

An Electrochemical Biosensor Based on a Myoglobin-specific Binding Peptide for Early Diagnosis of Acute Myocardial Infarction

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In this study, a simple, highly sensitive electrochemical biosensor for myoglobin was developed using a myoglobin-specific binding peptide as a sensing probe. A peptide (Myo-3R7, CPSTLGASC, 838 Da) identified by phage display and that specifically binds to myoglobin was covalently immobilized on a gold electrode functionalized *via* a dithiobis(succinimidyl propionate) (DSP) self-assembled monolayer (SAM). The peptide immobilization was confirmed with fluorescence microarray scanning and cyclic voltammetry (CV). The electrochemical performance of the biosensor with respect to myoglobin was characterized by CV and differential pulse voltammetry (DPV) using $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ as a redox probe. We successfully detected myoglobin in a broad working range of 17.8 to 1780 ng mL⁻¹ with a correlation coefficient (R^2) of 0.998. The estimated limit of detection (LOD) was fairly low, 9.8 ng mL⁻¹ in 30 min. The electrochemical biosensor based on a myoglobin-specific binding peptide offers sensitivity, selectivity, and rapidity, making it an attractive tool for the early detection of cardiac infarction.

Keywords Electrochemical sensor, myoglobin, myoglobin-specific binding peptide, gold electrode

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Introduction

Acute myocardial infarction (AMI), well known as a heart attack, is one of the most common reasons for unexpected sudden death, and may cause irreversible tissue injury or necrosis in the myocardium.¹ However, only 57% of AMI patients reveal electrocardiogram (ECG) changes, and a sizeable proportion of affected patients are “silent” without any noticeable symptoms. Therefore, the development of sensitive and reliable platforms for biomarker detection at an early stage has a significant impact on the medical diagnosis and prevention of AMI.² A heme-containing protein, cardiac myoglobin, is one of the markers that increase after AMI onset. Due to its small size of 17.8 kDa, myoglobin is quickly released into circulation within 1–3 h after symptom onset, and serves as a valuable screening molecule with high sensitivity and predictivity for AMI detection.^{3–5}

Optical biosensors, including a sandwich immunoassay with secondary labeled antibodies, enzyme-linked immunosorbent assay (ELISA), fluorescence, and surface plasma resonance (SPR), have been used for the detection of cardiac markers, such as troponin I, myoglobin, c-reactive protein (CRP), and

myeloperoxidase (MPO).^{6–10} Driven by a clinical need for point-of-care diagnostics, next-generation biosensors with improved sensitivity and specificity have attracted increasing attention in recent years, leading to the development of peptide-based sensing approaches.¹¹ Peptides derived from phage display are attractive alternatives to biosensor probes. Compared to other sensing probes (*e.g.*, antibodies), peptides can be readily synthesized and manipulated to optimize the target specificity; thus, peptides have high affinity and specificity for target analytes, even in a complex mixture and in minute amounts.^{11–13} More recently, biosensors based on peptides obtained through phage display have been applied for the detection of cardiac troponin I.¹⁴

In our previous work, we showed an electrochemical biosensor using a gold electrode through a self-assembled monolayer (SAM).¹⁵ Electrochemical biosensors hold great promise for point-of-care diagnostics, due to their advantages of high sensitivity, detection simplicity, cost efficiency, rapidity, and portability.¹⁶ In this study, we report a peptide-based electrochemical biosensor that enables rapid, sensitive, and selective analysis for the detection of cardiac myoglobin. As a biosensor peptide, we used a Myo-3R7 peptide with an amino acid sequence of CPSTLGASC that has been previously identified *via* biopanning of a phage displayed-peptide library against myoglobin.¹⁷ The selected peptides were immobilized on a gold electrode *via* a SAM involving dithiobis(succinimidyl propionate) (DSP). The characteristics of the peptide-immobilized biosensor and the electrochemical response to

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myoglobin were analyzed *via* cyclic voltammetry (CV) and differential pulse voltammetry (DPV).

Experimental

Reagents and chemicals

Tetrahydrofuran (THF), bovine serum albumin (BSA), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), and potassium chloride (KCl) were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was obtained from Junsei Chemicals (Tokyo, Japan), and potassium ferricyanide ($K_3[Fe(CN)_6]$) was purchased from Yakuri Pure Chemicals (Kyoto, Japan). Dithiobis(succinimidyl propionate) (DSP), *N*-hydroxy-succinimide (NHS), and a silver/silver chloride (Ag/AgCl) reference electrode were obtained from Thermo Fisher Scientific (Pittsburgh, PA). Cardiac-human myoglobin and myoglobin-free human serum were purchased from USBiological (Salem, MA). Fluorophores (FCR-456 amine; green, FCR-552 amine; red) were purchased from BioActs (Incheon, Korea).

Preparation of a myoglobin-specific binding peptide

The Myo-3R7 peptide (CPSTLGASC) was synthesized by an Fmoc method supplied by Pepttron Inc. (Daejeon, Korea). Five rounds of biopanning were performed to screen the M13 phage library against myoglobin using myoglobin-conjugated magnetic beads. The binding efficiency of the selected peptide to myoglobin was confirmed by Sandwich ELISA.¹⁷

Fabrication of peptide-based electrochemical myoglobin biosensors

The fabrication procedure for the gold electrode platform was slightly modified from a method previously reported by our group.¹⁵ A glass slide substrate was washed twice with ethanol to remove surface contaminations. The cleaned slide was covered by a shadow mask with a pattern of five multi-channels. The gold electrode was deposited on the slide using thermal evaporation (SNTEC, Korea) at about 1×10^{-5} Torr. For titanium deposition, a voltage of 4 V was applied to the slide, resulting in an electrical current of about 170 A. The deposition rate was about $2 \text{ \AA s}^{-1}$. After titanium deposition, gold was deposited at a voltage of 2.5 V and an electrical current of 100 A. The deposition rate was about $1.7 \text{ \AA s}^{-1}$. Glass substrates were positioned at a distance of 43 cm from the source and kept at room temperature.

For peptide immobilization, the gold electrode was first cleaned with distilled (DI) water and dried by a stream of nitrogen gas. The gold electrode was dipped for 3 h in a freshly made THF solution containing 1 mM DSP at room temperature. The activated DSP/gold electrode was immersed for 2 h in a phosphate buffered saline (PBS) containing 1 mM peptide at room temperature. Finally, the peptide/DSP/gold electrode was soaked in a 1 mg mL^{-1} BSA solution at room temperature for 1 h to block non-specific binding between the myoglobin and gold electrode surface. The final electrode was rinsed with DI water and dried in a stream of nitrogen gas. To visualize the DSP and peptide immobilization on the electrode, the surfaces of the DSP/gold and peptide/DSP/gold electrodes were activated with EDC/NHS, and then treated with green and red fluorescence dyes, respectively. Fluorescence images were obtained using a microarray scanner (GenePix 4000B, Molecular Devices, Sunnyvale, CA).

Electrochemical analysis

Electrochemical characterizations were performed *via* CV and DPV using an electrochemical analyzer (versaSTAT 3, AMETEK, Berwyn, PA). A three-electrode system was employed with gold as the working electrode, silver/silver chloride as the reference electrode, and platinum as the counter electrode. CV and DPV were carried out in a degassed $10 \text{ mM } K_3[Fe(CN)_6]$ solution (pH 7.4) containing 1 M KCl . A $K_3[Fe(CN)_6]$ solution was used as a mediator to produce electron flow from the redox reactions between ferrocyanide and ferricyanide. CV was performed in a potential range of -0.2 to 0.6 V at a scan rate of 50 mV s^{-1} . The electrochemical response of the immobilized peptide to myoglobin was analyzed by DPV with a pulse amplitude of 250 mV at a scan rate of 100 mV s^{-1} . The reaction time between the myoglobin and the peptide-immobilized electrode was 30 min. Myoglobin was diluted in PBS (pH 7.4) and used at different concentrations (17.8 , 178 and 1780 ng mL^{-1}).

Specificity to myoglobin

A myoglobin-free human serum was used as a myoglobin substitute. The serum was allowed to react with the peptide immobilized gold electrode for 30 min. CV was performed in a potential range of -0.2 to 0.6 V at a scan rate of 50 mV s^{-1} . DPV measurements were performed with a pulse amplitude of 250 mV at a scan rate of 100 mV s^{-1} . All electrochemical data were independently obtained in triplicate.

Results and Discussion

Fabrication of peptide-immobilized gold electrodes

Electrochemical biosensors have the advantages of high sensitivity and specificity as well as simplicity, and can be expanded into a multiplex detection platform.^{18,19} In particular, the combination of electrochemical sensing technology with biomolecule-specific peptides has significantly improved the performance of biosensors for biomarker detection.²⁰⁻²² Here, we used a short peptide with a sequence of CPSTLGASC (Myo-3R7) as a recognition molecule for capturing myoglobin, a cardiac biomarker. The Myo-3R7 peptide had been screened and identified *via* phage display.²³ The selected peptide had a high affinity to myoglobin, while the cross-binding activities of the peptide to other cardiac biomarkers, such as BSA, troponin I, and creatine kinase-MB, were minimal. The fabrication procedure for a peptide-based electrochemical myoglobin biosensor is illustrated in Fig. 1. A DSP-based SAM was formed on the surface of the gold electrode with five circular multi-channels, and the Myo-3R7 peptide was subsequently immobilized on the SAM-modified gold electrode.

In order to confirm immobilization of the DSP-based SAM and peptide on the gold electrode surface, the electrodes were observed with a fluorescence microarray scanner (Fig. 2). After the SAM treatment and peptide immobilization, the electrode surfaces were modified with NHS, and were then labeled with fluorescence dyes. As shown in Fig. 2, the green and red fluorescence signals were distributed over the SAM-modified (Fig. 2(A)) and peptide-immobilized gold electrodes (Fig. 2(B)), respectively. The fluorescence images of electrode surfaces imply that the myoglobin-specific binding peptides were successfully immobilized on the gold electrode.

Electrochemical analysis by CV

Cyclic voltammetry is an effective technique for measuring the electron transfer between a solution and an electrode surface.

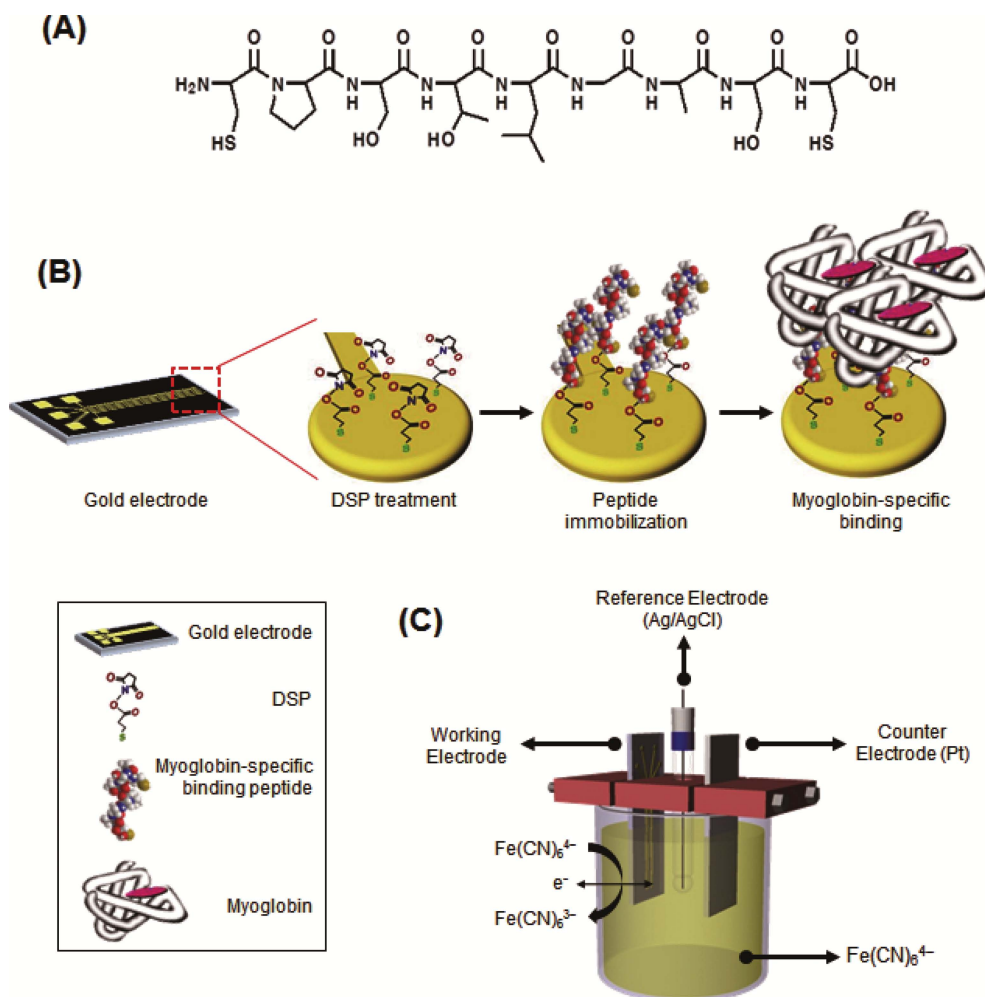


Fig. 1 (A) Structure of a myoglobin-specific binding peptide, Myo-3R7. (B) Schematic illustration of the fabrication process for an electrochemical sensor based on myoglobin-specific binding peptide immobilized on a gold electrode. (C) The mechanism of an electrochemical sensor for electrochemical signal production. Ag/AgCl and platinum electrodes were used as reference and counter electrodes, respectively. The peptide-immobilized gold electrode was used as a working electrode.

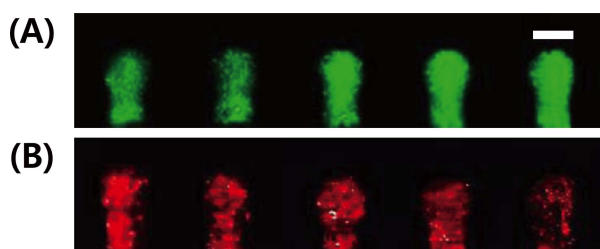


Fig. 2 Fluorescence images of electrode surfaces. (A) Schematic illustration for labeling fluorescence dyes. (B) DSP/gold electrode (green) and peptide/DSP/gold electrode (red). The scale bar represents 300 μm .

Changes in the peak current and the separation of the peak potentials in cyclic voltammograms are theoretically related to the electron-transfer rate constant.²⁴ Figure 3 shows cyclic voltammograms of $\text{K}_3[\text{Fe(CN)}_6]$ on bare gold, DSP/gold, and peptide/DSP/gold electrodes in a 10 mM $\text{K}_3[\text{Fe(CN)}_6]$ solution (pH 7.4) containing 1 M KCl. Data were obtained by scanning

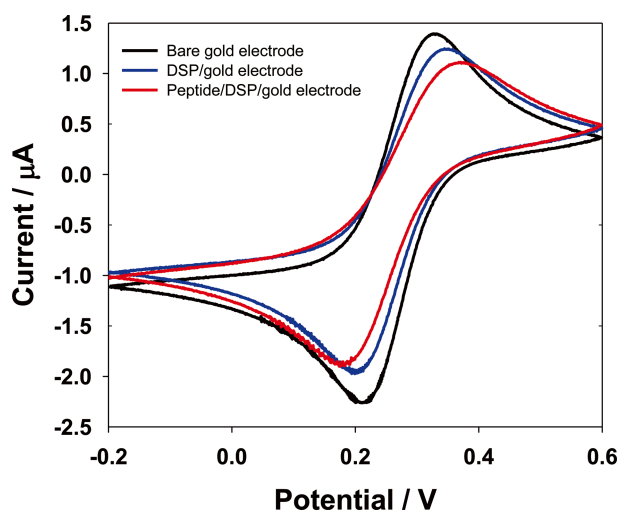


Fig. 3 Cyclic voltammograms of bare gold, DSP/gold, and peptide/DSP/gold electrodes at a scan rate of 50 mV s^{-1} .

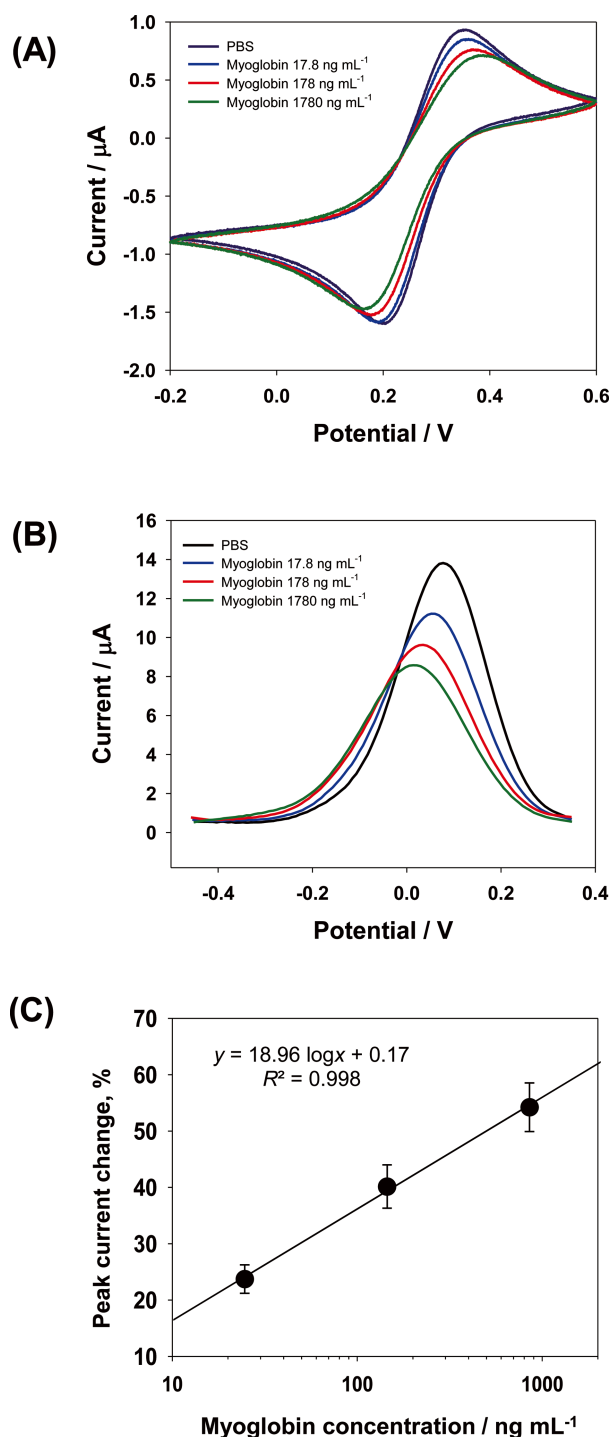


Fig. 4 Electrochemical response of the endotoxin sensor with respect to an increasing concentration of myoglobin. (A) Cyclic voltammograms of peptide/DSP/gold and BSA/peptide/DSP/gold electrodes at different myoglobin concentrations. (B) Differential pulse voltammograms of the electrochemical sensor at a scan rate of 100 mV s⁻¹ and different myoglobin concentrations. (C) Relationship between the peak current change and the myoglobin concentration.

from -0.2 to 0.6 V at 50 mV s⁻¹. K₃[Fe(CN)₆] is commonly used in electrochemical sensors as a mediator to produce electron flow from the redox reactions between ferrocyanide and ferricyanide. Generally, the signal intensity of a biosensor and the background level tend to increase with the potassium ferrocyanide concentration. Therefore, the concentration of

potassium ferrocyanide should be optimized for an electrochemical study considering the sensitivity of the sensor and the background level.²⁵ The voltammograms showed a typical reversible redox peak at the bare gold electrode surface. The redox peak currents of the bare gold electrode were -2.26 and 1.39 μA. The formation of a DSP layer on the gold electrode led to decreases in the redox peak currents to -1.97 and 1.25 μA, respectively. After peptide immobilization on the DSP/gold electrode, the redox peak currents further decreased to -1.90 and 1.11 μA, respectively. The separation between the two peaks in terms of the potential (ΔE_p) correspondingly increased from 0.117 V (bare gold) to 0.145 V (DSP/gold) to 0.195 V (peptide/DSP/gold electrode). Compared with a bare gold electrode, the decreases in the redox peak currents and the broadening of ΔE_p in the peptide/DSP/gold electrode can mainly be attributed to a blocking effect *via* partial inhibition of the diffusion of ions, such as [Fe(CN)₆]³⁻/[Fe(CN)₆]⁴⁻ anions. This result indicates the successful immobilization of peptide on the DSP/gold electrode.

Electrochemical detection of myoglobin

The electrochemical responses of the peptide-based electrochemical biosensors were investigated as a function of the myoglobin concentration (17.8 – 1780 ng mL⁻¹) using CV and DPV techniques (Fig. 4). The results showed that the redox peak currents in both CV and DPV gradually decreased with increasing myoglobin concentration. The formation of an insulating layer of myoglobin-peptide complexes retards the electron transfer of [Fe(CN)₆]³⁻/[Fe(CN)₆]⁴⁻, resulting in a decrease in the peak current and a broadening of ΔE_p .^{26,27} The calibration curve obtained as a function of the myoglobin concentration is shown in Fig. 4(C). The regression equation was $y = 18.96 \log x + 0.17$ with a correlation coefficient (R^2) of 0.998, where y and x represent the peak current change and the myoglobin concentration, respectively. Based on this result, the estimated limit of detection (LOD) was 9.8 ng mL⁻¹, as calculated on the basis of the IUPAC recommendations.²⁸ Recently, electrochemical biosensors for myoglobin detection have proven to be more sensitive and selective. The myoglobin detection limit of the immunosensor based on gold nanoparticles was 10 ng mL⁻¹,²⁹ while that for the immunosensor based on gold wire electrode was 5.2 ng mL⁻¹.³⁰ The electrochemical sensors using a carbon nanotube was able to detect myoglobin at 356 ng mL⁻¹.³¹ In this study, the LOD of the electrochemical biosensor using a myoglobin-specific binding peptide is comparable to those of other previous reports.

Myoglobin is quickly released into circulation as early as 1 – 3 h upon symptom onset. The myoglobin levels in normal serum range from 30 to 90 ng mL⁻¹. One hour after the onset of myocardial infarction, the myoglobin level in serum can increase to 900 ng mL⁻¹, or even higher.^{5,32} Therefore, rapid, sensitive and reliable methods for myoglobin detection can be very useful in clinical use. In our system, the peptide-based electrochemical biosensor showed excellent analytical performance, and the high sensitivity is attributable to effective myoglobin capture of the peptide. Interestingly, the LOD obtained from this work was comparatively lower than those in previous reports of myoglobin sensors based on modified electrodes in different methods.^{31,33,34}

Specificity to myoglobin

High specificity of a biosensor is one of the crucial parameters in developing a reliable biosensor. To evaluate the sensing specificity to myoglobin, a myoglobin-free human serum was used as a negative control. As shown in Fig. 5, changes in the

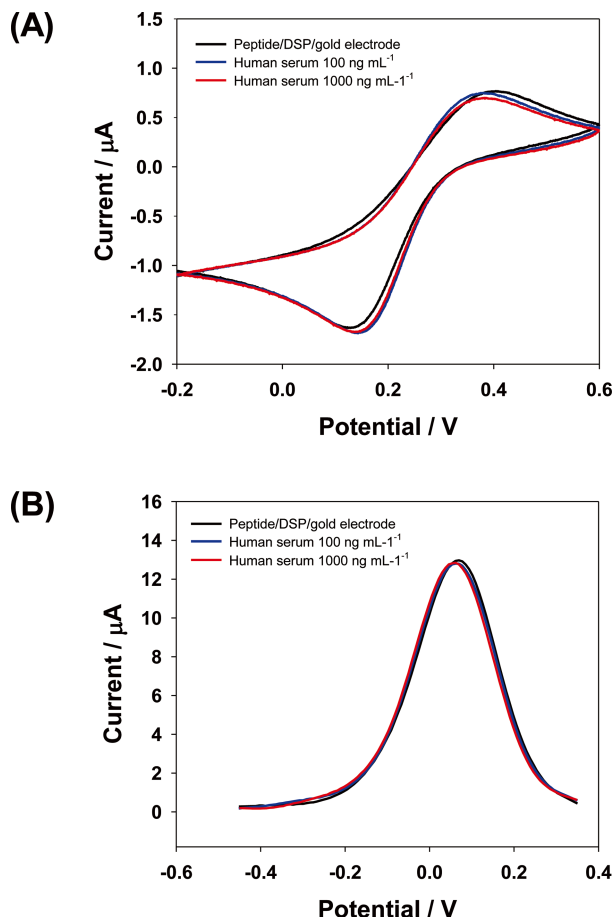


Fig. 5 Sensing specificity of the electrochemical sensor for myoglobin detection; human serum was used as a negative control. (A) Cyclic voltammograms at a scan rate of 50 mV s^{-1} and different human serum concentrations. (B) Differential pulse voltammograms at a scan rate of 100 mV s^{-1} and different human serum concentrations.

peak current signals in human serum at concentrations of 100 and 1000 ng mL^{-1} were negligible, supporting the high specificity of the proposed biosensor to myoglobin.

Conclusions

We fabricated an electrochemical myoglobin biosensor for the diagnosis of myocardial infarction using a myoglobin-specific binding peptide. A small peptide (Myo-3R7) selected from a phage display library was immobilized on a SAM-modified gold electrode. The CV and DPV techniques were used to characterize each stage involved in fabricating the peptide/DSP/gold electrode and the peptide-myoglobin interaction at the electrode surface. The fabricated electrochemical myoglobin biosensor was highly specific and sensitive, with a detection limit of 9.8 ng mL^{-1} . The peptide-based electrochemical myoglobin biosensor may provide an efficient platform for the early and point-of-care diagnosis of myocardial infarction.

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