



## Impedimetric CRISPR-dCas9 Based Biosensor System for Sickle Cell Anemia Mutation

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**Abstract:** Sickle cell anemia is one of the single point mutation diseases with symptoms such as stroke, lethargy, chronic anemia, and increased mortality, and it causes red blood cells to become sickle-shaped. In the study, a biosensor system was developed to detect this mutation quickly and cost-effectively. This biosensor system was prepared by forming a SAM layer with 4-Aminothiophenol (4-ATP) on the gold electrode, and coating it with amino graphene. It was then modified with SG-RNA with the sequence of the target mutation after CRISPR-dCas9 immobilization. The nanomaterial used in the preparation of the biosensor increased the sensitivity of the method by increasing the surface area. The biosensor prepared in this way was optimized and made to perform DNA analysis. As a measurement method, electrochemical impedance spectroscopy (EIS) was used. Electrochemical measurements were carried out in 50 mM pH 7.0 phosphate buffer solution, which includes 5 mM  $\text{Fe}(\text{CN})_6^{4-/3-}$  and 10 mM KCl, as redox probe solution by CV and EIS in this redox probe solution. EIS parameters were 10,000–0.05 Hz frequency, 10 mV AC and 180 mV DC potentials, and CV parameters were between - 0.2 to 0.5 V potential, 100 mV/s scan rate for 5 cycles. The DNA measurement time of the biosensor system was determined by the chronoimpedance measurements taken by applying a frequency of 500 Hz under 200 mV DC current. Measurement time of the biosensor was found to be 100 seconds. With the CRISPR-Cas9 based electrochemical biosensor system, which gives faster results compared to the measurement methods in the literature, a linear measurement between 40 pM and 1000 pM with a length of 400 base pairs was taken.

**Keywords:** Sickle cell anemia, CRISPR-dCas9, biosensor, electrochemical impedance spectroscopy.

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### INTRODUCTION

Sickle cell anemia (SCA) is an inherited disease that occurs when an abnormal form of hemoglobin, called Hemoglobin S (HbS), replaces the normal protein hemoglobin A (HbA) (1). The causative agent of HbS formation in SCA is a point (nucleotide) mutation in the beta globin gene (2). This mutation occurs in the beta globin gene, where the GAG triplet in the sixth position is transformed into GTG triplet, and valine is encoded instead of glutamine (3). When the amount of oxygen in the environment is low, HbS becomes insoluble and the polymeric structures they form by bonding to each other cause the deformation of red blood cells from

circular to sickle shape. If the red blood cell membrane is not too damaged, this deformation can be reversed by oxygenation, but this cycle damages the red blood cells, shortening their lifespan, and leading to anemia (4). Diagnosis of this mutation is made by conventional techniques such as polymerase chain reaction (5), liquid chromatography (6), and electrophoresis (7). Although these methods show high sensitivity, they are time-consuming and costly (4). In this study, a CRISPR-dCas9-based electrochemical biosensor system was developed to detect this mutation quickly and cost-effectively.

Biosensors are systems that are developed by the immobilization of highly selective biomolecules such as enzymes, receptors, antibodies, DNA, or proteins (biorecognition agents) produced by living systems on a physicochemical transducer and that allow measurement of complex substances (8). The CRISPR-dCas9 system is also used as a biorecognition agent in biosensor systems (9). dCas9 and synthetic guide RNA (sgRNA) have the capacity to specifically bind DNA sequences, and this binding can be used to detect DNAs (10). Biosensors are divided into two categories: bioaffinity and biocatalytic, according to the interaction feature between the biorecognition agent and the analyte molecule. Bioaffinity-based biosensors are based on the affinity between the biorecognition molecule and the analyte molecule. With this affinity, the measurement system is realized by measuring the affinity of the analyte molecule to the biorecognition molecule (11). Electrochemical systems precede other measurement methods (Optical, Piezoelectric) as they are cheap, fast, practical, and economical systems. Electrochemical systems with simplicity of transducers and easy measurement method can reach very low detection limits in biosensor systems modified with a suitable bioreceptor (12). Electrochemical biosensors can be classified as amperometric, voltammetric, or impedimetric.

Electrochemical impedance spectroscopy (EIS) is an electrochemical surface characterization technique and is widely used in sensors because it provides the advantage of label-free detection. EIS offers inexpensive measurement at low concentrations without sample pretreatment (13). Impedimetric measurement is well suited for affinity-based biosensor systems because the system does not require label, an electroactive secondary molecule, or biochemical reaction. With this method, quantitative analysis is possible only by determining the surface binding kinetics of the analyte molecule (13).

In the presented study, deactivated Cas 9 was immobilized by forming self-assembled monolayers on the gold electrode for the determination of sickle cell anemia and electrochemical impedance spectroscopy was used as the measurement method. SAM surfaces formed by the strong bond between gold and sulfur are very preferred in biosensor fabrication and provide stable surfaces (14). The sensitivity of the method was increased by obtaining a larger surface area with ammonium-functionalized graphene used in electrode immobilization.

## EXPERIMENTAL SECTION

### Chemicals and Equipment

dCas9 proteins were obtained from Applied Biological Materials (ABM) Inc. (Richmond, BC,

Canada), sgRNA sequences were obtained from Synthego Corp. Other chemicals were obtained from Sigma Aldrich (USA). Electrodes (Reference, Working, and Counter) were obtained from BASi. PalmSens 3 potentiostat system was used for electrochemical techniques (EIS and cyclic voltammetry (CV)) and PSTrace 5.6 was used as electrochemical interface software. Scanning Electron Microscopy (SEM) images obtained from COXEM EM-30 Plus (Daejeon, Korea). For FT-IR analysis, a Thermo Scientific NICOLET iS10 spectrometer was used.

### Method

#### *Experimental parameters*

All electrode modification steps were monitored by scanning electron microscopy (SEM), FT-IR, CV, and EIS. EIS and CV measurements were carried out in a solution of redox probe, which includes 5 mM  $\text{Fe}(\text{CN})_6^{4-/3-}$  redox couple and 100 mM KCl in 50 mM pH 7.0 phosphate buffer. All modifications and measurements were carried out at 37°C. EIS signals showed that the electrical circuit for EIS calculation was constructed as shown in Figure 1.

#### *Preparation of the electrode surface*

Prior to use, gold electrode (AuE) was polished 0.05  $\mu\text{m}$  alumina slurry for 2 minutes. Then, to remove any adsorbents, the electrode was washed ultrasonically with pure ethanol and pure water, respectively. EIS and CV were performed to obtain bare gold electrode as baseline. After each modification step, the electrode was washed by soaking in pure water and dried under nitrogen stream, gently. Subsequently, AuE was soaked in 50 mM 4-aminothiophenol (4-ATP) solution in ethanol for overnight to form Self-assembly Monolayer (SAM) for further immobilization via amino bonds (15). Then electrode was washed with ethanol and water to remove unbonded 4-ATP from AuE surface. In the next step, the electrode was incubated in 5% glutaraldehyde solution for 30 minutes to activate amino groups on the 4-ATPs. The ammonium functionalized graphene oxide (1 mg/mL) was sonicated until the particles dispersed homogeneously and dropped on the AuE/4ATP electrode and incubated for one hour. The glutaraldehyde step was performed again, and the modified electrode was incubated with dCas9 (250 pmol) protein for one hour. Then the electrode was incubated with sgRNA for 30 minutes. After the biosensor was successfully produced, the optimization of modification steps, preparation of the calibration curve, determination of LOD and LOQ, reproducibility and repeatability optimization studies were carried out.

#### *Biosensor optimization*

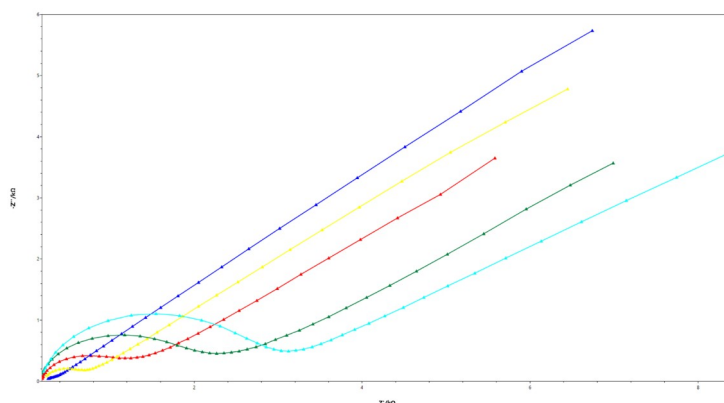
The first of the biosensor optimization steps was to determine the exact incubation time for modification and then measurement time. Chronoimpedance was used for obtaining the DNA detection time.

Chronoimpedimetric measurement is optimized in buffer solution and frequency was obtained from the Bode plot of impedimetric detection of the prepared biosensor (AuE-4ATP-GPH-dCas9-sgRNA) (16). The frequency is selected on Bode-Plot where the impedance shows an increasing character and the phase angle becomes constant. The applied potential should be low in order to prevent the oxidation of other compounds in real samples (17). After obtaining the exact DNA detection time, a calibration curve was performed by incubating the biosensor with DNA. The calibration curve was prepared with DNA standards against obtained EIS signals in redox probe solution. The LOQ was calculated by using  $10 S/m$  and LOD  $3.3 S/m$ .  $S$  is the standard deviation of EIS data at the lowest concentration of the calibration curve, seven times the obtained standard deviation, and  $m$  is the slope of the calibration curve. Reproducibility was obtained by preparing the calibration curves for six time and  $R^2$  results of the calibration curves with C.V%. Repeatability was also tested on three different DNA samples (80, 200, and 900 pM) with added artificial serums.

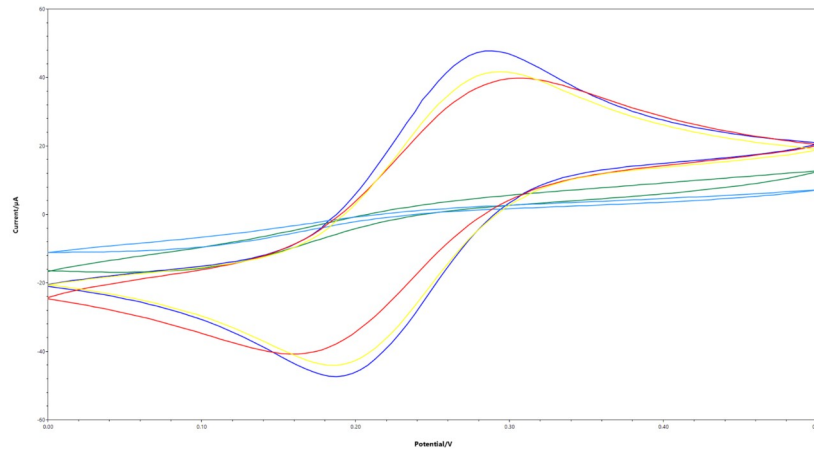
## RESULTS AND DISCUSSION

### Preparation of the Electrode Surface

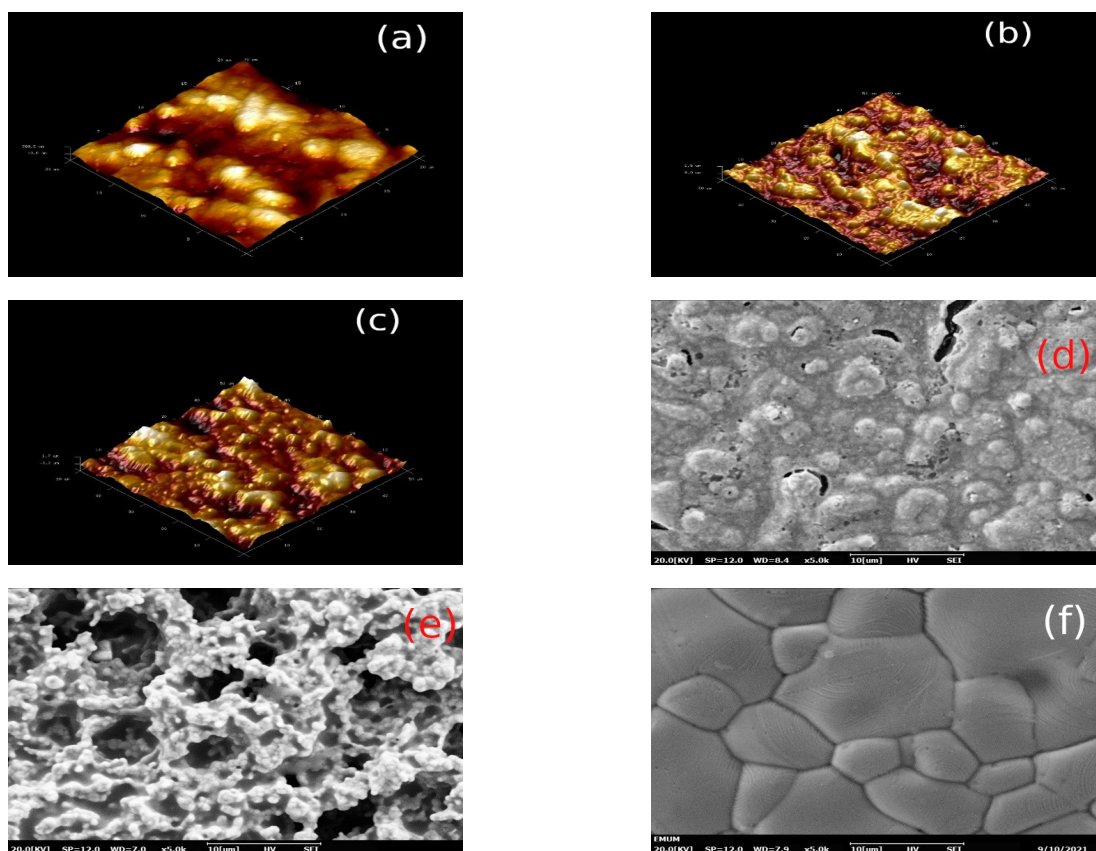
In order to develop a new generation genetic biosensor technology, CRISPR technology has been used with good accuracy. In this study, accuracy was increased by using nanomaterials with high sensitivity. AuE electrode modification steps were observed by EIS and in redox probe solution (Figures 1 and 2). AuE was chosen for its easy modification capabilities with SAM and observed as baseline in developing biosensors. The SAM layer was formed by 4-ATP, because of the benzene ring. Electrical conductivity is provided, and that increases the sensitivity with stability. The modification showed an increase in CV and decrease in EIS because the positively charged 4ATP groups attract the negatively charged redox probes, resulting in the release of more electrons that move towards electrode surface, which decreases the electron transfer resistance by increasing current. Afterwards, the amino graphene modification, the same results were observed. dCas9 and sgRNA modifications increased the EIS and decreased the CV peaks by forming insulation layers. The modification layers were also observed in Figure 3 as AFM and SEM images. There, it can be seen as topographic changes and changes in the surface characteristics can be seen (Figure 3).



**Figure 1:** EIS representation of the modification steps. a) Red EIS: AuE, blue EIS: AuE/4-ATP, yellow EIS: AuE/4-ATP/GPH, green EIS: AuE/4-ATP/GPH/dCas9, light blue EIS: AuE/4-ATP/GPH/dCas9/sgRNA.



**Figure 2:** CV representation of the modification steps. a) Red EIS: AuE, blue EIS: AuE/4-ATP, yellow EIS: AuE/4-ATP/GPH, green EIS: AuE/4-ATP/GPH/dCas9, light blue EIS: AuE/4-ATP/GPH/dCas9/sgRNA.

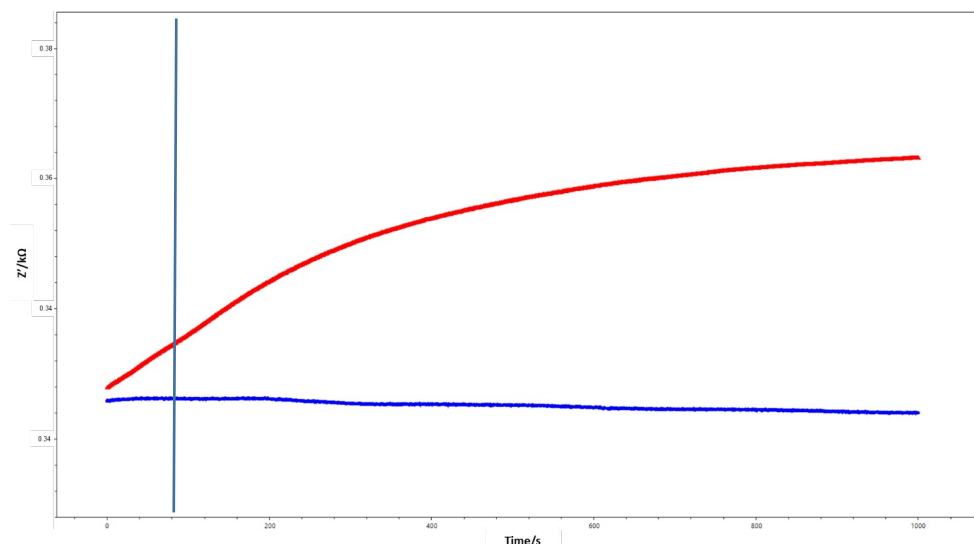


**Figure 3:** Atomic force microscopy and SEM images of the biosensor modifications. AFM images A; AuE, B; AuE-Cys-GPH-Cas9-sgRNA, C; AuE-Cys-GPH-dCas9-sgRNA-DNA, SEM images D; AuE, E; AuE-Cys-GPH-Cas9-sgRNA, F; AuE-Cys-GPH-dCas9-sgRNA-DNA.

### Biosensor Optimization

The first of the optimization steps was to determine the measurement time with chronoimpedance. The first linear increase was observed at 105 seconds, and biosensor saturation was observed at around

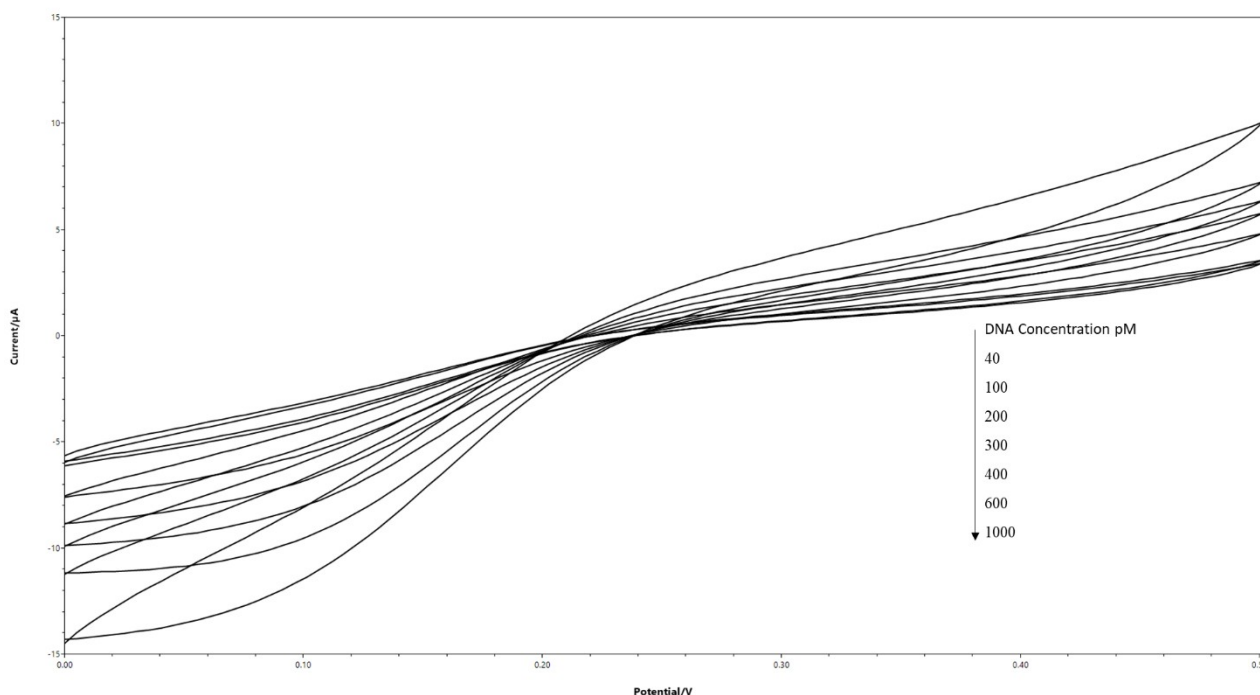
800 seconds (Figure 4). Therefore, detection time was determined to be 100 seconds, and this time was chosen for the determination of the DNA detection time.



**Figure 4:** Chronoimpedance curves of detection of the sickle cell anemia DNA. Red sickle cell anemia, blue blank solution (PBS).

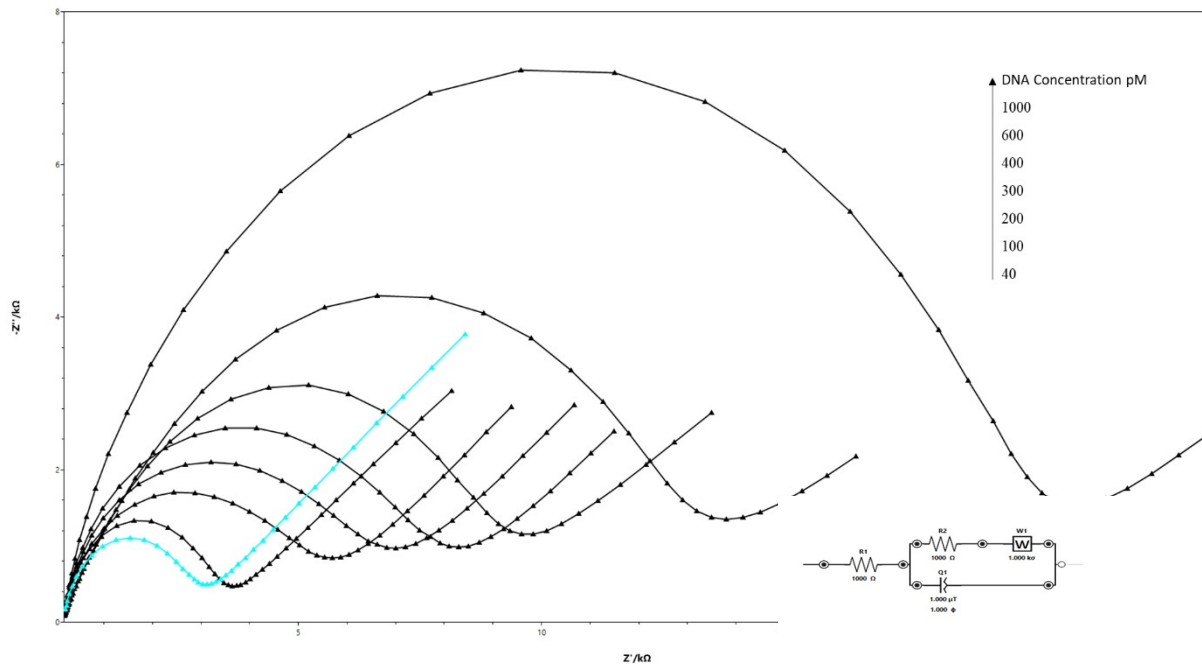
Afterwards, the calibration curve was prepared with the data of the electron transfer resistance of the biosensor, which was calculated by fitting EIS data

onto a proper circuit model (9). The biosensor showed good linearity between 40-1000 pM DNA (400 bp) and  $R^2=0.9930$ .

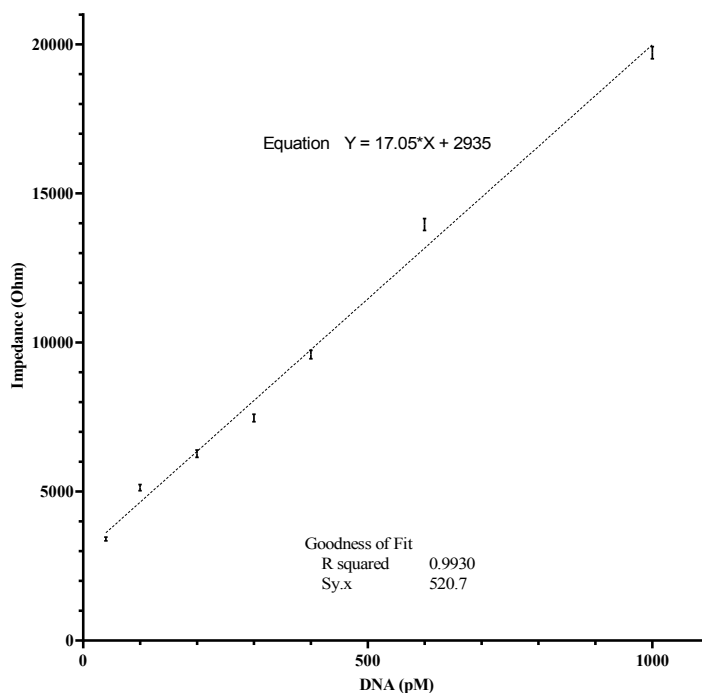


**Figure 5:** Representation of the calibration curve with CV.





**Figure 6:** Representation of the calibration curve with EIS.



**Figure 7:** Calibration curve of the biosensor (y-axis impedance, x-axis concentration of the DNA).

Sy.x, given in Figure 7, is the abbreviation of the Standard Error of Estimate. If this value is large, it indicates the goodness of fit value. It shows the agreement between the measured values and the theoretical values.

Optimization and characterizations are all performed using EIS in a redox probe solution. Reproducibility was also tested with different calibration curves and

$R^2=0.9930\pm 0.0027$ . Therefore, reproducibility is high because of the stability of self-assembly monolayer and the graphene layers. In order to find out the lower limit of detection (LOD) and lower limit of quantification, 40 pM concentration was tested and calculated as  $3412\pm 64.59$  ohm. LOD and LOQ were calculated as 12.50 pM and 37.88 pM, respectively. Performance parameters were shown in Table 1.

**Table 1:** Sickle cell anemia biosensor performance parameters.

Parameter		Performance	
Linearity		40-1000 pM	
LOD		12.50 pM	
LOQ		37.88 pM	
Reproducibility		R <sup>2</sup> =0.9930±0.0027 (2.7%)	
Repeatability and Recovery	Added	Found	C.V %
	80 pM	87 ± 6 pM	6.90
	200 pM	224 ± 11 pM	7.74
	900 pM	940 ± 21 pM	10.11

Methods such as Complete Blood Cell Count, Peripheral Blood Smear, Hemoglobin Electrophoresis, Isoelectric Focusing, High Performance Liquid Chromatography, and Polymerase Chain Reaction (PCR) are used to determine sickle cell anemia (18). These methods are time consuming and expensive. Also, the prepared biosensor is more feasible and sensitive than PCR to diagnose sickle cell anemia.

## CONCLUSION

In conclusion, in this study, a biosensor system was developed to detect the point mutation that causes sickle cell anemia. A gold electrode was used as working electrode and modified with 4-ATP and ammonium functionalized graphene oxide for sensitive measurement of the point mutation. Detection time was determined with chronoimpedimetric measurement. The biosensor showed good linearity between 40 and 1000 pM. As a result, a very selective and specific CRISPR-dCas9 based biosensor system for the determination of sickle cell anemia point mutation was developed and reported successfully. This study represents the easy to use, low cost and selective Sickle Cell DNA detection method. Moreover, there is no need for pre-sample applications and modifications. It can easily be used to detect sickle cell anemia.

## CONFLICT OF INTEREST

The author declares no conflict of interest.

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