Electroanalysis

WILEY-VCH

ELECTROANALYSIS
An International Journal Devoted to Electroanalysis, Sensors and Bioelectronic Devices

RESEARCH ARTICLE OPEN ACCESS

Molecularly Imprinted Nanoparticle-Based Polymer (Acrylamide-N-Vinyl imidazole)/Nafion Film Modified Screen-Printed Electrode System for Rapid Determination of Hemoglobin

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Received: 17 July 2024 | **Revised:** 24 August 2024 | **Accepted:** 28 August 2024

Keywords: electrochemical sensor | hemoglobin | molecularly imprinted polymer | nafion

ABSTRACT

In medical diagnostics, variations in hemoglobin levels can reveal a variety of prevalent health conditions. Abnormal hemoglobin is known to be connected with diseases like anemia, diabetes, hematemesis, hematuria, and hemoglobinuria. Consequently, there is a significant demand for advanced detection technologies and precise methodologies to accurately track and assess hemoglobin levels. This study demonstrates the potential of a novel molecularly imprinted nanoparticle-based sensor system to rapidly analyze hemoglobin levels without the need for a laboratory environment. Hemoglobin imprinted-poly(acrylamide-*co*-vinyl imidazole) [Hb-imp-p(AAm-*co*-VIM)] nanoparticles with high affinity and selectivity for hemoglobin were synthesized and loaded onto the surface of a screen-printed carbon electrode (SPCE) with a nafion nanofilm. Nafion has selective ion exchange properties and allows for the enhancement of the electrochemical signal. An increase in signal was observed in the presence of 0.5% Nafion. The Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE system was employed as the sensor surface for the detection of hemoglobin levels in blood. The Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE system was characterized by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements. The linear working range for hemoglobin was observed to be 0.73–15.54 μM (R^2: 0.9934), with a calculated limit of detection (LoD) value of 0.24 μM (3.3 S/N). The sensor system exhibited high efficiency at the biological pH value of 7.4. Furthermore, percent recovery values for hemoglobin in blood samples were observed to be between 90.34% and 103.68%. To determine the selectivity of the Hb-imp-p(AAm-co-VIM)/Nafion-SPCE electrode system, the current values of the system were investigated in the presence of ascorbic acid, cysteine, glucose, and IgG. The system exhibited high selectivity. Based on the data obtained, it is evident that the Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE system can detect hemoglobin in biological environments and has the potential for use in disease monitoring systems.

1 | Introduction

Often referred to as "globins," hemoglobin (Hb) and related heme proteins (such as myoglobin; Mb) are respiratory proteins that reversibly bind gaseous diatomic ligands (such as O_2) via a porphyrin ring contains iron, crucial for the binding and transport of O_2 [1]. Globins have been found in various organisms across all families, including bacteria, protists,

plants, fungi, and mammals. Humans have been found to possess four different forms of globins: myoglobin (Mb), neuroglobin (Ngb), cytoglobin (Cygb), and hemoglobin (Hb) [2–4]. Over the years, Hb has been the subject of extensive research and has garnered the most significant attention among the globin family [5]. Hb performs vital biological functions. With a molecular weight of 64.5 kDa, hemoglobin is a significant constituent of red blood cells (RBCs) [6]. The heme or iron groups, also known as bound prosthetic groups, are essential for the rapid binding of oxygen in the lungs and its reversible delivery to the tissues [7]. Additionally, the heme group contributes to the unique color of muscles and blood [8].

Hemoglobin (Hb) diseases are generally divided into two major types as follows: 1) quantitative fault in the production of one of the globin subunits, known as thalassemia syndromes, 2) structural defect in one of the globin subunits [9]. The World Health Organization (WHO) states that the average Hb level for men (15 years of age and older) should be 130.0 g/L (~8.07 mmol/L) or higher, 120.0 g/L (~7.45 mmol/L) or higher for non-pregnant women (15 years of age and older), and 110.0 g/L (~6.83 mmol/L) or higher for pregnant women [5, 10]. Variations in hemoglobin levels can precipitate the onset of certain pathological conditions. For instance, anemia, characterized by diminished oxygen-carrying capacity of blood, results in tissue hypoxia. Furthermore, anemia can be defined by reduced hemoglobin concentrations (below 13.5 g/dL (~8.38 mmol/L) (in males and 12.0 g/dL (~7.45 mmol/L) in females) or decreased hematocrit values (less than 41.0% in males and 36.0% in females), or a decline in red blood cell count [43]. Hematocrit terminology is more commonly used in daily clinical practice than red blood cell (RBC) count. Additionally, there are different lower limits for the normal range based on ethnic origin, gender, and age [43]. In cases of anemia, varying hemoglobin levels can range from mild (below 10.0 g/dL (~6.21 mmol/L)) to moderate (between 8.0 (~4.97 mmol/L) and 10.0 g/dL(~6.21 mmol/L)), severe (between 6.5 (~4.03 mmol/L) and 7.9 g/dL (~4.89 mmol/L)), and even life-threatening (below 6.5 g/dL (~4.03 mmol/L)) [44].

These results show that, in clinical applications, hemoglobin concentrations are closely associated with various diseases typically present in the human body. Several illnesses, including anemia, diabetes, hematemesis, hematuria, and hemoglobinuria, are linked to hemoglobin abnormalities [11]. Therefore, sensitive and accurate detection platforms, along with appropriate methods, are required to monitor concentrations of the protein marker hemoglobin. This monitoring is vital for early diagnosis and reliable prediction of diseases.

Developing and implementing fast, reliable, sensitive, selective, and economically attractive analysis methods are critical. In this context, biosensors offer advantages over traditional analysis methods [12]. Biosensors interact selectively with the target analyte using a biologically active identifier and then convert the result of this interaction into a measurable signal [13, 14]. Electrochemical biosensors allow the measurement of the electroactive signal released in the bio-interaction process involving electrochemical species [15]. Additionally, electrochemical sensors are among the most widely used biosensors due to their small, portable, and cost-effective nature [16]. Electrochemical methods are techniques used to study redox reactions and charge transfer at the electrode-solution interface. Modified electrodes are electrodes altered with coatings or materials to enhance performance. These modifications can include nanoparticles, polymers, or biological molecules. The advantages of modified electrodes include increased sensitivity for detecting low concentration analytes, improved selectivity towards specific analytes, enhanced stability and reproducibility of measurements, and the ability to tailor electrode properties for various applications [54, 55]. At this point, the presence of more sensitive and selective systems allows the emergence and preparation of measurement systems providing different applications every day, especially for the diagnosis and treatment of diseases [17, 18].

Significant progress has been made in sensor technology with the use of nanoscale materials. Nanomaterials increase the electroactive surface area due to their high surface area/volume ratios [19–21]. After functionalization of nanoparticles with affinity ligands, they can play significant roles in analysis and diagnostic applications [22]. For bioapplications of particles, affinity ligands must interact with the surface. Binding affinity ligands to particles enables the selective binding of biological targets to the particle surface in chemically complex mixtures [23]. Currently, systems prepared with molecular imprinting technology can provide exceptional results in meeting this need [24, 25]. Molecularly imprinted polymers (MIPs) are very suitable for molecular recognition as they have more binding regions than one or more binding regions of biological receptors [26, 27]. Moreover, due to their chemical stability against various environmental conditions, they are durable and can be preserved for a long time [28].

As mentioned above, detection of hemoglobin in blood samples using electrochemical sensors based on protein-imprinted nanomaterials is necessary. This is because a hemoglobin concentration above established limits can lead to serious disorders such as anemia or polycythemia. The World Health Organization (WHO) defines anemia when the hemoglobin concentration is less than 12.0 g/dL (~0.186 mmol/L) for women and greater than 13.0 g/dL (~0.201 mmol/L) for men [29, 30].

This proposed study demonstrates the detection of change in hemoglobin levels in blood using a Hb-imprinted nano polymer-based electrochemical biosensor. This allows changes in hemoglobin levels to be detected cheaply, quickly, sensitively, and specifically. Additionally, the synthesized novel polymer for the sensor contains acrylamide (AAm), which is preferred as the main monomer due to its high electrophilic reactivity and minimal toxicity. In addition, N-Vinyl imidazole (VIM), which is preferred for its chelation properties with metals, has been selected as a functional monomer without using any coordinating metal group to facilitate complex formation with Hb. It also assumes the role of a pseudo-specific ligand, and it is anticipated to increase its interaction with Hb chromatographically [42]. From this perspective, the originality and selectivity function of the used nanopolymer system have been enhanced. The study has the potential of a methodology that utilizes MIP-based sensor systems to rapidly analyze hemoglobin levels in blood without the need for a laboratory environment.

2 | Experimental

2.1 | Material and Reagents

All chemicals were of the highest purity available. The synthesis of Hb-imp-p(AAm-*co*-VIM) nanoparticles involved the use of various chemicals obtained from different sources. Hemoglobin (Hb) was obtained from bovine blood. The following chemicals were utilized in the synthesis process: Acrylamide (AAm), N-Vinyl imidazole (VIM), Polyvinyl alcohol (PVA), Sodium dodecyl sulfate (SDS), Sodium bicarbonate ($NaHCO_3$), Ethylene glycol dimethacrylate (EGDMA) (98% w/w), Ammonium persulfate (APS), Sodium bisulfite ($NaHSO_3$), Sodium chloride (NaCl), and for the solution used in sensor studies, Potassium chloride (KCl), Potassium hexacyanoferrate (III) $K_3[Fe(CN)_6]$, Potassium hexacyanoferrate (II) trihydrate $K_4[Fe(CN)_6]$, Potassium dihydrogen phosphate (KH_2PO_4). The reagents for selectivity studies included IgG (95% w/w) obtained from human serum, glucose (99.5% w/w), cysteine (97% w/w) and ascorbic acid are purchased from Sigma-Aldrich. Nafion 117 (5.0% w/v) solution was sourced from Chemours. All chemicals used were of analytical purity.

2.2 | Instrumentation

All electrochemical analyses were conducted using a Potentiostat/Galvanostat (Palm Sens 4) equipped with a screen-printed carbon electrode (SPCE) system. The SPCE was modified with an Hb-imp-p(AAm-*co*-VIM)/Nafion film and served as the working electrode.

Various laboratory equipment was utilized in the preparation of nanopolymers, including a shaking water bath (Memmert, WiseBath WSB-30), centrifuge (Centrion Scientific Benchtop centrifuges K2015R and Beckman Coulter Avanti centrifuge J–E), precision balance (KERN&Sohn GmbH ABS220-4, 0.1 mg accuracy), pH meter (ISTEK, NeoMet 240-L), oven (Memmert, UNB400, Germany), and a heated magnetic stirrer (Dragon Lab, MX–F). A detailed description of the instrumentation for nano polymer preparation can be found in our previous publication [31–33].

2.3 | Synthesis of Imprinted Polymeric Nanoparticles (MIP) and Non-imprinted Polymeric Nanoparticles (NIP)

Hemoglobin (Hb)-imprinted polymeric nanoparticles were prepared through the following process: Initially, the polymerization was conducted in a biphasic mixture of water and organic phases. In the water phase, a mixture of PVA (143.0 mg), SDS (64.0 mg), and sodium bicarbonate (12.5 mg) was obtained by stirring 110.0 ml of water at 60°C. In the organic phase, AAm (70.0 mg) and EGDMA (1.0 mL) were prepared. The Hb (10 mg)-VIM (271.5 μL) pre-complex was added to the organic phase. The two phases were stirred at 37°C and 500 revolutions per minute using a magnetic stirrer. Subsequently, the mixture was exposed to a nitrogen gas atmosphere for 15 min. Following this step, polymerization continued at 37°C for 24 h by adding 100.0 mg of APS and 50.0 mg of $NaHSO_3$ as polymer initiators (Figure 1) and detailed procedures have been thoroughly described in previous studies [42]. The resulting Hb imprinted nanoparticles were washed several times with water and methanol to remove residual monomers, surfactants, and initiators. After the washing process, the template molecule Hb was removed from the polymeric structure using a 0.5 M NaCl solution. The cleaned nanoparticles were redispersed in deionized water and stored at +4°C. Template-free p(AAm-*co*-VIM) nanoparticles (NIP) were prepared in the polymerization environment using the same recipe, but without the template molecule (Hb). The detailed synthesis procedure has been previously published by the project team [42].

2.4 | Preparation of Hb-imp-p(AAm-co-VIM)/Nafion-SPCE

Under optimized conditions, the modified screen-printed carbon electrode (Hb-imp-p(AAm-*co*-VIM)/Nafion SPCE) was prepared using the imprinted polymer. A suspension of Hb-imp-p(AAm-*co*-VIM) polymer was created in a 0.5% Nafion solution at a total concentration of 0.6 mg/mL. Fifteen microliters of the obtained Nafion-polymer mixture were dropped onto the electrode surface, and a film was formed by incubating it in the dark at room temperature for 2 h (Figure 2). The modified Hb-imp-p(AAm-*co*-VIM)/Nafion SPCE system was then stored at +4°C for later analyses.

3 | Results and Discussion

3.1 | Electrochemical Characterization

3.1.1 | Detection of the Optimum Scan Rate

Hb-imp-p(AAm-co-VIM)/Nafion-SPCE′s electrochemical behavior was investigated in the potential range of −0.20 to +0.80 V using 5.0 M $Fe(CN)_6^{3-/4-}$ in 0.1 M PBS with scan rates (ν) ranging from 10 to 100 mVs^{-1} (Figure 4A). The $Fe(CN)_6^{3-/4-}$ system exhibited a reversible single-electron oxidation. Additionally, it was observed that the oxidation and reduction peaks increased linearly within the studied scan rate range. Thus, the scan rate at which the modified SPCE electrode could operate without experiencing diffusion issues was illuminated. For achieving both fast analysis and high accuracy in experimental studies, the optimal scan rate was chosen as 60 mVs^{-1}.

In a diffusion-controlled system, the observed CV (Cyclic Voltammetry) response is determined above the potential scan rate because it determines the oxidation rate of $Fe(CN)_6^{4-}$ to $Fe(CN)_6^{3-}$. Investigation of the diffusion coefficient is a crucial factor. As shown by the Randles-Sevcik equation (Equation 1), the current is directly proportional to concentration and increases with the square root of the scan rate in diffusion-controlled systems [49]. Determining the charge transport rate is essential to understand the conversion of $Fe(CN)_6^{4-}$ to $Fe(CN)_6^{3-}$ in the solution. The charge transport rate can be quantified by measuring D_{ct} (apparent diffusion coefficient charge transport rate) using CV. In theory, the diffusion coefficient acts as a link between the molar flux resulting from

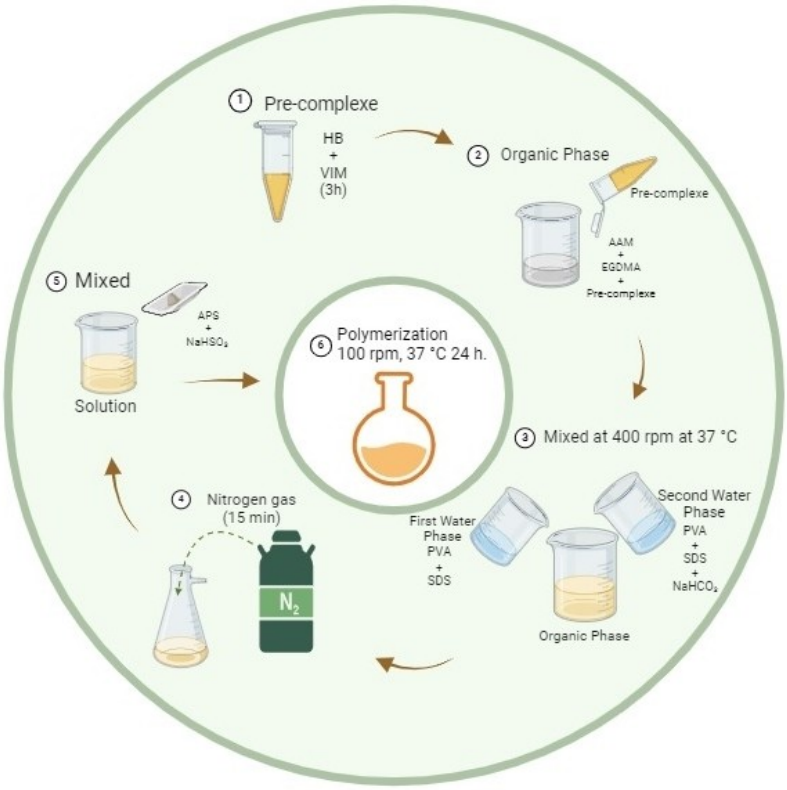

FIGURE 1 | Schematic illustration of imprinted polymeric nanoparticles (MIP-NP).

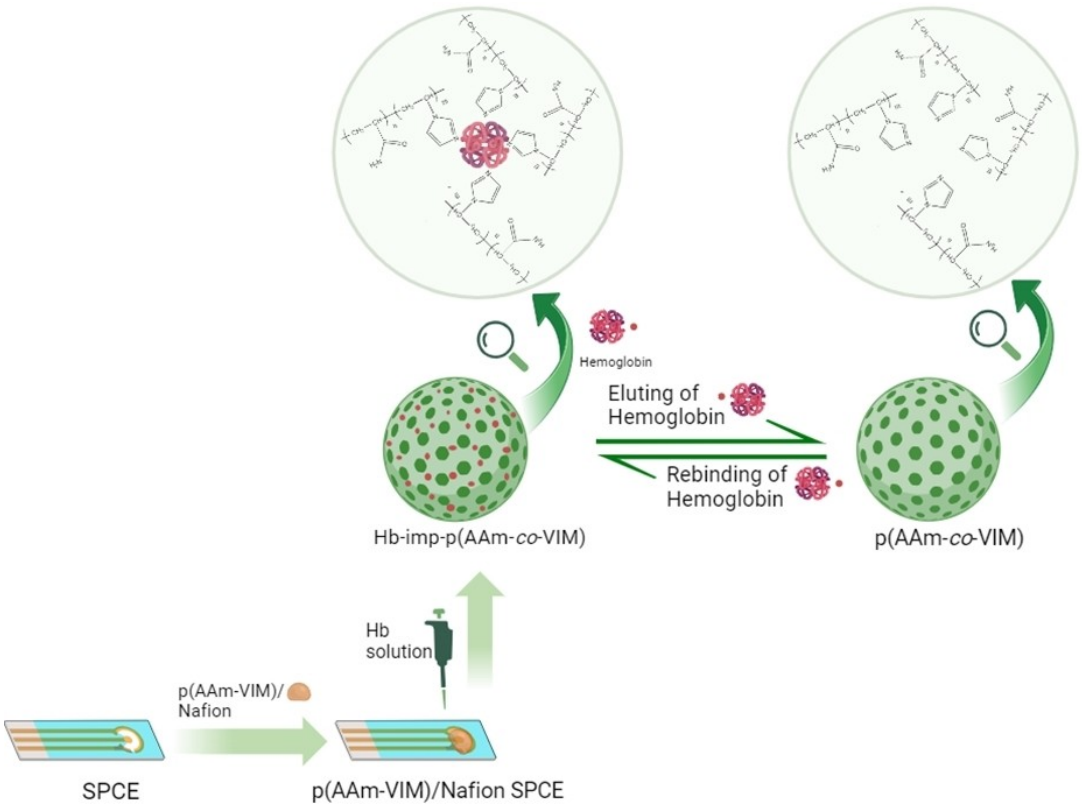

FIGURE 2 | Schematic illustration of the removal and rebinding of the template molecule from hemoglobin-imprinted polymeric nanoparticles.

molecular diffusion and the concentration gradient of the species [51]. A higher diffusion coefficient implies a quicker rate of diffusion between $Fe(CN)_6^{4-}$ to $Fe(CN)_6^{3-}$. This parameter can typically be obtained from an i_p vs. $\nu^{1/2}$ plot under diffusion-controlled conditions, which is often preferred by higher scan rates. In this study, these conditions were met in the range of 10 to 100 mVs^{-1}, and typical cyclic voltametric scan rate dependencies and Randles-Sevcik plots, where the peak current changes linearly with the square root of the scan rate in this range, are shown in Figure 4A.

$$i_p = 2.65 \times 10^5 n^{3/2} A D_{ct} \nu^{1/2} C \quad (1)$$

Using Equation (1), the D_{ct} values for CV results at different scan rates in Figure 3 were calculated ($D_{ct\text{-cathodic}}$ and $D_{ct\text{-anodic}}$). The linear relationship between the D_{ct} values obtained from varying scan rates is shown in Figure 3. As observed, nafion and polymer modified SPCE electrode exhibits linear changes in oxidation and reduction peaks within 5 mM Fe(CN) solution, solely based on the scan rate (ranging from 10 to 100 mV/s).

This behavior indicates that the modified SPCE electrode allows the molecules to diffuse into the polymer without encountering diffusion problem. In instances where there is no linear relationship between the D_{ct} values, which are obtained from variations in the scan rate, it indicates that the polymer on the electrode surface restricts the passage of molecules. This leads to irregular diffusion. This prevents the electrochemical signal (current) from increasing or decreasing linearly in response to changes in concentration [49]. The absence of a linear D_{ct} relationship makes the modified SPCE unreliable and unsuitable for determining hemoglobin concentration. According to the results from Figure 3, D_{ct}(Cathodic) and D_{ct}(Anodic) are identical in this study suggests that the density of the redox centres is uniform throughout the layer thickness.

The $D_{ct\text{-cathodic}}$ and $D_{ct\text{-anodic}}$ values have been calculated as $5.34\times10^{-8}\ cm^2s^{-1}$ and $5.16\times10^{-8}\ cm^2s^{-1}$, respectively (from Figure 3). The D_{ct} values observed in this study are identical ($D_{ct\text{-cathodic}}$ and $D_{ct\text{-anodic}}$). This data indicates that $Fe(CN)_6^{4-}$ and $Fe(CN)_6^{3-}$ centres can transport in the solution, leading to the conversion of $Fe(CN)_6^{4-}$ to $Fe(CN)_6^{3-}$ and vice versa. Also, the data shows the electrode system is electrochemically reversible. Electrochemically reversible systems possess the feature of multiple reusability [51]. The obtained D_{ct} values demonstrate that the designed sensor system has a high potential for reusability.

Therefore, it is clean that molecules (conversion of $Fe(CN)_6^{4-}$ to $Fe(CN)_6^{3-}$ in the solution) can pass through the nafion polymer film on the SPCE without experiencing diffusion problems, and the designed system allows for peak changes based on the scan rates studied. In the interpretation of D_{ct} values in Nafion film-based studies in the literature, it has been observed that the variation is based not on the Nafion film loading of the system but rather on the concentration of the electrolyte used [53]. The D_{ct} values were found to occur independently of the electrode surface loaded with nafion and polymer, and only varied depending on the concentration of $Fe(CN)_6^{4-}$ and $Fe(CN)_6^{3-}$. Thus, it is understood that the D_{ct} values in our study can be used for the determination of reversible and low-concentration Hb. These results demonstrate that the developed modified SPCE electrode can operate stably, providing a reliable system for determining hemoglobin concentration.

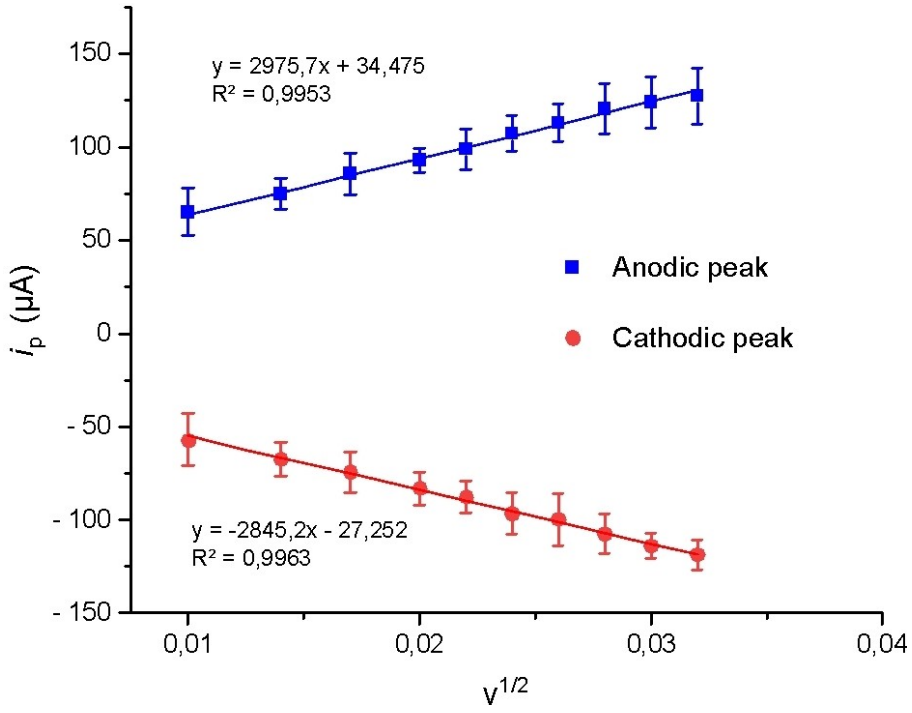

FIGURE 3 | Effect of increasing scan rate (from 10 to 100 mVs^{-}) on anodic and cathodic peak current from CVs in Figure 4A.

3.1.2 | The Influence of Different Surface Modifications on Sensor Performance

The scan rate, nafion concentration, Hb-imp-p(AAm-*co*-VIM) concentration, and film thickness at the obtained optimal values were observed for each surface modification through CV measurements. As seen in Figure 4B, the current value of the bare SPCE electrode increased with the addition of 0.5% nafion. This increase in current values is an expected change due to the conductive nature of nafion polymer [34, 50, 51]. When 0.6 mg/mL of Hb-imp-p(AAm-*co*-VIM) polymer was added to the electrode surface, a decrease in current values was observed, and this decrease is expected due to the non-conductive nature of the polymer. The modification of the Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE electrode was elucidated and monitored based on the CV results.

3.3 | Effect of Nafion Concentration

To overcome this issue, the potential use of immobilized Hb-imp-p(AAm-*co*-VIM)/Nafion films on the working electrode surface was investigated. Nafion polymers are widely used due to their resistance to chemical attacks under challenging conditions such as high temperatures and the presence of strong oxidants. Nafion is preferred over other films due to its high selectivity for large hydrophobic cations. This preference is based on the soluble proton of the Nafion polymer, allowing the inclusion of electroactive cations from the solution phase [35, 45–47].

Nafion is one of the most commonly used cation exchange polymers. The structure of Nafion includes an electrically neutral fluorocarbon backbone (polytetrafluoroethylene) and a pendant ionic group, SO_3^-, in a randomly attached side chain. Nafion has garnered special interest due to its appealing electrochemical and thermal properties such as proton transport, selective ion exchange, efficient catalyst support, and chemical stability [36, 49]. Other fluorinated commercial membranes similar to Nafion have also been developed, including Hyflon, Flemion, 3P membranes, Gore-Tex, and Gore Select. Additionally, membranes similar to Nafion but with weak acid groups $-COOH$ instead of $-SO_3H$ can be found, such as Aciplex [34, 45–47].

Hb-imp-p(AAm-*co*-VIM)/Nafion film solution was prepared using Nafion 117 solution containing methanol (ranging from 0.1 to 1% (w/v)). Subsequently, 15 µL of these methanol-containing solutions were applied to the surface of each SPCE. The solution on the electrode surface was allowed to dry for 2 h at room temperature. The SPCEs were then stored at +4°C for later use in the analysis. When examining the Differential Pulse Voltammetry (DPV) results at a scanning rate of 60 mV/s within the potential range of −0.20 to +0.60 V, the highest signal was observed at the concentration of 0.5% Nafion (Figure 5). Beyond the 0.5% Nafion concentration, a decrease in current signals was presumed due to the diffusion problem of $Fe(CN)_6^{3-}/^{4-}$ ions. These observed values were consistent with the literature [34, 45, 50, 51].

3.4 | Effect of Polymer Concentration

The characteristic low conductivity of the designed Hb-imp-p(AAm-*co*-VIM) polymer results in a relatively decreased overall conductivity of the electrode system when loaded onto the electrode surface. Therefore, it is essential to investigate the amounts of polymer loaded onto the electrode surface in order to find the working concentrations that yield the best current values. Polymer concentrations ranging from 0 to 1 mg/mL of Hb-imp-p(AAm-*co*-VIM) were loaded onto the electrode surface with the addition of 0.5% Nafion polymer. As shown in Figure 6, a concentration of 0.6 mg/mL Hb-imp-p(AAm-*co*-VIM) was determined as the optimum value, providing both a sufficient active area for the determination of hemoglobin levels and the best observation of electrochemical signal (current value).

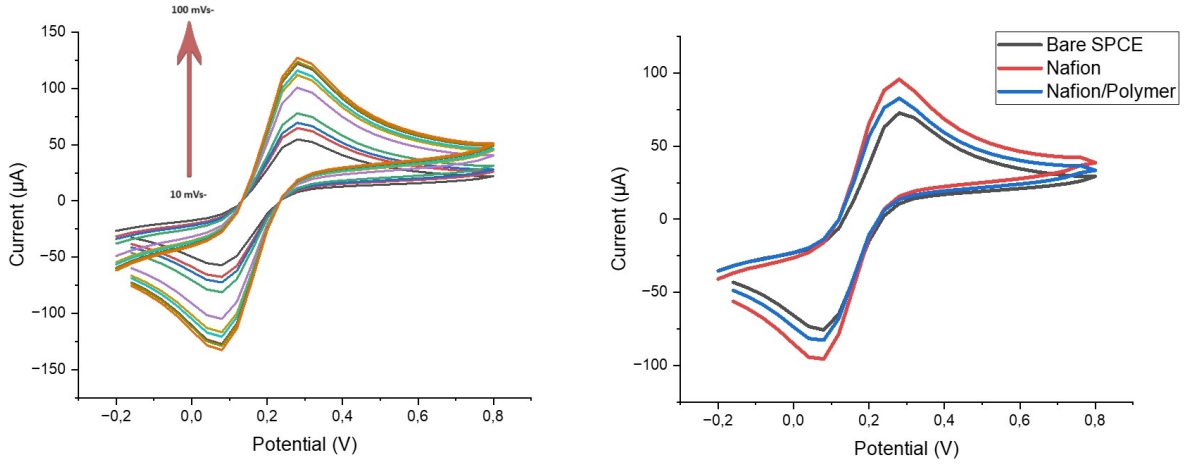

FIGURE 4 | A) Cyclic Voltammograms recorded at scan rates of 10 to 100 mVs^-, B) CVs of Bare Screen-Printed Carbon Electrode (SPCE), Nafion/SPCE, and Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE in 5 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M PBS.

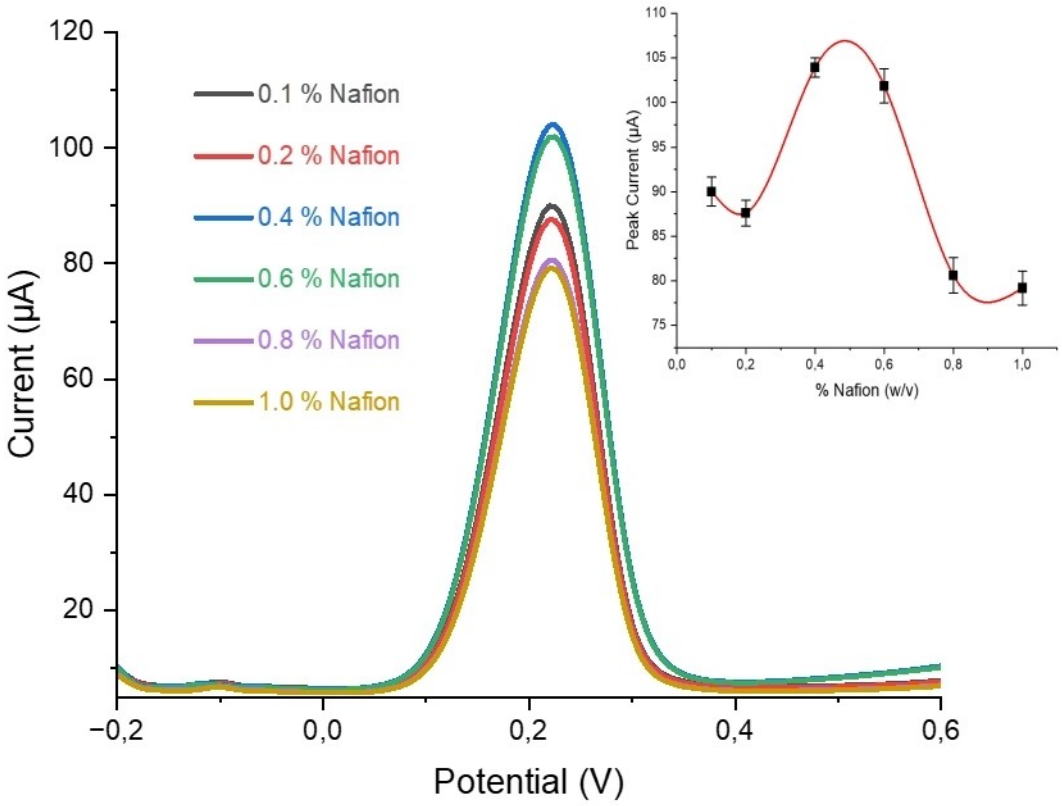

FIGURE 5 | DPVs of 5 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M PBS were recorded using various concentrations of Nafion ranging from 0.1% to 1%.

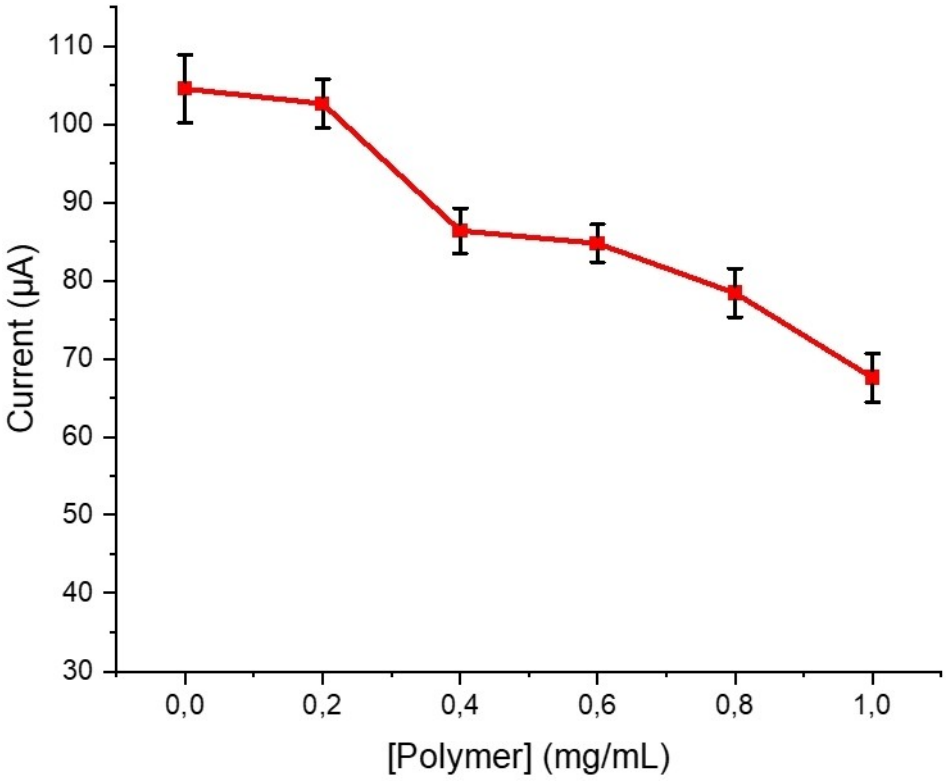

FIGURE 6 | DPV peak values of Hb-imp-p(AAm-*co*-VIM)/Nafion-modified SPCE in $Fe(CN)_6^{3-/4-}$ in 0.1 M PBS were recorded using varying concentrations of Hb-imp-p(AAm-*co*-VIM) (ranging from 0 to 1 mg/mL).

3.5 | Effect of Nafion Film Thickness

The thickness of nafion film directly effects current peak and repeatability [48, 50–52]. To achieve the best performance of the Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE electrode, the optimization of the film thickness to be used has been investigated. Solutions containing 0.5% Nafion and 0.6 mg/mL Hb-imp-p(AAm-*co*-VIM) polymer in volumes of 5, 10, 15, and 20 μL were applied to the SPCE electrode surface. As seen in Figure 7, the best current value was observed when a 15 μL solution was applied. A decrease in current values was observed at the 20 μL mark, indicating diffusion problems of $Fe(CN)_6^{3-/4-}$ ions due to film thickness, slowing down the reaction. Therefore, for the designed Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE, the optimum film thickness was selected as 15 μL.

In the literature, Nafion/platinum modified electrode for detection of trace iron was developed. The Pt electrodes coated with various Nafion films underwent electrochemical measurements in an electrolyte containing 100 ppb Fe(III). As the Nafion film thickness increased from 70 to 210 nm (detected by AFM), the signal response also increased. However, despite providing the highest current, the 210 nm Nafion film exhibited poor measurement repeatability. In contrast, the 90 nm film not only delivered relatively high current but also demonstrated excellent repeatability [45]. Our results also exhibited a similar trend based on Nafion volume. There was an increase in DVP response till 15 μL of % 0.5 nafion solution. For 20 μL of nafion solution, a decline in DPV response has been observed (Figure 7).

3.6 | pH Effect

The modified Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE electrode system aims to detect hemoglobin at biological levels in human blood. To elucidate the working performance of our system at biological pH values, experiments were conducted in the pH range of 6.0 to 9.0. A 10 mM PBS buffer was used for pH values between 6.0 and 8.0, while a Na_2CO_3-$NaHCO_3$ buffer was employed for pH 9.0. As shown in Figure 8, the modified Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE electrode system exhibited the highest current values in the pH range of 7.0 to 7.5. This range is notably compatible with the physiological pH value of 7.4. Furthermore, relatively high current values were observed at pH 6.5 and above, indicating that the electrode system operates with high efficiency over a broad pH range.

3.7 | Analytical Performance of Hb-imp-p(AAm-co-VIM)/Nafion-Modified SPCE

Under the obtained optimal conditions, the analytical performance of the Hb-imp-p(AAm-*co*-VIM)/Nafion-modified SPCE system was elucidated in the presence of different hemoglobin concentrations. To perform analytical analyses, eleven spikes were prepared with Hb concentrations of 0.5, 1.0, 2.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, and 14.0 μM. The resulting DPV current values were statistically calculated, and the graphs in Figure 9 were obtained. Within the scope of the study, the Limit of Detection (LoD) and Limit of Quantification (LoQ) values were statistically determined. Under the defined optimal conditions (in 5.0 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M PBS), the LoD value for Hb-

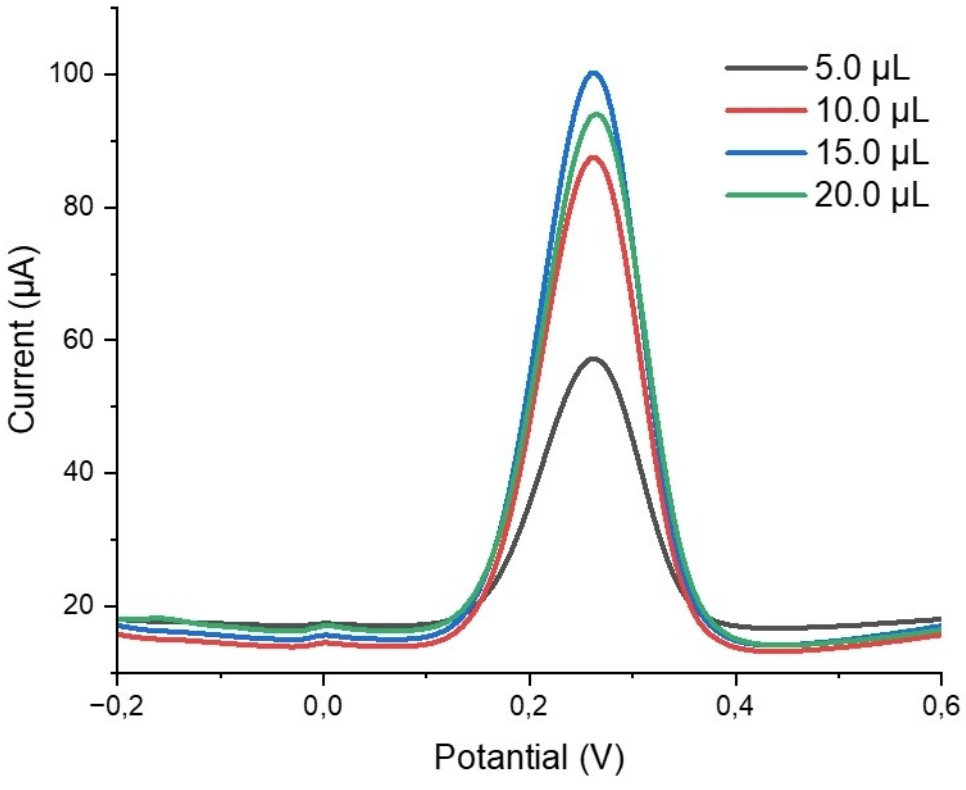

FIGURE 7 | The influence of varying volumes of Hb-imp-p(AAm-co-VIM)/Nafion, ranging from 5 to 20 μL, on the recorded DPV results in 5 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M PBS.

imp-p(AAm-*co*-VIM)/Nafion-modified SPCE was found to be 0.24 μM, and the LoQ value was 0.73 μM. The linear working range was observed to be 0.73 to 15.54 μM (R^2: 0.9934) (Figure 9).

$$[\text{Hb-Fe}^{\text{III}}]^{3+} + H^+ + e^- \leftrightarrow [\text{Hb(H)-Fe}^{\text{II}}]^{2+} \quad (2)$$

The given reaction illustrates the redox transformation associated with the iron center of hemoglobin (Hb) (Equation 2). In this transformation, the oxidized form of hemoglobin (Fe^{3+}), denoted as [Hb-Fe^{III}], converts into the reduced form of hemoglobin (Fe^{2+}), denoted as [Hb(H)−Fe^{II}], by accepting a proton (H^+) and an electron (e^-). During this process, the proton can interact with a histidine group or another ligand near the iron center. The reaction is electrochemically reversible, meaning that the hemoglobin molecule can transition between oxidation and reduction states. This allows for the observation of an electrochemical signal [54, 55].

Hb-imp-p(AAm-*co*-VIM)/Nafion-modified SPCE's analytical results were compared with published similar studies in Table 1. Upon examination of the results, the LoD value for this study was calculated as 0.24 μM, which appears to be a better value than many other results in the literature. Additionally, the observed linear working range from 0.73 μM to 15.54 μM highlights the potential use of Hb-imp-p(AAm-*co*-VIM)/Nafion-modified SPCE in human blood samples. When evaluating the results presented in Table 1, it is evident that Hb sensor systems typically exhibit LoD values ranging from 0.01 pM to 11.84 μM. The LoD value of this study (0.24 μM) indicates satisfactory results compared to many similar studies. Similarly, the linear working ranges of Hb sensor studies in the literature are observed to be between 0.01 pM to 100.0 mM and 0.50 mM to 10.0 mM levels. The linear working range of this study, however, falls between 0.73 μM and 15.54 μM. In comparison with studies in the literature, it is understood that Hb concentrations in the μM range can be effectively utilized.

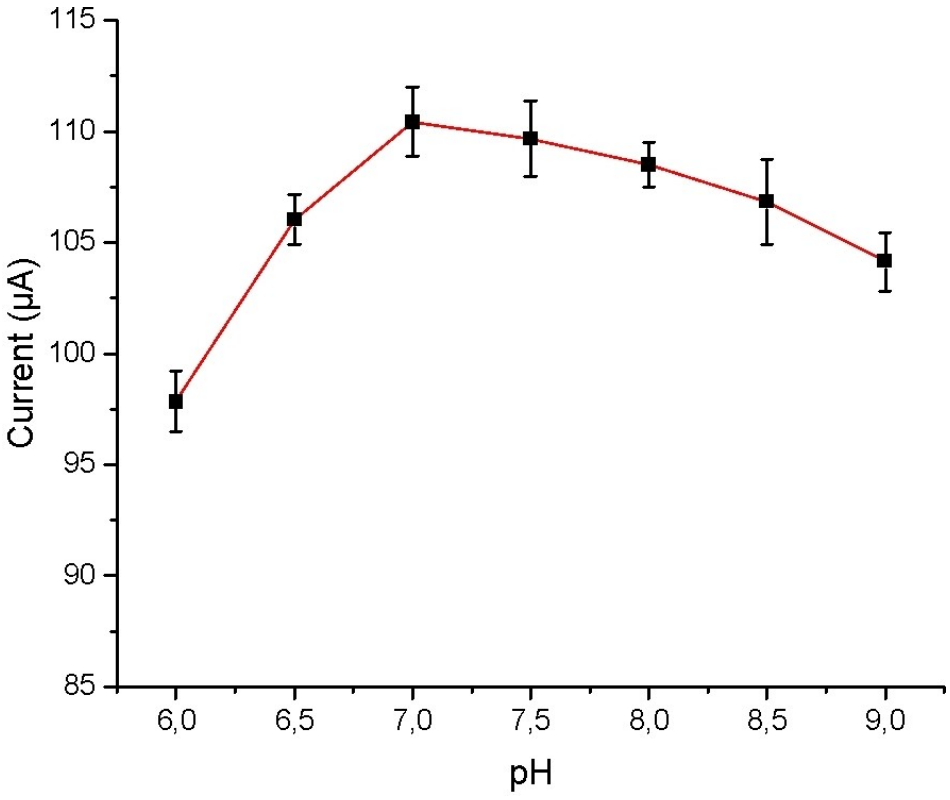

FIGURE 8 | pH effect on Hb-imp-p(AAm-co-VIM)/Nafion-modified SPCE and DPV results in 5.0 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M PBS.

TABLE 1 | Comparison of this study with previously reported voltametric hemoglobin sensor.

Electrode	Method	LoD	Linear range	Ref.
Hb-imp-p(AAm-co-VIM)/Nafion-SPCE -This study	DPV	0,24 μM	0,73 μM to 15,54 μM	–
Polarization-Differential SP	Spectrophotometry	1,1 μM	2,1 μM to 5,5 mM	[37]
NIP-NPs-MGCE	DPV	11,84 μM	0,50 mM to 10 mM	[38]
NiTe Nano-rods	CV	0,012 nM	0,025 nM to 0,9 nM	[39]
WS2-MIP/SPE	DPV	0,01 pM	0,01 pM–100 mM	[40]
GPE/rGO/Fe3O4	LSV	15 μM	100 μM to 1,80 mM	[41]

These results highlight the capability to be applied directly to biological matrices, making it ideally suited for portable and point-of-care measurements.

3.8 | Blood Sample Analysis

The performance of the designed Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE electrode system in blood samples was elucidated by conducting studies with appropriately prepared blood samples, following literature guidelines [56]. In this context, blood samples containing 2.5 mg/mL, 5.0 mg/mL, and 7.5 mg/mL hemoglobin were prepared, and the % recovery values were examined using the optimized Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE electrode system (Figure 10B). Thus, the performance of the designed modified electrode system in blood samples was detailed. When DPV measurements were examined, % recovery values for 2.5 mg/mL, 5.0 mg/mL, and 7.5 mg/

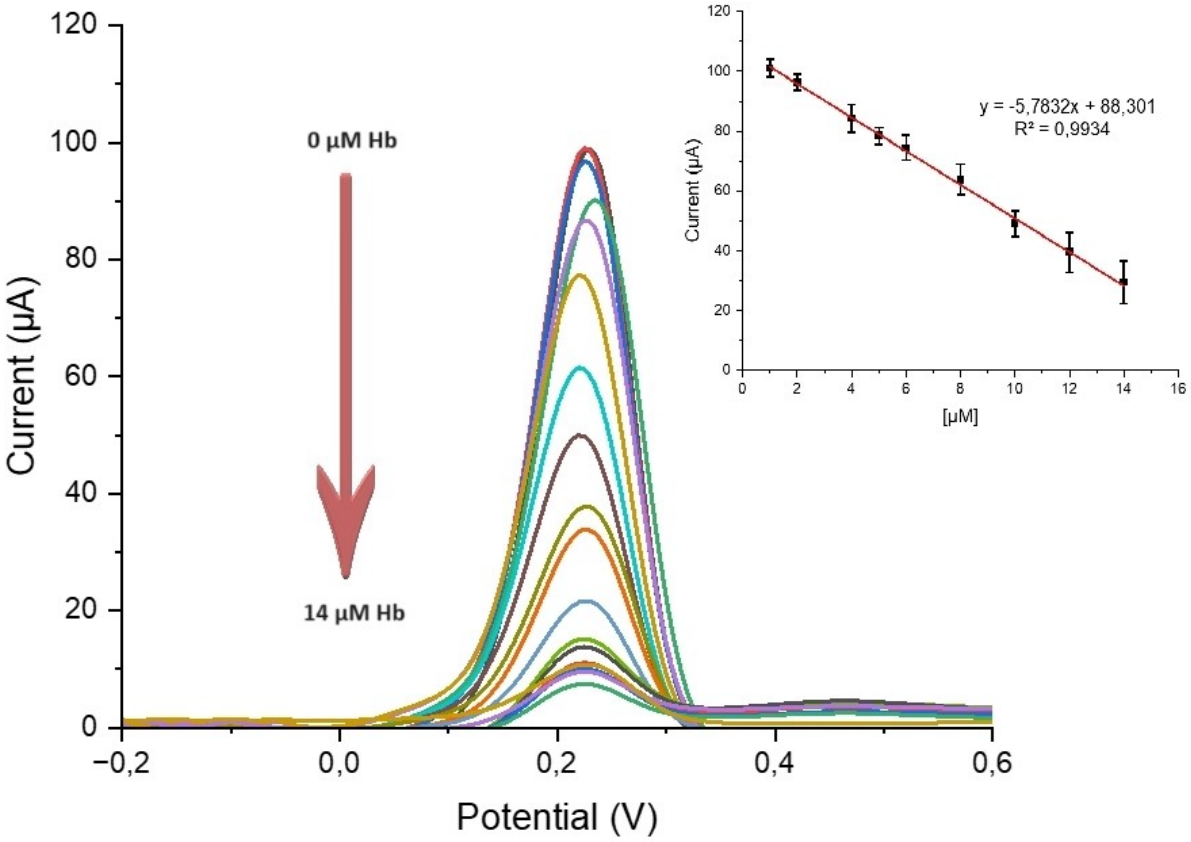

FIGURE 9 | DPV response for increasing hemoglobin in 5.0 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M PBS, pH 7.5 scanned over the potential range $-0.2 \leq E \leq 0.6$ V at a scan rate of 60 mVs^{-} and linear working range.

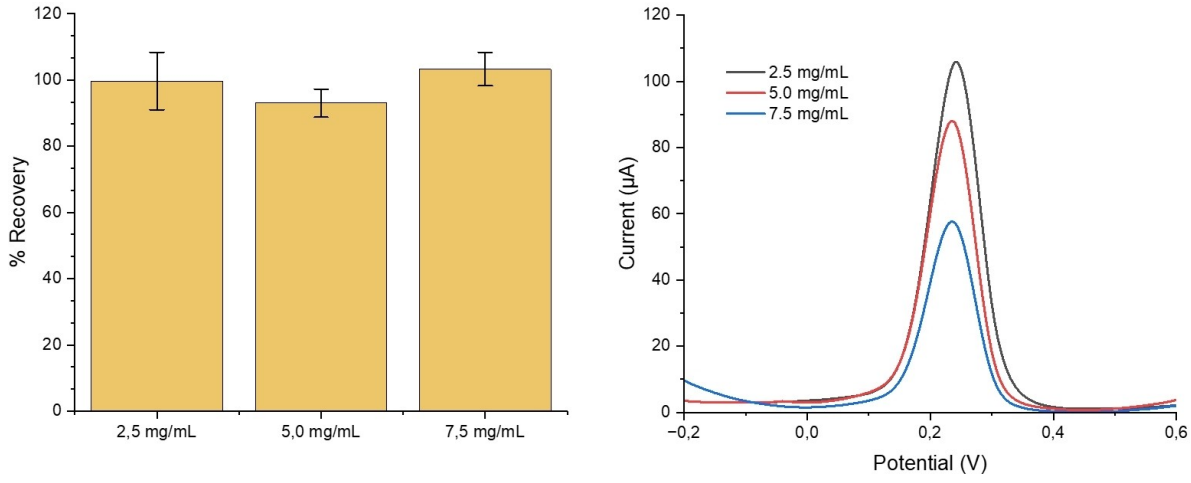

FIGURE 10 | **A)** % recovery and **B)** DPVs of different concentrations of hemoglobin in blood samples.

mL hemoglobin were observed to be % 98.6, % 90.34, and % 103.68, respectively (Figure 10A). The modified sensor system showing recovery values above 90% for hemoglobin concentrations in the linear working range indicates that the Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE electrode system can provide accurate results in real blood samples.

3.9 | Selectivity Study

In order to determine the selectivity of the Hb-imp-p(AAm-co-VIM)/Nafion-SPCE electrode system, the current values of the system were investigated in the presence of ascorbic acid, cysteine, glucose, and IgG, which are potential interferences in blood samples [40, 41]. The hemoglobin results represent real blood samples without spiking. Thus, the working performance of the designed electrode system in blood samples will be detailed. In this context, blood samples containing 2.5 mg/mL, 5.0 mg/mL, and 7.5 mg/mL ascorbic acid, cysteine, glucose, and IgG were prepared by the standard addition method. The recovery values of the optimized Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE electrode system was examined and compared with hemoglobin results (Figure 11).

When the selectivity results were examined, it was seen that the biosensor responses due to the hemoglobin selectivity of hemoglobin imprinted nano polymers; It has been found that it is much more selective than the biosensor responses obtained in the case of ascorbic acid, cysteine and glucose molecules. As a result of this, the effectiveness of the molecular imprinting method comes into play here, and the highest selectivity of our Hb-imprinted polymer is reported in this study, showing specificity for Hb in blood samples.

3.10 | Repeatability

In order to assess the reusability of the designed SPCE electrode system, repeated DPV measurements were performed on a stable blood sample with a constant hemoglobin concentration under the optimized conditions. After 20 repeated DPV measurements, the sensor system retained 85.52% of its initial current value, as illustrated in Figure 12. A comparison with the literature indicates that the designed sensor system exhibits a remarkably good number of reusability cycles [37, 41]. This finding underscores its commercial potential and usability of the developed sensor, making it suitable for repeated applications and providing reliable results over multiple measurements.

4 | Conclusion

This study underscores the importance of cost-effective sensor systems for monitoring disease groups, necessitating consistent and thorough surveillance. Here, we demonstrated the potential of molecularly imprinted nanoparticle-based sensor systems for rapid hemoglobin level analysis without laboratory

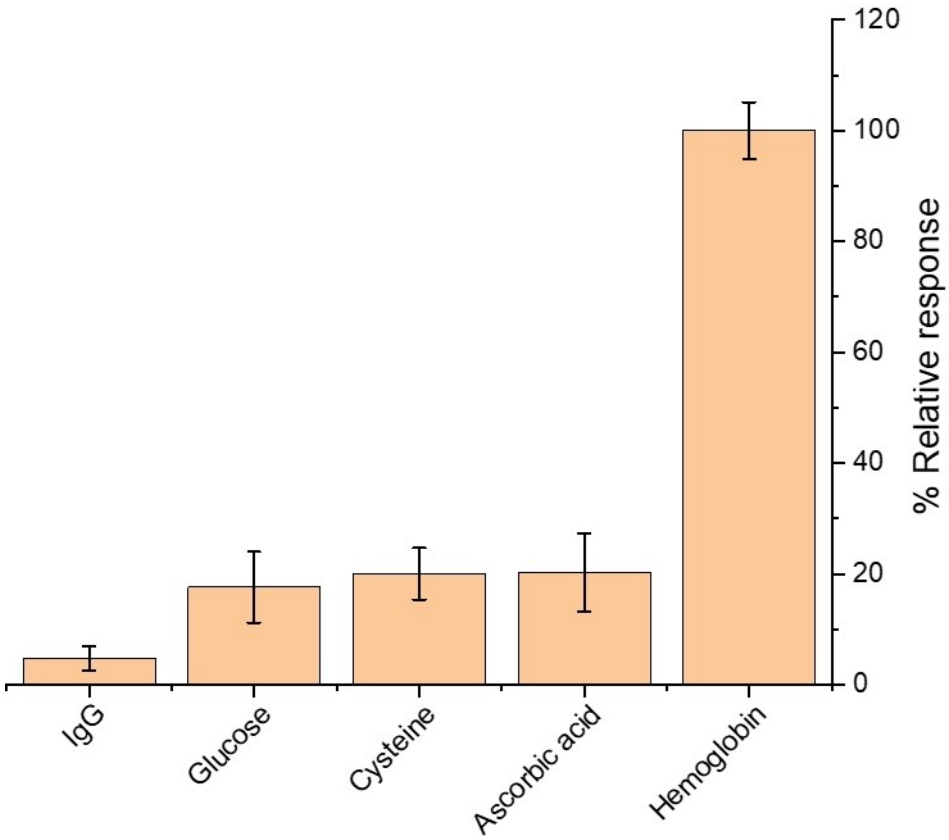

FIGURE 11 | % Relative response of 6 mg/mL ascorbic acid, cysteine, glucose, IgG, hemoglobin in 0.1 M PBS (pH: 7.5) scanned over the potential range $-0.2 \leq E \leq 0.6$ V at a scan rate of 60 mVs^{-}.

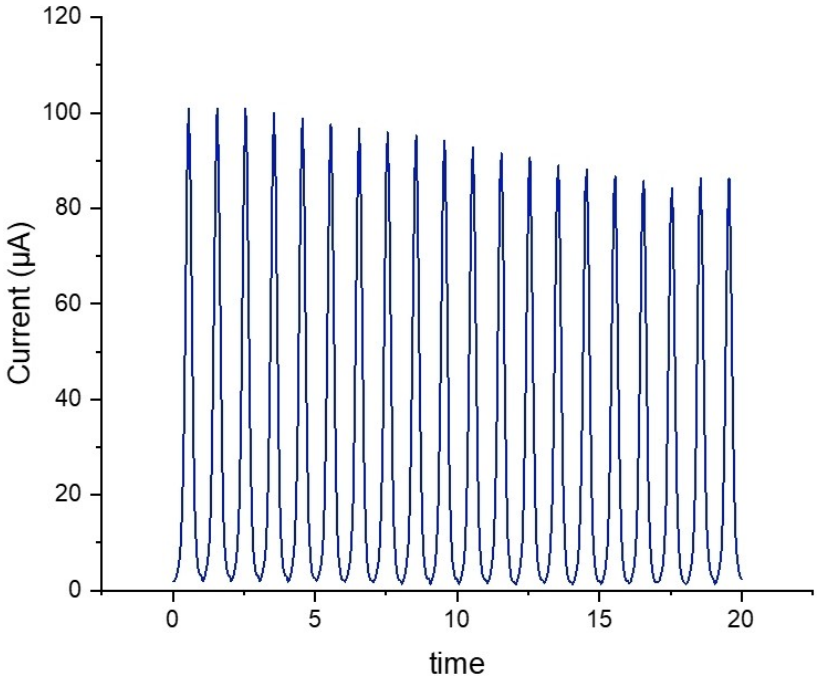

FIGURE 12 | Repeatability of the Hb-imp-p(AAm-*co*-VIM)/Nafion-SPCE modified electrode in an blood sample (pH 7.5) containing 1 mg/mL hemoglobin achieved by DPV method performed over the potential range of −0.2 to 0.6 V at a scan rate of 60 mVs^{-}.

infrastructure. Hemoglobin imprinted-poly(acrylamide-co-vinyl imidazole) [Hb-imp-p(AAm-co-VIM)] nanoparticles were synthesized and loaded onto a screen-printed carbon electrode (SPCE) with a nafion nanofilm, forming the Hb-imp-p(AAm-co-VIM)/Nafion-SPCE system.

The system′s performance was evaluated using cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The linear working range of the Hb sensor was found to be within an appropriate range from the literature, and the sensor demonstrated efficient operation at biological pH 7.4. Additionally, satisfactory recovery values were observed for hemoglobin in blood samples. Furthermore, detailed characterization of the synthesized polymer (Hb-imp-p(AAm-co-VIM)) has been provided in a previously published study by the group to better understand and evaluate the stability and performance of the sensor system [42]. Thus, both the affinity performance and electrochemical performance of the sensor system have been elucidated.

Overall, the Hb-imp-p(AAm-co-VIM)/Nafion-SPCE system shows promise for hemoglobin detection in biological environments and potential applications in disease monitoring systems. Determining the amount of hemoglobin with the sensor system is highly beneficial for portable and point-of-care detection. This approach, utilizing Hb-imp-p(AAm-*co*-VIM)/Nafion film-modified SPCE, highlights the capability to be applied directly to biological matrices with almost no sample preparation or extraction requirements, making it ideally suited for portable and point-of-care measurements. This study showcases the potential of MIP-based SPCE sensors for portable detection.

Acknowledgments

We would like to extend our deepest gratitude to the Department of Biochemistry and Department of Bioengineering at Ege University, Sabancı University (SUNUM) as well as the Department of Chemistry at Pamukkale University, for their invaluable collaboration and support throughout this research. The resources and facilities generously provided by these institutions were crucial in the successful execution of this study. We also wish to express a special acknowledgment to all the dedicated members of the research team for their significant contributions.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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