

Label-Free Electrochemical Biosensor for the Detection of Glycophorin A (CD235a)

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Abstract

Electrochemical biosensors offer an alternative method for identification of specific proteins in a biological sample by producing an electrical signal that is dependent on molecular recognition. Herein, we present a label-free electrochemical biosensor that focuses on Glycophorin A (CD235a). The biosensor is based on gold microelectrodes which were functionalized with a self-assembled monolayer (SAM) of mercaptoundecanoic acid (MUA), then activated with EDC/NHS. Non-specific binding on the oriented immobilized anti-CD235a antibodies was blocked using bovine serum albumin (BSA). These sequential surface modifications yielded a very low characteristic current by cyclic voltammetry (CV): The peak current significantly decreased by 93.4% (anodic) and 96.9% (cathodic) between the bare electrode and BSA-blocked surface. Analysis of the impedance spectra revealed a charge transfer resistance (R_{ct}) of 6 k Ω for bare electrode. This increased significantly to 434 k Ω following the detection of CD235a, indicating successful target binding. This substantial increase in R_{ct} confirms the biosensor's high sensitivity. Furthermore, the biosensor demonstrated high specificity towards the CD235a surface antigen.

Keywords: biosensor, electrochemistry, glycophorin A, impedance spectroscopy

Introduction

Identifying the presence of specific proteins in a biological sample is an essential task for both basic and applied research. Although classical analytical methods such as flow cytometry, Enzyme-Linked Immunosorbent Assay (ELISA) and western blotting are accurate and can conduct multi-parameter analysis, they have several disadvantages, such as invasiveness and time-consuming sample preparation. They are also not useful for continuous monitoring, since they only provide discrete snapshots of the status [1, 2]. This is critical for dynamic bioprocess control, where continuous data is required.

Electrochemical biosensors offer a promising approach to overcoming these limitations, as they allow for label-free, sensitive, real-time detection [3]. They convert molecular recognition events into measurable electrical signals, providing quantitative information about the concentration of the target analyte. Such sensors capable of continuously capturing biological signals are important for systems that rely on feedback-driven adaptation. This includes biological-technology interfaces, where both processes interact in a dynamic and reciprocal manner. Within the

concept of co-evolution, innovative technologies and sensor-based approaches which are able to adapt to alterations in biological systems, lay the groundwork for self-learning systems.

A crucial step for electrochemical sensors is the proper immobilization of biorecognition elements (typically antibodies) to guarantee their effectiveness, as this retains their specificity for target analytes and ensures they are oriented in an optimal position for binding. Self-assembled monolayers (SAMs) of organothiols on gold electrodes, coupled using carbodiimide chemistry (EDC/NHS), are a well-established platform for developing electrochemical immunosensors. This method enables the stable covalent attachment of antibodies while maintaining bioactivity and oriented immobilization [4, 5].

Here, we describe the development and characterization of a label-free electrochemical biosensor targeting glycophorin A (CD235a), a major transmembrane sialoglycoprotein of erythrocytes which is widely used as a marker of commitment to an erythroid lineage [6, 7]. Glycophorin A is expressed at a high density on mature red blood cells (RBCs) (approximately one million copies per cell).

To the best of our knowledge, this study presents the first practical demonstration of a label-free electrochemical immunosensing platform for the detection of CD235a.

2. Materials and Methods

2.1 Materials and Reagents

Screen-printed gold electrodes (DRP-C223BT-U75, Metrohm, Germany) served as substrates for surface functionalization. The following chemicals were used: mercaptoundecanoic: mercaptoundecanoic acid (MUA, 95%), 2-(N-Morpholino) ethanesulfonic acid (MES, $\geq 99\%$), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, $\geq 99.0\%$), N-hydroxysuccinimide (NHS, 98.0%), phosphate-buffered saline (PBS, pH 7.4), potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$), and potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6] \cdot 3H_2O$) all purchased from Sigma-Aldrich (Merck, Germany). A recombinant rabbit monoclonal anti-CD235a antibody (clone 27A8, Thermo Fisher Scientific, USA) was selected as the biorecognition element and Bovine serum albumin (BSA) was applied as a blocking agent, and potassium chloride (KCl) functioned as a supporting electrolyte. The electrodes were cleaned with absolute ethanol ($\geq 99.8\%$). The recombinant human Glycophorin A protein (Sino Biological Europe GmbH) served as the target biomarker (antigen) in this study. All aqueous solutions were prepared using deionized (DI) water, and all chemicals were used without further purification.

2.2 Electrode Preparation and Surface Functionalization

The gold microelectrodes were cleaned by rinsing them with ethanol, followed by a thorough wash with ultrapure water, then drying them under a stream of nitrogen. Surface functionalization was carried out using a sequential modification strategy (see Figure 1). First, a self-assembled monolayer (SAM) structure was formed by immersing the cleaned electrodes in a 1 mM solution of mercaptoundecanoic acid (MUA) in ethanol for 18 hours at room temperature in the dark to promote the formation of a well-organized monolayer. The thiol group of MUA creates stable Au-S bonds with the gold surface, and the terminal carboxyl groups serve as functional sites for further antibody coupling. The electrodes were then washed with ethanol, and ultrapure water, and dried under nitrogen.

The carboxyl groups were activated using a freshly prepared EDC/NHS solution (200 mM EDC and 50 mM NHS in MES at pH 6) by immersing the electrodes in it for 30 minutes at room temperature. This carbodiimide coupling chemistry generates reactive NHS esters from the carboxyl groups, which then react with the primary amines of the antibody's lysine residues to form stable amide bonds. After washing with PBS to remove any remaining reagents, 20 μ L of an anti-CD235a solution (100 μ g/mL in PBS) was dropped onto the electrode surface and left to incubate for 2 hours at room temperature. This approach enables the stable, covalent immobilization of antibodies on the electrode surface. The electrodes were then rinsed with PBS to remove any unbound antibody.

To block non-specific binding sites, the electrodes were incubated in 1% BSA in PBS for 30 minutes, afterwards they were rinsed three times with PBS. The BSA then acts as a passivating agent for any residual reactive sites, reducing non-specific protein adsorption in the next step of antigen detection. Subsequently, 20 μ L of recombinant human Glycophorin A protein (0.1 ng/mL in PBS) was pipetted onto the working electrode surface and incubated for 30 minutes at room temperature. After incubation, the electrodes were rinsed three times with PBS to remove any unbound antigen before electrochemical measurements.

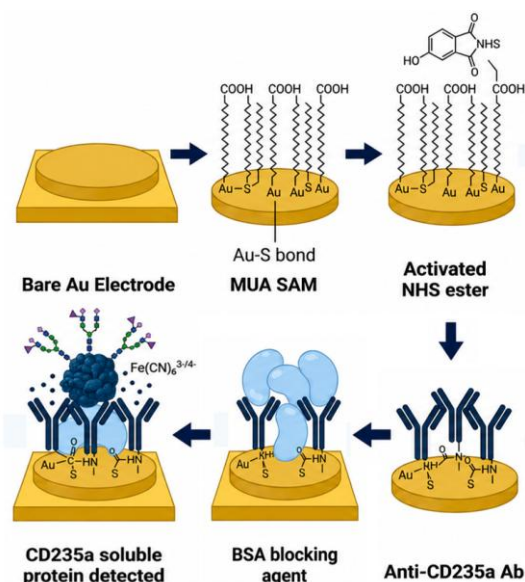


Figure 1: A schematic diagram of the Glycophorin A modification method and its detection.

2.3 Electrochemical Characterization

Electrochemical measurements were performed using a Gamry Interface 1010E potentiostat (Gamry Instruments, USA) in a three-electrode configuration consisting of a 1.6 mm diameter screen-printed gold working electrode (WE), a gold counter electrode (CE), and a Ag/AgCl pseudo-reference electrode. Measurements were performed at room temperature in a 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution prepared in PBS (pH 7.4).

Cyclic voltammetry (CV) was conducted over a potential range of -0.2 to $+0.6$ V versus Ag/AgCl. EIS measurements were performed at a DC potential of $+0.23$ V (vs. Ag/AgCl) which is the formal potential of the redox couple, with an AC amplitude of 10 mV, over a frequency range of 100 kHz to 0.01 Hz. Echem Analyst software (Gamry Instruments) was used to analyze the impedance data and fit the R_{ct} values obtained from a Randles equivalent circuit model.

3. Results

3.1 Cyclic Voltammetry Analysis

Cyclic voltammetry measurements were performed using the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe to evaluate the changes in electron transfer properties during the stepwise modification of the electrode surface (see Figure 2). The bare gold electrode produced well-defined, and quasi-reversible redox peaks with high current intensity (anodic peak current $I_{pa} = +46.0$ μA and cathodic peak current $I_{pc} = -41.8$ μA) as well as small peak-to-peak separation. Following EDC/NHS-based activation of the MUA SAM, the peak current decreased considerably ($I_{pa} = +8.4$ μA and $I_{pc} = -8.8$ μA), representing an 81.7% and 78.9% decrease in the anodic and cathodic currents, respectively compared to the bare electrode. This resulted in a marked increase in peak separation. Additionally, immobilizing the anti-CD235a antibody decreased the peak currents to $I_{pa} = +7.0$ μA and $I_{pc} = -8.7$ μA (reductions of 84.9% and 79.1%, respectively), compared to the bare electrode, followed by antibodies immobilization.

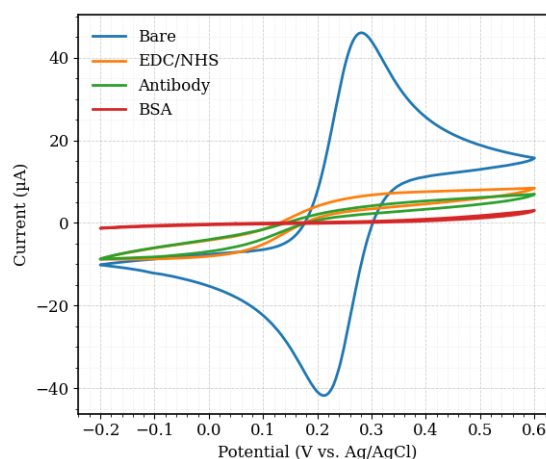


Figure 2: Cyclic voltammograms with modification of the electrode surface from bare gold through the formation of a self-assembled monolayer, the activation with EDC/NHS, the immobilization of antibodies and the blocking with BSA.

The final BSA blocking yielded the smallest measured currents ($I_{pa} = +3.0$ μA , $I_{pc} = -1.3$ μA), representing total reductions of 93.4% and 96.9% from the values for the bare electrode, respectively.

3.2 Electrochemical Impedance Spectroscopy

Electrochemical impedance spectroscopy (EIS) was utilized to analyze the changes in interfacial charge transfer resistance that occurred during the consecutive functionalization steps. This provided complementary quantitative data (see Figure 3). The Nyquist plots ($-Z''$ versus Z') showed an increase in semicircle diameter as the charge transfer resistance (R_{ct}) values increased. In Nyquist plots, the semicircle diameter is proportional to R_{ct} , reflecting resistance to electron transfer across the electrode–electrolyte interface. For the bare gold electrode, only a small semicircle with a low R_{ct} value of 6 k Ω appeared, due to the fast electron transfer occurring on the clean metal surface. Following MUA SAM formation (see Figure 3), the R_{ct} increased to 39 k Ω . Subsequent to EDC/NHS activation of the carboxyl-terminated SAM, R_{ct} increased further to 74 k Ω . The immobilization of the anti-CD235a antibody led to an increase in R_{ct} to 151 k Ω .

After BSA blocking, the R_{ct} increased slightly to 342 k Ω .

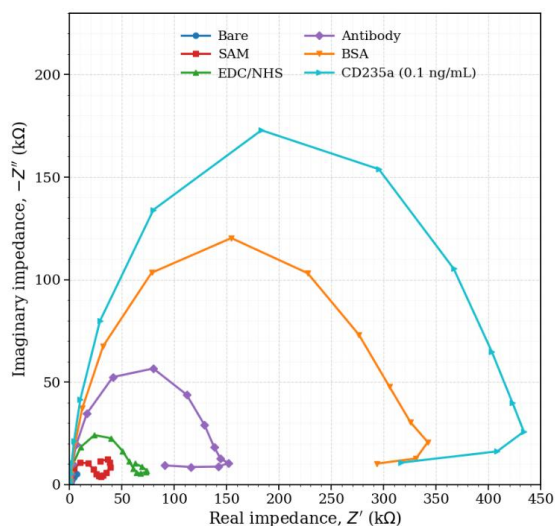


Figure 3: Nyquist plots with impedance changes during sequential electrode functionalization and CD235a detection.

3.3 Biosensor Performance for CD235a Detection

A continued increase in R_{ct} to 434 kΩ was observed after BSA-blocked electrodes were incubated with a 0.1 ng/mL solution of CD235a antigen in PBS (see Figure 4). This increase reflects the impact of antigen binding on interfacial resistance. An 27% increase in R_{ct} relative to the BSA-blocked state (corresponding to an absolute change of 92 kΩ) indicates the presence of specific antigen–antibody interactions. Although this increase is small compared to previous functionalization steps, it demonstrates molecular recognition between the immobilized anti-CD235a antibodies and their cognate antigen and confirming the sensitivity of the sensor toward low antigen concentrations.

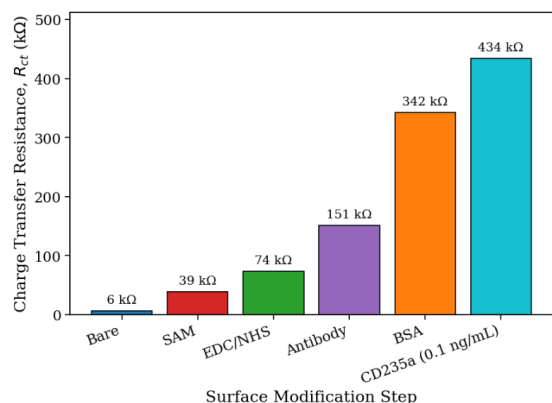


Figure 4: The charge transfer resistance (R_{ct}) values at each functionalization step.

4. Discussion

4.1 Surface Functionalization Strategy

The bare gold electrode exhibited quasi-reversible redox behavior with high peak currents and a small peak-to-peak separation (ΔE_p), indicating efficient electron transfer and a clean, electrochemically active gold surface. Such a surface provides an ideal foundation for the formation of a well-ordered self-assembled monolayer (SAM), which is essential for the stable immobilization of biomolecules. Accordingly, a carboxyl-terminated surface was established using a mercaptoundecanoic acid (MUA) self-assembled monolayer. Alkanethiol SAMs spontaneously assemble on gold through the formation of strong Au–S covalent bonds [8], resulting in highly stable monolayers. The 11-carbon alkyl chain promotes ordered molecular packing through van der Waals interactions, while the terminal carboxyl groups provide reactive sites for subsequent antibody immobilization.

The successful stepwise surface modification observed by cyclic voltammetry was further confirmed by electrochemical impedance spectroscopy, which provided complementary information on the interfacial electron-transfer properties during each functionalization step. The significant increase in R_{ct} following SAM formation indicates successful surface coverage by an insulating alkanethiol monolayer, which hinders electron transfer between the redox probe and the electrode surface [9,10]. This confirms the formation of a stable self-assembled monolayer suitable for subsequent biofunctionalization.

Activation of the terminal carboxyl groups using EDC/NHS chemistry enabled the covalent immobilization of antibodies through amide bond formation with their primary amine groups. The subsequent increase in R_{ct} after antibody immobilization confirms the successful attachment of the antibody layer. The relatively large size and insulating nature of the antibody molecules further impede interfacial charge transfer while preserving the integrity of the underlying SAM [11].

Finally, blocking with bovine serum albumin (BSA) occupied the remaining active sites, thereby minimizing non-specific adsorption [12]. The comparatively small increase in R_{ct} after BSA treatment indicates effective surface passivation without substantially altering the architecture of the biofunctional layer. Overall, the progressive increase in R_{ct} throughout each modification step confirms the successful stepwise fabrication of the biosensor and the formation of a stable biofunctional interface.

suitable for the specific recognition of the target antigen.

4.2 Biosensor Sensitivity and CD235a Detection

These results, along with the concurrent improvement in R_{ct} demonstrate the sensitivity of the biosensor and the successful incorporation of all functionalization steps from the bare electrode to the recognition of CD235a. Although the observed 27% increase in impedance upon antigen binding is modest relative to previous functionalization steps, it is indicative of specific molecular recognition and provides a reliable and distinguishable signal response. This increase is comparable to those reported for other electrochemical immunosensors for protein biomarkers and supports the effectiveness of the antibody immobilization strategy [3, 7].

Glycophorin A, the single most abundant protein in erythrocyte membranes, is present at approximately one million copies per mature red blood cell (RBC) [13] and accounts for ~2% of total membrane protein [14]. It is first expressed at the proerythroblast stage, and its levels increase during erythroid differentiation. With an effective concentration of 0.1 ng of CD235a, this biosensor shows potential for detecting CD235a expression.

The specificity of the biosensor is primarily attributed to the high affinity and selectivity of the recombinant anti-CD235a antibody (clone 27A8). Sensor performance was evaluated using a recombinant human CD235a (glycophorin A) protein corresponding to the extracellular domain (Met1–Glu91) as the target antigen [15]. Furthermore, the BSA blocking step minimized nonspecific adsorption by passivating the remaining active sites on the electrode surface, thereby reducing false-positive signals and improving the overall specificity of the biosensor.

Several factors contribute to the increase in impedance following antigen binding. First, the binding of CD235a, a heavily glycosylated membrane glycoprotein with an apparent molecular mass of approximately 40–45 kDa, increases the steric hindrance at the electrode interface, thereby restricting electron transfer [16]. Second, the extensive glycosylation of CD235a introduces a highly hydrated carbohydrate layer that further impedes charge transfer [17]. In addition, antigen binding may induce conformational changes in the antibody–antigen complex and modify the local dielectric environment at the electrode surface,

resulting in a further increase in the charge transfer resistance [18]. The clear distinction between the BSA-blocked and antigen-bound states demonstrates that the biosensor is capable of detecting CD235a at nanogram-per-milliliter concentrations, confirming the effectiveness of the antibody immobilization strategy.

4.3 Limitations of the biosensor

This study is a proof of concept showing the functionality of the biosensor under idealized conditions with a purified CD235a antigen in a buffer solution. However, prior to practical implementation, several key aspects must be addressed. First, a systematic dose-response characterization is necessary to determine the detection limit, linear dynamic range, and saturation concentration. Although preliminary results indicate sensitivity at the sub-nanogram per milliliter (ng/mL) level, a systematic dose-response analysis is required to accurately determine the detection limit and dynamic range. Second, the biosensor's performance should be evaluated in complex biological samples containing mixtures of different components such as serum proteins, growth factors and/or metabolites that could contribute to nonspecific interactions or interfere with antigen-antibody interactions. The BSA blocking step is expected to mitigate this interference; however, it must be validated under realistic conditions. Third, the long-term stability and reproducibility on different electrodes, as well as the possibility of fully regenerating the sensor, should be evaluated. Because the binding of antibody to antigen is irreversible under physiological conditions, the biosensor may function as a single-use device. Finally, validation in biologically relevant systems is essential. Such validation would demonstrate the ability of the biosensor to detect membrane-associated CD235a from live cells, as opposed to only soluble antigen.

4.4 Future directions: Real-time monitoring of CD235a

The reduced complexity associated of the label-free, real-time detection renders this biosensor beneficial for future monitoring systems, in which changes to the concentration of CD235a may be time dependent. Unlike ELISA, which requires periodic sampling, antibody labeling, and specialized instrumentation, electrochemical detection enables non-invasive, continuous, in situ observation. Making the developed biosensor an ideal tool for real-time monitoring of CD235a.

Continuous impedance measurements over a frequency range (100 kHz to 0.01 Hz) could provide information about CD235a levels. Additionally, the electrical readout allows for integration with automated control systems for closed-loop bioprocess control, which combined with the small (1.6 mm diameter) electrode footprint, enables easy integration into microfluidic or miniaturized bioreactor systems. Furthermore, automated strategies for electrode regeneration, reagent addition, sampling and testing under dynamic flow conditions could also be considered.

Ultimately, developing multiplexed sensor arrays that monitor multiple biomarkers simultaneously may provide a comprehensive bioprocess characterization information and enable advanced control strategies.

5. Conclusion

We demonstrated the development of an electrochemical biosensor that enables the label-free electrochemical detection of glycophorin A (CD235a), a key erythroid differentiation marker. This biosensor exhibits high sensitivity and specificity. The sequential functionalization strategy involves mercaptoundecanoic acid (MUA) self-assembled monolayer formation, EDC/NHS activation, anti-CD235a antibody immobilization, and bovine serum albumin (BSA) blocking. This results in a stable biofunctional interface favorable for specific antigen recognition.

Progressive surface modification via cyclic voltammetry resulted in a consistent decrease in peak currents, reaching up to 93.4% (anodic) and 96.9% (cathodic) from the bare electrode to the BSA-blocked surface. This indicates successful layer-by-layer assembly. The electrochemical impedance spectroscopy showed that charge transfer resistance increased significantly, from 6 k Ω for the bare electrode to 434 k Ω after CD235a detection, suggesting high sensitivity of the sensing platform. Clear differentiation from the BSA-blocked baseline upon the detection of 0.1 ng of the CD235a antigen further supports the specificity of the antigen-antibody interactions. Unlike conventional monitoring techniques, such as flow cytometry, this platform enables continuous, noninvasive, real-time monitoring without cell labeling, sampling, or disruption of the culture environment. Its small electrode footprint and electrical readout allow for integration into microfluidic devices with automated controls. This modular platform can easily be adapted to other biomarkers, making

it promising for new bioprocess monitoring technologies.

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6. References

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