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Spinal cord injury immunosensor: Sensitive detection of S100β on interdigitated electrode sensor

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ABSTRACT

A spinal cord injury is damage to the nerves and cells that receive and provide a signal from the brain to the rest of the body. Spinal injury causes changes in movement, sensation, and strength, affect the body functions near the injury site, and may lead to paralysis. S100β was found as a suitable biomarker for identifying spinal cord injury and its causing problem. Herein, S100β immunoassay was developed on interdigitated electrode sensor to diagnose spinal cord injury. For effective anti-S100β antibody immobilization, the antibody was premixed with 3-Aminopropyl) triethoxsilane and then attached to the hydroxylated interdigitated electrode surface. This method of antibody immobilization enhanced the antibody attachment two-times than the method without premix. Antibody-attached surfaces increased current responses as S100 concentrations increased, and the limit of detection was seen to be 1 pg/mL on the linearity until 3000 pg/mL at an R^2 value of 0.9907 [y = 7x - 6.4667]. Further, biofouling experiments with glial fibrillary acidic protein and γ-aminobutyric acid failed to enhance the current response, indicating the specific detection of S100β. This immunoassay identifies S100β at its lower level and helps to diagnose spinal cord injury and its related problem.

1. Introduction

A spinal cord injury is damage to the nerves and spinal cord at the end of the spinal canal, which leads to permanent changes in the functions of the body near the injury site. Each year, approximately half a million people are affected by a spinal cord injury [1–3]. Primary spinal injury is the result of mechanical damage to the spinal column, and subsequent spinal injury manifests as hemorrhagic necrosis, hypoxia, ischemia, apoptosis, and edema [4,5]. The secondary injury releases various biological markers from the injury site into the blood and the cerebrospinal fluid, which are utilized to identify the condition of injury [6]. Among them, S100β is one of the extensively studied biomarkers for central nervous system injuries, including spinal cord injury. S100β plays a vital role in developing the brain, stimulates astroglia maturation and proliferation, and is neuroprotective. It helps to promote spinal cord plasticity after the injury [7]. S100β is mainly expressed by glia and found as a suitable biomarker for spinal cord injury [8]. The research proved that the

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presence of S100β is higher during glial cell injury [9]. In addition, the concentration of S100β in cerebrospinal fluid and serum helps to predict the lesion outcome and prognosis [8]. Identifying and quantifying the level of S100β in biological samples help in diagnosing and monitoring the condition of spinal cord injury. The past studies attested with the methods of detecting S100β in head-injured patients by cranial computed tomography (CCT) at a serum S100β cut-off level (0.10 μg/L) [10]. Peskind et al. [11] revealed the level of S100β in older healthy patients to be 0.81 ± 0.13 ng/mL of cerebrospinal fluid. In this context, the expected level of detection of S100β in the biological fluids is in the range from 0.1 μg/L-0.81 ng/mL (8 pM-67 nM) and most of the current biosensors could achieve this range. Towards this current direction, at present several biosensing systems have been generated and demonstrated well [12,13].

S100β is a protein that is often used as a biomarker for brain injury, as it is released into the bloodstream when there is damage to the brain. Several analytical methods can be used for S100β detection. ELISA is a commonly used method for detecting S100β levels in blood or other bodily fluids. It involves using antibodies that are specific to S100β to capture the protein and then detecting it using an enzyme-linked secondary antibody [14,15]. Until now, ELISA is considered one of the gold standard methods and a complement to other analytical methods. Western blotting is another method that can be used to detect S100β. It involves separating proteins based on their size using gel electrophoresis and then transferring them onto a membrane. The membrane is then probed with an S100β-specific antibody to detect the protein [16]. Immunohistochemistry is a technique used to detect specific proteins in tissue sections. It involves using antibodies that are specific to S100β to detect the protein in tissue samples [17]. Mass spectrometry is a sensitive method that can be used to detect and quantify S100β in bodily fluids. It involves ionizing the protein and then analyzing the resulting mass-to-charge ratio to identify and quantify the protein [18]. Fluorescence-based assays, such as fluorescence resonance energy transfer (FRET) and fluorescence polarization (FP), can also be used for S100β detection. These methods involve using fluorescent probes that bind specifically to S100β, and the resulting fluorescence signal is used to quantify the target [19]. However, the involvement of current volt-based measurement, which can give high-performance output is limited.

This research work focused to develop an immunosensor for detecting the level of S100β on the interdigitated electrochemical sensor. Interdigitated electrode (IDE) sensor is an attractive electrochemical technology in biological assays due to its rapid detection, low cost, high selectivity, and specificity [20,21]. Recent IDE sensors are focusing to identify various targets to diagnose a wide range of diseases [22–24]. Researches used various chemical, and physical surface functionalization to improve the surface coverage on the sensing electrode surface as it is a critical step [25]. Apart from that, nanomaterials have also recently been focused on by researchers for surface functionalization, which increase the biomolecule binding on the sensor surface and also gives stability and proper orientation to the molecules [26–28]. IDE used in this study is working based on dual electrodes with interdigitated arrangements with gaps and finger regions. The ions on the surface changes upon surface modification or molecular interaction and reflects by the current changes between two electrodes, as a result of change in dipole moment [29]. Recently, several evidences have been provided by the protein biomarker-based analysis for clinical applications [30]. The recorded demonstrations indicate high-performance detection with higher sensitivity and selectivity with IDE. The primary advantage of IDE is flexibility in designing with the desired numbers of gaps and fingers and their size ranges from nano to micrometers [31]. However, IDE has limitations with the gap size, as it makes a short-circuit if reduced to a low-nanometer range. On the other hand, molecular immobilization is also noticed to reduce the gaps between the fingers. Considering the available immobilization methods, they involve covalent/non-covalent attachment, and makes the right-oriented as well as non-oriented positions of antibodies [32–34].

In this work anti-S100β antibody was attached to IDE through the amine linker 3-Aminopropyl)triethoxsilane (APTES) and then S100β was identified and the electric signal was recorded. Since the detection of S100β is highly dependent on the immobilization of antibodies on IDE, amine functionalization was conducted to immobilize the antibody and then interacted with S100β for diagnosing spinal cord injury.

2. Materials and methods

2.1. Reagents and biomolecules

Thermo Fisher Scientific (USA) supplied S100β (10 μg and prepared 0.1 mg/mL stock) and anti-S100β antibody (100 μg and prepared 1 mg/mL stock). Sigma Aldrich (USA) provided (3-Aminopropyl)triethoxsilane (APTES), and Life Technologies (USA) provided the 1x PBS buffer. The General Science Corporation received a request for PEG-b-PAAC (Japan). Glial fibrillary acidic protein (GFAP) and Gamma-aminobutyric acid (GABA) were bought from Sigma Aldrich (USA). All measurements on the dielectrode surface were made by the power supply through Picoammeter at the range of 0–2 V with an interval of 0.1 V. The complete experiment was performed at room temperature with wet conditions using 10 mM PBS (pH 7.4) and washings were carried out between each surface modification. The modified surface was observed under 3D-nanoprofiler and Atomic force microscope.

2.2. Sensing electrode fabrication

Using AutoCAD software, an electrode model was initially designed with the proper gap size, length, and thickness before being transferred to the photomask [23]. The following stages are involved in creating an interdigitated electrode (IDE). (a) The base substrate of the Si-wafer was cleaned with the solutions 'RCA1 and RCA2'; (b) Thermal oxidation was then carried out at 500 °C for 1 h to produce a layer of SiO_2; (c) A layer of Al was then further deposited on SiO_2 by using an Al-coil and thermal evaporator; (d) After applying spin coating technology to the SiO_2/Al surface, a uniform positive photoresist was created. (e) The drawn pattern was then transferred onto the surface of the photoresist using UV-light exposure. (f) The final surface was obtained and then submerged in

photoresist developer and Al-etching solution to clear the exposed area. (g) The obtained IDE was then rinsed with acetone and distilled water.

2.3. Comparison of Anti-S100β immobilization on IDE through amine linker

Amine surface was used to capture anti-S100β on the IDE surface and two different methods were compared for antibody immobilization. In the first method without pre-mixing APTES and antibody, initially the electrode was rinsed with distilled water before being immersed in diluted potassium hydroxide solution (1%, diluted in water). Further, the KOH-treated substrate was functionalized by amine groups by treating with 5 μl of 2% v/v APTES at 60 °C for 1 h to reach the maximum process of salinization. The amine-modified surface was rinsed with distilled water to remove the unbound APTES. At the same time, an anti-S100β antibody (5 μl of 4 μg/mL) was combined with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide(EDC) and N-Hydroxysuccinimide (NHS; 10 min at 37 °C). The obtained EDC cross-linked antibody was dropped on the amine-modified substrate and incubated at 37 °C for 1 h. Finally, the antibody-coated substrate was washed with by10 mM PBS to remove the excess antibody.

The second strategy (with pre-mixing APTES and antibody) was followed with the same steps until KOH activation. After that, anti-S100β antibody (5 μl of 8 g/mL in PBS) was combined in a 1:1 (v/v) ratio with 1% APTES. On the KOH-treated substrate, the final antibody concentration of 4 μg/mL in 0.5% APTES was applied, and the substrate was then left at room temperature for 30 min. Each experiment's current response was noted.

2.4. Immunoassay for S100β identification

After the optimization of antibody immobilization, an immunoassay was conducted on antibody attached IDE sensor. Before that, the surface was covered with PEG-b-PAAC to reduce the biofouling of S100β, and the corresponding current response was recorded. Afterward, the lowest concentration at 1 pg/mL of S100β was added to the antibody immobilized surface and rested for 30 min at room temperature. After washing the surface with PBS, the current response was recorded again. The difference in current response indicated the interaction of S100β with its antibody. Then, increasing concentrations of S100β from 1 to 3000 pg/mL were interacted on antibody-modified surfaces and the current responses were registered. The detection limit of S100β was determined by computing and plotting the differences among the current responses. The limit of detection (LOD = standard deviation of the baseline + 3σ) is considered the lowest concentration of an analyte against the background signal. For the measurements, the current supply at the range from 0 until 2 V with the interval of 0.1 V was given through probe station by the picoammeter. The dimension of the device is 6000 × 18,000 μm and square region for probing is with 2000 μm. Sixteen pairs finger and gap regions were made to perform the surface biomolecular interaction. All experiments were performed at room temperature with the sensing surface maintained to be wet-condition. Washings were carried out between each surface modification or interaction using 10 mM PBS (pH 7.4).

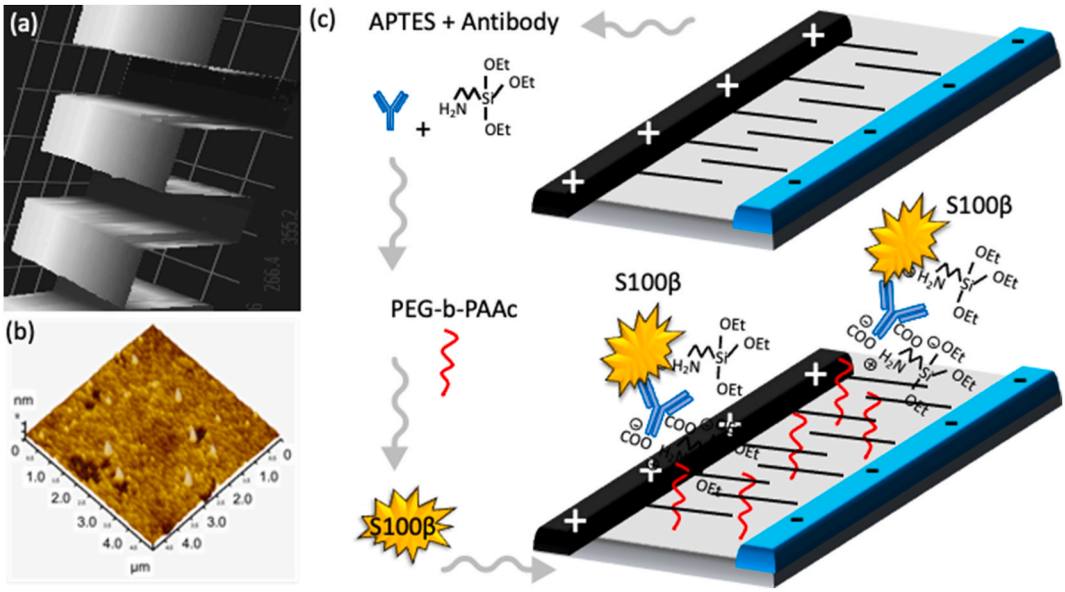

Fig. 1. Surface morphology of interdigitated electrode. (a) Observed under 3D nanoprofiler. (b) Observed under atomic force microscope. (c) Schematic illustration of S100β immunosensing on IDE. Initially, IDE was hydroxylated by KOH and then APTES-anti-S100β antibody conjugates were attached. The uncovered area was masked by a blocking agent PEG-b-PAAc. On the antibody's modified surface, S100β interaction with the antibody was monitored.

2.5. Control performances for specific identification of S100β

Control tests were carried out by using non-immune antibodies and non-matching proteins (GFAP and GABA). After mixing the non-immune antibody with APTES and attaching it on the IDE surface, S100β was interacted. Prior to and following the addition of S100β, the current reflections were recorded. In a different experiment, GFAP and GABA were introduced to the surfaces immobilized with anti- S100β antibody in place of S100β, and subsequent current responses were measured. Current responses from the control experiment were compared with a specific S100β detection on the IDE.

3. Results and discussion

Fig. 1a and b shows the surface morphology of interdigitated electrode observed under 3D-nanoprofiler and atomic force microscope, respectively. Both observations clearly displayed the intactness of the sensing surface fabricated, and the uniformity of the surface. The schematic illustration for S100β immunoassay on interdigitated electrode sensor (IDE) is outlined (Fig. 1c). A single-step antibody immobilization process through the amine linker was carried out to attach the anti-S100β antibody to IDE. Initially, IDE was hydroxylated with KOH to enhance the initial adsorption chain reaction for APTES binding on IDE [35]. With this reaction, surface-tethered hydroxyl groups are undergoing silane reaction with APTES and APTES ends with amine groups. On the KOH surface APTES-anti-S100β antibody conjugate was attached. This mix rendered the amine group in APTES to be charged positively and allowed to bind with the carboxylate group in the antibody [36]. Apart from that, the silanol group in APTES are displaying inter- and intra-ionic interactions between the amino group of APTES and antibody. The amino and silanol group in APTES displays hydrogen bonding with the carboxylic and amino groups of antibodies to form the stable APTES-antibody conjugates. After antibody attachment, the uncovered surface was covered with the blocking agent PEG-b-PAAc. PEG-b-PAAc proved as an excellent blocking agent for amine surface. Since we used APTES to link antibodieson the IDE surface, excess APTES attract the other biomolecule non-specifically, which may lead to an increase in the signal-to-noise ratio and affects the analytical performances. PEG-b-PAAc and derivatives are synthetic polymers that are easily producible and these densely packed polymers display higher resistance to biofouling and increase the sensitivity of the biomolecules [37–40]. In this work, PEG-b-PAAc was utilized to cover the excess amine surfaces by multiple van der Waals contacts and to facilitate the specific interaction of S100β with the antibody.

The above system is a dielectrode (aluminum) sensor that measures changes in the electrical properties of a material, such as its dielectric constant or electrical conductivity. When an electric field is applied to the material, the electrical properties of the material change and this change is detected by the dielectrode sensor. The magnitude of the change depends on the type of material being measured and the strength of the electric field. The electric field is typically applied using the low power supply by a picoammeter and is connected to the sensor. The electrical properties of the sample, such as its dielectric constant or electrical conductivity, will change in response to the electric field (Fig. 2). Because of the dipole moment, surface behaviour reflects the altered ionic movements between positive and negative electrodes. Variations in surface functionalization can be monitored based on molecular charges.

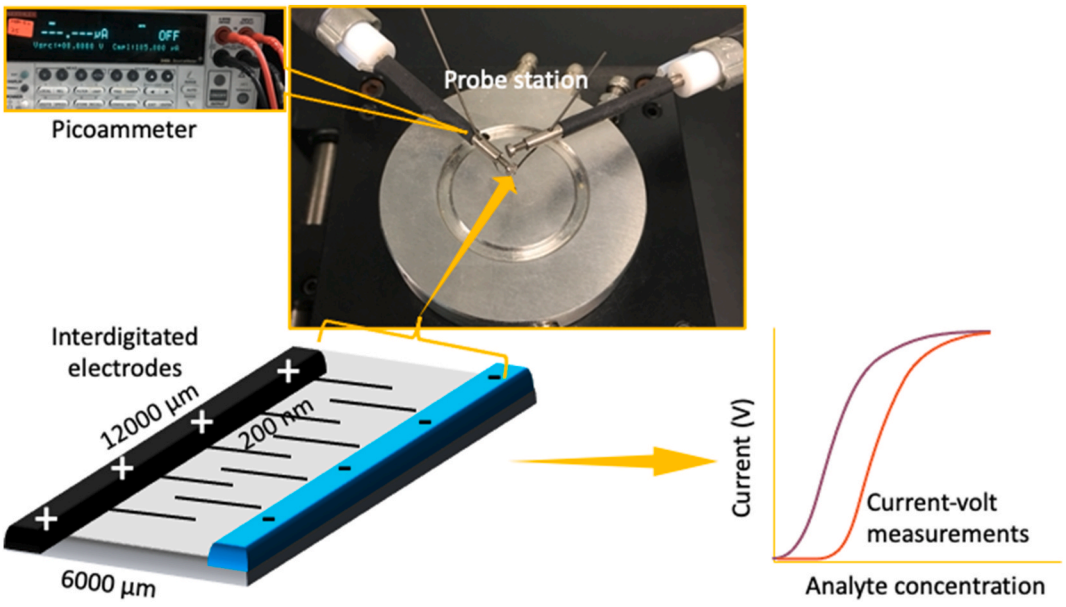

Fig. 2. Working principle on dielectrode sensor and the devise infrastructure. The basic system arrangement is shown with the complete set-up, including power supply by picoammeter, probe station, and dielectrode.

3.1. Comparison of anti-S100β antibody immobilization on IDE

Anti-S100β antibody was immobilized on IDE in two ways. In method 1, after the KOH treatment, IDE was modified with APTES and then the antibody was attached to the amine in APTES through COOH in the anti-S100β antibody by means hydrogen bonding. As shown in Fig. 3a, the bare electrode surface shows the current value as 2.44 pA, after treating with KOH the current was changed to 13.4 pA. Further, when IDE was modified by APTES and antibody, current values were increased to 24.4 and 48.1 pA, respectively. Higher changes in current were recorded after the surface was modified with the antibody, indicating the immobilization of the anti-S100β antibody on IDE through the amine linker. Finally, PEG-b-PAAc was added, then the current was increased further to 61.2 pA.

In method 2, after KOH treatment the surface current was increased from 2.43 to 0.137 pA (Fig. 3b). And then the mixture of APTES and antibody was dropped on IDE, the current value increased drastically to 72.9 pA. This was due to the higher number of antibodies attached to IDE through this method of mixing APTES and antibody prior to adding on the substrate. The enhancement in current response with antibody attachment in methods 1 and 2 are 24.4 and 59.5 pA, respectively, which clearly shows two times difference between the responses (Fig. 4a). This might be due to the stronger interaction happening between APTES and antibody in the liquid phase compared to the antibody attached on the pre-formed APTES modified surface. Apart from that, method 2 did not involve any cross-linking agent to form the amide coupling between APTES and antibody, physical adsorption was attributed to the binding of antibody on IDE. Further, it was noted that the current response with (poly(ethylene glycol) poly(acrylic acid) block polymer) (PEG-*b*-PAAc) in method 1 was higher compared to method 2 due to the higher occupancy of antibody with APTES in method 2, which reduces the chance of interaction of PEG-b-PAAc with APTES (Fig. 4a). By using method 2, higher immobilization of antibody was achieved by enhancing the physical adsorption of preformed APTES-antibody conjugate on the IDE surface. So, an immunoassay to identify S100β on anti-S100β antibody immobilized IDE was conducted by using method 2 to attain maximum sensitivity.

3.2. Immunoassay for S100β identification: Interactive analysis

After the antibody optimization, S100β immunoassay was carried out on the antibody attached IDE. S100β concentrations from 1 to 3000 pg/mL were diluted in 10 mM PBS (pH 7.4) and individually dropped on the antibody-modified electrode surface. After washing the surface, current responses were recorded for each experiment. PEG-b-PAAc modified IDE displays the current level of 0.76 nA, and after dropping 1 pg/mL of S100β, the current level was increased to 1.02 nA (Fig. 4b). This increment of current confirms the interaction of S100β with its antibody. Further increments with S100β concentrations from 10 until 3000 pg/mL, current responses were increased concomitantly until 2000 pg/mL. After the level of 2000 pg/mL, the current response has attained the level of saturation until 3000 pg/mL. It was noticed that, with enhancing S100β concentration, current responses also increased gradually. The differences in current levels with each concentration of S100β were calculated and plotted in a linear regression graph and calculated the limit of S100β detection as 1 pg/mL with an R^2 value, 0.9907 [$y = 7x - 6.4667$] (Fig. 5). This lower level of detection limit was achieved due to the higher number of antibody immobilization with the proper orientation on the IDE sensor, assisted by PEG-b-PAAc.

3.3. Control performances for specific S100β detection

To evaluate the biofouling (non-specificity) on the sensing electrode surface, control experiments were performed with relevant biomarkers such as, glial fibrillary acidic protein (GFAP), Gamma-aminobutyric acid (GABA), and non-immune antibodies on PEG-b-PAAc modified IDE. It has been suggested that GFAP could be a good option for biofluid-based indicators for a variety of neurological disorders, particularly after stroke and severe brain/spinal cord injury. GABA is an amino acid that serves as the central nervous

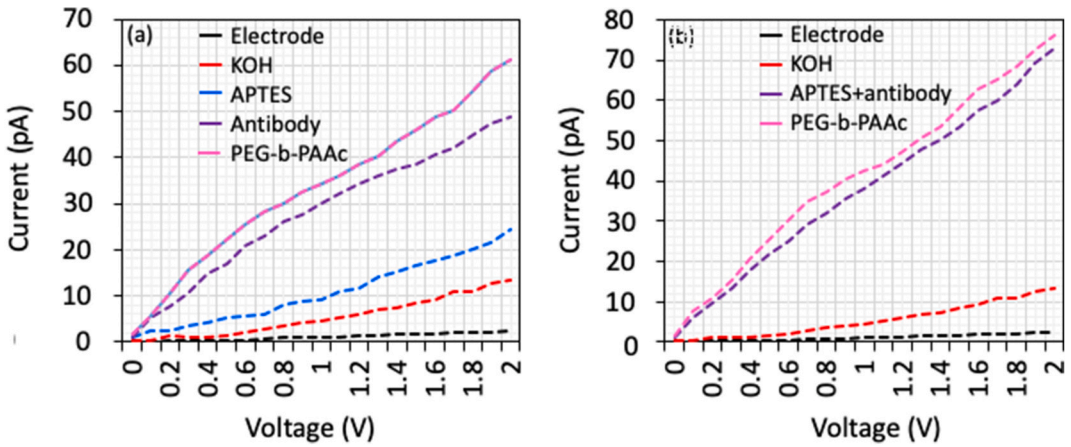

Fig. 3. Comparison of anti-S100β antibody immobilization on IDE. (a) Method 1: antibody was immobilized on APTES modified electrode. The current response was drastically increased after adding the antibody to IDE; (b) Method 2: APTES premixed antibody was immobilized directly on IDE. Higher current responses were achieved after adding to the IDE surface.

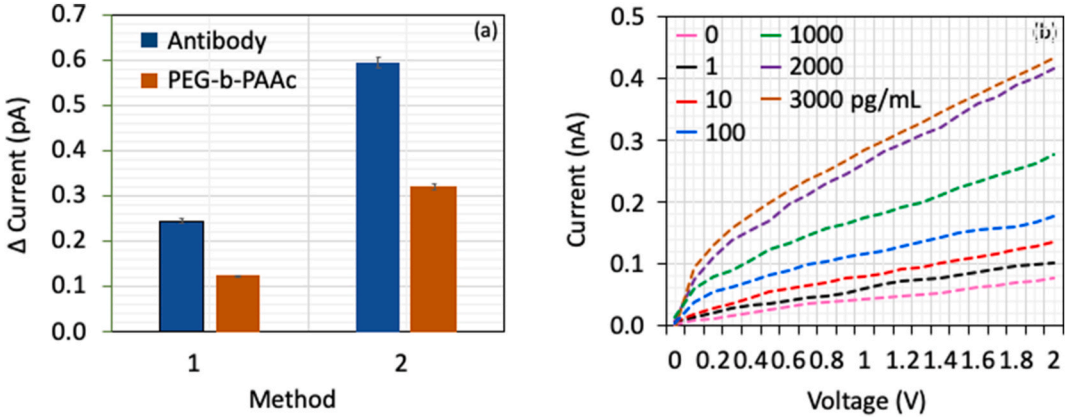

Fig. 4. (a) Difference of current of antibody immobilization on IDE by methods 1 and 2. Method 2 shows the highest changes in current compared with method 1. Error values were averaged with triplicates. (b) Immunoassay of S100β. S100β concentrations from 1 to 3000 pg/mL were diluted in 10 mM PBS and individually dropped on antibody modified electrode. The increment of current confirms the interaction of S100β with the antibody.

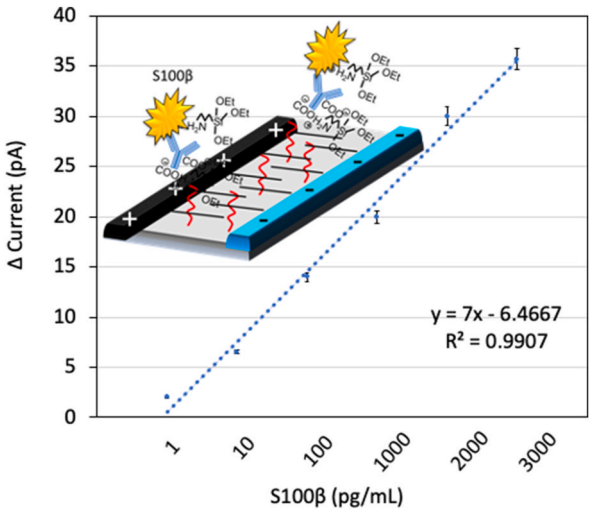

Fig. 5. Current responses of different S100β concentrations. Interacted with its antibody. With increasing S100β concentration, current responses were also increased gradually. Error values were averaged with triplicates. (b) The differences in each concentration of S100β were calculated and plotted in a linear regression graph and calculated the detection limit of S100β is 1 pg/mL with an R^2 value of 0.9907.

system's main inhibitory neurotransmitter and by preventing nerve transmission, it works to lower neuronal excitability. As shown in Fig. 6, relevant control proteins and antibodies are not showing noticeable current changes. In general amine-modified surfaces easily attract other biomolecules non-specifically and lead to false positive results. PEG-b-PPAc bound with the excess amine surfaces and reduced the non-specific binding of other proteins and antibody [29]. Previous research proved that PEG-b-PPAc controls the nonspecific binding of gold nanoparticles on the amine-modified surface and increases the specific binding of the target and analyte. In another research, PEG-b-PPAc increases the probe (aptamer) immobilization on the gold surface with a proper orientation and enhances the sensitivity of target molecular interaction [29–31]. Herein, PEG-b-PAAc modified surface does not increase with the current response and prevent the non-specific binding, indicating the specific detection of S100β.

4. Conclusion

A spinal cord injury damages the spinal cord and nearby bones and tissues. Depending on the severity of injury, it causes various problems with the functions of different parts of the body. In this research high-affinity spinal cord injury immunosensor was developed by detecting the biomarker, 'S100β' on interdigitated electrode sensor. A higher level of anti-S100β antibodies on IDE was achieved by premixing antibodies with APTES, which shows 2-time higher current responses than the usual method. As a result, the detection limit of 1 pg/mL was reached by improving the interaction of S100β with its antibody on the sensor surface. The specific identification of S100β was confirmed by control studies that did not show any notable binding with current responses. This

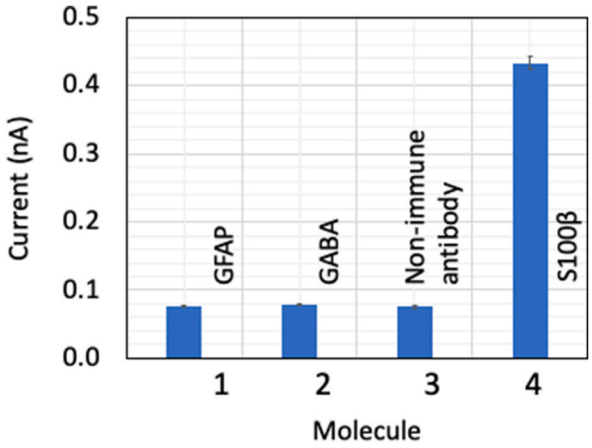

Fig. 6. Determining biofouling on the sensing electrode. Control proteins GFAP and GABA and non-immune antibodies on PEG-B-PAAc modified IDE did not increase the current responses. PEG-B-PAAc reduces biofouling, indicating the specific detection of S100β. Error values were averaged with triplicates.

immunoassay aids in locating spinal cord damage and detecting S100β at its lower level.

Author contribution statement

Hao Zhang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Subash C.B. Gopinath: Analyzed and interpreted the data. Yajun Hu: Contributed reagents, materials, analysis tools or data..

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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