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ACS Sens., **Just Accepted Manuscript** • DOI: 10.1021/acssensors.8b01126 • Publication Date (Web): 24 Dec 2018

Neutral Charged Immunosensor Platform for Protein based Biomarker Analysis with Enhanced Sensitivity

Yifan Dai,†ϕ Liang-yuan Chiu,‡ Yinuo Chen,ϕ Shiyi Qin,ϕ Xiaowei Wu,ϕ Chung Chiun Liu*ϕ†

†Electronics Design Center, ϕ Department of Chemical and Biomolecular Engineering, ‡ Department of Chemistry, Case Western Reserve University, Cleveland, OH, 44106, USA

ABSTRACT: A non-invasive, highly sensitive universal immunosensor platform for protein based biomarker detection is described in this report. A neutral charged sensing environment is constructed by an antibody with an oppositely charged amino acid as surface charge neutralizer. By adjusting the pH condition of the testing environment, this neutral charged immunosensor (NCI) directly utilizes the electrostatic charges of the analyte for quantification of circulating protein markers, achieving a wide dynamic range covering through the whole pico-mole level. Comparing with previous studies on electrostatic charges characterization, this NCI demonstrates its capability on analyze not only the negatively charged biomolecules but also positively charged analytes. We applied this NCI for the detection of HE4 antigen with a detection limit at 2.5 pM and Tau antigen with a detection limit at 0.968 pM, demonstrating the high-sensitivity property of this platform. Furthermore, this NCI possesses a simple fabrication method (less than 2hrs) and a short testing turnaround time (less than 30min), providing an excellent potential for further clinical point-of-care applications. **KEYWORDS**: *universal antigen detection method, electrochemistry, electrostatic charge interaction, universal biosensor, point-of -care*

The development of single-use biosensors for biomarkers detection is critical to effective medical care. Screening of circulating protein markers has been widely recognized as a valid non-invasive approach for medical diagnosis[1-3]. Instead of traditional sophisticated medical screening methods, such as Western blot, mass spectroscopy, electrochemical platform has been evaluated as a promising, rapid method for protein based biomarkers detection[4-7]. However, most of the electrochemical platforms require complex fabricated sensing substrate or extra enzymatic tags in order to achieve high-sensitivity performance. Therefore, these methods are not suitable for routine clinical care, which requires not only high detection sensitivity but also a simple immunosensor fabrication method and fast turnaround time[8]. Thus, the development of a simple, cost-effective, valid and universal method for single-use biomarker diagnostic tools remains elusive and demanding. Herein, we report a novel neutral charged detection system, utilizing the electrostatic charge of the analyte itself to achieve pico-molar level sensing of protein based biomarkers within hours.

Antibody based immunosensor fabrication methods are recognized as the most specific way for analyte recognition[5, 9-10]. Typically, self-assemble monolayer (SAM) on noble metal for antibody immobilization is commonly used for immunosensor fabrication[11-12]. However, the complex and time-consuming process for the formation and activation of SAM and further crosslinking with an antibody make this method undesirable for single-use biosensor fabrication for routine clinical usage. Also, the inhomogeneous surface layer of SAM typically causes nonspecific binding and alignment issues[13-14], which are not ideal for measurement of electrostatic charge interaction. Herein, we apply a bioconjugation method for biosensor fabrication, which provides an external thiol linker onto the biomolecules needed for immobilization[7, 15-16]. The external thiol linked biomolecules are directly incubated onto the electrode surface without any surface treatment, providing a highly-aligned and exposed biomolecule layer suitable for electrostatic charge interaction on the electrode surface. Moreover, this method provides a simple immunosensor fabrication method suitable for rapid clinical requirement.

In order to achieve a highly sensitive detection of an analyte, we designed a neutral charged immunosensor (NCI) in order to fully utilize the electrostatic charge on the analyte for the quantification of biomarkers. Previous studies demonstrated the effective utilization of electrostatic interaction for high-sensitivity electrochemical immunosensor by the application of external charge-labeled materials, such as charged gold nanomaterials[17,18], DNA decorated gold nanoparticles[19]. The construction of such sensing platforms required external introduction of charged species to achieve electrostatic interaction with selected redox systems. However, instead of introducing labeled-charged materials, we directly utilized the charged analyte as an interaction target based on a comprehensive understanding of its electrostatic property. Electrostatic charge of an analyte is determined by its isoelectric point (pI) and the pH value of the environment (the testing buffer). Therefore, it is pivotal to comprehend the electrostatic charge of the analyte for general immunosensor application. In order to electrochemically quantify the electrostatic charge, a redox

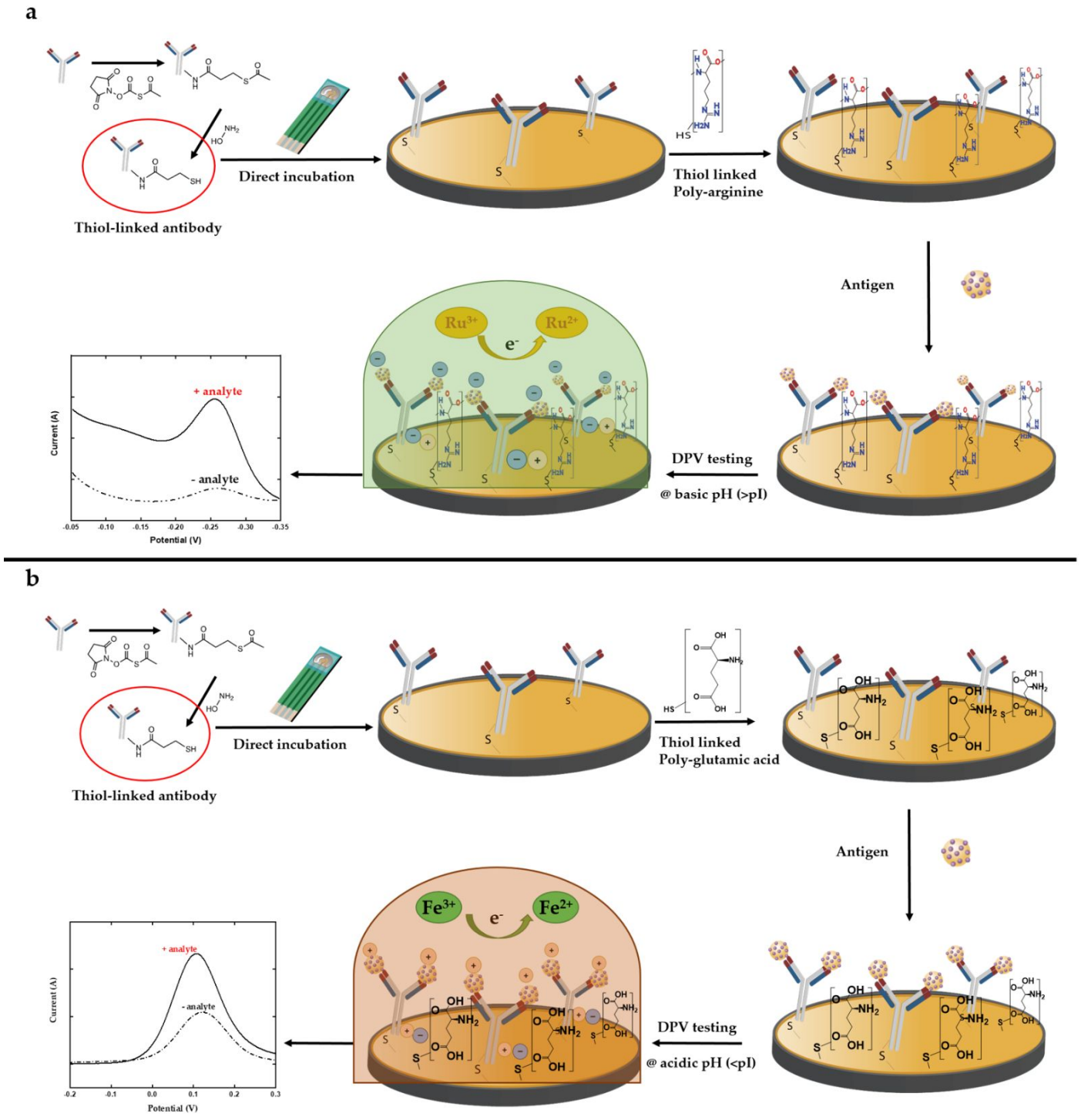

Figure 1.a) Formation process of the neutral charged immunosensor (NCI) for acidic pI analyte. Bioconjugation reaction through N-succinimidyl S-acetylthioacetate (SATA) to provide an external thiol linked antibody; thiol-linked antibody was directly incubated onto the gold electrode; thiol-linked poly-arginine was further immobilized to neutralize any electrostatic charge through formation of hydrogen bonding; this fabricated NCI platform was treated with biological samples containing target analyte; Differential pulse voltammetry (DPV) was performed with the presence of $[Ru(NH_3)_6]^{2+/3+}$ at basic pH condition to fully excite the negative electrostatic charge from the target analyte; DPV measurement demonstrated the difference between +analyte and –analyte. b) Formation process of the NCI for basic pI analyte. SATA conjugated antibody under through direct incubation onto the gold electrode; thiol-linked poly-glutamic acid was further immobilized to neutralize positive electrostatic charge through formation of hydrogen bonding; NCI was further treated with biological samples containing target analyte; DPV was performed with the presence of $[Fe(CN)_6]^{3-/4-}$ at acidic pH condition to fully excite the positive electrostatic charge from the analyte; DPV measurement demonstrated the difference between +analyte and –analyte.

coupling solution, such as $[Ru(NH_3)_6]^{2+/3+}$, is typically applied to interact with the analyte[20-24]. However, when encountering with positive charged analyte, repulsion of the cationic probe in the liquid-electrode interface leads to an increase of diffusion length and a decrease in sensitivity[17, 25]. Therefore, in this study, based on the charge condition of the analyte, we present the proper applications for both cationic and anodic probes for recruiting its corresponding charged substance. Also, for accurate quantification of electrostatic charge, any charged substance (other than the analyte) on the electrode surface needs to be neutralized in order to provide a neutral environment before the introduction of the analyte. For further directing the charge of the analyte, the buffer pH value of the redox coupling solution is altered. Therefore, there are two circumstances of the electrostatic charge based on electrochemical detection using a redox probe solution. First, for an analyte with an acidic pI value (< pH=7), a testing buffer with a pH over 7 is used to promote a negatively charged analyte. Second, for an analyte with a basic pI value (>pH=7), a testing buffer with a pH below 7 is used to promote a positively charged analyte. Most of the previous studies were limited to investigate the electrostatic performance only based on negatively charged substances[26-29]. We aimed to provide a comprehensive and simple approach to meet the detection demands for all different analytes (with acidic/basic pI) by using electrostatic charges interaction. Consequently, with the purpose of detection of a wide range of analytes, we developed this NCI for both described conditions as a universal immunosensor platform.

RESULTS AND DISCUSSION

A thin gold film based sensor fabricated by chemical vapor deposition was used as the immunosensor developing platform. This gold sensor prototype was produced on an industrial scale; therefore, it is ideal for single-use immunosensor development. The detail description of this sensor configuration and characterization of the stability and reproducibility of this sensor prototype were reported in previous studies[5, 7]. In order to immobilize the antibody onto the gold sensor surface, N-succinimidyl S-acetylthioacetate (SATA) was applied to conjugate the lysine side chain, producing an external thiol linker that reacted with the gold electrode surface. The characterization of SATA based bioconjugation method is shown in a previous study[7].

NCI Construction for Analyte with Acidic pI. For an analyte with an acidic pI value, the principle that underlies the NCI is shown in Figure 1a. The bioconjugated antibody was immobilized on the gold electrode surface through the gold-thiol reaction, forming a single layer of antibody. Therefore, prior to the introduction of antigen, the charge condition of the immunosensor surface was only determined by the antibody itself. After incubation of antigen onto the immunosensor, the surface charge was then controlled by both the electrostatic charges of the antibody and the antigen. Consequently, for conventional electrochemical sensor development, tedious study for a balanced antibody concentration was always required in order to minimize the effects of electrostatic charge interaction within a certain electrochemical redox system[5, 30-31]. Therefore, in order to utilize the electrostatic charge for quantification of the biomarker, the background signal from the antibody needs to be reduced or even neutralized to prevent the possibility that the background signal is dominant comparing with that of analyte[20], because the degrees of charges of antibody and antigen are different. In order to resolve this research difficulty, we utilized a naturally highly-charged amino acids[32], poly-arginine as the surface charge neutralizer for negatively charged antibody under the pH condition of the electrochemical testing environment. Arginine possessed the highest isoelectric point (pI) among amino acids[33]. It maintained a positive charge for pH under 10.76[34]. After immobilization of poly-arginine, the electrostatic charge from antibody on the immunosensor surface was then neutralized. The whole surface charge condition was now only determined by the electrostatic property of the analyte, and therefore enhancing the detection accuracy and sensitivity. The whole construction time of this NCI was approximately 1 hr, agreeing with the requirement for quick turnaround time for clinical routine applications.

An electrochemical redox system based on the cationic probe, $[Ru(NH_3)_6]^{2+/3+}$ was applied as the indicator of the intensity of negative charges on the immunosensor surface. $[Ru(NH_3)_6]^{2+/3+}$ was commonly used for electrochemical signal readout for nucleic acids[8, 35] owing to its ability on corporation with negative charged nucleic acids backbone. For the detection of negatively charged analyte, we used the electrostatic interaction between $[Ru(NH_3)_6]^{2+/3+}$ and negatively charged amino acids (Asp & Glu). The cationic probe was attracted into the antigen binding layer instead of being repulsed to the outer layer of the liquid interface. Therefore, the diffusion distance of the electron was decreased, providing a higher degree of electron accessibility of the Au electrode and leading to a higher sensitivity[25]. In order to fully utilize the negative charges from the amino acids, a basic buffer (pH=9.6), carbonate bicarbonate, while maintaining the positive charges of poly-arginine, was used as the composition for the redox solution. Differential pulse voltammetry (DPV) was used as high-sensitivity electrochemical transduction mechanism[5, 36].

NCI Construction for Analyte with Basic pI. For an analyte with a basic pI value, the principle of this NCI is shown in Figure 1b. An analyte with a basic pI value possesses positive charges under physiological condition. Therefore, in order to interact with positively charged targets, a negatively charged redox system, $[Fe(CN)_6]^{3-/4-}$, was applied. In this case, the anodic probe was attracted into the positively charged antigen layer, decreasing the electron diffusion distance and increasing the sensitivity. To further fully utilize the positively charged amino acids, the anodic probe was prepared in an acidic buffer, Bis-Tris

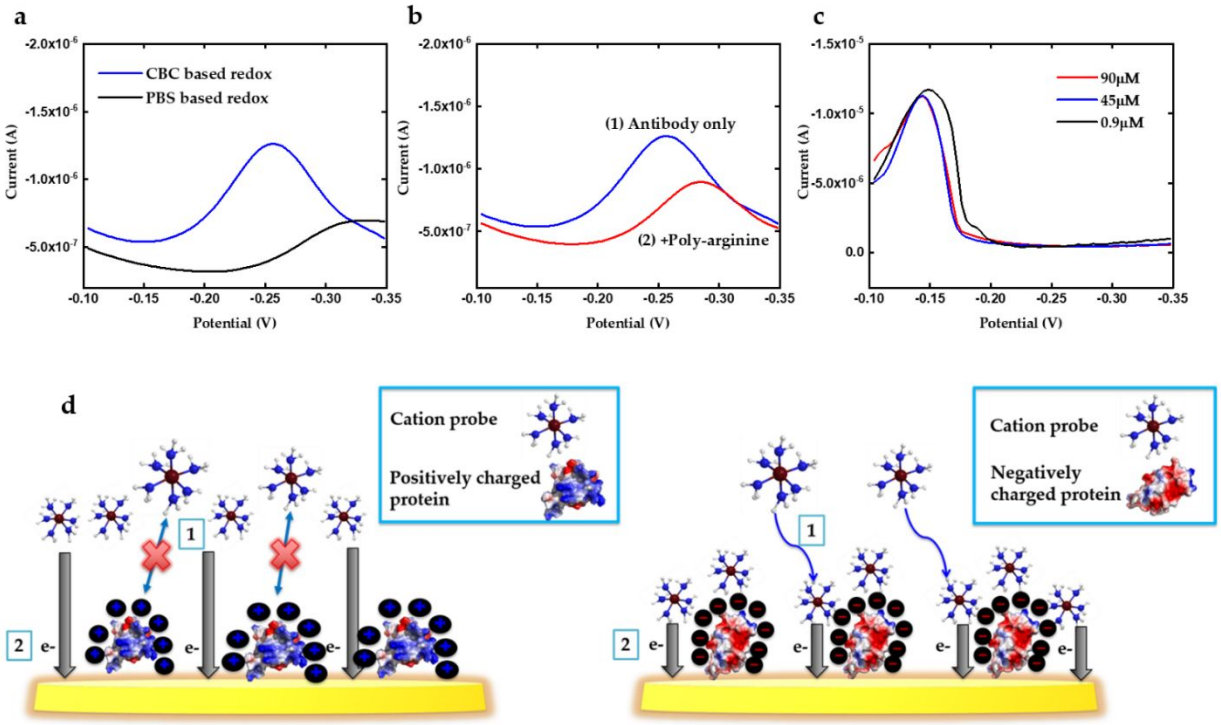

Figure 2. a) Comparison of DPV measurements of electrostatic interactions of HE4 antibody with $[Ru(NH_3)_6]^{2+/3+}$ in CBC buffer (pH=9.6) and in PBS buffer (pH=7.4). b) DPV measurement by $[Ru(NH_3)_6]^{2+/3+}$ in CBC buffer (pH=9.6) on HE4 antibody with the addition of poly-arginine as negative charge neutralizer. c) DPV measurements by $[Ru(NH_3)_6]^{2+/3+}$ in CBC buffer (pH=9.6) on various concentrations of poly-arginine alone. d) Proposed cation probe interaction models for positively charged protein and negatively charged protein.

buffer (pH=6.2). After immobilization of the antibody, under acidic pH condition, the antibody was partially positively charged. Therefore, in order to minimize the background signal, negatively charged poly-glutamic acid was applied as positive charges neutralizer owing to the low isoelectric point of glutamic acid (pI=3.08). Once the NCI was constructed, the electrostatic charge readout from DPV would only be determined by the charge interaction between the positively charged analyte and the anodic probe $[Fe(CN)_6]^{3-/4-}$ under acidic condition. To demonstrate the validity of this NCI platform, we applied this NCI (1) to detect HE4 (WFDC2) protein, a biomarker for ovarian cancer[37], based on the $[Ru(NH_3)_6]^{2+/3+}$ redox interaction with negative charged analyte. We also used this NCI (2) to detect T-tau protein, a biomarker of neuro-degenerative disorders[38], based on the $[Fe(CN)_6]^{3-/4-}$ redox interaction with positive charged analyte. The performance of other non-charged immunosensor platform was also compared with that of NCI, demonstrating the high-sensitivity and low-detection limit advantages of this NCI platform. This study provided a novel perspective on a simple development method for a universal single-use immunosensor platform with highly enhanced sensitivity.

NCI Development for HE4 Detection. First, we demonstrated this NCI for the detection of HE4 antigen, which possessed an acidic pI value. In order to utilize the electrostatic charge for the detection, the charge condition of the antibody while testing, needed to be comprehended. The surface charge of the antibody was derived from the pH difference between its own isoelectric point and the pH value of the testing buffer. Therefore, in order to fully induce the negative charges from the antibody, the influence of basicity of the testing buffer on electrostatic charge was firstly evaluated based on the HE4 antibody. Two different buffers prepared redox coupling, phosphate buffer saline (pH=7.4) and carbonate bicarbonate (pH=9.6), were investigated using DPV in the presence of 50μM of $[Ru(NH_3)_6]^{2+/3+}$. As shown in Figure 2a, using the HE4 antibody layer covered immunosensor, the carbonate bicarbonate dissolved redox coupling resulted in a higher current outputs comparing with that of phosphate buffer saline dissolved redox coupling, indicating that a more basic buffer medium improved the electrostatic charge interaction between the protein and the ruthenium based redox probe. Therefore, CBC buffer based redox coupling solution was chosen for further application in HE4 detection. As shown in Figure 2b, the blue line (1) demonstrates the electrostatic charge signal based on the negatively charged

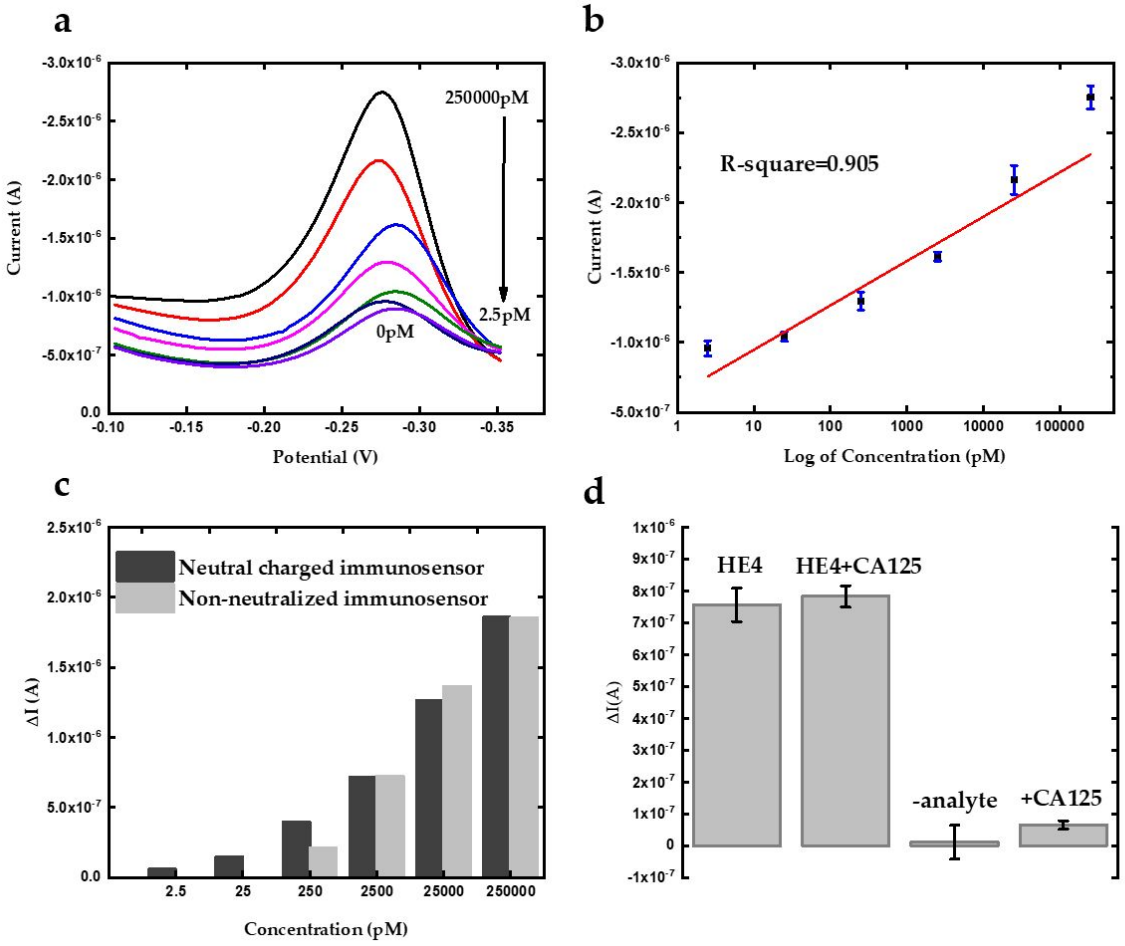

Figure 3. a) DPV measurements of various concentrations (2.5pM-250000pM) of HE4 antigen using HE4 NCI. b) Calibration curve for the DPV measurements of NCI. c)Comparison of detection resolution of NCI with non-neutralized immunosensor in response to various HE4 concentrations (2.5pM-250000pM). d) Interference study using CA125 antigen on the HE4 NCI.

antibody (2.43μM) covered electrode surface. To investigate the charge compensation ability of poly-arginine, we further incubated SATA conjugated poly-arginine onto the HE4 antibody covered electrode, producing a neutral charged surface under testing condition. In order to mediate the charge from antibody, multiple concentrations of thiol linked poly-arginine were tested and shown in Figure S1. The red line (2) in Figure 2b presents the electrostatic charge signal based on negatively charged antibody electrode surface plus the selected concentration (30.4μM) of positively charged poly-arginine. The decrease of current output was caused by that the negative charged amino acids in the antibody, which were interacted with poly-arginine instead of $[Ru(NH_3)_6]^{2+/3+}$, due to the formation of hydrogen bonding between negative charged amino acids and positive charged poly-arginine. Consequently, the reduction process of $[Ru(NH_3)_6]^{3+}$ to $[Ru(NH_3)_6]^{2+}$ was weakened. To verify that electrostatic interaction was the dominant component for the decrease of current outputs, we further evaluated the interaction of $[Ru(NH_3)_6]^{2+/3+}$ with different concentrations of poly-arginine alone on the gold sensor surface. As shown in Figure 2c, various concentrations of poly-arginine did not cause significant decrease of current outputs based on the cation redox reaction, indicating that the cation redox reaction process was not impeded by the addition of poly-arginine. Therefore, we concluded that the decrease of current in Figure 2b was not initiated by the electron transfer hindrance from the addition of poly-arginine. Furthermore, this phenomenon (Figure 2c) can be explained as that the repulsion between cation probe and positively charged biomolecules creates a gap preventing their interactions.

Based on above observations, we proposed an electrostatic interaction model as shown in Figure 2d. After the redox solution is placed on the sensor surface, if the immobilized biomolecules are oppositely charged, the attractive force moves the cation probe closer to the electrode surface, decreasing the electron transfer distance during the redox reaction. Consequently, the repulsion and attraction of cation probe with differently charged biomolecules lead to different electron diffusion distance, further delivering different intensity of current outputs from the redox reaction. With the understanding of the electrostatic interaction, this developed NCI was further applied to investigate the electrostatic detection of HE4 antigen.

For electrostatic quantification of HE4 antigen, the amino acids sequence of HE4 antigen were firstly evaluated and shown in the SI, which was used for calculation of the isoelectric point of the HE4 antigen. The pI for HE4 antigen is 4.50, which is lower than the pH value of CBC buffer, indicating that under the CBC buffer based redox coupling

solution, it possesses strong negative charges. Multiple concentration of HE4 antigen in human serum was incubated on the HE4 NCI for 30 min at room temperature. After this short incubation time, DPV measurements were performed using CBC based $[Ru(NH_3)_6]^{2+/3+}$ redox solution. The incubation time of the $[Ru(NH_3)_6]^{2+/3+}$ redox based CBC solution was also investigated and shown in the Figure S2. A quiet time of 15s was selected, allowing the electrostatic cooperation between $[Ru(NH_3)_6]^{2+/3+}$ and negative charged HE4 antigen. As shown in Figure 3a, the concentrations of HE4 antigen show a positive correlation with the current outputs. A low detection limit of HE4 antigen was observed at 2.5 pM. Comparing with recent report on nano-molar level HE4 detection[39], the results of this study demonstrated a detection limit of three order of magnitude lower, confirming the high sensitivity property of this NCI. The calibration curve is shown in Figure 3b with a R-square value of 0.905 and RSD value of 3.066%, indicating a good linear relationship and the high reproducibility of this detection platform. To further demonstrate the high-sensitivity property owing to the application of charge neutralizer, we compared the performance of this NCI platform with a non-neutralizer platform, which only possessed the thiol-linked antibody on the gold sensor surface. These two types of immunosensor employed identical antigen incubation process and tested by the same electrochemical method. As shown in Figure 3c, the changes of current referring with the base line are presented. The NCI platform not only demonstrated a higher resolution for the same concentration range of detection, but also defined a lower detection limit comparing with those of the non-neutralizer platform, indicating a lower possibility of false positive results and a higher sensitivity of this NCI platform. The specificity of this HE4 NCI platform was further assessed by using CA-125 protein, also a biomarker of ovarian cancer[37]. Mixed CA-125 (11000pM) and HE4 antigen (2500pM) samples were applied to incubate on the prepared HE4 NCI. We also applied only CA-125 (11000pM) incubation on the HE4 NCI. As shown in Figure 3d, the change of current was compared between the interference samples and the only HE4 sample and non-analyte baseline. The change of current was lower than the RSD of this NCI platform. Therefore, the interference test demonstrated that there was no signal based on only CA-125 antigen and also proved that there was no interference from CA-125 antigen on the recognition of HE4 antigen. These two experiments validated the high-selectivity using the NCI platform.

NCI Development for Tau Detection. In order to demonstrate the NCI on analysis of analyte with basic pI value, T-Tau protein was employed as target for NCI development. We first analyzed the influence of the pH condition of the detection buffer by comparing the DPV measurement of the immobilized antibody using PBS based redox (pH=7.4) and Bis-Tris based redox (pH=6.2). As shown in Figure 4a, with the application of more acidic buffer, the electrostatic charge performance of the antibody (6.67 nM) was enhanced based on the DPV measurement using $[Fe(CN)_6]^{3-/4-}$. In order to produce NCI based on a positively charged condition, a highly-negative charged, hydrophilic amino acid, aspartic acid was applied as the charge neutralizer. In order to immobilize the poly-aspartic acid, a bioconjugation mechanism aiming at modification of side chain carboxylic group was applied by reacting with 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), cystamine, and 2-Mercaptoethylamine•HCl (2-MEA) sequentially, providing an external thiol linker (Figure S3). Figure S4 shows the optimization of DPV signal based on different concentrations of poly-aspartic acids. As shown in Figure 4b, after incubation of selected concentration (3.10 nM) of thiol-linked poly-aspartic acid, the DPV signal produced by the electrostatic interaction was significantly decreased based on the positively charged amino acids interaction with $[Fe(CN)_6]^{3-/4-}$. To verify that the decrease of DPV signal was caused by the formation of salt bridge (between negatively charged glutamic acid and positively charged amino acids of the antibody at the testing environment) instead of electron hindrance due to addition of surface molecules, we further analyzed the interaction between $[Fe(CN)_6]^{3-/4-}$ and different concentrations of poly-glutamic acid. As shown in Figure 4c, the interaction of anion redox probe with negatively charged poly-glutamic acids did not change significantly with a large increase of concentration of poly-glutamic acid. This phenomenon first confirmed that the decrease of DPV signal shown in Figure 4b was not caused by increase of surface impedance. Second, the unchanged DPV signal in Figure 4c indicated that the redox reaction process was not interfered by the immobilized poly-glutamic acid, because there was repulsion force between the anion probe and negatively charged biomolecules. Moreover, based on our observations, an interaction model between the anion probe and charged biomolecules was suggested as shown in Figure 4d. We proposed that the difference in electron diffusion distance caused by electrostatic interaction and incorporation led to different current outputs behavior. This fabricated NCI was further applied for incubation of T-Tau antigen.

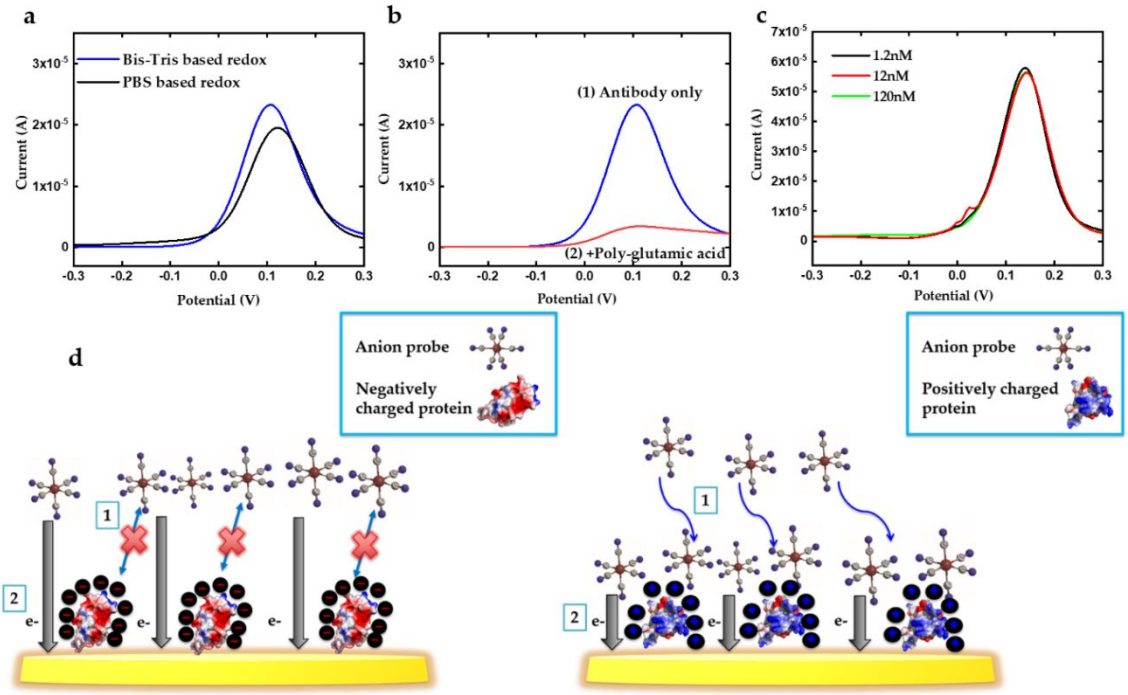

Figure 4. a) Comparison of DPV measurements of electrostatic interactions of Tau antibody with $[Fe(CN)_6]^{3-/4-}$ in Bis-Tris buffer (pH=6.2) and in PBS buffer (pH=7.4). b) DPV measurements by $[Fe(CN)_6]^{3-/4-}$ in Bis-Tris buffer (pH=6.2) on Tau antibody with the addition of poly-glutamic acid as positive charge neutralizer. c) DPV measurements by $[Fe(CN)_6]^{3-/4-}$ in Bis-Tris buffer (pH=6.2) on various concentrations of poly-glutamic acid alone. d) Proposed anion probe interaction models for negatively charged protein and positively charged protein.

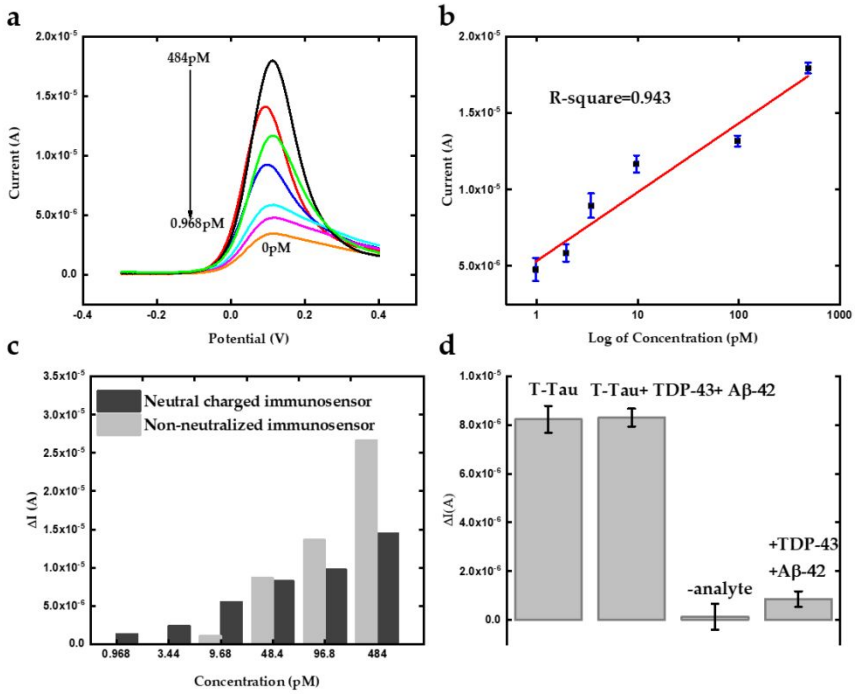

Figure 5. a) DPV measurement of various concentrations of T-Tau antigen (454pM-0.968pM) using NCI. b) Calibration curve of the DPV measurements of T-Tau NCI. c) Comparison of detection resolution of NCI with non-neutralized immunosensor in response to various T-Tau antigen concentrations of 454pM-0.968pM. d) Interference study using TDP-43 and Aβ-42 antigen on the T-Tau NCI.

T-Tau protein has a basic isoelectric point (pI=8.6). The amino acids sequence of T-Tau protein is shown in the SI. Therefore, under pH<8.6, T-Tau protein is highly positive charged[5, 38]. Multiple concentrations of T-Tau antigen were incubated onto the prepared T-Tau NCI for 30 min. After incubation, the immunosensors were tested by DPV in the presence of Bis-Tris based $[Fe(CN)_6]^{3-/4-}$ solution. As shown in Figure 5a, the DPV measurements demonstrated a positive correlation with the current outputs, indicating the positive electrostatic interaction between the T-Tau antigen with $[Fe(CN)_6]^{3-/4-}$. Comparing with the results of recent studies on Tau detection that demonstrated a sensitivity at micro/nano-molar level[5, 40], our NCI platform establishes a low detection limit of 0.968 pM in human serum. The calibration curve is shown in Figure 5b with a R-square value of 0.943 and RSD around 3.6%.

We further compared this NCI with non-neutralized platform. Non-neutralized platform did not incorporate the charge neutralizer, poly-glutamic acid. As shown in Figure 5c, comparing with non-neutralized immunosensor, our T-Tau NCI demonstrated one order lower of the detection limit, confirming the high-sensitivity of this NCI platform. For interference study, 9.68pM T-Tau antigen sample was mixed with TAR DNA binding protein 43 (133pM) and β-amyloid 42 antigen (153pM), which are confirmed biomarkers for neurodegenerative disorders[41-43]. As shown in Figure 5d, all the interference currents produced by non-target analyte were within the range of RSD from the DPV calibration curve. Therefore, this T-Tau NCI also confirmed its high-specificity on electrochemical detection.

CONCLUSION

In summary, the developed neutral charged immunosensor (NCI) provides a simple, rapid immunosensor fabrication protocol suitable for routine clinical point-of-care usages. By triggering the electrostatic charge of the target analyte, we provide two perspectives for the NCI, covering the detection methods for both negative charged and positive charged analytes. Therefore, this NCI platform provides a universal method for electrochemical quantification of antigen. The pico-molar level detection limit of this simple immunosensor platform is sufficiently low for quantification of most of the circulating protein biomarkers.

AUTHOR INFORMATION

Corresponding Author

* Email: cxl9@case.edu

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.
Materials, bioconjugation procedure for Anti-HE4, Bioconjugation procedure for poly-arginine, Amino acid sequence of HE4 antigen, HE4 NCI preparation, Differential pulse voltammetry (HE4 measurement), Bioconjugation procedure for Anti-T-Tau, Bioconjugation procedure for poly-glutamic acid, Amino acid sequence of T-Tau antigen, T-Tau NCI preparation, Differential pulse voltammetry (T-Tau measurement), Evaluation of different concentrations of thiol-linked poly-arginine (Figure S1), Incubation time of the [Ru(NH3)6]3+ redox for optimization of electrostatic charge readout (Figure S2), Bioconjugation method for providing thiol-linker onto glutamic acid (Figure S3), Evaluation of different concentrations of thiol-linked poly-glutamic acid (Figure S4).

Author Contributions

Y.D and C.C.L designed and conceived the research. Y.C, S.Q, X.W conducted the experiments. Y.D, C.C.L and L.Y.C analyzed data. Y.D and C.C.L drafted the manuscript. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

Wallace R. Persons research fund of Case Alumni Association.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

We acknowledge the funding support from Wallace R. Persons research fund of Case Alumni Association. Yifan Dai gratefully acknowledges Dr. Harihara Baskaran from Department of Chemical and Biomoleculear Engineering and Department of Biomedical Engineering, Case Western Reserve University, and Dr. Blanton Tolbert from Department of Chemistry, Case Western Reserve University, and Laurie Dudik from Department of Chemical and Biomolecular Engineering, Case Western Reserve University for their insightful discussion over the course of this research.

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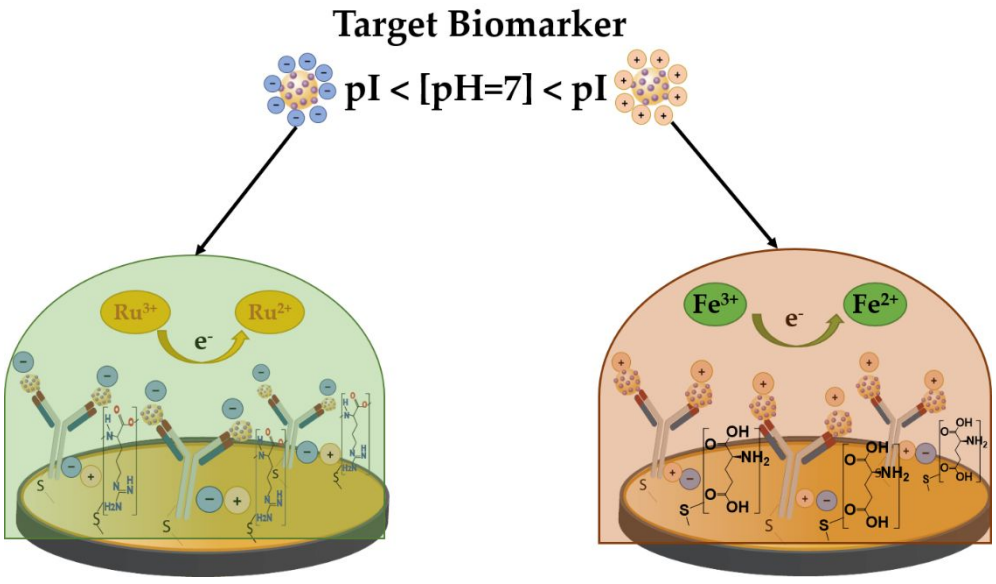
FOR TOC ONLY:



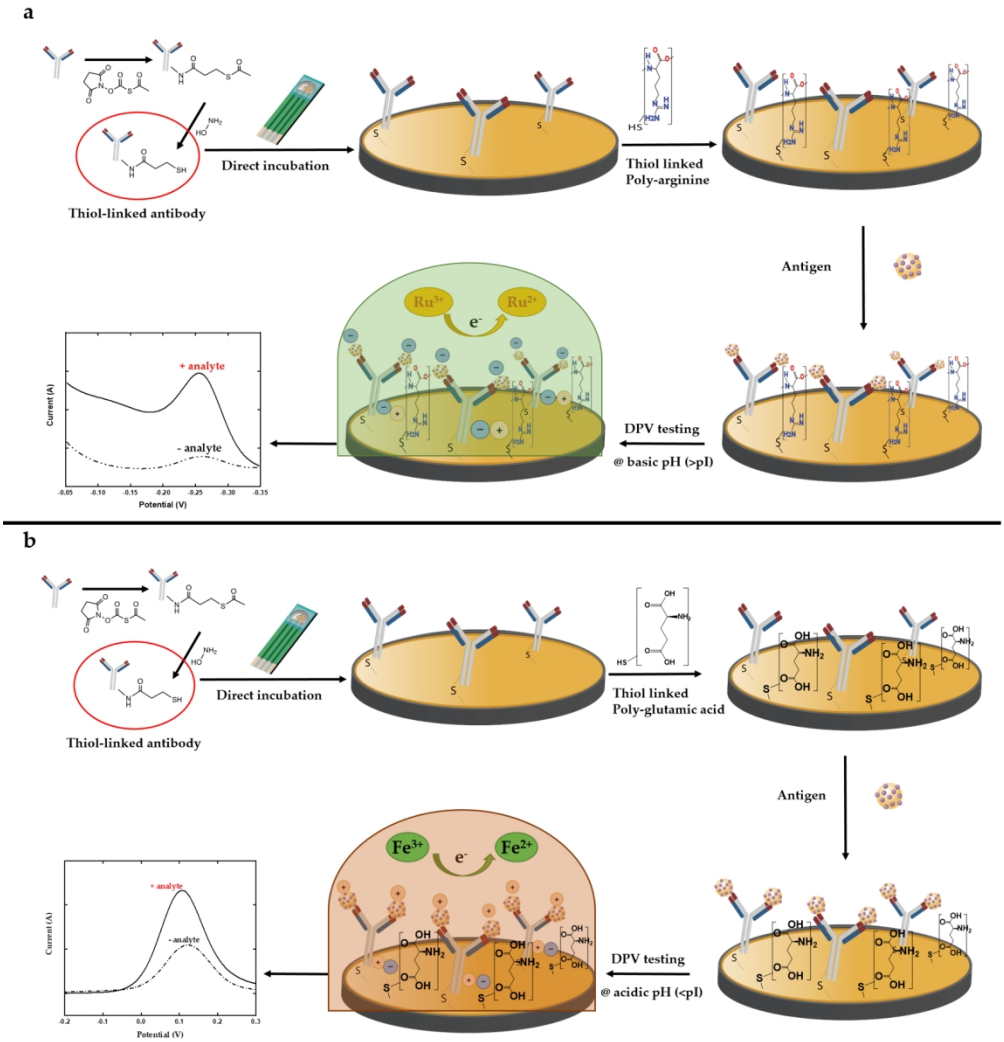


Figure 1.

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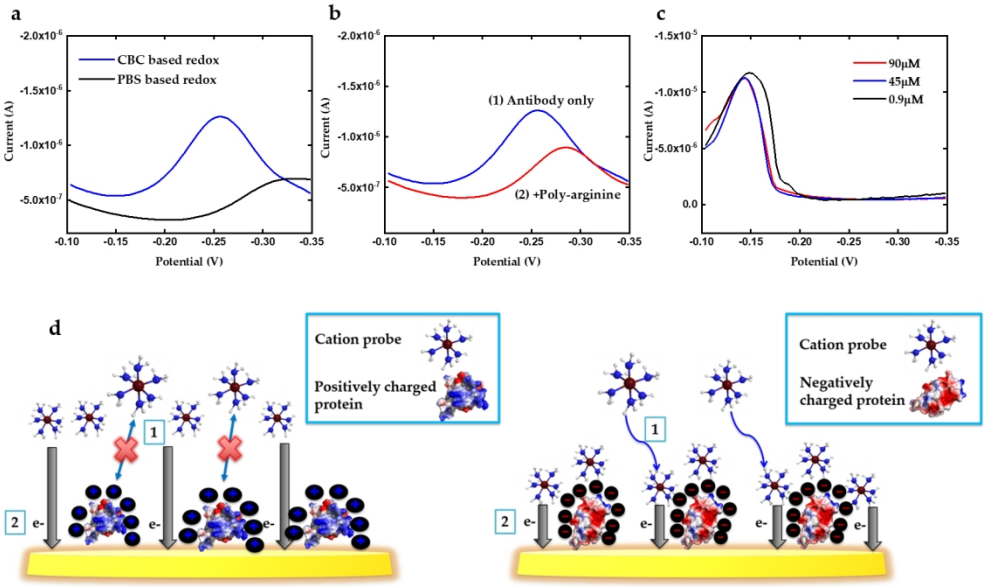

Figure 2

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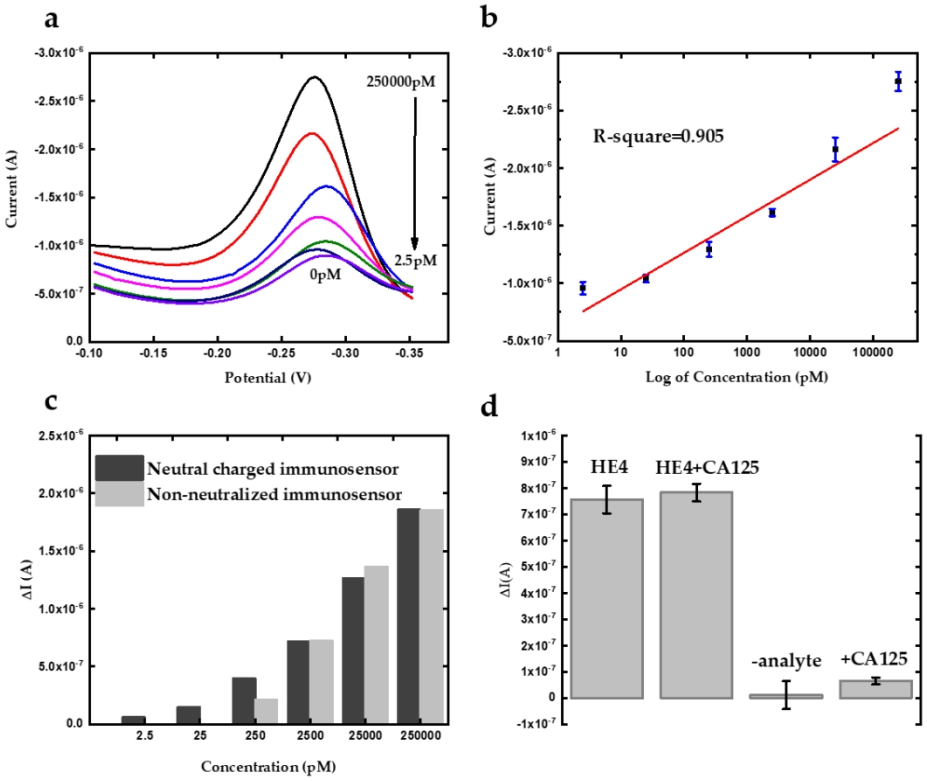

Figure 3

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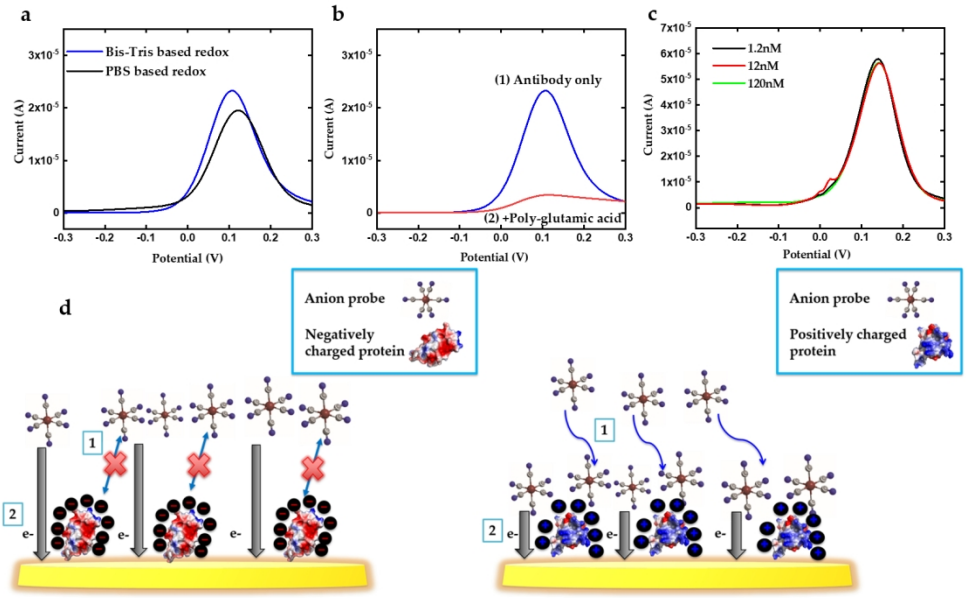

Figure 4

265x162mm (168 x 168 DPI)

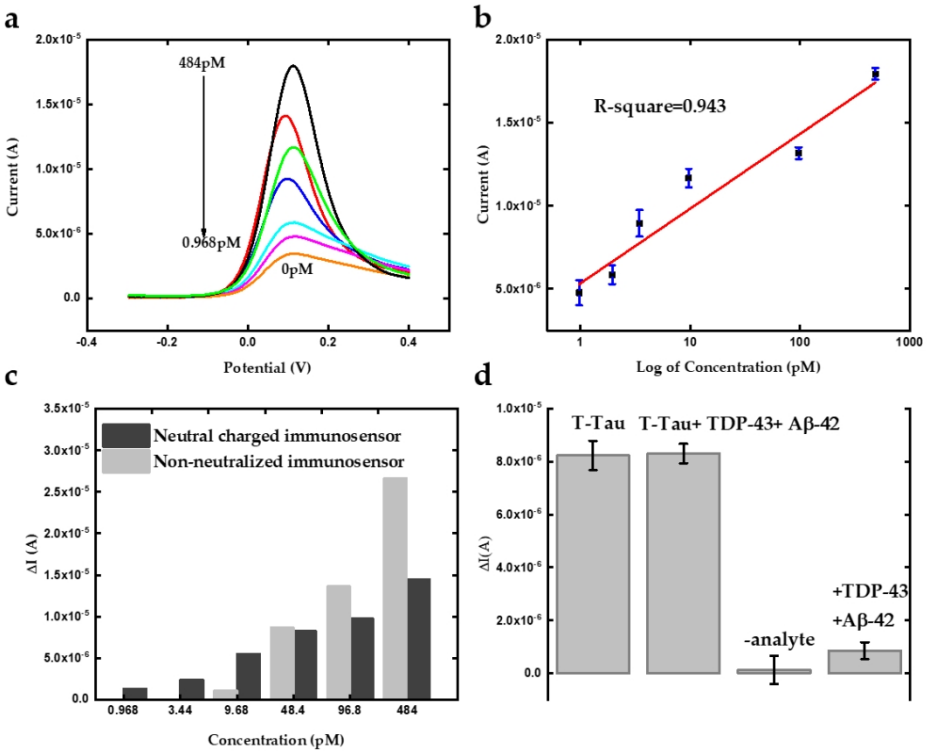

Figure 5

182x142mm (168 x 168 DPI)