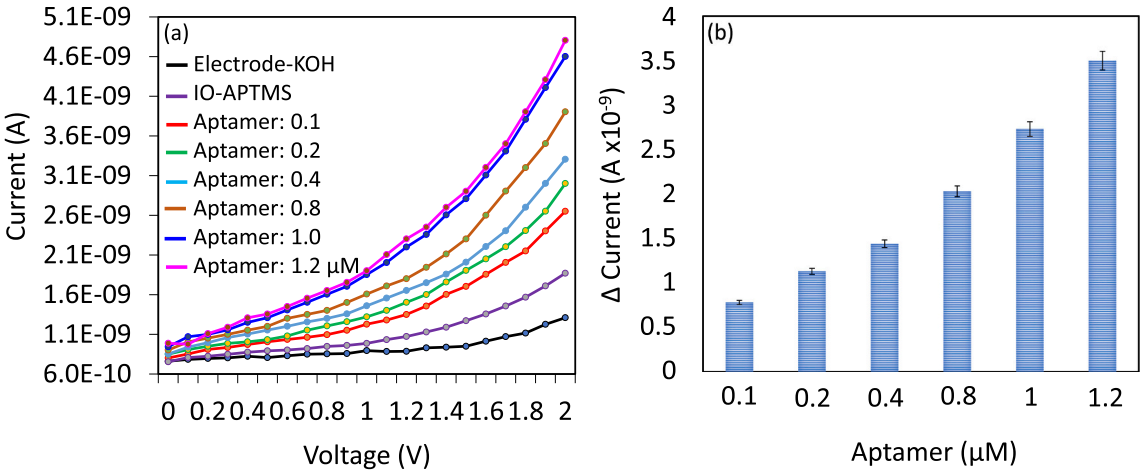

Fig. 3. Immobilization of anti- alpha-synuclein aptamer on IDE. (a) Graph of current-volt measurement for the immobilization of different concentrations of aptamer. After each immobilization, a clear response of current was recorded; (b) Differences in current responses for different concentrations of aptamer immobilization. With increasing the aptamer concentration, the current levelw were also increased, from 1000 nM saturation of current was noticed.

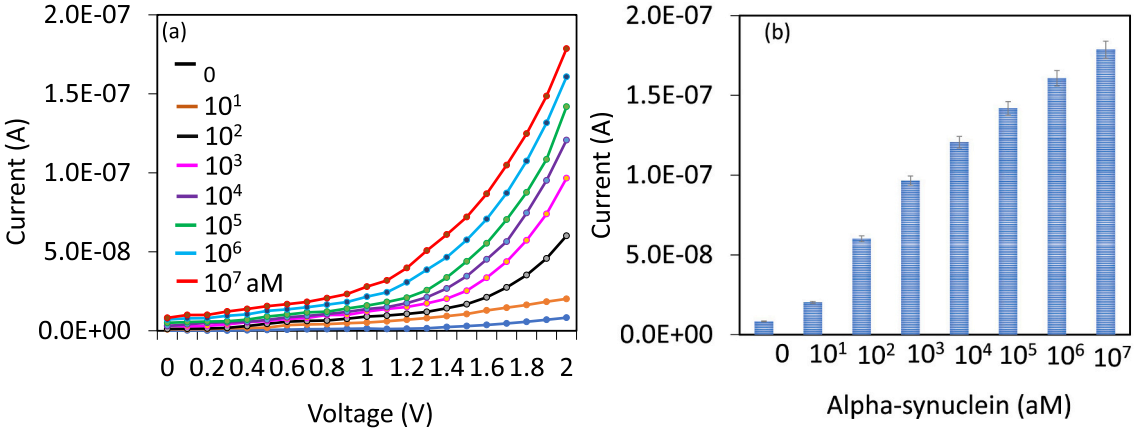

Fig. 4. Detection of alpha-synuclein by aptamer-antibody sandwich assay. (a) alpha-synuclein concentrations from 10 aM to 10 nM were determined. A clear response of current was recorded after increasing the concentration of alpha-synuclein. (b) Current response of different concentrations of alpha-synuclein. Increasing the α-synuclein concentrations, enhances the current levels gradually.

10^1,10^2,10^3,10^4,10^5,10^6,10^7 aM, were calculated as 1.22×10^{-8}, 5.18×10^{-8}, 8.83×10^{-8}, 11.27×10^{-8}, 13.37×10^{-8}, 15.27×10^{-8}, and 17.07×10^{-8} A, which are plotted in an excel sheet and estimated the limit of detection of alpha-synuclein was 10 aM with an R^2 value of 0.9729 (Fig. 5a).

3.4. Selective and specific detection of alpha-synuclein

To identify the selective detection of alpha-synuclein, different concentrations of alpha-synuclein were diluted in an artificial CSF and detected by sandwich probes on IO modified IDE. Fig. 5b shows the current response of alpha-synuclein spiked artificial CSF by a sandwich assay. The current responses for 10 aM,100 aM, 1 pM, 10 pM, 100 pM, 1 nM, and 10 nM were recorded as 1.52×10^{-8}, 5.51×10^{-8}, 8.86×10^{-8}, 1.10×10^{-7}, 1.22×10^{-7}, 1.51×10^{-7}, and 1.71×10^{-7} A. The current response was slightly decreased at the lower concentration of alpha-synuclein and shows the similar ranges at the higher concentrations. At the same, the limit of detection was maintained as 10 aM in the CSF spiked alpha-synuclein, which confirms the detection of alpha-synuclein without any interference. Further, to identify the specific detection of alpaha-synuclein, four control experiments were performed, Experiments with complementary aptamer instead of specific-aptamer sequence, non-immune antibody instead of anti-alpha-synuclein antibody, with control proteins namely, PINK 1 [20], and DJ-1 [21]. Fig. 6 shows the current response of control and specific experiments. As shown in the figure, the current response did not increase with the control performance and shows a good increment of current responses in the specific experiment, indicating the specific detection of alpha-synuclein.

4. Conclusion

Parkinson's disease (PD) is a neurological degenerative, life-changing disorder affecting one in every 500 people over the age of 60. PD affects the daily lifestyle with the symptoms of fatigue, difficulty with movement and walking, limb rigidity, and loss of smell. Alpha-synuclein is the presynaptic neuronal protein that plays a major role in developing PD, this work was focused to develop a biosensor for alpha-synuclein. On the iron oxide nanomaterial-modified interdigitated electrode, an aptamer-antibody sandwich assay was conducted. To enhance the interaction of alpha-synuclein with its antibody, antibodies were modified with gold nanoparticles. This sandwich assay detects the alpha-synuclein as low as 10 aM with an R^2 value of 0.9729. Further specific detection of alpha-synuclein was confirmed with control molecules and selective performances were confirmed with CSF spiked alpha-synuclein. This sandwich assay aids to identify the level of alpha-synuclein and suggests the

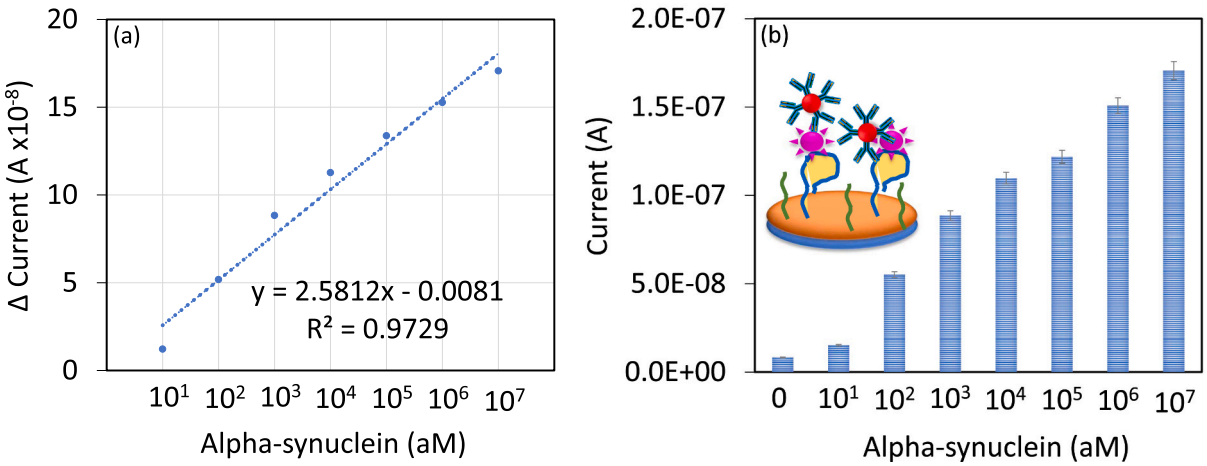

Fig. 5. (a) Limit of detection of alpha-synuclein. The difference in current response from the background was calculated and plotted in an Excel sheet and estimated the limit of detection tobe 10 aM with an R^2 value of 0.9729. (b) Selective detection of alpha-synuclein. Different concentrations of alpha-synuclein were diluted in an artificial CSF and detected by a sandwich probes. Increasing alpha-synuclein concentrations, elevates the current level gradually, which confirms the detection of alpha-synuclein without any interference.

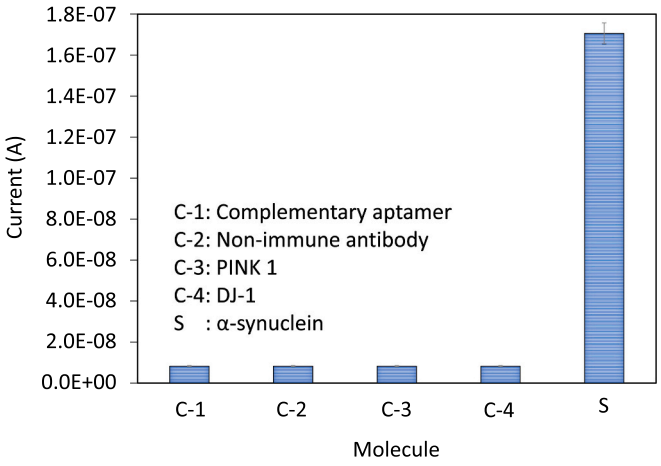

Fig. 6. Specific detection of alpha-synuclein. Experiments with complementary aptamer sequence instead of aptamer, non-immune antibody instead of anti-alpha-synuclein antibody, and with control proteins namely PINK 1, and DJ-1. The current response did not increase in the control performance and shows a good increment of current responses in the specific experiment, indicating the specific detection of alpha-synuclein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proper tailored treatment to suppress the progression of PD.

CRediT authorship contribution statement

Xi Zhang: Conceptualization, Data curation, Formal analysis, Investigation, Funding acquisition, Resources, Validation, Writing – original draft, Writing – review & editing. **Menghai Wu:** Data curation, Methodology, Visualization, Validation, Writing – review & editing. **Subash C.B. Gopinath:** Validation, Visualization, Resources, Project administration, Supervision, Writing – review & editing. **Yeng Chen:** Data curation, Validation, Visualization, Resources, Writing – review & editing.

Declaration of Competing Interest

No conflict of interest.

Data availability

No data was used for the research described in the article.

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