Metal Enhanced Electrochemical Cyclooxygenase-2 (COX-2) Sensor for Biological Applications

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Abstract
Pain measurement is commonly required in biomedical and other emergency situations, yet there has been no pain biosensor reported in literature. Conventional approaches for pain measurement relies on Wong-Baker face diagrams, which are grossly inadequate for situations involving children or unconscious people. We report a label-free immunosensor for monitoring the pain biomarker cylooxygenase-2 (COX-2) in blood. The sensor is based on the concept of metal-enhanced detection (MED). MED relies on the idea that the immobilization of underpotential deposition (upd) metallic films deposited either as a monolayer or electrostatically held onto a solid gold substrate could significantly amplify bimolecular recognition such as involving antigen-antibody (Ab-Ag) interactions. The surface bound Ab-Ag complex insulates the electrode; causing a decrease in concentration-dependent redox signals. A linear detection range of $(3.64–3640.00)\times10^{-4}$ ng/mL was recorded with a detection limit of 0.25×10^{-4} ng/mL, which was 4 orders of magnitude lower than that reported for ELISA for the same biomarker. The immunosensor exhibited selectivity of less than 6% for potential interferents.

Keywords: COX-2, Cyclic voltammetry, Immunosensor, Pain biomarkers, Real samples, Self-assembled-monolayer

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1 Introduction

Pain is a complex response involving the interaction of multiple inflammatory mediators released at sites of tissue injury. Assessment of pain is necessary in clinical setting for diagnosis, pain management, choice of treatment and for the evaluation of treatment efficacy. Current methods used to assess pain include visual analog, Wong-Baker faces and verbal numeric scales [1]. However, studies have found that these scales may be subjective with discrepancies recorded in pain intensity measurements [2]. Hence there is a need for developing an objective scale that relies on the fundamentals of chemical and biological mechanisms of pain transduction.

Arachidonic acid (AA) is one of the major pain biomarkers. Its products of arachidonic acid (AA) which include enzymatically generated molecules such as thromboxanes and prostaglandins, among others, are the major substances that play a pivotal role in both inflammation and pain [3,4]. The most important step in the production of prostaglandins and thromboxanes is the metabolism of AA to prostaglandin H_2 (PGH_2) by the enzyme prostaglandin H_2 synthase, also referred to as cyclooxygenase-2 (COX-2) [5]. COX-2 is normally undetectable in healthy tissues but, rapidly induced in response to inflammatory stimuli such as bacterial endotoxin [6,7]. In the presence of two oxygen molecules, it catalyzes the conversion of AA to form prostaglandin G_2 (PGG_2) and a peroxidase (POX) reaction in which PGG_2 subsequently undergoes a two-electron reduction to (PGH_2) [8].

Currently, the Wong-Baker faces pain rating scale is used as the standard method for pain measurement but it allows for a subjective and a rather qualitative indicator of pain [10]. Studies have shown that the amount of AA and the levels of COX-2, PGG_2 or PGH_2 at the point of inflammation may ultimately translate to the degree of inflammation [9], thus providing a means of directly assessing the level of pain. The long-term goal of this work is to utilize biochemical transduction mechanisms as a means of developing a pain biosensor. The immediate goal is to develop a label-free approach to pain measurement using COX-2 antibody-modified electrode as a sensor for the antigenic COX-2. We recently reported the electrochemical characterization of different pain biomarkers [11] but the development of electrochemical sensors for assessing the level of pain or pain biomarkers has not been realized. We hereby report a label-free, MED-based sensor for monitoring COX-2, a major pain biomarker.

1.1 Immunosensor Concept

Developed in our laboratory, MED relies on the integration of advanced nanomaterials with electrochemical

techniques such as cyclic voltammetry, differential pulsed voltammetry and UPD of silver. Underpotential deposition is an electrochemical process whereby a single metal adlayer is electroplated on a dissimilar metal [12]. The UPD phenomenon is observed when there are strong adatom-substrate interactions that are energetically more favorable than that formed during bulk electrodeposition. In MED, a solid-phase monolayer of silver is deposited onto a gold electrode using UPD [12–14]. MED signal is based on the redox peaks from the UPD silver, which significantly enhances the sensitivity of the biomolecular recognition [12,13]. By oxidizing the silver monolayer, and in the presence of immobilized Ab molecules, highly reactive oxides of silver are generated in situ causing a change in the electronic properties of the immobilized monolayer. By scanning in the reverse direction, current arising from the of the reduction of the oxide layers is measured. If an antigen is introduced into the medium, the molecular recognition occurring between the antibody and the antigen is evidenced by a corresponding change in the redox currents of the silver monolayer, which dependents on the concentrations of the antigen. The concentration-dependent signals are attributed to the interfacial charge transfer barrier and related "site-blocking effects" of the non-electroactive COX-2 antigen.

We have established that MED reactive surfaces could be fabricated using metalized silver-on-gold (mSOG); either as continuous films, nanoparticles, colloids or monolayers. The mSOG surface is predicted to significantly amplify the electrochemical signals following molecular recognition without an intentionally added electroactive species in solution [13,14]. As a result, MED phenomena allowed for label-free detection of bioaffinity recognition, precise and reproducible control of surface coverage while simultaneously increasing the sensitivity of electrochemical detection.

We have developed MED-based biosensors for other bioaffinity reagents such as DNA-DNA, DNA-small molecule detection and genetic mismatch of DNA [14,15]. In this work, we extend the MED concept for the detection of pain biomarkers as the first step towards realizing an objective measure of pain for AA, COX-2 or PGG_2. We report the development of an immunosensor for COX-2 with subsequent validation in model real samples. Quantitative ELISA was first carried out to establish the molecular recognition between goat anti-COX-2 and COX-2 enzyme (antigen). The capture antibody is immobilized on the electrode via thiol groups of a thiolated Protein G which helps with the antibody orientation. To the best of our knowledge, this is the first electrochemical sensor to be reported for monitoring important pain biomarkers such COX-2 proteins.

2 Experimental

2.1 Reagents

Prostaglandin H synthase (Human recombinant COX-2, antigen), COX-1, goat anti-human COX-2 and rabbit anti-COX-2 polyclonal antibodies were purchased from Cayman Chemicals (Santa Cruz Ann Arbor, MI) and stored at −20 °C. Bovine serum albumin (BSA) and flat-bottom polystyrene 96-well microplates were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). The following chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA)-Sodium hydrogen phosphate, sodium phosphate, sodium carbonate, sodium hydrogen carbonate, rabbit anti-goat IgG alkaline phosphatase enzyme conjugate, tris HCl, tris base, human serum albumin, ovalbumin, diethanolamine and phosphoric acid. P-nitrophenyl phosphate disodium (PNPP) salt was purchased from Pierce Chemicals (Rockford, IL USA). Sodium chloride, sulfuric acid, sodium hydroxide and ethylenediaminetetraacetic acid (EDTA) were purchased from J.T Baker Chemicals (Philipsburg, N.J). Recombinant Protein G and succinimidyl-6-[3′-(2-pyridyldithio)-propionamido] hexanoate (LC-SPDP) were purchased from Thermo Scientific. Dithiothreitol (DTT) was purchased from Fermentas Life Science (Glen Burnie, Maryland, USA). Dimethyl sulfoxide (DMSO) was purchased from EMD Chemical (Gibbstown, NJ, USA). Simulated blood was obtained from forensics source (Jacksonville, FL, US).

2.2 Enzyme Linked Immunosorbent Assay (ELISA) Protocol

The ELISA protocol used was based on the method described in literature [15] with minor modifications: Flat-bottom polystyrene 96-well microplates were coated overnight at 4 °C with 100 μL/well of 1 μg/mL of polyclonal goat anti-COX-2 (prepared in 0.05 M bicarbonate buffer pH 9.6) as capture antibody. The plates were washed three times with phosphate buffer saline (PBS) buffer containing 0.05 % Tween 20 (PBS-T, pH 7.6) and the same washing procedure followed in each subsequent stage of the assay. The plates were then blocked with 200 μL of 1 mg/mL Bovine serum albumin (BSA) prepared in PBS (pH 7.6) and incubated overnight at 4ºC. The same washing procedure was followed and 100 μL of 0.0126 ng/mL–1260 ng/mL of COX-2 enzyme (antigen) in PBS-BSA buffer was applied to the wells. Two sets were used as a blank (PBS-BSA buffer) and a control respectively. The control was treated similarly to the sample wells but no antigen was added. All the other wells were filled with the COX-2 enzyme before an overnight incubation at 4 °C. Plates were subsequently washed and 100 μL of 1 μg/mL rabbit polyclonal COX-2 antibody was applied except blank. Following incubation for 2 h at ambient temperatures, another round of washing was carried out before adding 100 μL of goat anti-rabbit IgG-alkaline

phosphatase in Tris buffer (pH 8.0) excluding blanks. This step was followed by additional incubation for 1 h at room temperature. After the final rinsing with Tris buffer (pH 8.0), a 100 μL of 1 mg/mL PNPP solution prepared in 10% diethanolamine buffer (DEA) was added except blank and the plates incubated for 30 minutes at room temperature. Optical densities (OD) of the solutions were measured at 405 nm using Synergy HTRDR multi-detection microplate reader.

2.3 Thiolation of Protein G using LC-SPDP

Thiolation of Protein G was carried out as described in literature [16]. Briefly, a five-fold excess of LC-SPDP dissolved in PBS-EDTA containing 10% DMSO was reacted with Protein G that was dissolved in PBS-EDTA. Excess LC-SPDP and unwanted by-products were immediately removed by centrifugal filtration and the buffer was exchanged with sodium acetate. DTT was added to a final concentration of 50 mM and reacted for 30 min at room temperature. The reaction mixture was then loaded onto a dialysis bag that placed in a beaker containing PBS-EDTA buffer. The buffer was changed after every two hours twice and then left to dialyze overnight. Overnight dialysis was carried out in order to remove the unbound Protein G and the LC-SCPD. The dialyzed sample was removed from the dialysis bag and stored at 4 °C until required. Scheme 1 illustrates thiolation of the protein G and subsequent formation of assembled layer on the silver modified gold electrode.

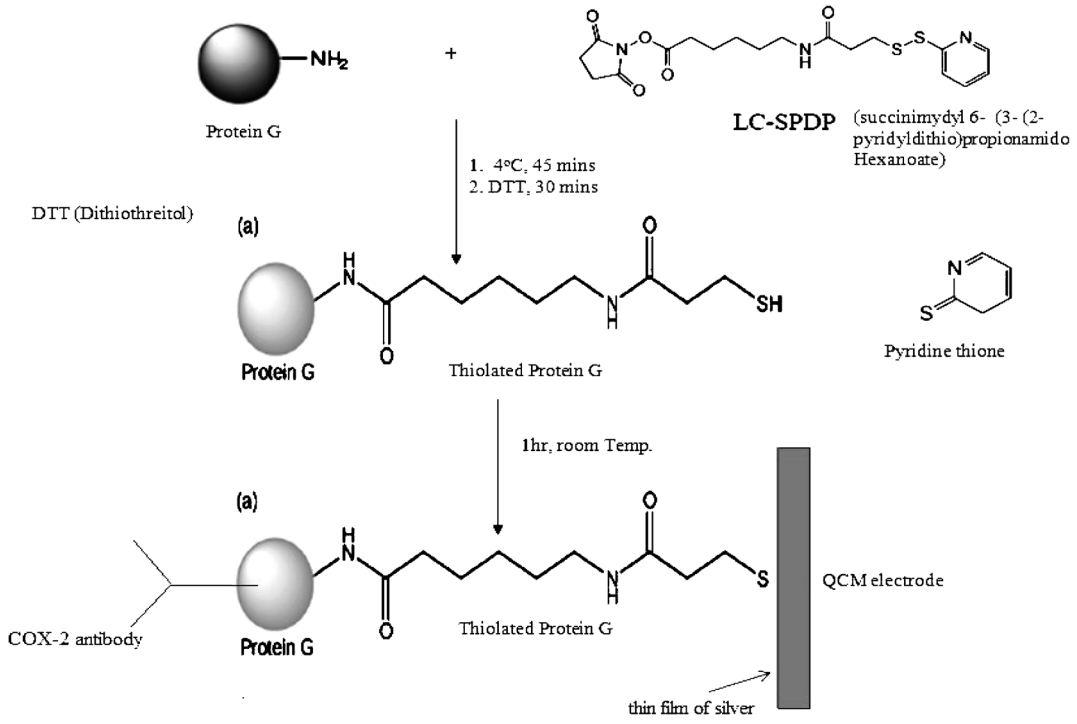
2.4 Instrumentation

All electrochemical measurements were performed in a conventional three electrode set up using an EG & G PAR 263A potentiostat equipped with an EG & G M270 software for data acquisition. A T-cut gold-quartz crystals (9 MHz, Area = 0.2 cm^2) purchased from International Crystal Manufacturers (OK, USA) were used as the working electrodes. Saturated silver electrode (Ag/AgCl-saturated) and platinum wire were used as reference and counter electrode respectively. A well-type electrochemical cell, made from Teflon with a capacity of 300 μL was used. All solutions were purged with nitrogen for 5 min before any electrochemical measurements were taken.

2.5 UPD of Silver and further Modification of Gold Electrode (UPD-Ag)

The gold quartz crystal were cleaned in freshly prepared piranha solution (30% H_2O_2:H_2SO_4, 1:3 V/V), rinsed with water and ethanol and finally blow dried with nitrogen gas. The deposition procedure was as previously described [13,14,17]. Briefly, silver was deposited on a gold quartz crystals using 1 mM silver nitrate solution at the following potentiostat settings: initial potential 503 mV, potential step 1 of 303 mV (49.90 s), and potential step 2 of 503 mV (10.11 s).

Surface pretreatment of the UPD-Ag was achieved using the LC-SPDP crosslinker. Succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate, is a cleavable, water insoluble, amino and thiol (sulfhydryl) reactive heterobi-



Scheme 1. A flow diagram illustrating thiolation of protein G and subsequent formation of self-assembled layer on the silver modified gold electrode.

functional protein crosslinker. The "long chain" LC-SPDP crosslinking reagent has a 15.7 angstrom spacer arm and it facilitates the attachment of thiolated Protein G on the gold surface [18]. This surface linkage allows a maximum interaction between the antibody and the antigen. The antibody provides the best orientation with reduced steric hinderance [18]. Similar surface conjugation using LC-SPDP with Protein G had been reported for oriented immobilization of the antibody [16,19,20]. In order to achieve the oriented surface conjugation described, a 200 μL of the thiolated Protein G was applied on the surface of the electrode in the Teflon well and left to incubate for one hour. The electrode was rinsed thoroughly with PBS (pH 7.6) buffer before applying a solution of 200 μL of 1 μg/mL COX-2 goat antibody; which was followed by additional incubation for two hours. The COX-2 immobilized electrode was subsequently rinsed three times with PBS buffer before applying 1 mg/mL BSA prepared in PBS (pH 7.6) to block any non-specific sites for one hour. Finally, the COX-2 immobilized electrode was utilized as a sensor via incubation at varying concentrations (3.64×10^{-4} ng/ mL – 3640000×10^{-4} ng/ mL) with COX-2 enzyme (prepared in PBS-BSA) for 10 min and then characterized using cyclic voltammetry.

2.6 Cross-Reactivity Studies and Detection of COX-2 in Model Real Samples

The effect of potential interferants such as human serum albumin (0.1 μg/ mL), ovalbumin (0.1 μg/mL), and COX-1 (3.64×10^{2} ng/ mL) on the MED immunosensor was tested. The change in the redox currents in the presence of the interferents was compared with that obtained for COX-2. Also, to investigate the efficiency of the MED immunosensor, simulated blood that was designed to mimic the consistency of real human blood was employed as real sample. Known concentrations of COX-2 were spiked in the simulated blood and then detected at the immunosensor. The amount of COX-2 recovered was compared to the amount spiked in the sample in order to ascertain the efficiency of the immunosensor.

3 Results and Discussion

This work seeks to develop a label-free MED immunosensor for COX-2, a major pain biomarker. As discussed earlier, the resulting immunosensor was validated for COX-2 using simulated blood real samples. Prior to the development of the MED immunosensor, a quantitative ELISA was carried out to establish the molecular recognition between goat anti-COX-2 and COX-2 enzyme. The COX-2 enzyme (antigen) standards used to generate a working calibration plot for the ELISA ranged from 0.0126 to 1260 ng/ mL. The ELISA results as shown in Fig 1 revealed an increase in the recorded absorbance as the concentration of the COX-2 was increased. This suggests a biomolecular recognition of the COX-2 enzyme by its antibodies, thus providing a direct measure of COX-2 protein. The linear range, as shown in the inset of Figure 1, was found to be between 1–126 ng mL^{-1} and a

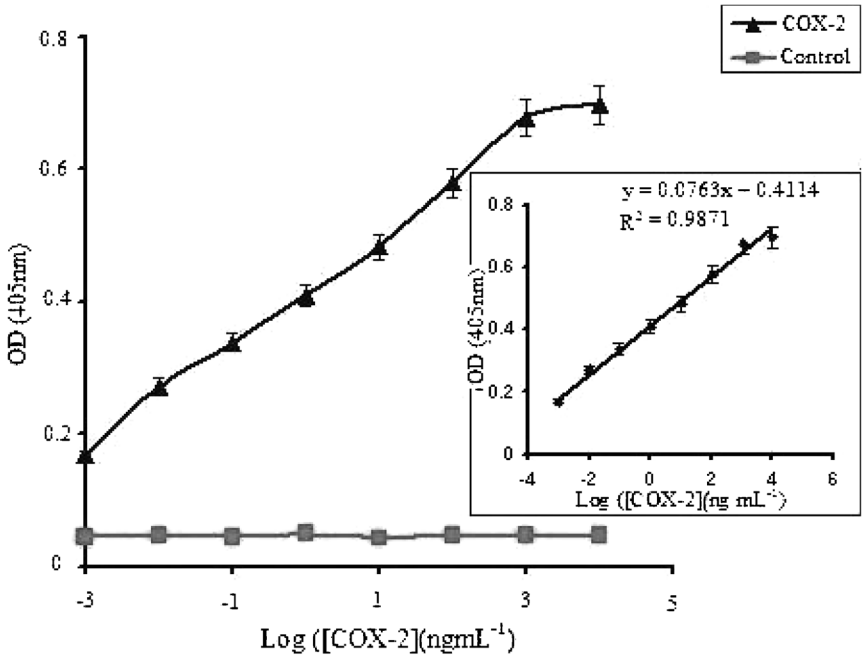


Fig. 1. ELISA results. The results shows the measurable range of the COX-2 enzyme and inset shows a linear standard calibration curve obtained in the sandwich ELISA. Inset shows a linear detection range of between 1–126 ng mL^{-1} and a detection limit of 0.24 ng mL^{-1} was recorded.

detection limit of 0.24 ng mL^{-1} was estimated based on 3 times the standard deviation of the blank.

3.1 Immunosensor Design

Thin film of silver monolayer was deposited on the surface of the gold electrode using as described in the experimental section. A self-assembled monolayer of thiolated Protein G was then assembled on the silver modified electrode either through attachment to the silver or uncovered gold surface. Silver deposited on the surface of the electrode has been found to increase the stability of the alkanethiol SAM. Jennings et al. demonstrated that alkanethiol-based SAMs on gold surfaces modified by UPD to contain a thin layer of silver exhibited greater stabilities toward desorption than did SAMs on native gold surfaces [12]. A layer of goat anti-COX-2 polyclonal antibody was then assembled onto the silver modified gold electrode via its antibody binding sites of the thiolated protein G which then formed the immunosensor surface.

Cyclic voltammetry was used to characterize the electrode surface. Oxidizing the silver monolayer in the presence of the goat anti-COX-2 antibody resulted in reactive silver oxides accompanied by electrons producing a redox signal. It showed anodic and cathodic peaks at around 135 mV and −2 mV respectively. The peak currents recorded indicated no significant change even after several cycles were run, indicating the stability and reproducibility of the goat anti-COX-2 immunosensor. Incubation of the goat anti-COX-2 antibody modified electrode with different concentrations of COX-2 enzyme prepared in PBS buffer (pH 7.6) resulted in biospecific molecular recognition, forming an antibody-antigen complex evidenced by the change in the redox current. The complex formation on the surface of the electrode resulted in insulation of the surface toward the redox couple as predicted by MED concept. This insulation reflected the differences in the effective diffusion of ions to the electrode surface due to the Ab-Ag interaction.

The extent of insulation was found to be proportional to the concentration of COX-2 enzyme used indicated by the reduction in the intensity of both the anodic and cathodic peak currents as the concentration was increased (Figure 2A). To ascertain that the reduction in peak current was due to the antibody-antigen interaction, a control containing buffer solution without incubation of the COX-2 enzyme was performed. The peak current did not show any significant change indicating that the observed current decrease was due to the interaction between COX-2 enzyme and its antibody. A plot of the change in the anodic peak current versus the COX-2 enzyme concentration showed a linear range of between 3.64×10^{-4} to 36400×10^{-4} ng/mL with a detection limit of 2.54×10^{-5} ng/mL based on 3 times the standard deviation of blank (Figure 2B). Interassay precision of the immunosensor was calculated based on results of three independent experiments using equation 1 and a percent coefficient of variation (%CV) of between 8.45–13.12 % was obtained.

$$\%CV = \frac{\text{standard deviation of the mean of triplicates}}{\text{mean of triplicates}} \times 100 \quad (1)$$

3.2 Cross-Reactivity and Detection of COX-2 in Real Samples

The effect of potential interferents (human serum albumin, ovalbumin and COX-1) tested was found to be insignificant since no major change in the redox currents was observed using the interferents as analytes. The cross selectivity, calculated using COX-2 response as 100 % and equation 2 was found to be of less than 6 %

$$\%\ \text{Cross selectivity} = \frac{\text{interferent response}}{\text{COX-2 response}} \times 100 \quad (2)$$

To investigate the efficiency of the MED immunosensor, COX-2 enzyme was detected in simulated blood using the MED immunosensor. Simulated blood obtained from forensics source (Jacksonville, FL, US) was diluted in PBS buffer and different known concentrations of COX-2 spiked in the samples.. Calibration curve was first constructed using standard COX-2 concentrations between 3.64×10^{-4} to 36400×10^{-4} ng/mL. The immunosensor response of each spiked sample was compared with the response of the standard COX-2 concentration and used to calculate the % recovery. To further validate the system, a control experiment whereby the simulated blood without spiking the COX-2 was performed. Both the anodic and cathodic peak currents decreased as the COX-2 concentration spiked in the samples increased. A plot of the anodic current change versus the COX-2 concentrations spiked in the sample showed a linear range of between 3.64×10^{-4} to 36400×10^{-4} ng/ mL (Figure 3). However, there was no significant change in both the cathodic and anodic peak currents for the control experiment. This showed that the simulated blood did not have any interferents which can interfere with the performance of the immunosensor when analyzing real sample. As predicted by the MED, this suggests that the decrease in the peak currents was due to the interaction between the spiked antigenic COX-2 and the surface-immobilized antibody.

The % recovery of the COX-2 determined from the spiked samples was calculated as shown in Equation 3 and was found to be between 66–102 % with an average value of 81.31 %. These values show that the MED immunosensor is selective to interferents in the simulated blood and it can be used to detect COX-2 in real-life blood samples from patients suffering from acute and chronic inflammation pain.

$$\%\ \text{Recovery} = \frac{\Delta I_{pa}(\text{spiked sample})}{\Delta I_{pa}(\text{standard sample})} \times 100 \quad (3)$$

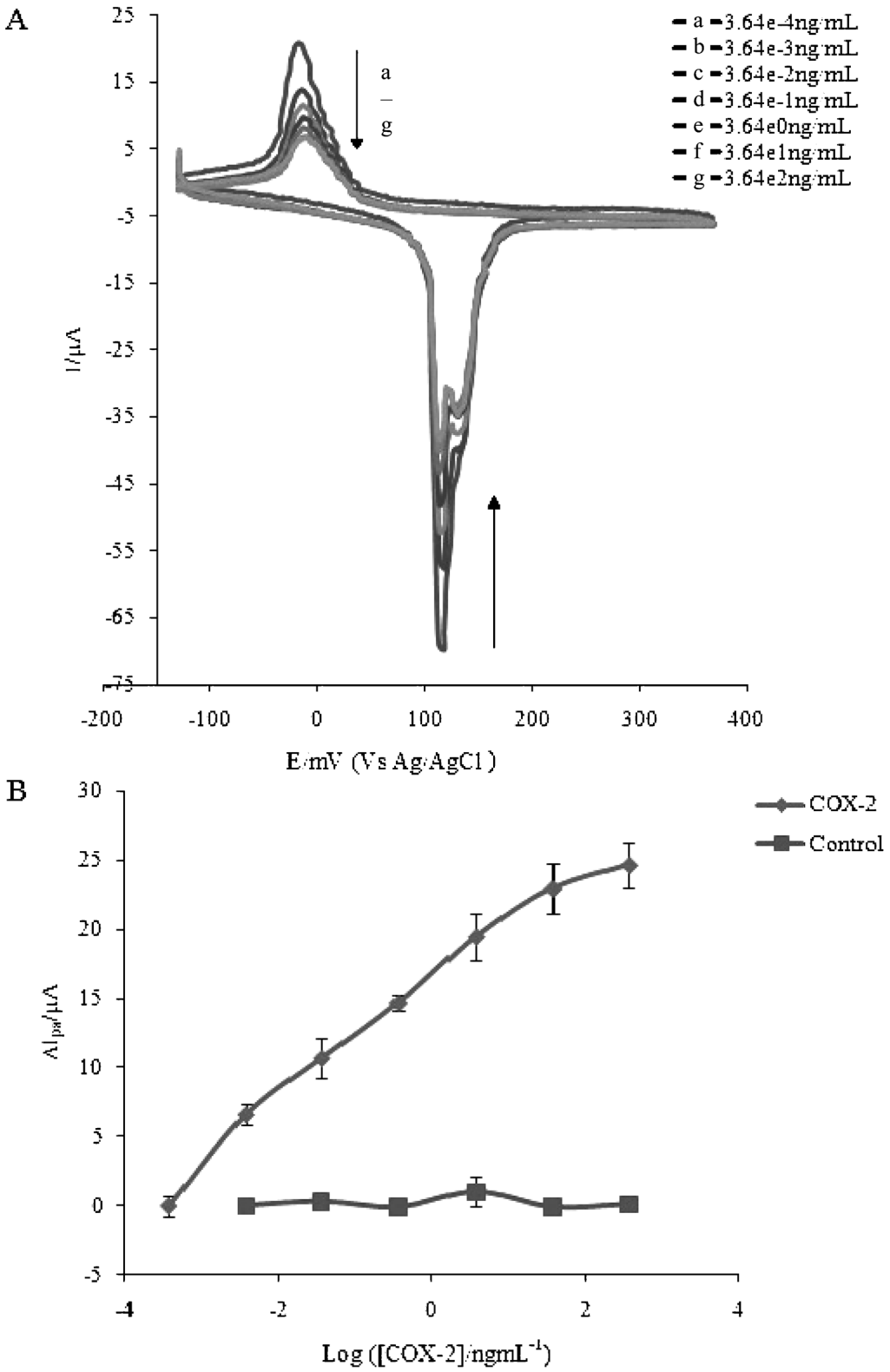


Fig. 2. Representative Cyclic voltammograms and Calibration curve. (A) Voltammograms obtained after incubating the goat anti-COX-2 modified electrode with different concentrations of COX-2. (B) MED calibration curve using a plot of the anodic peak current change ($\Delta I_{pa} = I_{pa} - I_{pa}(\text{ref})$ where I_{pa} (ref) $= -63$ μA) versus COX-2 concentration. $LOD = 2.54 \times 10^{-5}$ ng mL^{-1}, $LDR = 3.64 \times 10^{-4}$ to 36400×10^{-4} ng mL^{-1}

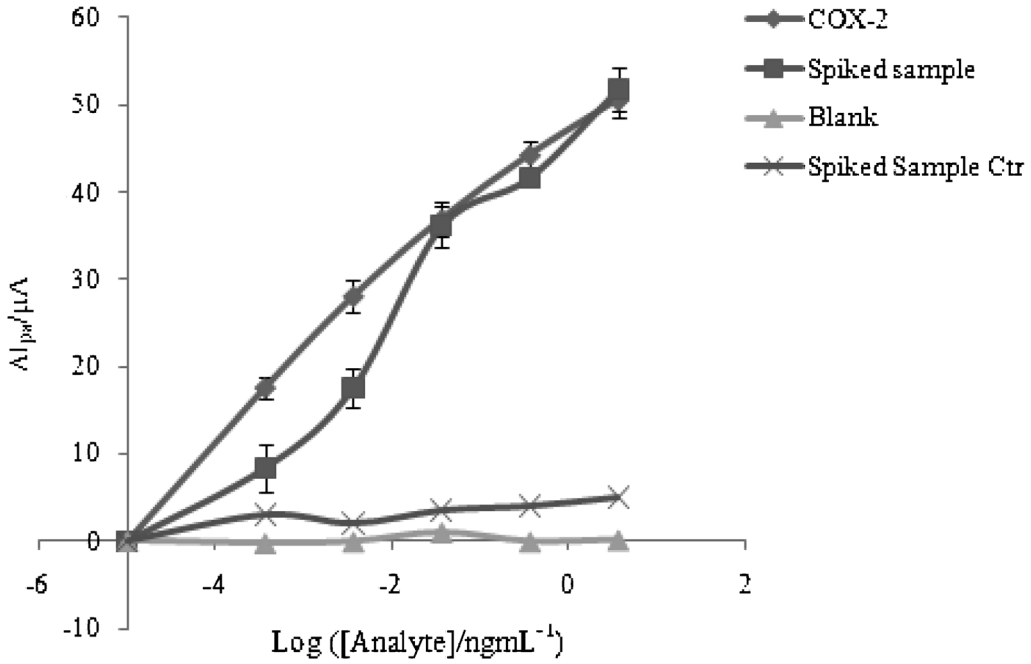


Fig. 3. A plot of the anodic peak current change ($\Delta I_{pa} = I_{pa} - I_{pa}(\text{ref})$ where $I_{pa}(\text{ref}) = -80\ \mu A$) versus concentration of COX-2 in both standard and spiked samples.

4 Conclusions

Label free metal enhanced electrochemical detection biosensor is reported for monitoring COX-2, a major pain biomarker. In this method, monodispersed silver ions served as the redox probe. The cathodic and anodic peak current response with or without COX-2 indicated a concentration-dependent response which was due to the COX-2 enzyme. The concentration-dependent response revealed a linear detection range of between 3.64×10^{-4} to 3.64×10^{1} ng mL^{-1} with a detection limit of 2.54×10^{-5} ng mL^{-1} which was about 4 orders of magnitude lower than that recorded for ELISA technique. The immunosensor exhibited a remarkable selectivity of less than 6% and good recovery of 81.31% of COX-2 spiked in simulated blood used as real sample. This was an indication that there were no interferents in the simulated blood which may interfere with the detection of COX-2 in real samples. This immunosensor may thus serve as a basis for the quantification of COX-2 and other pain biomarkers such as AA and PGG_2. Future work will involve testing this immunosensor in real-life biological applications such as using blood samples from patients with pain.

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