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$CoFe_2O_4$-Chitosan and Gold Nanoparticles Based Label-Free Electrochemical Immunosensor for Determination of Leptin

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Highlights

- A novel label-free immunosensor was fabricated for the quantification of leptin protein.
- The sensing platform was based on GCE/$CoFe_2O_4$-CHI/AuNP.
- This platform showed good electrochemical response due to the synergistic impact of these materials.
- The developed immunosensor has a wide dynamic range for leptin.

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Abstract

Herein, a label-free electrochemical leptin immunosensor was demonstrated. The sensing platform consists of the immobilizing of the anti-leptin antibody on a glassy carbon electrode (GCE) modified with cobalt iron oxide ($CoFe_2O_4$) nanoparticles, chitosan (CHI), and gold nanoparticles (AuNPs). A simple and rapid leptin determination was achieved by measuring the change of current response in a redox probe solution before and after the immunocomplex formation. SEM examined the surface morphologies of the prepared electrodes. The electrochemical performance of the leptin immunosensor was commented on via EIS, CV, and DPV. Under optimized circumstances, a linear response was found between the current peaks acquired from DPV and the logarithm concentration of leptin in the range of 1─4000 ng mL^{-1} with a detection limit (LOD) of 0.1 ng mL^{-1}. The subjected immunosensor demonstrated satisfactory reproducibility.

1. INTRODUCTION

Obesity is a significant and incurable health problem that can occur with irregular eating habits, sedentary lifestyles, and consumption of high-calorie diets. It contributes to the formation of insulin resistance, cardiovascular disease, and type 2 diabetes [1–5]. Leptin, a peptide hormone of the obesity gene, is synthesized primarily in adipose tissue. It plays a noteworthy role in the physiological organizing of energy intake and expenditure and is directly proportional to the body's fat percentage [6,7]. Alterations in the levels of leptin are related to obesity, metabolic abnormalities, cancer, and infertility. For this reason, determining leptin is essential in understanding its role and connection with diseases [8]. Various methods, such as capillary electrophoresis [9], radioimmunoassay [10], enzyme-linked immunosorbent assay [11], and surface plasma resonance [12] have been reported for quantification of leptin. However, the methods have drawbacks, requiring expensive instruments and expert personnel and including troublesome and time-consuming procedures. Biosensors can also be an alternative because they have features such as easy and low-cost preparation, rapid determination, and high-sensitivity [13–15].

Electrochemical immunosensors are compact analytical instruments with many advantages, such as high selectivity and sensitivity, primarily due to antibodies that bind to the antigen of interest. Therefore, they provide a promising analytical method for the determination of biomolecules [16]. They can be classified as label-free and labelled formats (sandwich-type). In the sandwich type, the antigen is trapped between the primary and the labelled secondary antibody [17]. However, the labelling step has restrictions, such as being time-consuming and costly and can cause the denaturation of antigen molecules. Thus, label-free immunosensors have also become a crucial bioanalytical method for determining antigen molecules [17–

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20]. Many analytes, such as antibodies and antigens, do not inherently act as redox couples; therefore, electron mediators must be connected to the electrode surface or added to the sensing solution to generate an electrochemical response. Besides, as with many other sensor systems, nanomaterials provide a larger surface area, can increase the signal and conductivity, and are biocompatible [21,22].

Magnetic nanoparticles are materials of interest in applications such as separation/purification, catalysis, and sensors. In addition, as a spinal type of ferrite, which is a low-cost material with surface catalytic activity, cobalt ferrite ($CoFe_2O_4$) finds the opportunity to be used in recyclable nanocatalyst [23], photocatalyst [24], and sensor applications [25]. $CoFe_2O_4$ nanoparticles have potential owing to their unique electrical, optical, catalytic, medical, and magnetic features [26]. Gold nanoparticles (AuNPs) have been an attractive nanomaterial, especially in chemical sensor applications, as they have many characteristics, such as small particle size, large surface area, and excellent electrical conductivity [27,28]. Chitosan (CHI) is a natural polymer isolated mainly from the exoskeleton of crustaceans, especially shrimp. It is a copolymer of D-glucosamine and N-acetyl-glucosamine obtained from the complete or partial deacetylation of chitin [29,30]. CHI is considered a non-toxic, biodegradable, biocompatible, and amine-rich polymer, emphasizing its good film-forming ability. CHI has reactive amino and hydroxyl functional groups in each polymer chain. It can be utilized as an immobilization platform in the amperometric biosensors [31–33].

In this study, we aimed to prepare a sensing platform based on $CoFe_2O_4$-CHI/AuNP to detect leptin. This label-free electrochemical immunosensor combined the characteristics of $CoFe_2O_4$, chitosan, and gold nanoparticles. The GCE/$CoFe_2O_4$-CHI/AuNP electrode offered a matrix for immobilizing anti-leptin antibodies. After antibody-antigen complex formation, DPV is performed via a well-known redox mediator, $[Fe(CN)_6]^{3-/4-}$. In light of the literature review, it has been seen that there are no studies on the determination of leptin with the immunosensor aimed to be prepared in this study.

2. MATERIAL METHOD

2.1. Reagents and Solutions

Polyclonal anti-leptin antibody and leptin protein were both purchased from Abcam. Chitosan, $CoFe_2O_4$ nanoparticles, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), N-Hydroxysuccinimide (NHS), and Bovine Serum Albumin (BSA) were obtained from Sigma-Aldrich. Hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4.3H_2O$) was the product of Acros Organics. Disodium monohydrogenphosphate dihydrate ($Na_2HPO_4.2H_2O$) and sodium dihydrogenphosphate dihydrate ($NaH_2PO_4.2H_2O$) were obtained from Riedel de Haën. Potassium ferric cyanide ($K_3Fe(CN)_6.H_2O$ and potassium ferro cyanide ($K_4Fe(CN)_6.H_2O$ were also bought from Acros Organics. All other chemicals were of analytical grade and were utilized without another treatment. An anti-leptin antibody solution was prepared by diluting the 1 mg/mL polyclonal anti-leptin antibody solution with 0.1 mol L^{-1} pH 7.0 phosphate buffer solution (PBS). Leptin solutions were obtained by diluting the recombinant human leptin protein solution with PBS. The prepared solutions were aliquoted and stored at -20 °C. Electrochemical measurements were realized using 0.1 mol L^{-1} PBS, including 5.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol L^{-1} KCl. This electrochemical working medium was referred to as the PBS-redox in the following sections.

2.2. Apparatus

Autolab-PGSTAT 204 potentiostat/galvanostat was utilized to conduct cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements. For the impedance spectroscopy (EIS) experiments, Ivium CompactStat was utilized, and data analysis was performed using the ZSimpwin program. A three-electrode cell was operated using an unmodified GCE or modified GCE as the working electrode, an Ag/AgCl electrode (3 mol L^{-1} NaCl) as the reference electrode, and a Pt wire as the counter electrode. The surface images of the electrodes were recorded by a FEI Nova NanoSEM 650 scanning electron microscope.

2.3. Preparation of Immunosensor

Before the electrode fabrication, the bare GCE was first polished on a cleaning pad using 0.05 μm alumina suspension. Subsequently, the electrode was kept in distilled water, ethanol, and nitric acid for 3 minutes in an ultrasonicator and dried in air. Then, electrodes were subjected to electrochemical treatment with CV in 0.5 mol L^{-1} H_2SO_4, scanning the voltage between -0.5 and 1.6 V at a sweep speed of 0.1 Vs^{-1} with 20 cycles. The electrode was treated several times with distilled water and allowed to dry at room temperature. Firstly, chitosan (CHI) solution (0.5 w/v %) was prepared by dissolving CHI powder in acetic acid solution (1 v/v %) with magnetic stirring for 3 hours. $CoFe_2O_4$ nanoparticles (2 mg) were weighed and dispersed in 1 mL of double distilled water for 2 hours in an ultrasonicator. The obtained dispersion was added to the same volume of CHI solution and mixed in an ultrasonicator for 10 minutes. To prepare the GCE/$CoFe_2O_4$-CHI, 10 μL of $CoFe_2O_4$-CHI solution was dripped onto the GC electrode surface. Afterward drying at room temperature, AuNPs were electrodeposited on the GCE/$CoFe_2O_4$-CHI surface by CV scanning from (-0.2) to (+1.2) V (100 mV s^{-1}, 15 cycles) in a 0.5 mol L^{-1} H_2SO_4 solution including 0.6 mmol L^{-1} $HAuCl_4$. The GCE/$CoFe_2O_4$-CHI/AuNP was then left to air dry. The anti-leptin antibody (Ab) was immobilized onto the GCE/$CoFe_2O_4$-CHI/AuNP electrode via EDC/NHS chemistry, and the electrode was incubated at 37°C overnight. A 0.2% bovine serum albumin (BSA) solution was dripped on the GCE/$CoFe_2O_4$-CHI/AuNP/Ab electrode and incubated for 40 minutes to prevent non-specific interactions. Finally, the GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA electrode was set at diverse concentrations of the leptin antigen solution. The preparation operation of label-free leptin immunosensor was demonstrated in Figure 1.

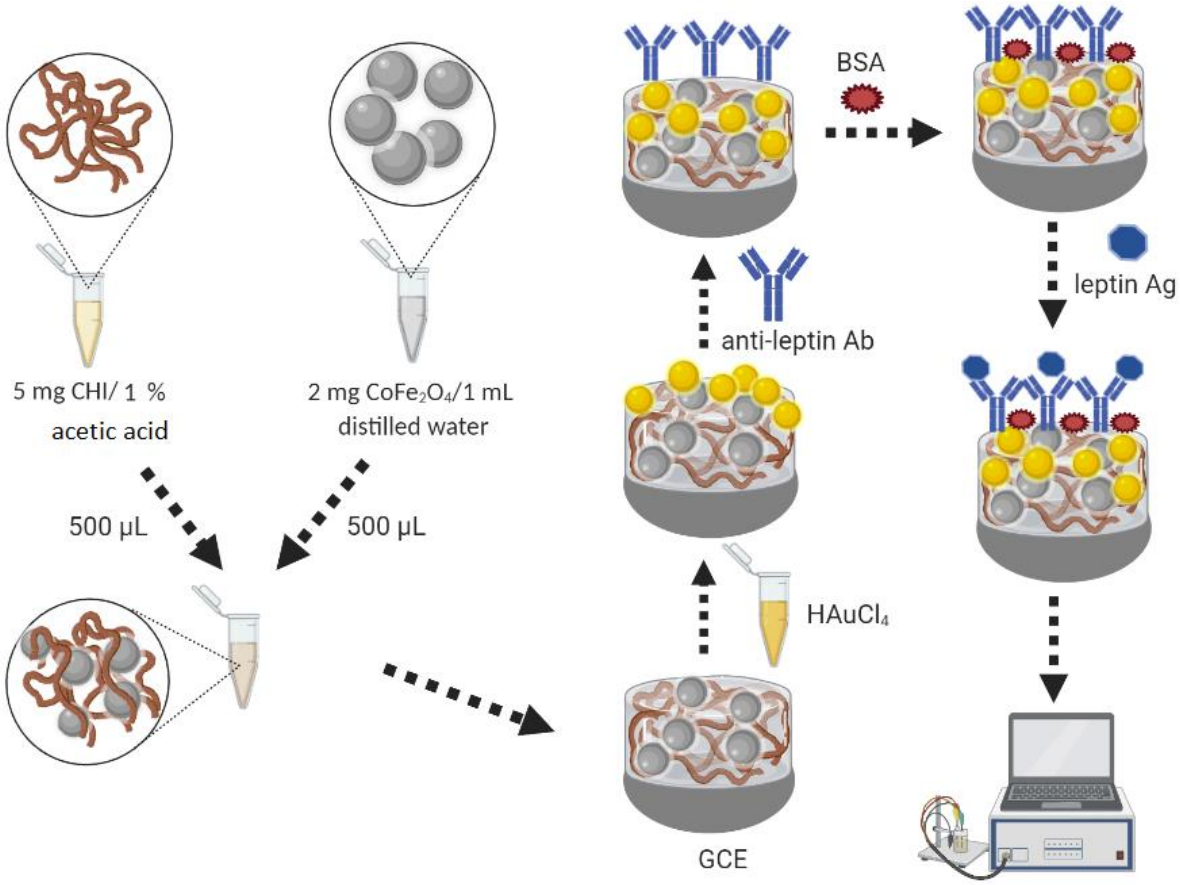


Figure 1. *Preparation scheme of label-free leptin immunosensor*

2.4. Electrochemical Studies

The electrochemical determination of leptin was conducted as follows: the prepared GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA immunosensor was treated with diverse concentrations of leptin antigen (Ag) for 40 minutes to obtain antibody-antigen immunocomplex. After an incubation period, DP voltammograms were recorded in the PBS-redox using the following parameters: Potential interval is between (-0.2) and (+1.1)

V, sweep rate is 0.05 V s^{-1}. The immunological reaction between leptin antigen and leptin antibody was monitored by determining the change in peak currents of the redox species $[Fe(CN)_6]^{3-/4-}$ (Figure 2).

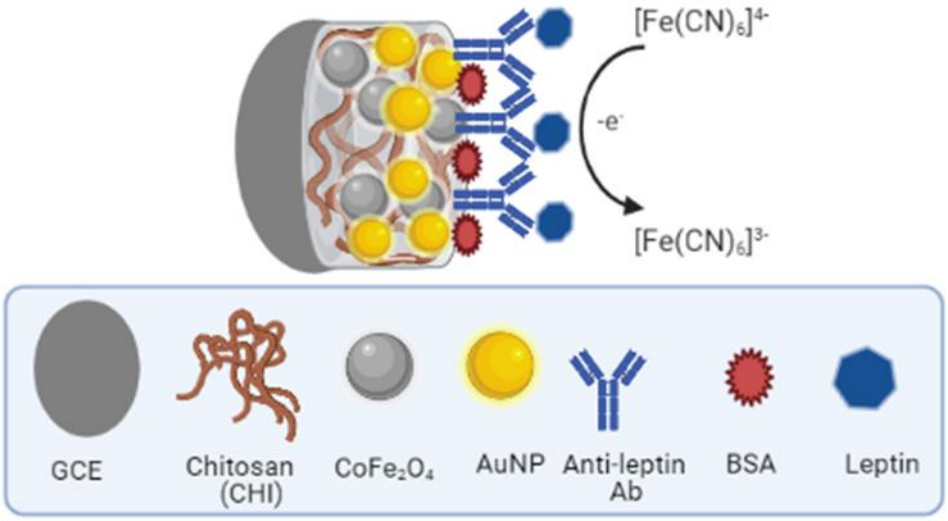
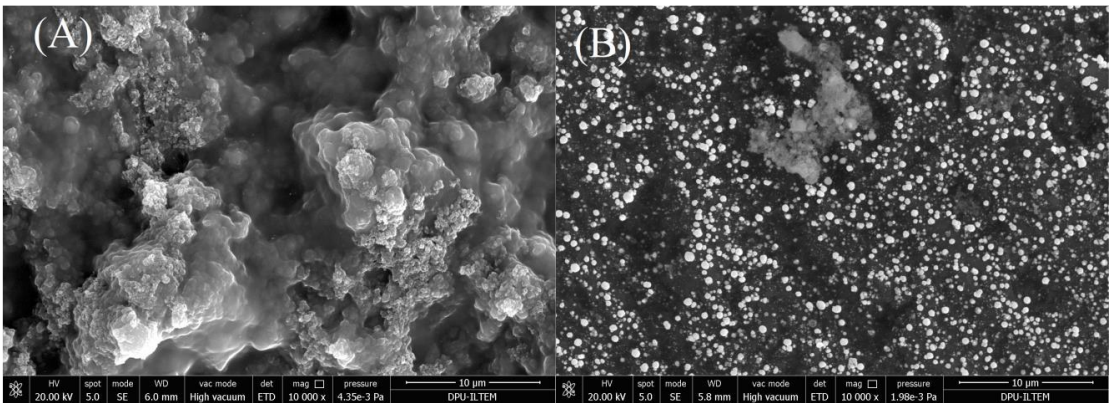


Figure 2. *Electrochemical measurement scheme of label-free leptin immunosensor*

3. THE RESEARCH FINDINGS AND DISCUSSION

3.1. Surface Image of the Immunosensor

Scanning electron microscopy is a widely utilized technique for investigating the surface properties of electrodes [25,34]. The surface properties of the immunosensor were evaluated during the preparation steps. When Figure 3 is examined, a very porous and rough structure is observed due to the suitable film-forming characteristic of CHI with the electrode modified with $CoFe_2O_4$-CHI (Figure 3 (A)). When AuNPs are deposited on the GCE/$CoFe_2O_4$-CHI surface, particles that tend to form a spherical shape without significant aggregation can be seen to accumulate on the surface (Figure 3 (B)). It can be said that a smoother surface structure was formed in the electrodes prepared by adding leptin antibody, BSA, and leptin to the GCE/$CoFe_2O_4$-CHI/AuNP surface, respectively, and the morphology of the surface changed (Figure 3 (C)-(E)).



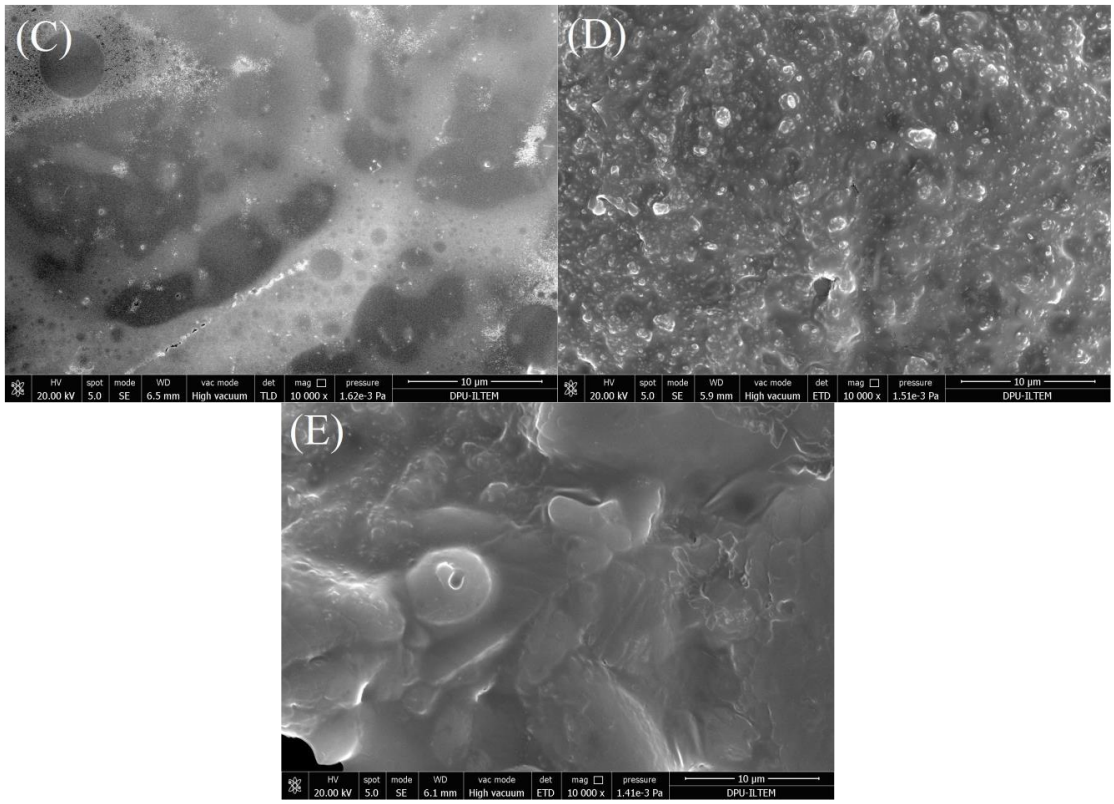


Figure 3. *SEM images of (A) GCE/$CoFe_2O_4$-CHI, (B) GCE/$CoFe_2O_4$-CHI/AuNP, (C) GCE/$CoFe_2O_4$-CHI/AuNP/Ab, (D) GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA and (E) GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA/Ag*

3.2. Electrochemical Characterization of Immunosensor

CV is a valuable method to provide information about the electrochemical properties of modified electrodes [35]. For this purpose, the promising electrodes were immersed into the PBS-redox, and voltammograms were recorded at a potential range of (-0.2) - (+0.6) V (*vs*. Ag/AgCl) at a scan rate of 50 mV s-1 (Figure 4). In addition, the peak currents and peak potentials determined from voltammograms are given in Table 1. Considering Figure 4 and Table 1, a single oxidation and reduction peak was obtained with each electrode. However, the peak currents obtained with GCE/$CoFe_2O_4$-CHI were higher than those obtained with GCE (Figure 4 (a) and (b)). This result shows a facilitated redox process in the GCE/$CoFe_2O_4$-CHI compared to the unmodified GCE. After AuNPs electrodeposition on the GCE/$CoFe_2O_4$-CHI surface, the highest peak currents were gained because of the synergistic impact of the electrode components of $CoFe_2O_4$ and AuNPs (Figure 4(c)). $CoFe_2O_4$, a member of the ferrite family, is used in sensing platforms due to high electrocatalysis activity, high surface area, outstanding biocompatibility, and excellent chemical stability [36]. AuNPs are widely used nanostructures in electrochemical fields owing to their superb features, such as the ability to facilitate electron transfer and large surface area [37].

After demonstrating the effect of the GCE/$CoFe_2O_4$-CHI/AuNP electrode on the behavior of the redox couple, it was investigated whether the proposed electrode could be used to prepare an immunosensor for leptin. Anti-leptin antibody was immobilized onto GCE/$CoFe_2O_4$-CHI/AuNP to obtain GCE/$CoFe_2O_4$-CHI/AuNP/Ab. As stated above, the prepared electrode was placed in a cell containing the PBS-redox, and CVs were taken at a voltage range of (-0.2) - (+0.6) V (*vs*. Ag/AgCl) (Figure 4 (d)). It was seen that the peak currents diminished, and the peak potentials changed to more negative potentials with this electrode compared to the modified electrode. The decrement in current response can be ascribed to the immobilization of the antibody on the electrode surface [38]. Subsequently, non-specific binding places were blocked by treating the GCE/$CoFe_2O_4$-CHI/AuNP/Ab electrode with BSA, and peak currents continued to decline (Figure 4(e)). This decrease in peak currents indicates that a layer that blocks electron transfer is formed on the electrode surface, thereby disrupting the electrochemical reaction of $[Fe(CN)_6]^{3-/4-}$ [39]. In conclusion, it can be said that the immunosensor was successfully prepared.

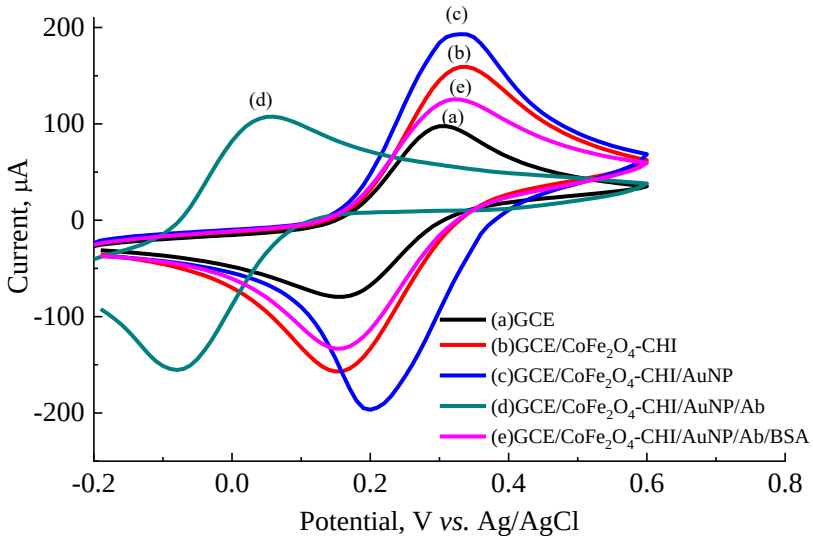
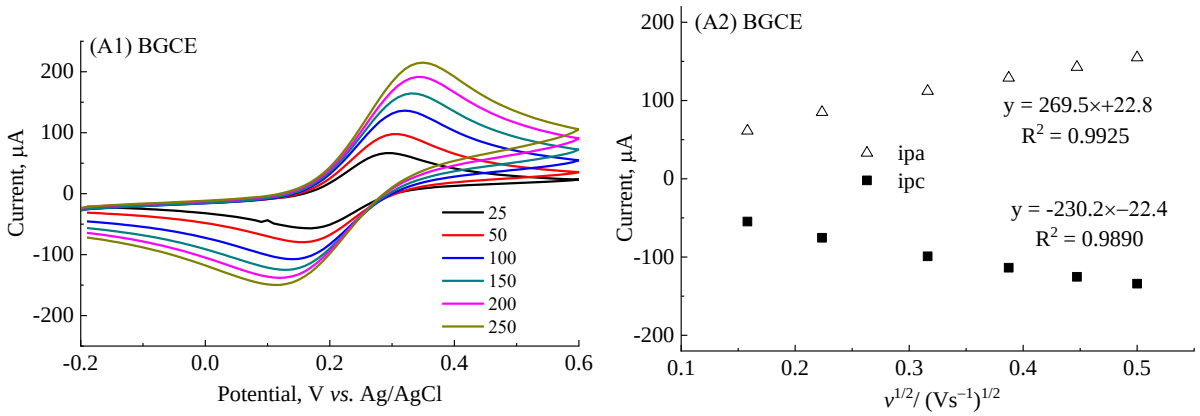


Figure 4. *CVs of (a) GCE, (b) GCE/$CoFe_2O_4$-CHI, (c) GCE/$CoFe_2O_4$-CHI/AuNP, (d) GCE/$CoFe_2O_4$-CHI/AuNP/Ab and (e) GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA*

Table 1. *Comparison of the electrochemical properties of the prepared electrodes*

Electrode	i_{pa}, **µA**	i_{pc}, **µA**	E_p, **V**
GCE	85.0	-75.4	0.310/0.160
GCE/$CoFe_2O_4$-CHI	126.3	-156.2	0.340/0.150
GCE/$CoFe_2O_4$-CHI/AuNP	157.7	-187.2	0.330/0.200
GCE/$CoFe_2O_4$-CHI/AuNP/Ab	108.8	-96.3	0.06/-0.09
GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA	97.5	-120.6	0.320/0.150

Electrode reaction kinetics were evaluated by studying the impact of sweep rate on peak currents. For this purpose, using GCE, GCE/$CoFe_2O_4$-CHI/AuNP, and GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA electrodes, CVs were taken at different sweep rates (25-250 mV s^{-1}) in the PBS-redox (Figure 5). It was monitored that the peak currents raised with the enhancement of the sweep rate in each electrode. As depicted in the inset of Figure 5, both anodic and cathodic peak currents were linearly proportional to the square root of the sweep rate. This consequence suggests that the electrode reaction was primarily driven by a diffusion-controlled process. Diffusion-controlled processes are an ideal mass transfer method, especially for quantitative determinations [40].



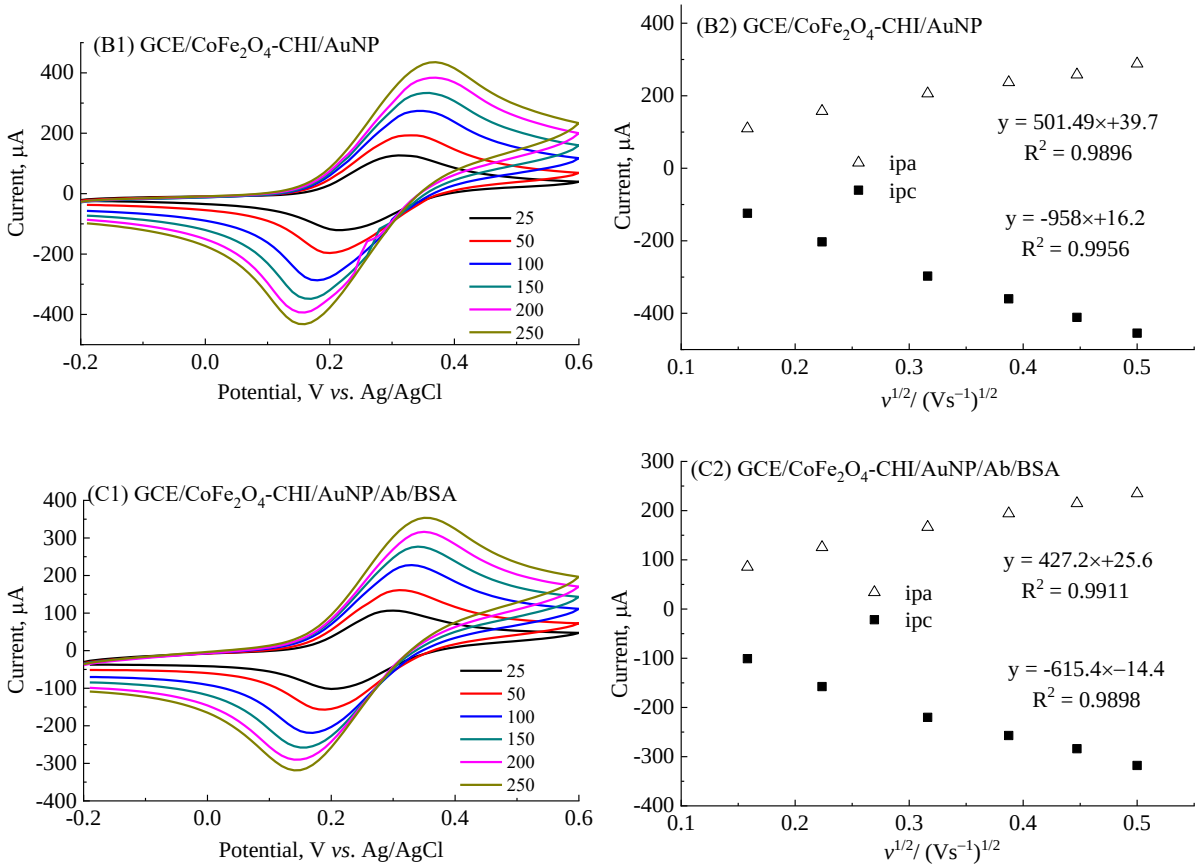


Figure 5. *CVs at different sweep rates and plots of the peak current vs. the square root of scan rate of (A1-A2) BGCE (B1-B2), GCE/$CoFe_2O_4$-CHI/AuNP, and (C1-C2) GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA*

EIS technique helps provide information about interfacial alterations of the modified electrode surface. The semicircular part of the EIS spectra shows the electron transfer process at high frequencies, and the linear fragment shows the diffusion process at low frequencies. The diameter of the half-cycle equals the electron-transfer resistance (R_{et}) [41]. The electrochemical properties and preparation steps of the immunosensor were also explored with the EIS. Impedance spectra were taken in the PBS-redox in the frequency range of 10^5 to 0.01 Hz (Figure 6 (A)). Electron transfer resistances (R_{et}) between the solution and the electrode interface were determined as approximately 1220 ohms, 156 ohms, and 47 ohms for the GCE, GCE/$CoFe_2O_4$-CHI, and GCE/$CoFe_2O_4$-CHI/AuNP electrodes, respectively (curve a, b, and c). It is seen that lower electron transfer resistance and a higher conductivity are obtained with the GCE/$CoFe_2O_4$-CHI/AuNP electrode. This may be due to the prepared film's effective surface area, excellent electrocatalytic behavior, and good conductivity properties. It is thought that the prepared GCE/$CoFe_2O_4$-CHI/AuNP can be effectively utilized to prepare an immunosensor. Afterward, when the anti-leptin antibody was immobilized on the electrode surface, the diameter of the Nyquist curve increased to approximately 345 ohms (curve d). This may be ascribed to the hydrophobic antibody layer blocking the electron flow between the electrode solution interface [42]. After BSA treatment, the GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA electrode had a higher R_{et} value (452 ohms (curve e)). It can be said that the hydrophobic layer consisting of the electrode surface causes an increment in the R_{et} value and prevents electron transfer [18]. The results obtained from CV and EIS techniques agree, and the immunosensor was prepared successfully. Figure 6(B) shows the equivalent circuit model: R_{et} is the electron transfer resistance, R_s is the solution resistance, CPE_{dl} is the stationary phase element, and W is the Warburg resistance.

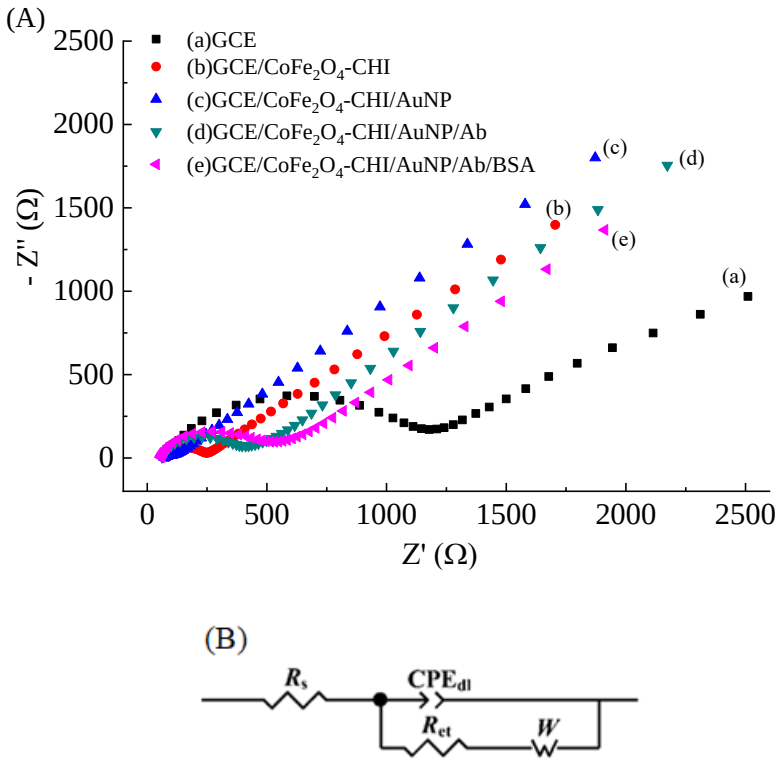


Figure 6. *(A) Nyquist plots of (a) GCE (b) GCE/$CoFe_2O_4$-CHI (c) GCE/$CoFe_2O_4$-CHI/AuNP (d) GCE/$CoFe_2O_4$-CHI/AuNP/Ab (e) GCE/ $CoFe_2O_4$-CHI/AuNP/Ab/BSA (B) The equivalent electrical circuit suggested for fitting Nyquist plots*

3.3. Leptin Response of Immunosensor

In label-free immunosensors, since antibodies and antigens are not redox-active structures, the response is interpreted using a mediator. Generally, $[Fe(CN)_6]^{3-/4-}$ redox pair is known as one of the mediators used in label-free electrochemical immunosensor systems. This redox mediator has a low ground current, gives a well-defined double redox peak, and the electrochemical signal obtained with this couple is very stable [43]. In various studies, the $[Fe(CN)_6]^{3-/4-}$ redox couple is utilized as a mediator, and the electrochemical response was based on decreasing peak currents after immunocomplex forming [44–46]. To investigate whether the prepared immunosensor could be utilized in leptin determination, a certain amount of leptin antigen solution was dripped on the electrode surface. GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA/Ag electrode was obtained. After the incubation step for 40 minutes, differential pulse voltammograms (DPVs) were taken in the voltage range between (-0.2) and (+1,1) V at a sweep speed of 50 mV s^{-1} in the PBS-redox. As depicted in Figure 7, the peak currents enhanced in the modification steps, but the peak currents decreased after the antibody and BSA treatment (curve (a)-(e)). Similarly, the peak currents are further reduced with the emergence of the hydrophobic immunocomplex on the electrode surface (curve f). The immunocomplex on the electrode surface acts as an electron transfer and mass transfer insulating layer, as electron transfer between the electron mediator and the electrode is inhibited, resulting in a reduction in the signal current response [47]. Accordingly, as the surface properties change, the oxidation of the redox species on the electrode surface is prevented, and the peak currents decrease. Because the antigen and antibody interactions are particular, the resulting signal can be used for detection.

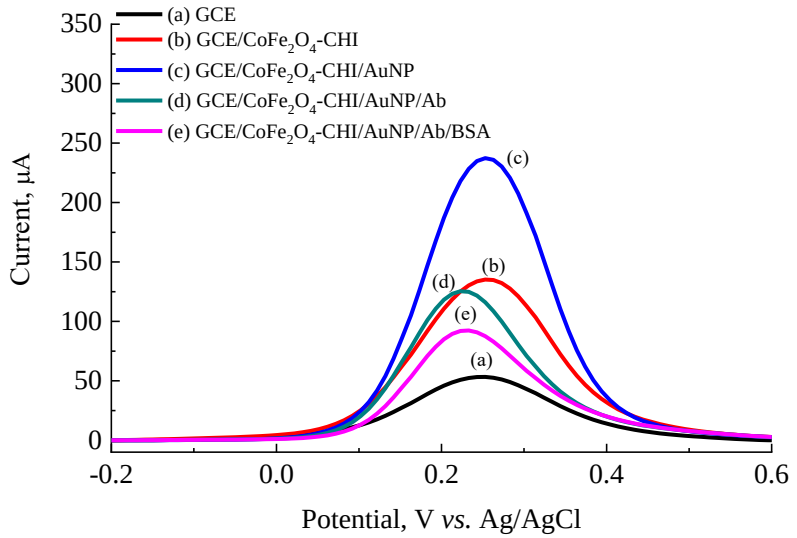


Figure 7. *DPVs of (a) GCE, (b) GCE/$CoFe_2O_4$-CHI, (c)GCE/$CoFe_2O_4$-CHI/AuNP, (d) GCE/ $CoFe_2O_4$-CHI/AuNP/Ab, (e) GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA and (f) GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA/Ag*

3.4. pH Optimization

The impact of pH on leptin determination was explored by recording the DPVs at different pH values of the redox probe solution ranging from 5.0 to 8.0 in the voltage range from (-0.2) to (+1.1) V (scan rate of 50 mV s^{-1}). The peak currents determined from the voltammograms were plotted against the solution pH (Figure 8). When the Figure is examined, the high peak currents obtained at pH 5.0 and 6.0 indicate that the response of the redox couple is not inhibited adequately on the immunosensor surface. In addition, the currents of the redox couple at pH 7.0 seem to decrease due to the proper binding of the antibody on the electrode surface and the successful formation of the immunocomplex. When the pH of the solution was increased to 8.0, it was determined that there was not much change. The more acidic and basic redox solutions have not been studied because they do not create a suitable environment for biomolecules and may lead to decreased biological activity of the antibody and dissociation of the antigen-antibody complex [48]. For these reasons, the pH of the redox solution was chosen as 7.0, and subsequent experiments were carried out at this pH.

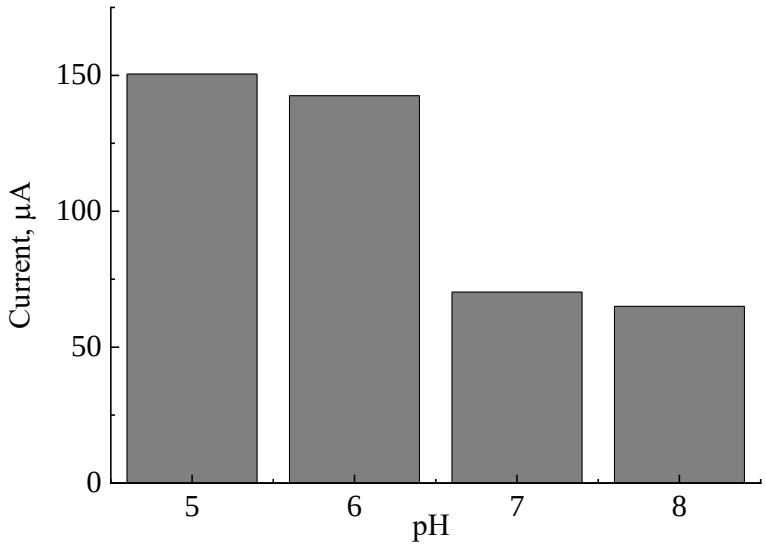


Figure 8*. Effect of pH of redox probe solution on DPV responses*

3.5. Anti-leptin Antibody Concentration Optimization

The antibody concentration is a vital factor for the efficiency of immunosensors. To gain a convenient electrochemical response, the effect of the anti-leptin antibody concentration (0.1 ─ 20 μg/mL) was studied using DPV. In Figure 9, the peak currents of the redox probe reduced with the increment in antibody concentration. It can be interpreted as the emergence of a more effective immunocomplex layer on the electrode surface with the rise in the antibody concentration and preventing the oxidation of the redox species. However, antibody concentrations greater than 10 μg/mL were accompanied by an insignificant decrease in peak current. Thus, at increasing concentrations, it can be said that the reaction between antibody and antigen reaches saturation [49]. The appropriate antibody concentration, 10 μg/mL, which is below the saturation concentration and at which adequate response is obtained, was selected, and this value was used in all experiments.

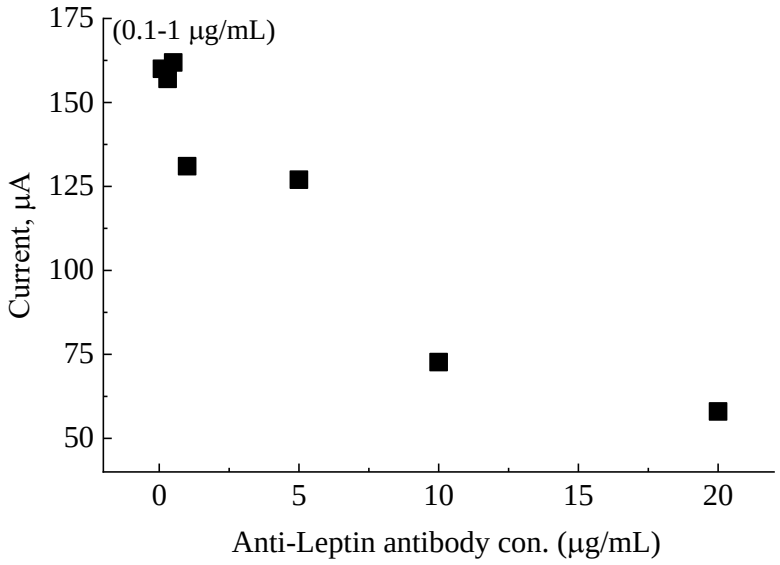


Figure 9. *Effect of antibody concentration on DPV responses*

3.6. Analytical Performance of Immunosensor

Distinct concentrations of leptin antigen (Ag) solutions were added to the GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA electrodes to obtain the working range of the subjected immunosensor. After incubation, DPVs were taken at a scan rate of 50 mV s^{-1} in the voltage interval (-0.2) to (+1.1) V *vs.* Ag/AgCl in the PBS-redox. In Figure 10(A), the oxidation peak currents of the redox species reduced with the enhancement in leptin concentration. It can be attributed to forming a protein-insulating layer on the electrode. There was a non-linear relationship between leptin concentration and peak currents. This is the usual behavior for ligand-based biosensor systems that rely on species interaction, such as antibody-antigen [50]. One approach to linearizing the graph is to reconstruct it by taking the logarithm of the concentration values. Figure 10(B) shows a calibration graph acquired by plotting the peak currents against the leptin concentration, equating to $y = -20.7x+119.9$ ($R^2 = 0.9927$). Thus, the linear working range obtained for the determination of leptin with the prepared immunosensor was determined as 1-4000 ng mL^{-1}, and the LOD was defined as 0.1 ng mL^{-1}. These results are comparable with those of other leptin immunosensors reported in the literature (Table 2).

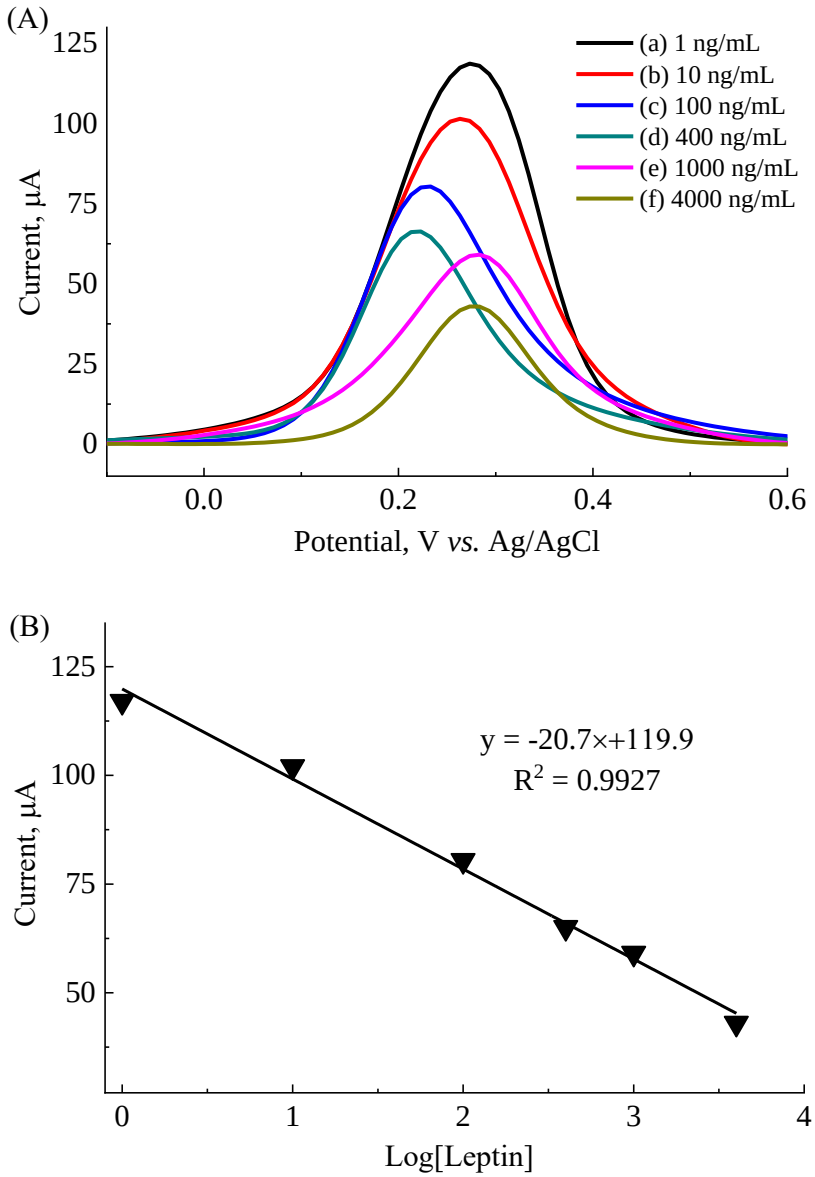


Figure 10. *(A) DPVs obtained at different antigen concentrations (B) Calibration curve based current value vs. logarithm of antigen concentration*

Table 2. *Comparison of electrochemical immunosensors for leptin determination*

Electrode/Label	**Working Range**	**DetectionLimit**	**Ref.**
BSA/Glu/Cys/AuNPs/PG-BP/GCE (Label-free)	0.150–2500 pg/mL	0.036 pg/mL	[51]
G/PPy/PPa/Au (Label-free)	10–100000 ng/mL	10 ng/mL	[52]
AP-IgG/anti-LP/LP/LP-Biotin/Strept-MB (ALP)	5-100 pg/mL	0.5 pg/mL	[53]
AP-anti-Ab/anti-LP/LP-SWCNTs/CS/GCE (ALP)	0–1000 ng/mL	5 pg /mL	[54]
SPGE/4-MBA/LP-Ab/BSA (Label-free)	100 pg/mL-1 µg/mL	100 pg/mL	[55]
GP/PGA/anti-LP/BSA (Label-free)	0.02 -20 pg/mL	0.00813 pg/mL	[56]
TEGCnSH/ HOOCC10SH/ALP-Ab (ALP)	100 pg/mL-10 ng/mL	13.6 pg/mL	[57]
SPGE/DTSSP/ab/LP/B-Ab/Streptavidin HRP/oPD (HRP)	0.1-20 ng/mL	0.033 ng/mL	[58]
GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA/Ag (Label-free)	1-4000 ng/mL	1 ng/mL	This work

LP: Leptin, ALP: Alkaline phosphatase, BSA: Bovine serum albumin, Glu: Glutaraldehyde, Cys: Cysteine, AuNPs: Gold nanoparticles, PG-BP: Porous graphene functionalized black phosphorus, GCE: Glassy carbon electrode. G: G protein, PPy:

Polypyrrole, PPa: Pyrrole Propylic Acid, Au: Gold. AP-IgG: IgG labeled with alkaline phosphatase, Strept-MB: streptavidin-functionalized magnetic beads. AP-anti-Ab: alkaline phosphatase labelled antibody, SWCNTs: Single-walled carbon nanotubes, CS: chitosan. SPGE: Screen printed gold electrode, 4-MBA: 4-mercaptobenzoic acid, BSA: Bovine serum albumin. GP: Graphite paper electrode, PGA: Polyglutamic acid, BSA: Bovine serum albumin. TEGCnSH: Ttri(ethylene glycol) terminated short alkanethiol, HOOCC10SH: 10-Carboxy-1-decanthiol, ALP-Ab: Alkaline phosphatase labelled antibody. SPGE: Screen printed gold electrodes, DTSSP: 3,30-dithiobis (sulfosuccinimidyl propionate), B: Biotin, HRP: Horseradish peroxidase, oPD: *o*-Phenylenediamine dihydrochloride

3.7. Determination of Reproducibility

To verify the reproducibility of the prepared immunosensor, three different sensors were fabricated for each leptin concentration. DPVs were recorded at a voltage range of (- 0.2) to (+1.1) V *vs.* AgCl/AgCl and at a sweep rate of 50 mV $s^{-1,}$ and the relative standard deviation (RSD) of the responses was given in Table 3. The reproducibility of the prepared immunosensor was found to be suitable.

Table 3. *Reproducibility of the prepared immunosensor*

Leptin Con. (ng mL^{-1})	RSD %
1	0.60
10	3.80
100	6.07

4. RESULTS

In conclusion, a novel label-free immunosensor, which can be an alternative to medical tests with its sensitive and easy-to-use features based on GCE/$CoFe_2O_4$-CHI/AuNP/Ab/BSA electrode, is produced for leptin determination. The response of the immunosensor was linear in the range of 1-4000 ng mL^{-1} with a detection limit of 0.1 ng mL^{-1}. The prepared immunosensor utilized the properties of components as follows: *i)* $CoFe_2O_4$ NPs provide high surface area and an increased electrical response, *ii)* CHI provides a biocompatible environment for immobilization of antibody, *iii)* AuNPs facilitate electron transfer. The reproducibility of the prepared immunosensor was satisfactory. The simplicity of the manufacturing and measurement processes makes this immunosensor a promising tool for clinical analysis.

CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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