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Application of Bioconjugation Chemistry on Biosensor Fabrication for Detection of TAR-DNA binding Protein 43

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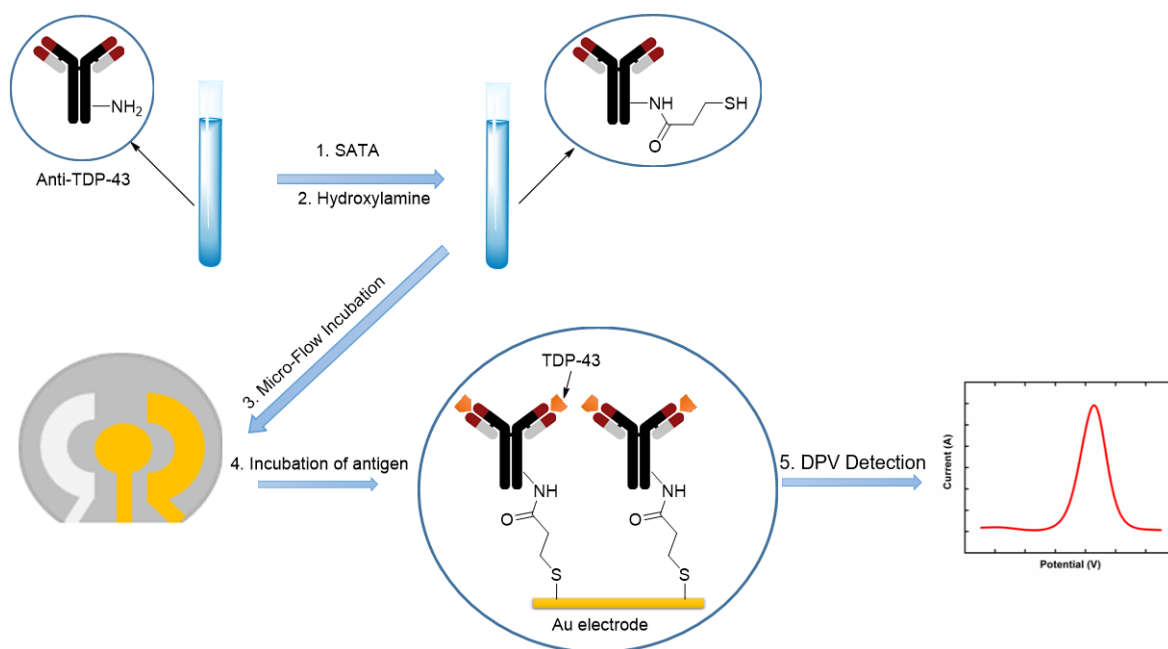
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Abstract:

A simple-prepare, single-use and cost-effective, *in vitro* biosensor for the detection of TAR DNA-binding protein 43 (TDP-43), a biomarker of neuro-degenerative disorders, was designed, manufactured and tested. This study reports the first biosensor application for the detection of TDP-43 using a novel biosensor fabrication methodology. Bioconjugation mechanism was applied by conjugating anti-TDP 43 with N-succinimidyl S-acetylthioacetate (SATA) producing a thiol-linked anti-TDP 43, which was used to directly link with gold electrode surface, minimizing the preparation steps for biosensor fabrication and simplifying the biosensor surface. The effectiveness of this bioconjugation mechanism was evaluated and confirmed by FqRRM12 protein, using nuclear magnetic resonance (NMR). The surface coverage of the electrode was analyzed by Time-of-Flight-Secondary Ion Mass Spectrometry (TOF-SIMS). Differential pulse voltammetry (DPV) was acted as the detection transduction mechanism with the use of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe. Human TDP-43 peptide of 0.0005 $\mu\text{g/mL}$ to 2 $\mu\text{g/mL}$ in undiluted human serum was analyzed using this TDP-43 biosensor. Interference study of the TDP-43 biosensor using β -amyloid 42 protein and T-tau protein confirmed the specificity this TDP-43 biosensor. This bioconjugation chemistry based approach for biosensor fabrication circumvents tedious gold surface modification and functionalization while enabling specific detection of TDP-43 in less than 1hr with a low fabrication cost of a single biosensor less than \$3.



Key Word:

TAR DNA-binding Protein 43 (TDP-43), bioconjugation, electrochemical detection, differential pulse voltammetry

1. Introduction

The population affected by neuro-degenerative diseases has been growing for decades. Just for Alzheimer's disease, the affected number of individuals in the United States is expected to be around 13.2 million by 2050 (Forman et al. 2004). Early detection of neuro-degenerative disorders is pivotal for disease control. The determination of neuro-degenerative disorders can be assisted by monitoring of specific biomarkers, which are recognized highly involving in the neuron central system through the pathological process of neuro-degenerative disorders, such as Tau protein, beta-amyloid 42, TAR DNA binding protein 43, and others. Ubiquitination of misfolded protein in central nervous system is a key character of neuro-degenerative disorders, including Alzheimer's disease, motor neuron disease, frontotemporal dementia and amyotrophic lateral sclerosis (Chen-Plotkin et al. 2010; Forman et al. 2004). In pathophysiological conditions, certain protein transfers out from nucleus and enters into cytoplasm. Neumann et al. found the accumulation and ubiquitination process of a specific protein, named TAR

DNA-binding protein 43 (TDP-43) during the pathophysiological process (Neumann et al. 2006). Furthermore, Neumann et al evaluated the protein components of the ubiquitinated inclusions, and TAR DNA-binding protein 43 (TDP-43) was identified as the main protein constituent found in patients of amyotrophic lateral sclerosis (Kasai et al. 2009; Neumann et al. 2006) (Sreedharan et al. 2008). Consequently, TDP-43 was recognized as a neuropathological hallmark in many neuro-degenerative disorders, including frontotemporal lobar degeneration and Alzheimer's disease. TDP-43 protein was presented in cerebrospinal fluid and human blood (Feneberg et al. 2014) (Goossens et al. 2015) (Irwin et al. 2013). Thus, the detection of TAR DNA-binding protein 43 is pivotal not only for early treatment of neuro-degenerative disorders but also for monitoring therapy effects of TDP-43 related drugs treatment.

Traditionally, mass spectrometric analysis was used for quantitative determination of TDP-43 (Kametani et al. 2016). This type of analysis was expensive, time-consuming and required complicated process. Common biomarker analytical techniques, such as ELISA and Western Blot, were less expensive, but remained to be time-consuming and required complex preparations. Hence, biosensor became a feasible approach for development of detection of TDP-43. Yet, the preparation of common biosensor was a complex process. The process involved the formation of self-assembled monolayers (SAM) for immobilization and functionalization by thiol group at one end and carboxylic group at the other end, crosslinking and activation treatment for binding of antibody (Rushworth et al. 2014) (Love et al. 2005) (Choi et al. 2005) (Dai et al. 2017a). These processes typically required a couple of days. The completeness of sensor coverage and overall sensitivity were always the technical concerns in the common preparation process (Love et al. 2005) (Bonroy et al. 2006), such as pin holes for electrode coverage, the alignment of antibody and others. Therefore, a different and effective preparation process for a simple, cost-effective single-use biosensor fabrication was desirable and applied in this study.

Bioconjugation technique was used for the preparation of this TDP-43 biosensor. Bioconjugation is a chemical strategy forming a stable covalent link between biomolecule and organic molecule. This formation results in a zero length linkage between protein and electrode for this study. This innovative bioconjugation technique was used for the preparation of biosensor with the advantages on shortening the preparation process, enhancing the coverage of sensor surface/minimizing pinhole effect, and improving the practical usage for clinical application. Antibody and antigen interaction is still the crucial mechanism for ensuring the selectivity of the biosensor. N-succinimidyl S-acetylthioacetate (SATA) was typically used in preparation of antibody-enzyme conjugates (Hermanson 2013). In this study, SATA was selected to conjugate monoclonal anti-TAR DNA-binding protein, which formed a SATA-acetylated antibody. Through the reaction between the SATA-acetylated antibody and hydroxylamine, a thiol labeled monoclonal anti-TAR DNA-binding protein was produced and then applied onto gold sensor forming the gold-sulfur (Au-S) bond. Comparing with complex multiple-day preparation process of most biosensors on detection biomarkers, this unique procedure was a single-step process. Also, the total chemical cost from bioconjugation process including fabrication of the sensor prototype is around \$2.10/biosensor, which is lower than that of the SAM (around \$3.03/biosensor), showing the cost-effectiveness of the bioconjugation method fabricated biosensor. This method allowed electrochemical detection of TDP-43 antigen without any extra treatment for signal amplification or cumbersome surface functionalization steps in the fabrication of conventional immunobiosensor. The bonded TDP-43 antigen antibody complex was then quantified using differential pulse voltammetry with a $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox system (Dai et al. 2017b; Koo et al. 2016; Sina et al. 2014). Comparing with commonly used redox system ($[\text{Ru}(\text{NH}_3)_6]^{3+} / [\text{Fe}(\text{CN})_6]^{3-}$) (Das et al. 2012; Das et al. 2015; Kelley et al. 2014), which showed

great sensitivity in DNA detection owing to the ability to electrostatically attract to the negative charged back bone of nucleic acids, $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox system alone was capable of quantifying surface antibody-antigen complex based on electrochemical surface impedance difference. The combination of this bio-conjugation preparation process and the unique thin gold film biosensor with differential pulse voltammetry (DPV) resulted in the production of a single-use, effective, sensitive and cost-effective biosensor for the detection of TDP-43.

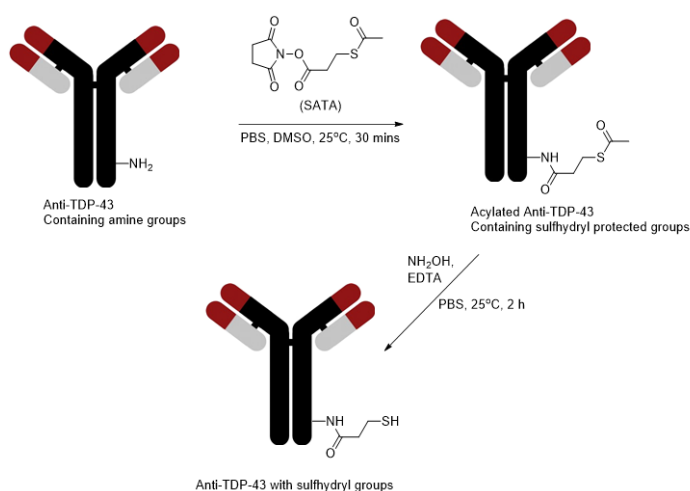
2. Methods

2.1 Materials and Apparatus

Human TDP43 peptide (Cat. ab41970) was purchased from Abcam (Cambridge, MS). Monoclonal Anti-TARDBP antibody produced in mouse (Cat. WH0023435M1), phosphate-buffer saline (PBS) 1.0 M (pH 7.4), undiluted human serum, ethylenediaminetetraacetic acid (EDTA) (Cat. EDS), 11-Mercaptoundecanoic acid (11-MUA) (Cat. 450561), 3-Mercaptopropionic acid (3-MPA) (Cat. M5801), N-(3 dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS), dimethyl sulfoxide (DMSO), Amicon ultra-15 10K and amicon ultra-0.5 10K filters were purchased from Sigma Aldrich (St. Louis, MO). N-succinimidyl S-acetylthioacetate (SATA) (Cat. PI26102), potassium hydroxide pellets, concentrated H_2SO_4 (95.0 to 98.0 w/w %), and concentrated HNO_3 (70% w/w %) were obtained from Fisher Scientific (Pittsburgh, PA.). A CHI 660C Electrochemical Workstation (CH Instrument, Inc., Austin, TX) was used for DPV and EIS investigations. Time-of-Flight Secondary Ion Mass Spectroscopy (TOF-SIMS) was performed with a Physical Electronics TRIFT V nanoTOF TOF-SIMS (Chanhassen, MN). The expression and purification of hnRNP F protein is shown in the Supplementary Information for the evaluation of the reaction mechanism and prove the reaction principle of SATA with protein.

2.2 Synthesis of Thiol-linked Anti-TAR DNA-binding Protein 43 (Anti-TDP-43)

Thiol-linked anti-TAR DNA-binding protein 43 was synthesized for the biosensor fabrication. 0.5mg of SATA was firstly dissolved in 1mL of DMSO. 1 μ L of the prepared SATA solution was mixed with 50 μ L of anti-TAR DNA-binding protein 43 product in 0.1M PBS solution based on a molar ratio between SATA and antibody of 20:1(Hermanson 2013), and incubated together for 30min at room temperature. The solution was then filtered by Amicon ultra-0.5 10k filter. The filtered solution was diluted to 0.5 mL with 0.1M PBS and centrifuged at 12000rpm for 15min at 10°C producing a concentrated modified antibody sample of a total volume of 50 μ L. This filtered antibody solution was stored at 4°C condition and ready for further deacetylation process. The deacetylation process aimed at generation of sulfhydryl group linked protein by the reaction with a prepared deacetylation solution (0.5M hydroxylamine, 25mM EDTA in 0.1M PBS solution with pH at 7.2. EDTA was added during the reaction to prevent crosslink between sulfhydryl groups. 5 μ L of the deacetylation solution was mixed with 50 μ L filtered antibody solution and incubated for 2hrs at room temperature. Amicon ultra-0.5 10k filter was applied again, and the deacetylated antibody solution was diluted to 0.5mL with 10mM EDTA in 0.1 M PBS solution and



centrifuged at 12000rpm for 15min to a volume of 50 μ L. This dilution process was repeated for 3 times to remove excessive reagents. Thiol-linked anti-TAR DNA-binding protein 43 was produced through this process. The produced thiol-linked antibody can be stored at -20°C to maintain the bio-reactivity of the antibody for further usage of TDP-43 biosensor fabrication. Figure1 shows the synthesis process described above.

Figure1. Free primary amine groups on Anti-TDP-43 react with SATA to generate acylated Anti-TDP-43 containing thioester groups. After treatment with hydroxylamine, thioester groups hydrolyzed to sulfhydryl groups.

Quantitatively, the concentration of the produced thiol-linked protein was determined by the absorptivity at 280 nm by ultraviolet light. As shown in table1, the concentration of the produced thiol-linked antibody was at 0.12mg/mL.

Table 1. Concentration profile of the thiol-linked Anti-TDP43.

	Volume (μL)	Abs. at 280nm	Conc. (mg/mL)
Antibody as purchased	20	0.221	0.5
Antibody in PBS buffer before reaction	50	0.083	0.19
Antibody with –SH group	50	0.052	0.12

2.3 Preparation of TAR DNA-binding Protein 43 (TDP-43) Biosensor Using a Micro-Flow Incubation System

TDP-43 biosensor was fabricated based on a three-electrode sensor prototype with thin gold film working and counter electrodes and Ag/AgCl reference electrode (Dai and chiu Liu 2017; Dai et al. 2017a, b). The thin gold film was deposited by sputtering technique with a thickness of 50nm and the Ag/AgCl reference electrode was produced by thick-film printing technology. The gold sensor prototype was manufactured using a roll-to-roll process, ensuring the production of biosensor cost-effective. The manufacture cost for 100 sensors was approximately \$120. The detail configuration of the thin gold film sensor prototype and its electrochemical characterization of the sensor are given in the Supplementary Information. A chemical cleaning procedure was applied to remove any oxides and particles on the biosensor surface decreasing the electrode charge transfer resistance. Typically, a row of 8-10 biosensors were immersed individually in 2M KOH solution, 0.05M H_2SO_4 solution (95.0 to 98.0 w/w %), and 0.05M HNO_3 solution (70 w/w%) in sequence for 5min each. The row of biosensors was rinsed by DI water between each cleaning solution. After cleaning, nitrogen air was used for drying the biosensor. The effectiveness of this cleaning process was demonstrated in a previous study (Dai et al. 2017b). Prepared thiol-linked anti-TDP-43 (Anti-TARDBP43) solution was then diluted by 0.1M PBS buffer with 10mM EDTA and 0.15 M NaCl to a concentration of 0.25 $\mu\text{g}/\text{mL}$. A micro-flow incubation system was applied for incubation of thiol-linked antibody solution. The configuration of the micro-flow incubation system is shown in the Supplementary Information. Continuous flow incubation process can maximize the surface coverage of protein and enhance the homogeneity and reproducibility of the incubation results comparing with static dropping incubation. The continuous flow system was made with stainless steel and designed to accommodate 10 biosensors inside the flow system. Also, protein is typically sensitive to temperature (Somero 1995). Thus, an investigation of the relationship between incubation temperature of antibody and the interaction intensity of antigen-antibody is shown in the Supplementary Information. An ideal incubation time was identified at 4°C. The flow rate was set at 80 $\mu\text{L}/\text{min}$ with a retention time for 3 hours at 4°C. After incubation, the biosensors were rinsed with 0.1M PBS and dried with nitrogen gas and stored at 4°C.

2.4 Electrochemical Spectroscopy Impedance (EIS)

The EIS was applied at a frequency range of 0.1-10000Hz with an amplitude of 0.01V. After biosensor incubated by samples and cleaned by DI water and dried by nitrogen gas, EIS test was then applied using

20 μ L of a redox solution with 5mM in each component of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ to measure the resistivity on the gold sensor surface.

2.5 Time-of-Flight-Secondary Ion Mass Spectrometry (TOF-SIMS)

TOF-SIMS was performed under negative polarity to use the better sensitivity of the instrument to Au, S and their fragments. Element maps for Au and Au-S demonstrated the distribution with a primary source of both C60 and Ga ions. The images acquired with Ga source were selected to demonstrate the electrode coverage with better spatial resolution. Experimentally, the primary source was a Ga^+ beam accelerated to 30 KV and bunched to a pulse size of 7 ns and an acquisition rate of 8 KHz. At this setting, the surface of the electrode was mapped with a spatial resolution of 500 nm. Map stitching was then used to generate ion maps with a total area of 2×2 mm.

2.6 Differential Pulse Voltammetry

Differential pulse voltammetry was used as the transduction mechanism for TDP-43 detection. Typically, before testing, the antigen incubated biosensors were firstly cleaned by DI water and dried by nitrogen gas. For DPV measurement, 20 μ L of a redox probe solution with 5mM in each component of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ was applied onto the biosensor. A voltage ranges of -0.25 V to +0.35 V was applied for DPV measurement with increment of 0.004V, amplitude of 0.05V, pulse width of 0.05s, sampling width of 0.0167s and pulse period of 0.2s.

3. Results and Discussion

3.1 NMR Analysis of SATA Modified FqRRM12 Protein

In order to examine the efficiency and prove the principle of sulfhydryl modification on protein, a protocol to monitor the extent of modification by NMR spectroscopy was developed using the N-terminal fragment of the hnRNP F protein (hnRNP F quasi-RNA Recognition Motifs 1 and 2, FqRRM12, residues 1-194) as a model system as shown in figure2a. Since Anti-TDP-43 purchased from Sigma Aldrich is IgG type, its molecular weight is about 150 kDa, which is beyond the characterization limit by NMR (Frueh et al. 2013). So we introduced the FqRRM12 protein for NMR analysis in order to prove the principle of the reaction. The ^{15}N - 1H Heteronuclear Single Quantum Coherence (^{15}N - 1H HSQC) spectrum of a ^{15}N -labeled protein reports on the chemical environment of each amide group within a protein, and as such it provides a convenient analytical tool to monitor post-translational modifications (Theillet et al. 2012). Sulfhydryl modification is a classical type of lysine acetylation; therefore, we followed the reaction of FqRRM12 with SATA by comparing HSQC spectra of the unmodified and modified ^{15}N -labeled protein. Figure 2b shows the overlay of the ^{15}N - 1H HSQC spectra of unmodified and modified FqRRM12. Comparison of the spectra reveal that the correlation peaks of Lys72, Lys124 and Lys185 completely disappear in the SATA-modified protein. Interestingly, the signal intensity of Gly133 and Asp74 were also significantly reduced in the ^{15}N - 1H HSQC spectrum as shown in Figure2b. Analysis of the FqRRM12 three-dimensional structure (PDB ID: 2kfy for RRM1 domain; 2kg0 for RRM2 domain) shows that Gly133 and Asp74 are in close proximity to the side chains of Lys171 and Lys72, respectively as shown in figure2a, which might account for the observed perturbations to their correlation peaks. Of note, SATA modification of the epsilon amine groups of lysine residues result in formation of new amide bonds that

should be detected by NMR. Indeed, the ^{15}N - ^1H HSQC spectrum of SATA modified FqRRM12 shows additional correlation peaks relative to the unmodified protein as shown in figure2b.

The second step of the chemical reaction, which reduces the attached SATA to a thiol functional group, was also monitored by recording ^{15}N - ^1H HSQC experiments. Comparison of the spectra of the oxidized and reduced forms of the SATA modified FqRRM12 reveal that the reduction occurs site-specifically since the NMR chemical shifts of the two proteins are essentially identical as shown in figure2c. The absence of additional chemical shift perturbations to FqRRM12 upon reducing SATA is expected given that the site of reaction is more than four bonds away from the amide group.

3.2 Electrochemical Impedance Spectroscopy Evaluation of Modified Gold Surface

Electrochemical impedance spectroscopy (EIS) was conducted to confirm the existence of sulfhydryl modified FqRRM12 by examining the ability of the modified protein to bind with gold electrode surface. The gold binding ability of unmodified protein was also evaluated as a negative control. A relative large concentration of FqRRM12 protein (15 $\mu\text{g}/\text{mL}$) with incubation of 1hour on the gold sensor was used to evaluate the binding effect. Significant impedance difference on sulfhydryl modified protein immobilized sensor was observed comparing with the impedance on the bare electrode using the same redox solution. In figure 2d, the blue line represents the surface impedance from the sulfhydryl modified FqRRM12 produced electrode and the black line represents the surface impedance from a cleaned bare electrode. Unmodified protein immobilized sensor shows minor change of impedance comparing with that of bare electrode, confirming the gold binding ability of sulfhydryl modified protein. Taken together, the NMR and EIS results confirmed that FqRRM12 was site-specifically modified under our experimental conditions.

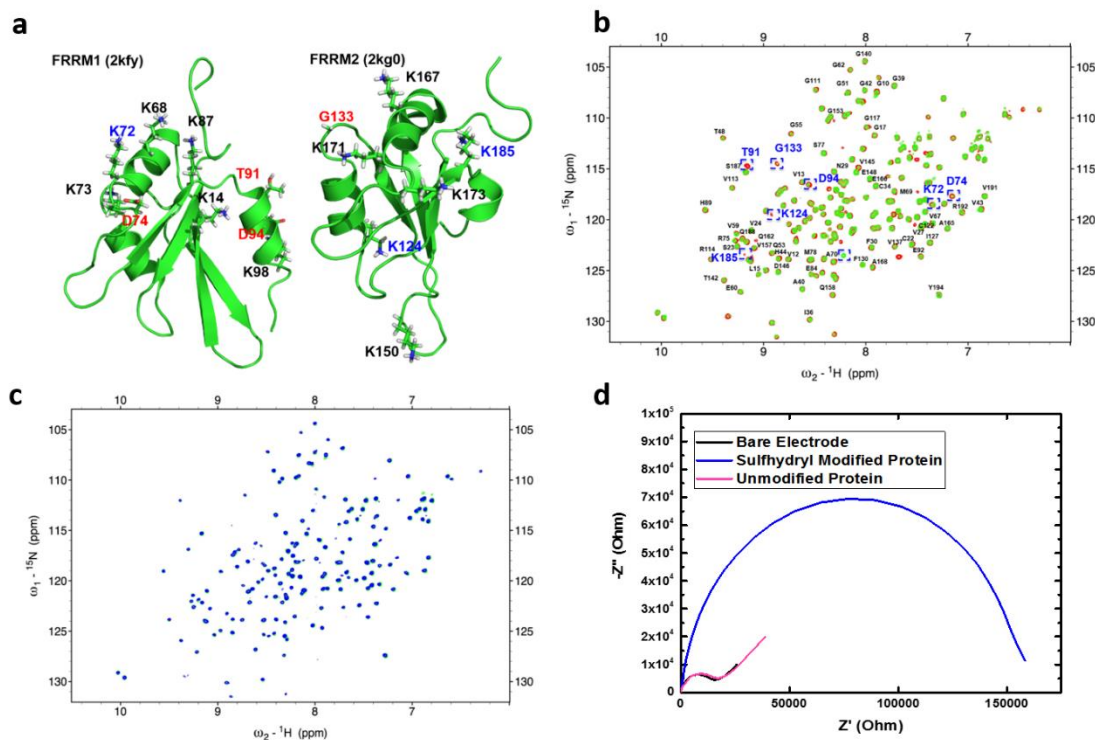


Figure 2. (a) Possible locations of amino acids modified by SATA on the 3D structure of FqRRM12.). (b) Overlay ^1H - ^{15}N HSQC spectrum of FqRRM12 (red) and acetylated FqRRM12 (green). Chemical shift perturbations to D74 and G133 were also detected upon SATA modification, likely due to their close proximity to K171 and K72. (c) Overlay ^1H - ^{15}N HSQC spectrum of SATA-acetylated FqRRM12 (green) and SATA modified FqRRM12 with reduced thiol groups (blue) (d) Nyquist plot presents the impedance differences between cleaned bare electrode, unmodified protein bonded electrode and sulfhydryl modified protein bonded electrode.

3.3 Time-of-Flight-Secondary Ion Mass Spectrometry (TOF-SIMS) Analysis of Thiol-linked Antibody Coverage

In order to confirm the effectiveness of the SATA and protein reaction for the practice of biosensor fabrication, the validity of gold sulfur (Au-S) bond and the coverage of the working electrode surface were investigated by TOF-SIMS technique. The working electrode with linked anti-TAR DNA binding protein 43 (anti-TDP43) were used for analysis. Two biosensors prepared separately by thiol linked anti-TDP43 and traditional 11-Mercaptoundecanoic acid (11-MUA) formed monolayer with cross-linked anti-TDP43 were analyzed and the electrode coverage of each sample was compared. The confirmation of Au-S bond on the gold electrode surface indicated the successful synthesis of thiol-linked protein as shown in figure 3a. Figure 3b shows the coverage of Au-S bond of the electrode using 11-MUA prepared monolayer with cross-linked antibody. The difference in coverage between the two samples was apparent. The counts percentage of Au-S bond based on total counts in the developed thiol-linked anti-TDP43 electrode was 63.3% higher comparing to that of the 11-MUA linked antibody covered electrode. The results of TOF-SIMS analysis proved the effectiveness of bioconjugation mechanism's ability on covering electrode surface, ensuring the reproducibility of further antigen incubation and detection.

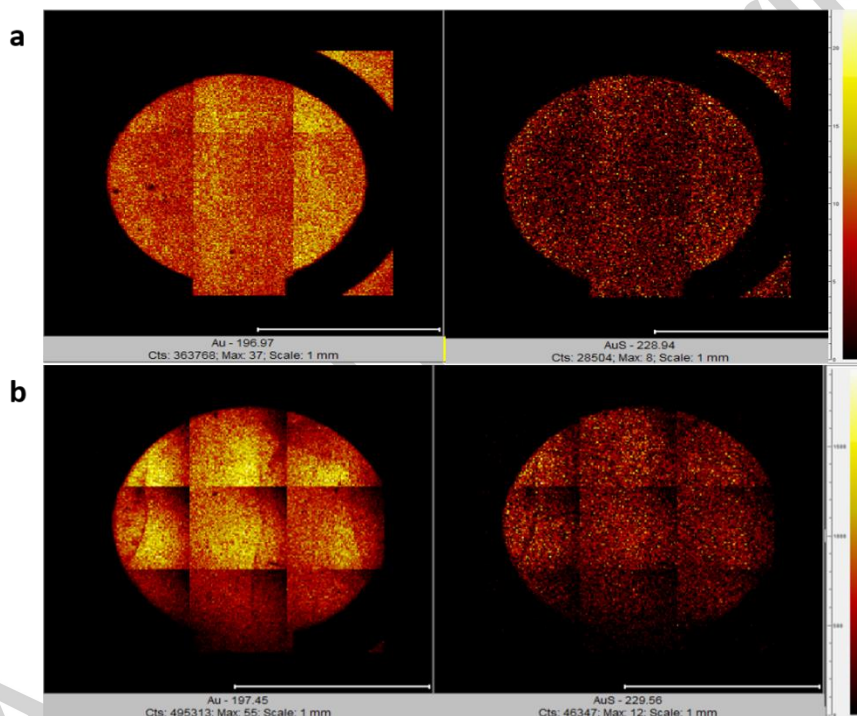


Figure 3. (a) The total secondary ions acquired at the negative polarity of gold (left) and gold-sulfur ion image (right) using a Ga^+ primary source for thiol-linked Anti-TDP43 covered electrode. **(b)** The total secondary ions acquired at the negative polarity of gold (left) and gold-sulfur ion image (right) using a Ga^+ primary source for 11-MUA linked Anti-TDP43 formed monolayer.

3.4 Detection of TAR DNA-binding Protein 43 (TDP-43) in Phosphate Buffer Saline (PBS)

Differential pulse voltammetry (DPV) was the transduction mechanism for the detection of TDP-43 in this study. Compared with common electrochemical voltammetry, such as cyclic voltammetry, differential pulse voltammetry applies pulse potential following with a potential drop and an immediate measurement of current outputs, in which the charge current is minimized and the sensitivity of the measurement is enhanced. Owing to its high sensitivity, DPV has been generously applied to different electrochemical detection systems (Dai et al. 2017a; Das et al. 2012; Das et al. 2015; Koo et al. 2016; Koo et al. 2015; Lapierre et al. 2003; Sina et al. 2014; Wan et al. 2014; Yang et al. 2009).

TDP-43 antigen samples prepared in PBS solution was firstly used for proving the validity of bioconjugation mechanism prepared biosensor. The detection sensitivity between conventional 3-MPA and 11-MUA monolayer prepared TDP-43 biosensors and bioconjugation mechanism prepared TDP-43 biosensor were compared and assessed. The preparation process of 3-MPA and 11-MUA based TDP-43 biosensors were described in the Supplementary Information and a total time of approximately 51 hours were required to prepare those self-assemble monolayer based TDP-43 biosensors, which were significantly longer than the preparation time of the bioconjugation mechanism based TDP-43 biosensor (3 hours for bioconjugation of antibody plus 3 hours for biosensor fabrication). In order to compare the sensitivity of these three types of TDP-43 biosensors, human recombinant TDP-43 antigen was diluted by 0.1M PBS solution to multiple concentrations ranging from 1 $\mu\text{g/mL}$ to 0.01 $\mu\text{g/mL}$. The 20 μL of prepared antigen solution was then applied onto both the bioconjugation prepared biosensors and the 3-MPA and 11-MUA based biosensors. The incubation time of TDP-43 antigen was 1 hour at room temperature. After incubation, each biosensor was rinsed by 1mL 0.1M PBS solution and dried by nitrogen. DPV was then applied to measure the conductivity on the biosensor surface; different current outputs were due to the impedance difference on the biosensor surface, which was provided by the various amount of incubated TDP-43 antigen. Therefore, the current outputs were used to quantify different concentrations of TDP-43 antigen. Figure 4a shows the DPV measurements for the comparison of the sensitivity of the three different types of biosensors based on PBS prepared antigen. 3-MPA prepared biosensor showed a higher current outputs comparing with 11-MUA based biosensors, because the 3-MPA system processes a shorter chain length providing less impedance on the biosensor surface. However, the bioconjugation method prepared TDP-43 biosensor showed relatively high current density outputs comparing with that of either the 3-MPA or 11-MUA prepared TDP-43 biosensor based on the same detection conditions, indicating that a higher sensitivity was achieved by the bioconjugation method prepared TDP-43 biosensor over conventional SAM type biosensors.

Also, the current density gradients between different concentrations of TDP-43 antigen were apparently larger from the results of bioconjugation based TDP-43 biosensor comparing with those of the 3-MPA and 11-MUA prepared TDP-43 biosensor. Hereby, we defined the value of the change of current density divided by concentration gradients ($\Delta i/\Delta C$) as the resolution of the biosensor. This term was used to quantitatively compare the ability of each biosensor on differentiating different concentrations. High resolution means the biosensor has less possibility to produce error signal under the detection concentration range. Derived from the DPV results shown in figure 4a, the bioconjugation method prepared biosensor showed the highest $\Delta i/\Delta C$ of 35.35 $\text{A}\cdot\text{mL}/(\text{m}^2\cdot\mu\text{g})$. However, 3-MPA and 11-MUA system prepared biosensor showed a relatively lower value of $\Delta i/\Delta C$ of 6.06 $\text{A}\cdot\text{mL}/(\text{m}^2\cdot\mu\text{g})$ and 6.25 $\text{A}\cdot\text{mL}/(\text{m}^2\cdot\mu\text{g})$, indicating a higher resolution achieved by bioconjugation method prepared biosensor. Moreover, bioconjugation chemistry produces the direct link of antibody and gold electrode surface, so no other chemicals (such as unreacted/unoccupied ester groups from activated 3-MPA/11-MUA system)

besides TDP-43 antibody can react with antigen ensuring the surface linked antibody is the only binding sites available for TDP-43 antigen to interact with. This simplified surface minimizes the possibility of non-specific binding resulting in a high-sensitivity and high-distinguish detection of biomolecules.

Electrochemical impedance spectroscopy was also conducted to measure the surface impedance in order to verify the principle of the detection results from DPV for PBS test. The EIS measurement was conducted with the same procedure as described above for the DPV measurement with the usage of a redox probe coupling. As shown in figure4b, dashed line shows the impedance of bare electrode and antibody coated electrode; solid lines show the impedance of different TDP-43 antigen concentration. Highest concentration of TDP-43 antigen indicates the highest impedance (biggest circle) on the sensor surface at low frequency region, which was consistent with the lowest current output through the DPV measurement.

3.5 Detection of TDP-43 in Undiluted Human Serum with Interference Study

Human recombinant TDP-43 antigen was also diluted by undiluted human serum to multiple concentrations, ranging from 0.0005 μ g/mL to 2 μ g/mL. The procedure for TDP-43 antigen in PBS measurement was also used in the serum test for DPV measurement. The calibration curve for the DPV measurement is shown in figure4c with a linear relationship of $Y = -9.607X + 4.662$ with a R-square value of 0.986 and RSD of 3.56%, indicating a high reproducibility with $n = 5$. Comparing with TDP-43 protein in PBS measurement, the measurement of TDP-43 in undiluted human serum showed decreased current peak for the same concentration range (1 μ g/mL to 0.01 μ g/mL), which may due to matrix effect caused by complex composition of undiluted human serum. Also, the biomolecules contained in undiluted human serum may form unspecific binding onto the gold surface, so the impedance on the surface would increase, causing a decreasing of the current output. This unspecific absorption is caused by the components in the solution medium (undiluted human serum) of the antigen solution, and these components affected identically on all the measurements in undiluted human serum as shown in figure4d comparing with figure4a. This kind of interference on detection signal caused by components other than the specific analyte is evaluated as matrix effect (Dams et al. 2003; Matuszewski et al. 2003). The relative matrix factor based on undiluted human serum over PBS on the same concentration range was around 0.43.

From the serum detection test, the limitation of detection of TDP-43 antigen was found at 0.0005 μ g/mL (pink line) in figure4d, which aligned closely to the zero concentration line (dark green line). The saturation limit of detection was found at 1 μ g/mL (light blue line) in figure4d, which overlaid with the 2 μ g/mL (orange line). In order to assess the specificity of this developed TDP-43 biosensor, β -amyloid 42 antigen and T-tau protein were selected for interference tests of this biosensor. 5 μ g/mL of β -amyloid 42 antigen solution in human serum and 1 μ g/mL of T-tau protein in human serum were incubated onto the TDP-43 biosensors for 1 hour at room temperature. After incubation, the biosensors were cleaned by DI water and nitrogen gas, and tested using the redox coupling as described in the previous DPV test. The current outputs based on the detection procedure are shown as the orange color line and dark blue line in figure4d, which overlapped with the detection response of non-TDP-43 antigen solution. Another interference test was conducted by a mixed solution of 5 μ g/mL of β -amyloid 42, 1 μ g/mL of T-tau protein with 0.1 μ g/mL of TDP-43 antigen. The mixed solution was incubated on the TDP-43 biosensor for one hour. The biosensors were then cleaned and tested as described in the previous DPV test. The current output of DPV measurement of this mixed solution was identical to the current output of DPV

measurement of only 0.1 $\mu\text{g/mL}$ of TDP-43 antigen as the green line signal in figure 4d (The DPV test graph is shown in the Supplementary Information Figure6). These two tests also proved that the non-specific absorption was resulted from the components in the undiluted human serum and also demonstrated the high specificity of the simple bioconjugation prepared TDP-43 biosensor in isolating TDP-43 antigen targets for electrochemical detection.

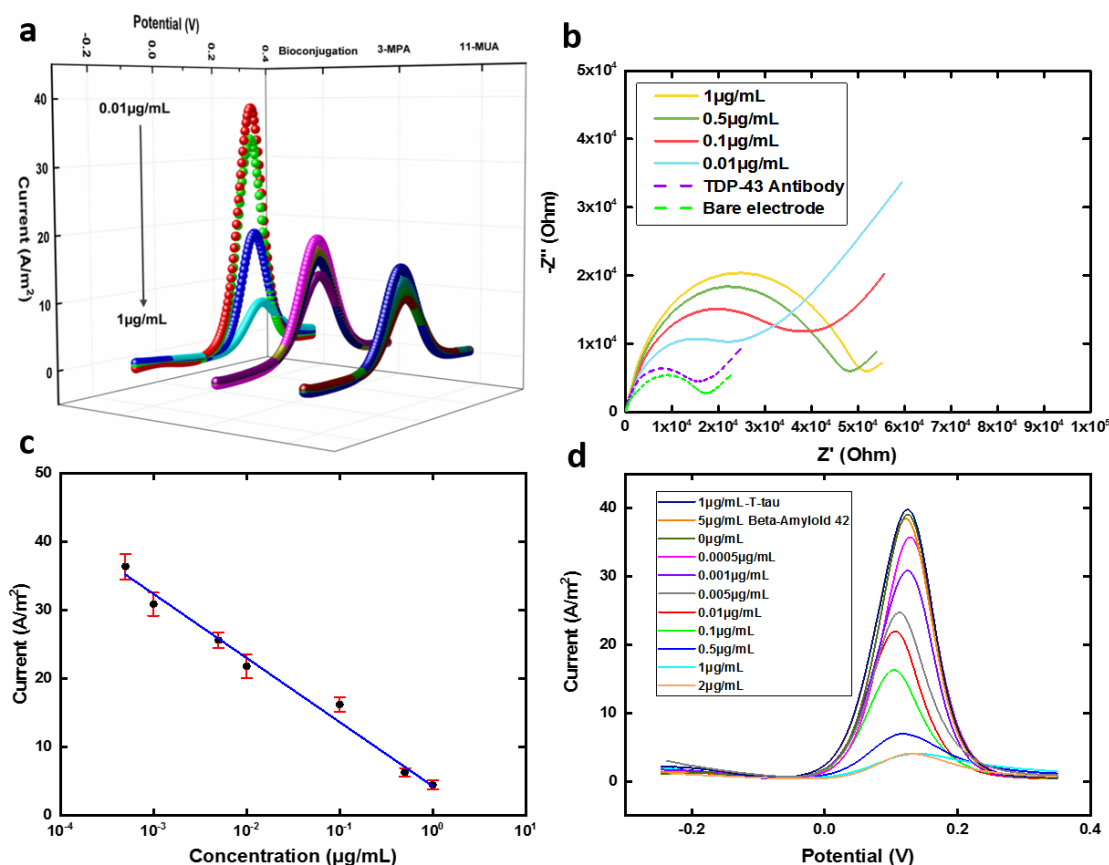


Figure 4. (a) Sensitivity comparison of 11-MUA & 3-MPA based TDP-43 biosensors and bioconjugation method based TDP-43 biosensor using DPV measurement of TDP-43 antigen in 0.1M PBS solution. (b) Nyquist Plot of TDP-43 antigen in 0.1M PBS solution. (c) Calibration linear curve based on DPV measurement of TDP-43 antigen in undiluted human serum. (d) DPV measurement of TDP-43 antigen in undiluted human serum with limitation and interference tests.

4. Conclusions

This work demonstrates a novel, simple, rapid, cost-effective bioconjugation approach for biosensor fabrication. We hereby developed the first biosensor for detection of TAR DNA-binding protein 43 (TDP-43) in undiluted human serum. Selectivity was confirmed by using β -amyloid 42 antigen and T-tau protein as potential interference biomolecules, and no interference by these compounds were observed. The bioconjugation technique used in this study demonstrated a time-efficient, simple method for fabrication of antibody based biosensor with a preparation time of 3hrs and a detection time around 1hr. The bioconjugation method for biosensor fabrication is highly versatile, as the bioconjugation molecule (such as N-succinimidyl-S-acetylthiopropionate, N-succinimidyl S-acetylthioacetate) can be varied based on different amino acid conjugation sites, which will be discussed in the future study. The versatility of this fabrication method displays promising potential on developing a universal sensing platform for various biomarkers detection.

Conflicts of interest

There are no conflicts to declare.

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Author Contributions

CCL, YY, GS, BT, YD conceived and designed the experiments. YD, CW, LYC, and KA conducted the experiments. YD, LYC, KA and CW analyzed data. YD, CW, LYC, KA, BT, GS, YY, CCL prepared this manuscript.

Highlights

- Single-use, cost-effective biosensor for the detection of TAR DNA binding protein-43, a biomarker of Neuromuscular Disorders.
- Bioconjugation chemistry based method provides a simple, rapid preparation process for biosensor fabrication.
- NMR, electrochemical methods and surface analysis (TOF-SIMS) characterization of the bioconjugation reaction between N-succinimidyl S-acetylthioacetate (SATA) and antibody.
- Differential pulse voltammetry was used to quantify the biomolecules.