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Biosensors

Electrochemical impedimetric detection of stroke biomarker NT-proBNP using disposable screen-printed gold electrodes

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Abstract

Stroke is the second top leading cause of death globally. It is caused by an abrupt interruption of blood flow to the brain. In that course, brain natriuretic peptide (BNP) and its derivative N-terminal pro b-type natriuretic peptide (NT-proBNP), neurohormones produced mainly by the heart ventricles in response to excessive stretching of cardiomyocytes (heart muscle cells), are proven to be good biomarkers for heart failure diagnosis. Moreover, there is growing clinical interest of the use of NT-proBNP for stroke diagnosis and prognosis because it is significantly associated with cardioembolic stroke and secondary stroke reoccurrence, with sensitivity >90% and specificity >80%. However, in diagnostic settings, there is still a need to address the encountered analytical problems, particularly assay specificity and set up. In this study, a novel approach for NT-proBNP detection is demonstrated using an electrochemical immunoassay method. A label-free impedimetry immunosensor for stroke biomarker was developed using modified disposable screen-printed gold electrodes (SPGE) hosting specific anti-NT-proBNP capture antibody. The performance of our immunosensor was studied in the presence of NT-proBNP in both buffered and mock (porcine) plasma samples. A linear relation between the relative total resistance (ΔR_{tot}) responses and the NT-proBNP concentrations in buffer was observed in a range from 0.1 to 5 ng mL^{-1} with a correlation coefficient (R^2) of 0.94656. Overall, the biosensor has demonstrated the capability to quantitate NT-proBNP and differentiate such concentrations in a low concentration range, especially among 0, 0.1, 0.5, 1, and 3 ng mL^{-1} in plasma samples within 25 min. This range is valuable not only for classifying cardioembolic stroke (higher or equal to 0.5 ng mL^{-1}), but also predicting the risk of secondary stroke reoccurrence (higher than 0.255 ng mL^{-1}). Our biosensor has the potential to be used as an easy-to-use point-of-care test that is both accurate and affordable.

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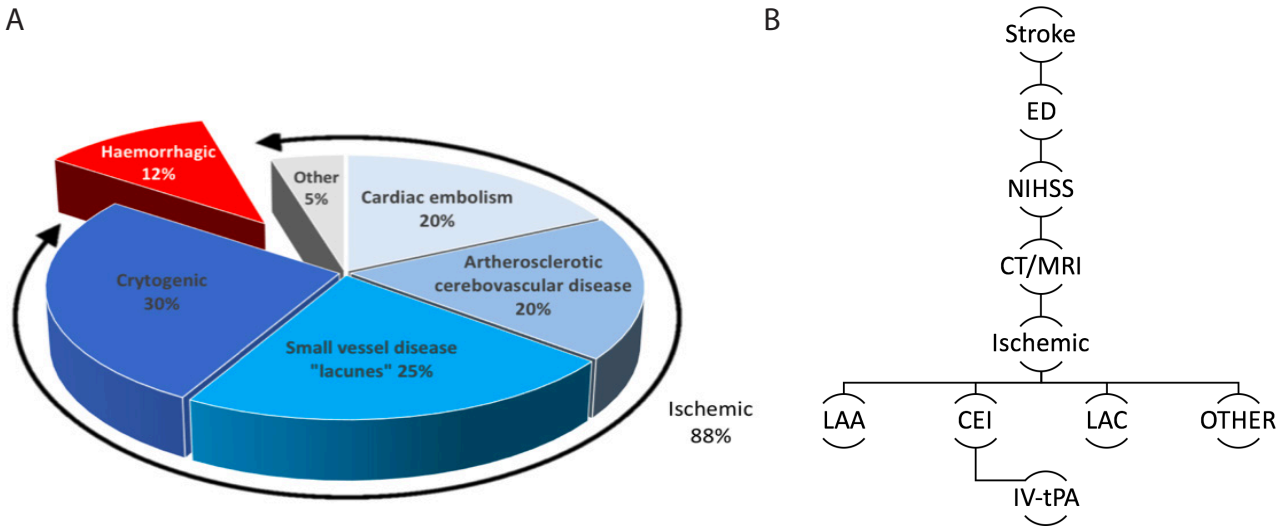
Introduction

Stroke is the second most common cause of death and a major cause of disability. Fifteen million people worldwide suffer from stroke each year with more than 30% of patients dying every year, while over 30% will suffer from permanent disability. Without intervention, the number of global deaths is projected to rise to 7.8 million in 2030 (1).

In the event of a stroke, time is of crucial importance because brain cells die rapidly after onset and hence any effective treatment must start as early as possible. This is the 'time is brain' rationale, where in the absence of blood perfusion during ischemia, a shortage of oxygen and glucose to feed cellular metabolism occurs, that can then cause irreversible damage. Timely diagnosis and treatment continue to be the major limitation of therapeutic approaches, especially when only a minority of eligible ischemic stroke patients receive recanalizing therapies (2-4).

Stroke diagnostics is complicated by the fact that not all strokes are alike. There are two main types: ischemic and hemmorhagic strokes (**Fig. 1A**). Our focus is on the ischemic stroke, particularly cardioembolic stroke (CEI) that is generally more severe and more prone to reoccurrence. In the case of a stroke emergency, a typical stroke diagnostic system, as illustrated in **Fig. 1B**, shows that the classification of CEI is of upmost importance (after differentiating between ischemic and hemmorhagic stroke) due to the time-limited



**LAA – large-artery atherosclerosis; CEI – cardioembolism; LAC – small-vessel occlusion (lacunar)*

Figure 1. (A) Stroke subtypes based on the TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification. (B) A typical stroke diagnostic system. When stroke symptoms occur (t=0), the patient is transported to an emergency department (ED) (t≤30min). The suspected stroke patient will then be evaluated by a neurologist using NIHSS (National Institutes of Health stroke scale) (t≤60min) and sent for a CT/MRI scan for initial stroke diagnosis (t≤90min). Thereafter, stroke subtype classification is conducted (t≤120min) and once CEI is confirmed, IV-tPA is administered (t≤180min). The average admission time of IV-tPA is 3 hr from symptoms onset. In addition, the main focus of post-stroke care is the outcome improvement and prevention of secondary stroke reoccurence; this is conducted using various tests, such as regular NT-proBNP monitoring.

window for treatment with the intravenous tissue plasminogen activator (IV-tPA) (3, 5-7). Although CEI has become a treatable disease by IV-tPA, success rates remain low because most patients are being treated at the late stage of the therapeutic window (2). Furthermore, another aspect of stroke care is post-stroke care, that is related to rehabilitation of stroke patients and most importantly prevention of secondary stroke episodes whereby classification of stroke subtype is detrimental (8-11).

Current clinical guidelines emphasize the need for early stroke care and its success lies in the timely recognition in the pre-hospital setting (ambulance) and a prompt diagnosis at the Emergency Room (ER) (e.g. identifying some neurologic symptoms after the sudden onset, such as facial asymmetry, arm and leg weakness, and slurring of speech). Therefore, it is necessary to utilize a point-of-care testing that will enable a faster and better approach for stroke patients' care in their most critical state, especially because it also provides the relevant needed information on-site, without sample processing and central-laboratory long testing and results analysis procedures (12).

Studies have suggested that the diagnosis of stroke could be complemented with the use of biomarkers, possibly enabling an important saving of time and resources. One such candidate biomarker that has been extensively studied and shown great diagnostic accuracy is the brain natriuretic peptide (BNP)(13). It is a neurohormone produced mainly by the heart ventricles in humans (14) and is secreted in response to excessive stretching of cardiomyocytes (heart muscle cells) (15). BNP, a biologically active 32-amino acid long cyclic polypeptide, is secreted along with its derivative, NT-proBNP, a biologically inactive 76-amino acid N-terminal fragment (16). However, BNP has a half-life of only 20 min, while NT-proBNP 1-2 h, thus making NT-proBNP more suitable for rapid, point-of-care diagnostics (17).

NT-proBNP is a known biomarker for diagnosis of heart failure (18). Recently, studies have suggested a new diagnostic role for this biomarker, which is the characterization of cardioembolic stroke. Serum levels of NT-proBNP have been shown to elevate in cardioembolic stroke patients up to 72 h from onset of symptoms (19-33). Increased NT-proBNP levels have been indicative of cardioembolic stroke with a sensitivity of 75.6% and specificity of 87.4% (21). Moreover, the mean serum NT-proBNP value has been significantly higher in cardioembolic stroke patients ($P<0.001$) (491.6 pg mL^{-1}; 95% confidence interval 283.7-852.0 pg mL^{-1}) than that in non-cardioembolic ischemic stroke patients (124.7 pg mL^{-1}; 86.3-180.2 pg mL^{-1}) (33).

In our preliminary study on 480 Singaporean patients, we have found that NT-proBNP is the best predictor of cardioembolic stroke among 7 other biomarkers tested (Table 1), such as Interleukin-6 (IL-6), S100, D-dimer, Neuron-Specific Enolase (NSE), Insulin, Cortisol, and Albumin. Using receiver operating characteristic (ROC) curve analysis, NT-proBNP plasma levels higher or equal to 500 pg mL^{-1} are associated with cardioembolic stroke, with a sensitivity >80% and specificity >80%, yet the exact cut-off will be determined during future studies. Furthermore, elevated NT-proBNP levels have also been shown to predict the reoccurrence of secondary stroke

Table 1. Summary of ischemic stroke biomarkers

No.	Biomarker	Family	Stroke Expression Association	Stroke Clinical Value	Ref.
1.	B-Type Natriuretic Peptide (BNP) / NT-proBNP	Heart muscle cells (cardio-myocytes) origin	Cardioembolic (CEI) ischemic stroke, increased mortality and second stroke indication	Prognosis[a] and recovery[c]	(17, 19-32, 34-45)
2.	Interleukin-6 (IL-6)	General inflammatory cytokines	Cardioembolic (CEI) and lacunar (LAC) ischemic stroke subtypes	Prognosis[a]	(46-54)
3.	S100-Beta	Glial cells origin	General blood–brain barrier (BBB) dysfunction	Diagnosis	(55-61)
4.	Neuron-Specific Enolase (NSE)	Neuronal cells origin	Neurological outcomes and infarct volume	Prognosis[b]	(46, 55-59, 62-64)
5.	D-dimer	Hemostatic proteins	Cardioembolic (CEI) ischemic stroke subtype and recurrence strokes	Prognosis[a]	(65-84)
6.	Albumin	Metabolic proteins	Cardioembolic (CEI) ischemic stroke subtype	Prognosis[a]	(85)
7.	Cortisol	Steroid hormones	Stroke severity and outcome prediction	Prognosis[b]	(86-89)
8.	Insulin	Metabolic proteins	Cardiovascular risk	Diagnosis	(90)

[a] *subtype classification;* [b] *outcome prediction;* [c] *second stroke*

(43-45, 91-93) whereby levels greater than 255 pg mL^{-1} pose significantly higher risk with a sensitivity of 76% and specificity of 60% (44).

Today, NT-proBNP level is routinely measured using various immunoassay techniques (94), especially enzyme-linked immunosorbent assay (ELISA) for heart failure diagnosis. However, ELISA is a multi-step, lab-based technique that requires sophisticated instrumentation operated by qualified and trained personnel. The analysis is also time-consuming and not well-suited for point-of-care measurements. Therefore, there is an urgent need for point-of-care tools. Currently, there are more than 10 FDA-approved NT-proBNP immunoassays in the 510(k) database, such as Roche Diagnostics GmbH (CARDIAC proBNP+), Abbott Diagnostics (AxSYM® BNP), and Alere Triage® BNP Test. For example, CARDIAC proBNP+ point-of-care kit employs a two-line detection system (i.e. test and control lines) whose quantitative results are obtained by an optical reader, Cobas h 232 (95). Even though the aforementioned commercial kits sufficiently provide quantitative results, the application is limited to the use of expensive optical readers (upward of USD 1,000). High initial cost makes them impractical for a widespread use as a point-of-care technology, especially in less developed healthcare settings.

In recent years, electrochemistry has gained tremendous interest in diagnostics due to its high sensitivity, simple instrumentation, and ease of miniaturization. Some electrochemical sensors have been developed for the determination of NT-proBNP involving sandwich formats. For example, capture antibodies were immobilized on gold and carbon nanotubes composite platform and bioconjugates of gold nanochains, while HRP-labeled secondary antibodies were employed for signal amplification (96). Another has employed only a single antibody/antigen pair in indirect competitive ELISA format with the help of magnetic nanoparticles (97). In this paper, we propose a novel and low-cost label-free electrochemical point-of-care test for NT-proBNP that not only principally eliminates any use of optical readers, but also forgoes magnetic nanoparticles and enzymatic reactions while still enabling reliable quantitation. The fabricated impedimetric electrochemical biosensor has successfully provided the detection and quantification of NT-proBNP in both buffered and plasma samples at hundreds of picogram concentration level (i.e. 10^{-1} ng mL^{-1}).

Experimental Section

Materials

Reagents

Phosphate buffered saline (PBS) tablet (Cat. P4417), N-Hydroxysuccinimide (NHS) (Cat. 130672), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (Cat. 03450), sulfuric acid 95-98% (Cat. 320501), 11-Mercaptoundecanoic acid (MUA) (Cat. 450561), 2-(N-Morpholino)ethanesulfonic acid (MES) sodium salt (Cat. M5057), and ethanolamine (Cat. 398136) were purchased from Sigma-Aldrich. Ethanol 95% (6417-5) was obtained from Aik Moh (Singapore). Ferrocene acetic acid (Cat. F0406) was purchased from Tokyo Chemical

Industry. All reagents were of analytical grade and used without further purification. All aqueous solutions were prepared using Milli-Q ultrafiltered (UF) H_2O (with a resistivity of 18.2 MΩ cm at 25 °C).

Immunoreagents

Monoclonal mouse anti-human N-terminal proBNP (NT-proBNP) (IgG) MAb.24E11 against a.a.r.61-76 (100 µg) (Cat. Orb79568), and NT-proBNP (10 µg) (Cat. orb81959) were purchased from Biorbyt Ltd., United Kingdom.

Porcine plasma

Porcine plasma was used as a medium to mimic the sample environment of human plasma, which is the preferred sample type for diagnostic testing of NT-proBNP. Porcine whole blood was purchased from Primary Industries Pte. Ltd. (Singapore), with an official permit from Singapore Agri-Food & Veterinary Authority (AVA). To prevent blood coagulation before plasma extraction, heparin solution was prepared by dissolving 100 mg heparin sodium salt in 20 mL cold 0.1 M Krebs-Ringer Phosphate buffer (pH 7.3) and then mixed with 1 L of the blood. Once collected, the blood was aliquoted to 50-mL falcon tubes and centrifuged for 30 min at 400 x *g*. Afterwards, the resulting plasma was purified once again with the same separation condition so as to remove any remains of red blood cells. For storage, the aliquoted porcine plasma stocks were frozen at -80 °C, and whenever needed, one aliquot was thawed in a 37°C-incubator for at least 30 min. Thereafter, various concentrations of NT-proBNP were spiked into the porcine plasma for biosensor validation.

Electrodes

Screen-printed gold electrodes (Cat. 220 BT) with a 4-mm working electrode diameter (WE: gold, CE: gold, RE: silver) and their connector to a potentiostat (Cat. DRP-DSC-4MM) were purchased from DropSens.

Instruments

A Solartron potentiostat (Model 1470E) was used for all electrochemical measurements. Centrifuge (Universal 320) was from Hettich Zentrifugen. Cobas e-411 and Cobas c-111 (Roche) were used for determining the biomarkers levels at NUHS.

Electrode cleaning

SPGEs were cleaned by cyclic voltammetry (CV) in the presence of 100 µL solution of 0.5 M H_2SO_4 for ten cycles in a potential window of -0.1 V to 0.9 V at a scan rate of 100 mV s^{-1}. Once rinsed with 40 mL deionized (DI) water, they were dried with compressed air.

Biofunctionalization of the SPGE with anti-NT-proBNP capture antibody

As previously reported (98), the cleaned SPGEs were incubated with 1 mM MUA-ethanolic solution (150 µL) for 18 h at 4 °C in the dark. Before electrochemical characterization, they were rinsed with ethanol (5 mL) and dried, which then was rinsed again with DI water (10 mL) and dried. The SPGEs-MUA were then exposed to an activation reagent solution (50 µL of 0.4 mM EDC/ 0.1 mM NHS in 10 mM MES buffer pH 5) for 1 h at room temperature, rinsed with MES buffer (5 mL) without drying, rinsed with 1X PBS buffer pH 7.4 (10 mL) and functionalized after with 25 µL specific anti-NT-proBNP IgG 0.1 mg mL^{-1} for 1 h at room temperature in PBS buffer. The modified SPGEs-antibody were sequentially rinsed with PBS buffer (10 mL) noting that drying should be avoided. All biofunctionalized SPGEs were then rinsed with DI water (60 mL) for electrochemical characterization. Afterwards, the remaining active sites of biofunctionalized SPGEs were passivated with 25 µL 50 mM ethanolamine (EA) in PBS buffer for 30 min at room temperature, then rinsed with PBS (10 mL) followed by DI water (60 mL) without drying. Prior to immunoassay, the biofunctionalized SPGEs were stored at 4 °C with a 100 µL drop of DI water.

Electrochemical characterization of biofunctionalized SPGE with ferrocene acetic acid

The functionalized electrodes were characterized by CV and EIS for each of the various steps during the preparation of the anti-NT-proBNP antibody immobilization. Prior to the measurement, all SPGEs were rinsed with DI water. Then, CV and EIS measurements were performed in the presence of 50 µL 5 mM ferrocence acetic acid in 1X PBS solution. All CV measurements were done in a potential window of -0.3 V to 0.5 V at scan rates of 100 mV s^{-1}, while EIS at 0.06 V (vs. pseudo Ag reference) and 10 mV AC amplitude occurred in a frequency range from 60 kHz to 0.5 Hz with 15 steps/decade.

Reproducibility of the constructed biosensor

Biofunctionalization was done across 28 independent SPGEs, thus the reproducibility was assessed. By using the impedance method (e.g. **Fig. 2**), a value of R_{tot} for each SPGE after immobilization of the capture antibody was calculated. The amount of capture antibody was assumed to be proportional to the value of resistance. The Relative Standard Deviation (RSD) of biofunctionalized SPGE was found to be 15.1%.

NT-proBNP detection

Twenty-eight independent biofunctionalized SPGEs were used for each concentration of NT-proBNP both in PBS buffer and porcine plasma, ranging from 0.1 ng mL^{-1} up to 5 ng mL^{-1}, in duplicates. As illustrated in **Fig. 2**, a drop of 50 µL NT-proBNP was applied during a 20-min incubation before the SPGE was washed with DI water (60 mL). Afterwards, CV and EIS were performed sequentially after applying 50 µL of 50 mM ferrocene acetic acid in 1X PBS on the electrode surface. All CV measurements were done in a potential window of -0.3 V to 0.5 V at scan rates of 100 mV s^{-1} for 8 cycles, while EIS at 0.06 V (vs. pseudo Ag reference) and 10 mV AC amplitude occurred in a frequency range from 60 kHz to 0.5 Hz with 15 steps/decade.

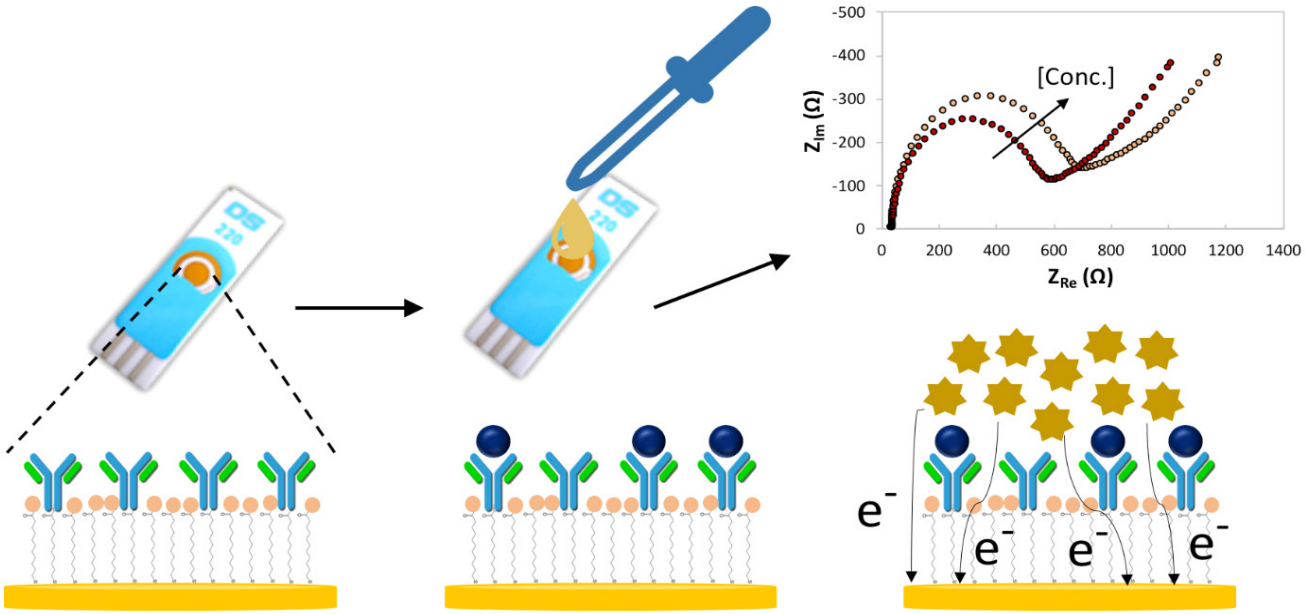


Figure 2. Immunoassay schematic and rationale. A MUA-activated surface of screen-printed gold electrodes (SPGE) was biofunctionalized with anti-NT-proBNP capture antibodies that was then passivated with ethanolamine to reduce non-specific bindings to the surface. A 50 µL drop of NT-proBNP analyte was applied to the SPGE for 20 min before washing. An electrochemical signal was recorded after applying 50 µL of 50 mM ferrocene acetic acid in 1X PBS on the electrode surface.

Results and Discussion

Electrochemical characterization of biofunctionalized SPGE

All electrochemical measurements were performed in 1X PBS buffer pH 7.4 containing 5 mM ferrocene acetic acid. Both CV and EIS spectra of ferrocene acetic acid reporter were utilized to investigate the changes of the SPGE behavior after each functionalization step. **Fig. 3** shows the cyclic voltammograms (A) and Nyquist plots (B) of each layer: bare screen-printed Au electrodes (a), Au-MUA (b), Au-MUA-Ab (c), Au-MUA-Ab-EA (d), and Au-MUA-Ab-EA-NT-proBNP of 0.1 ng mL^{-1} in PBS (e). As shown in **Fig. 3A**, stepwise functionalization of SPGE was accompanied by a decrease in the amperometric response and an increase in the peak-to-peak separation between the cathodic and anodic waves of the redox reporter, showing that the electron-transfer kinetics of ferrocene acetic acid was obstructed. Initially, the bare SPGE showed a high current signal, suggesting a facile electron process of ferrocene acetic acid to the electrode surface. After functionalization with MUA and capture antibody immobilization, the electron transfer between the electrochemical reporter and electrode surface was evidently inhibited. However, when ethanolamine was immobilized onto the remaining active sites of the SPGE, a slight increase of amperometric response was observed instead, owing to a net positive surface charge of the ethanolamine blocking layer (pKa = 9.5). Nevertheless, after NT-proBNP was captured on the surface, there was a further decrease of anodic and cathodic peaks due to an inert protein layer for electron and mass-transfer of ferrocene acetic acid toward the electrode surface. Furthermore, in addition to CV, EIS was employed as a suitable method for label-free biosensors because it also provides detailed information about impedance changes during the modification process. In general, an impedance spectrum is represented as a Nyquist plot which consists of a semi-circle and a linear portion. The semicircle portion that typically occurs at higher frequencies corresponds to an electron-transfer-limited process, while the linear portion at lower frequencies represents the diffusion-limited process. Moreover, the semi-circle diameter equates to electron-transfer resistance, R_{et}. **Fig. 3B** shows faradaic impedance spectra during the sequential stepwise modification process. The bare SPGE showed a low electron-transfer resistance, while the first layer consisting of the self-assembled monolayers of MUA showed an increase of resistance, owing to the layers of COO-terminal groups on the electrode surface generating a negatively charged surface that reduced the ability of the redox reporter to access the layer. Similarly, the subsequent biofunctionalization step with an additional layer of anti-NT-proBNP capture antibodies showed a further growth of the resistive semi-circle. However, the blocking step with ethanolamine which conferred a positive charge layer, promoting the attraction of ferrocene acetic acid and, hence, a small decrease in the resistance. The following step corresponds to the NT-proBNP immunoassay whereby the peptide was captured on the electrode surface and the insulated protein layer hindered the diffusion of the electrochemical probe toward the electrode surface resulting in a resistance increase. In short, the inversely proportional trend between increasing resistance and decreasing current signal showed a credible proof for each biofunctionalization step since the additional layers hindered the electron transfer and diffusion of the redox reporter from the electrolyte to the working electrode of the SPGE.

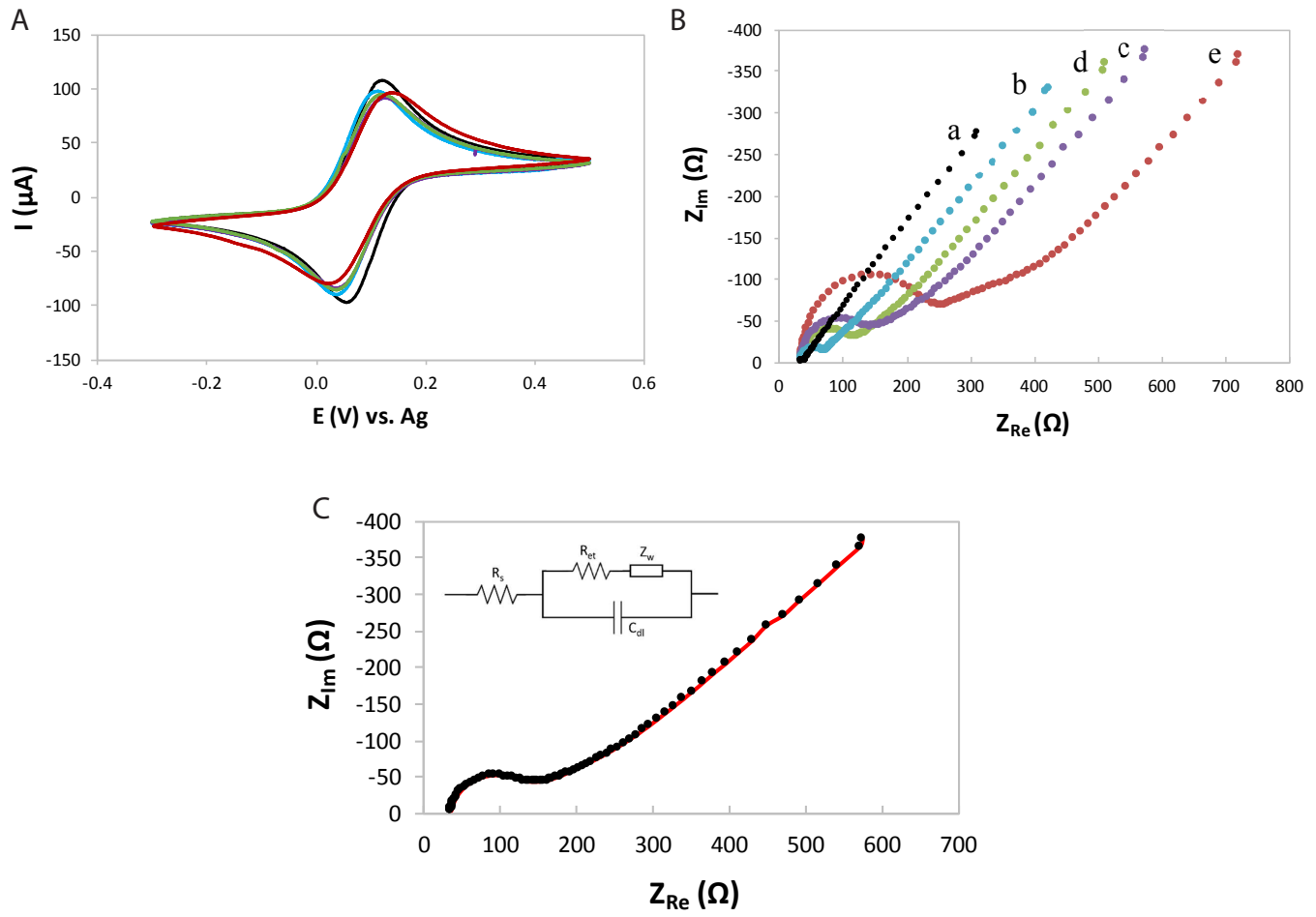


Figure 3. Cyclic voltammograms (A) and Nyquist plots (B) of each layer: bare screen-printed Au electrodes (a), Au-MUA (b), Au-MUA-Ab (c), Au-MUA-Ab-EA (d), and Au-MUA-Ab-EA-NT-proBNP of 0.1 ng mL^{-1} in PBS. The scan rate applied for CV was 100 mV s^{-1} while the potential for EIS was at 0.06 V (vs. pseudo Ag reference) and 10 mV AC amplitude in a frequency range from 60 kHz to 0.5 Hz with 15 steps/decade. A modified Randles circuit was presented together with the curve fitting for impedance spectra of capture antibody (C).

Equivalent circuit

The impedance data were fitted with commercial software ZView®. A modified Randle's equivalent circuit and the fitting of capture antibody spectrum to the equivalent circuit (solid line) are both shown in **Fig. 3C**, indicating good agreement with the circuit model over the frequency range. The circuit, which is often to model interfacial phenomena, consists of the following four elements: (i) ohmic resistance of the electrolyte solution at higher frequency, R_s; (ii) Warburg impedance, Z_w, representing resistance to mass transfer such as the diffusion of ions from bulk electrolyte to electrode interface; (iii) the double layer capacitance (C_{dl}) that corresponds to an electrical double layer (insulator) between an electrode and its surrounding electrolyte, relating to the surface condition of the electrode; and (iv) the electron-transfer resistance, R_{et}, which exists if a redox reporter is present in the electrolyte solution. The parallel elements (C_{dl} and $Z_w + R_{et}$) of the equivalent circuit were introduced since the total current through the working interface was the sum of respective contributions from the faradaic process and the double layer charging. Theoretically, Z_w and R_s represent the bulk properties of the electrolyte solution and the diffusion features of the redox probe in solution, thus are not affected by modifications on the electrode surface. However, a small yet negligible change in R_s was observed during the modification process. As shown in **Fig. 3B**, the ohmic resistance of the solution was evidently not affected by the modification of electrode while the changes in R_{et} were much larger than those in other impedance components. Therefore, it is safe to say that R_{et} was more useful for sensing the interfacial properties of the prepared immunosensor during all these assembly procedures, although it is mostly common to report the total resistance of the system ($R_{tot} = R_s + R_{et}$).

Data fitting of biofunctionalized SPGE

The fitting values of the circuit elements obtained for electrode assembly are presented in Table 2. For the bare SPGE, the value of R_{tot} is 40.06 Ω, exhibiting a nearly straight line in the Nyquist plot (**Fig. 3B**, **curve a**) which shows a predominance of the diffusive effect over the resistive one. After MUA functionalization, the value of R_{tot} was 65.46 Ω (**Fig. 3B, curve b**) which is slightly bigger than that of the bare SPGE. After immobilization of capture anti-NT-proBNP antibody, there was a significant increase in the resistive semi-circle leading to a R_{tot} value of 148.53 Ω (**Figu. 3B, curve c**). A small decrease in R_{tot} to

Table 2. Values of equivalent circuit elements obtained for experimental data fitting after each additional layer on the SPGE: Bare Au, MUA, capture antibody (Ab), ethanolamine (EA) and NT-proBNP at 0.1 ng mL^{-1}

	R_s (Ω)	R_{et} (Ω)	R_{tot} (Ω)
Bare Au	32.89 ± 0.13	7.17 ± 0.40	40.06 ± 0.53
Au-MUA	35.00 ± 0.33	30.46 ± 1.66	65.46 ± 1.99
Au-MUA-Ab	34.70 ± 0.25	113.83 ± 5.64	148.53 ± 5.89
Au-MUA-Ab-EA	34.61 ± 0.22	93.51 ± 4.83	128.12 ± 5.05
Au-MUA-Ab-EA-NTproBNP	31.99 ± 0.26	275.25 ± 19.35	304.24 ± 19.61

Table 3. Electrochemical impedance results for NT-proBNP detection in PBS buffer from Figure 4A

[NT-proBNP] in PBS (ng mL^{-1})	R_{tot} (Ω)	R_{tot} - R_{EA} (Ω)
0.1	304.24 ± 19.61	176.12 ± 14.56
0.3	377.82 ± 3.99	249.70 ± 1.06
0.5	400.20 ± 25.10	272.08 ± 20.05
0.7	408.25 ± 45.23	280.13 ± 40.18
1	470.64 ± 60.68	342.52 ± 55.63
5	512.02 ± 35.90	383.9 ± 30.85

128.12 Ω (**Fig. 3B, curve d**) was shown in the subsequent step of ethanolamine passivation. After the capture of NT-proBNP (0.1 ng mL^{-1}) on the electrode surface, R_{tot} continued to increase to 304.24 Ω (**Fig. 3B, curve e**). The reason is that the antibody and NT-proBNP acted as insulators, and they hindered the diffusion of the redox reporter toward the electrode surface. This confirms the formation of a compact homogenous immunocomplex on the electrode surface. Furthermore, as explained above, although changes in R_s were still reported, one should notice that R_{et} signified the changes associated with the impediment of electron transfer of the redox reporter, ferrocene acetic acid, to the modified electrode surface. This behavior was reflected in the growth of the semi-circle diameter at higher frequencies.

NT-proBNP detection

To evaluate the biofunctionalized SPGE as an immunosensor, we exposed it to various concentrations of NT-proBNP in PBS buffer. The corresponding Nyquist plots of the impedance spectra are shown in **Fig. 4A**, and the fitting values of R_{tot} are presented in **Table 3**. It was found that the diameter of the Nyquist circle, and consequently R_{tot}, increased with increments in concentration of NT-proBNP. This can be explained by the fact that by increasing the concentration, an increased amount of molecules were bound specifically to the immobilized capture anti-NT-proBNP antibodies, which thereafter acted as a definite kinetic barrier for the electron transfer. As shown in **Fig. 4B**, a linear relation between the relative R_{tot} responses and

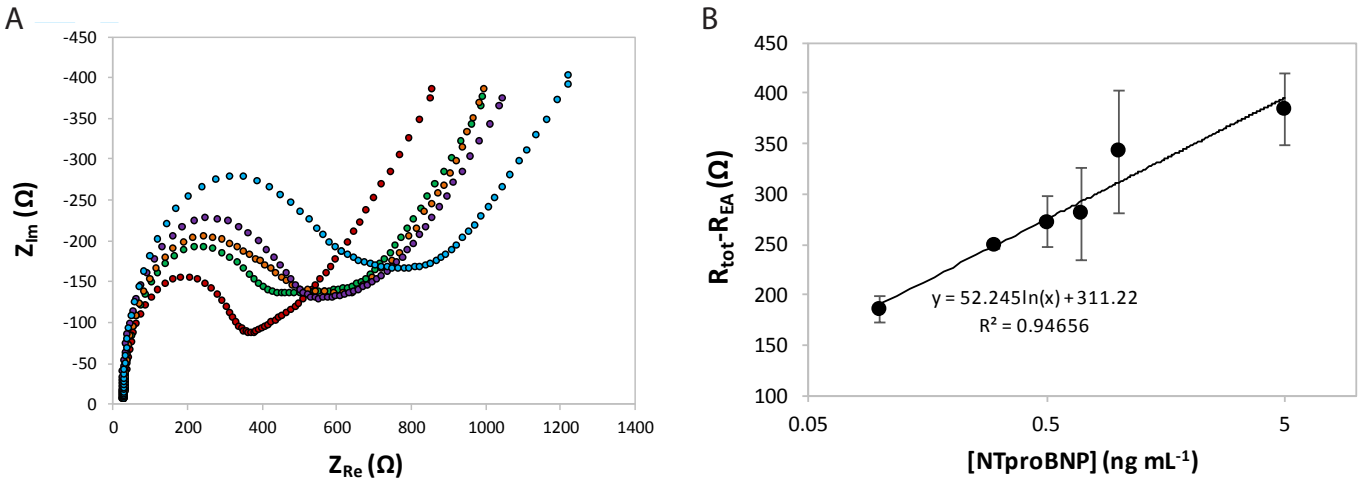


Figure 4. Impendence spectra (A) that correspond to the SPGE after incubating them with increasing concentrations of NT-proBNP in PBS buffer (10 mM, pH 7.4) from 0.1 to 5 ng mL^{-1}. Calibration curve for the NT-proBNP immunosensor (B).

the NT-proBNP concentrations in PBS buffer was observed in a range from 0.1 to 5 ng mL^{-1} with a correlation coefficient (R^2) of 0.94656. In **Table 3**, the change in R_{tot} (ΔR_{tot}) is calculated by subtracting the resistance of the last layer of ethanolamine (R_{EA}) from the corresponding R_{tot} of each NT-proBNP concentration. As can be seen, ΔR_{tot} increased with increasing NT-proBNP concentrations within the detection range. The lowest detection limit of NT-proBNP was 0.1 ng mL^{-1}. This result was determined by the minimum value attained from $(R_{tot} - R_{EA})_{min}$ =176.12 - 14.56 = 161.56 Ω, which was still larger than the 3σ value of R_{EA} (143.27 Ω). The electrochemical immunosensor altogether displayed a well-defined concentration dependence.

Sensor validation with spiked porcine plasma

In order to assess the potential clinical performance of the electrochemical immunosensor, NT-proBNP-spiked porcine plasma was used to mimic physiological fluid in real human samples. The NT-proBNP concentrations were predetermined and measured following the same aforementioned procedure. A drop of 50 µL spiked sample was applied on the SPGE and removed after a 20-min incubation which was followed by washing the electrode surface with DI water (60 mL). The SPGE was then slightly dried around its periphery. The results, as seen in **Fig. 5**, have confirmed the specificity of the biosensor toward NT-proBNP. Even though the plasma effect was conspicuous by the high ΔR_{tot} (316.8 Ω), in a low concentration such as 0.1 ng mL^{-1}, the fabricated sensor was still able to detect the peptide, shown by an increase of ΔR_{tot} (54.97 Ω) with respect to the blank (0 ng mL^{-1}). With subsequent additions of NT-proBNP, despite a marginal increase between 0.1 and 0.7 ng mL^{-1}, the ΔR_{tot} grew afterwards with a saturation at 5 ng mL^{-1}. Overall, the biosensor has demonstrated the capability to quantitate NT-proBNP and differentiate such concentrations in a low concentration range, especially among 0, 0.1, 0.5, 1, and 3 ng mL^{-1}.

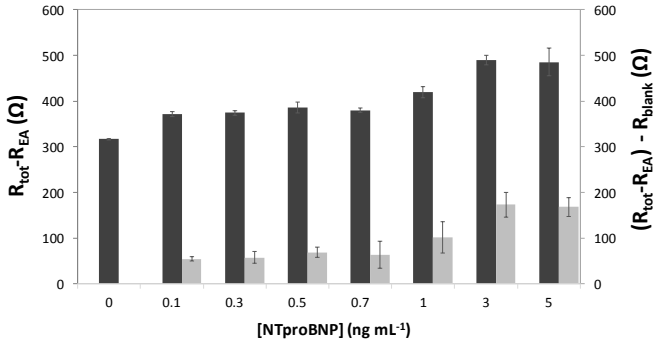


Figure 5. Immunosensor performance was assessed using NT-proBNP-spiked porcine plasma which was then evaluated based on the change of total resistance (ΔR_{tot}). The blank value was elicited from the plasma without any additional of NT-proBNP and it was then used to determine the values of NT-proBNP detection in different concentrations.

Conclusion

A label-free EIS immunosensor for stroke biomarker was developed after appropriate modifications of disposable screen-printed gold electrodes. Composed of deposited gold nanoparticles, the electrode surface provided a good microenvironment for the immobilization of antibody while retaining biological activity for the immunosensor. NT-proBNP, a cardioembolic stroke biomarker, was studied in our impedance immunosensor with both buffer and porcine plasma samples with the idea to develop a sensor that was versatile, sensitive, and specific, enabling a future NT-proBNP point-of-care kit to assess multiform cardiac failures. The detection range of our immunosensor was in accordance with the desired clinical NT-proBNP plasma level to classify cardioembolic stroke and predict the risk of secondary stroke reoccurrence. Therefore, our biosensor has shown the potential as a reliable point-of-care test that is both user-friendly and low-cost.

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