An Electrochemical Biosensor Based on Gold Nanoparticles/Carbon Nanotubes Hybrid for Determination of recombinant human erythropoietin in human blood plasma

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The aim of this study is to fabricate an electrochemical biosensor using a decreased recombinant human erythropoietin (rhuEPO)/Au NPs/CNTs nanohybrid modified glassy carbon electrode (GCE) to measure the amount of the peptide hormone rhuEPO in a sample of cyclists' blood plasma. The GCE surface was modified with an Au NPs/CNTs nanohybrid structure using the electrodeposition method, and rhuEPO was reduced on the nanohybrid structure using chronoamperometry. Studies on the structural properties of electrodeposited Au NPs/CNTs revealed that these particles had a suitable distribution on the surface of the CNTs and could be regarded as a hybrid nanoparticle nano-disperse ensemble. The development of decorated Au NPs on CNTs porous structure surface facilitated the functionalization of the nanoparticles with rhuEPO specific antibody as well as promoted the stability of rhuEPO biosensors, and reduced rhuEPO promoted the selectivity of biosensors, according to electrochemical analyses of rhuEPO biosensors by DPV and amperometry. According to amperometric experiments, the linear range was 0 to 22000 ng/l, and a sensitivity value of 4.3907 $\mu A/\mu gl^{-1}$ for reduced rhuEPO/Au NPs/CNTs/GCE toward rhuEPO was achieved. The detection threshold was set at 1 ng/l as well. These results demonstrated the wide linear range and adequate detection limit value of the decreased rhuEPO/Au NPs/CNTs nanohybrid when compared to recently reported rhuEPO sensors and biosensors. rhuEPO was extracted using magnetic beads and reduced on Au NPs/CNTs/GCE, and the results showed excellent agreement and validity between amperometry and ELISA analyses. The applicability and validity of reduced rhuEPO/Au NPs/CNTs/GCE were investigated for the determination level of rhuEPO in a blood plasma sample from cyclists. Additionally, the developed approach can be used to determine the level of rhuEPO in clinical samples because of the appropriate relative standard deviation ($\geq$4.69 %) and relative recovery ($\geq$99.00 %) values.

Keywords: Au nanoparticles; CNTs; Nanohybrid structure; Electrodeposition; Peptide hormones; Erythropoietin; Amperometry

1. INTRODUCTION

Any molecule made up of two or more amino acids connected by peptide bonds is referred to as a peptide [1, 2]. An amino acid's general structure is R-CH(NH$_2$)COOH [3, 4]. Each amino acid is a monomer that joins with other amino acids to form a peptide polymer chain when one amino acid's carboxyl group (-COOH) combines with another amino acid's amino group (-NH$_2$) to create a covalent connection [5, 6]. Thus, peptides are amino acids that are the building blocks of certain proteins needed by the skin, like collagen and elastin [7, 8].

Peptides function as enzymes, hormones, poisons, antibiotics, and structural elements of cells and tissues. The hormones oxytocin, glutathione (which encourages tissue growth), insulin from the pancreas, glucagon (a hyperglycemic factor), luteinizing hormone, steroid hormone, parathyroid hormone, and ghrelin are some examples of peptides [9-11]. Examples of peptide hormones that help the body deal with stress and control the synthesis of several tissues in the body, respectively, include corticotrophins and growth hormone [12-14].

The body naturally produces erythropoietin, a peptide hormone, primarily in the kidneys [15-17]. Red blood cell production in the bone marrow is prompted by it. Severe anemia may develop if the body does not create enough erythropoietin. Patients with chronic renal disease who have malfunctioning kidneys frequently experience this [18-20]. Red blood cells carry oxygen to the cells, including muscle cells, permitting them to function more efficiently [21-23]. This is why erythropoietin is helpful for athletes [24, 25]. Elite cycling studies have shown that erythropoietin improves athletic performance and gives users an unfair advantage over rivals [26, 27]. Therefore, according to the WADA forbidden list, erythropoietin is never allowed in sports [28, 29]. Recombinant human erythropoietin (rhuEPO), a biopharmaceutical medicine given to patients who have low hemoglobin due to chronic renal disease, cancer, or anemia, is one example of synthetic rhuEPO manufactured using recombinant DNA technology [30-32].

Because of this, measuring rhuEPO levels is crucial. Numerous studies have been conducted to identify rhuEPO levels as doping agents in athlete biological samples using electrochemical sensors and biosensors [33-41]. To improve the selectivity and sensitivity of rhuEPO sensors, these sensors and biosensors must be modified with the appropriate nanostructure and nanocomosites. Therefore, the aim of this study is to make an electrochemical biosensor via reduced rhuEPO, Au NPs, CNTs, and GCE that can be used to measure the level of rhuEPO in a sample of blood plasma from cyclists.

2. EXPERIMENT

The electrodeposition method was used for modification of the GCE surface [42, 43]. Before the modification, the GCE surface was successively polished with alumina powder (1.0, 0.3 and 0.05 μm, 99.99%, Sigma-Aldrich) on a buff until it got the mirror-smooth surface. After each polishing, the GCE surface was ultrasonically washed with a mixture of water and ethanol. For electrodeposition, 2.0 mg CNTs (99%, Luoyang Tongrun Info Technology Co., Ltd., China) were dispersed in 30 mL of 0.1 M KNO$_3$ (≥99.0%, Sigma-Aldrich) solution containing 0.2 g/L HAuCl$_4$ (99.5%,Merck, Germany). The

electrodeposition was performed using an electrochemical workstation potentiostat (Xiamen Tob New Energy Technology Co., Ltd., China) in a three-electrode electrochemical setup containing clean GCE, an Ag/AgCl (3 M KCl) and Pt mesh as working, reference and counter electrode, respectively. The electrodeposition of the CNTs was conducted at 1 V for 200 s. The electrodeposition of the Au NPs on the CNTs/GCE was performed at -0.24 V for 100 s.

The antibody-conjugated magnetic beads (Ab-MB) were prepared for careful extraction from rhuEPO of human blood. For preparation of Ab-MB, 200 µL of a mixture of equal volume ratios of 0.15M N-hydroxysuccinimide (NHS, 98%, Sigma-Aldrich) and 0.15M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 99.0%, Sigma-Aldrich) solution was ultrasonically added to amine derivatized (99%, Sigma-Aldrich). Then, 50 µL silanized iron oxide magnetic beads (size of 3µL, Thermo Fisher Scientific, USA) were added to the obtained mixture under magnetic stirring. After that, 20 µL of $0.2gL^{-1}$ rhuEPO antibody (EPOGEN, Amgen, Thousand Oaks, CA, USA) were added to the mixture. Subsequently, for conjugation of the magnetic beads by the antibody, the obtained mixture was stored at 5°C for 5 hours. The resultant magnetic beads were separated from the solutions using a magnetic separator followed by resuspendation in 200 µL of 0.1 M phosphate buffer solution (PBS) with pH =7.4. Finally, the obtained Ab-MB was stored at 5°C before use. 0.1 M PBS contained a mixture of equivalent volume ratio of 0.1M NaH_2PO_4 and 0.1M Na_2HPO_4 (99%, Merck, Germany).

The rhuEPO standard solutions were prepared in a concentration range from 1 ng/l to 1000 ng/l through serial dilution in 0.1 M PBS with pH =7.4. A 12ng/l of rhuEPO was added into human blood. Then, 200 µL of Ab-MB was added to 200 µL of plasma. The mixture was left for 90 minutes with mild shaking at room temperature. The MBs were separated from the mixture using a magnetic separator and washed several times with 150 µL of 0.1 M PBS with pH =7.4 to remove the impurities and avoid the adsorption of interferants. 250 µL of 1.0 M coupling buffer of glycine/KOH with pH 10.0 was added to the beads to release the captured rhuEPO from the magnetic beads followed by mild shaking for 30 minutes at room temperature. To remove the glycine buffer from the recovered solutions, they were loaded onto a size exclusion column and the extracted biomolecules were eluted using 500 µL of 0.1M PBS with pH 7.4.

For the modification of Au NPs/CNTs/GCE, CNTs/GCE and Au NPs/GCE with rhuEPO, 10 µl of standard rhuEPO solution with a concentration range of 1000 ng/l was loaded on nanostructured surface of electrodes, and then assembled and reduced through chronoamperometry at a potential of -1.0 V for 25min into 0.1M KOH. Finally, to electrochemically absorption the reduced protein on the surface of the electrode, the DPV experiment was performed at potential range from -1 V to -1.4 V at scan rate of 10mV/s for 15 cycles. The rhuEPO/Au NPs/CNTs/GCE, rhuEPO/CNTs/GCE and rhuEPO/Au NPs/GCE were stored in 4°C for electrochemical analyses. The human Erythropoietin Quantikine IVD ELISA Kit was also utilized for studies of rhuEPO content in human plasma.

Field emission scanning electron microscopy (FESEM) and X-ray diffraction (XRD) were used to conduct structural and morphological analyses of the electrodeposited nanostructure.

3. RESULTS AND DISCUSSION

Figure 1 displays SEM images of CNTs/GCE, Au NPs/GCE, and Au NPs/CNTs/GCE. High density CNTs electrodeposited on the surface of the GCE are visible in Figure 1a's SEM images of CNTs/GCE, and their diameters range from 60 to 70 nm. The electrodeposited CNTs have a large surface area and void spaces or gaps between them that can be thought of as macropores, and diffusion can occur easily through the pores [44, 45]. Figure 1b's SEM pictures of the Au NPs/CNTs/GCE combination demonstrate that the NPs are properly distributed over the surface of the CNTs and can be regarded as a nanoparticle hybrid nano-disperse ensemble [46, 47]. Moreover, there is a high aspect ratio interpenetrating CNTs which prevents nanoparticles aggregation [48-50], thereby enabling decorated Au NPs have an average diameter of 30 nm.
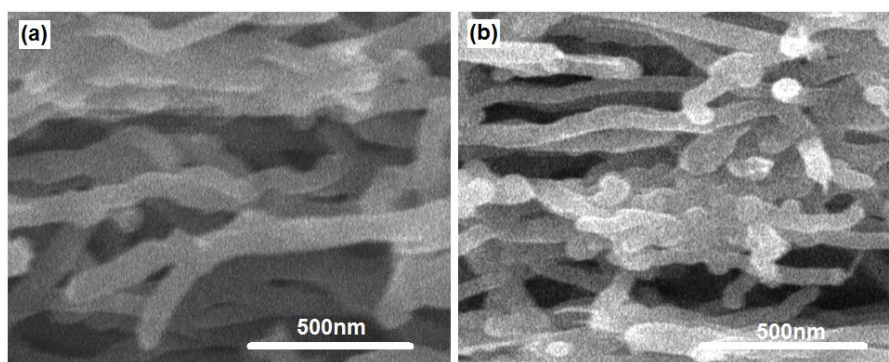

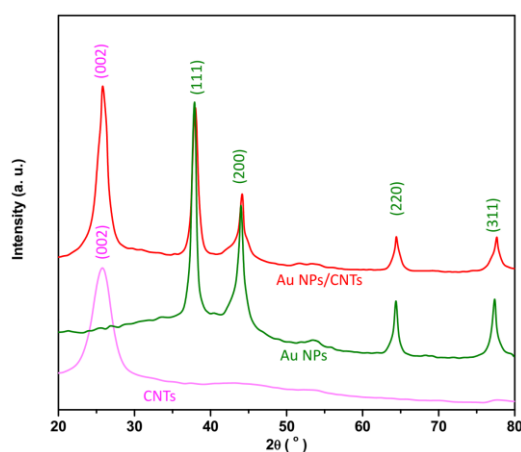**Figure 1.** SEMs of (a)CNTs/GCE, (b) Au NPs/CNTs/GCE

Figure 2. The XRD profiles of electrodeposited CNTs, Au NPs and Au NPs/CNTs.

XRD profiles of deposited CNTs, Au NPs and Au NPs/CNTs are displayed in Figure 2. The XRD profile of CNTs in Figure 2a illustrates a sharp diffraction peak at 25.75° which is assigned to the graphitic structure of CNTs with a plane of (002) [51, 52]. The XRD profiles of Au NPs and Au NPs/CNTs in Figure 2 show diffraction peaks at 37.78°, 43.91°, 64.36° and 77.59° which are indexed

to the face-centered cubic (fcc) structure of metallic Au with (111), (200), (220) and (311) planes(JCPDS No. 04-0784) [53-55]. The XRD profile of Au NPs/CNTs exhibits an additionally strong peak at 25.77° which demonstrates the successful deposition of Au NPs on CNT surfaces that further confirmed by the SEM analysis as shown in Figure 1c.

For erythropoietin, normal standards in healthy people are in a concentration range from 7 to 19 ng/l [56, 57]. Therefore, the electrochemical biosensor performance should be evaluated within this concentration range. The obtained DPV responses of bare GCE, CNTs/GCE, Au NPs/GCE, Au NPs/CNTs/GCE, reduced rhuEPO/CNTs/GCE, reduced rhuEPO/ Au NPs /GCE and reduced rhuEPO/Au NPs/CNTs/GCE in 0.1 M PBS with pH 7.4 and 100 ng/l rhuEPO at a scanning rate of 15 mV/s are depicted in Figure 3. It is found that bare GCE, CNTs/GCE, Au NPs/GCE and Au NPs/CNTs/GCE do not show any peak current at potential window of 0.0 V to 0.52 V. The observed peaks at 0.18 V, 0.17 V and 0.16 V are reduced rhuEPO/CNTs/GCE, reduced rhuEPO/ Au NPs /GCE and reduced rhuEPO/Au NPs/CNTs/GCE, respectively, indicating reduced rhuEPO important role in efficient reduction of the disulfide bonds in proteins and peptides [58-60].
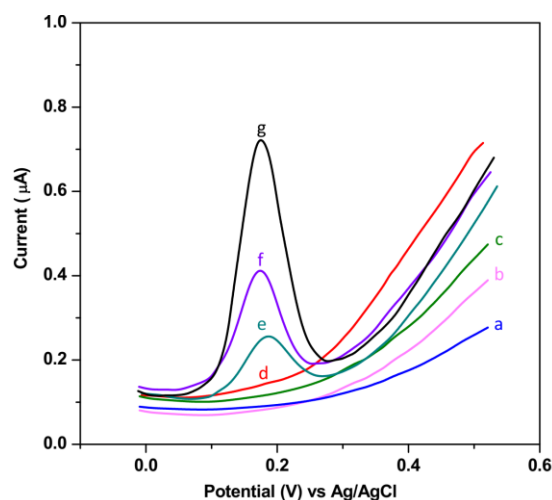

Figure 3. The obtained DPV responses of (a) bare GCE, (b) CNTs/GCE, (c) Au NPs/GCE, (d) Au NPs/CNTs/GCE, (e) reduced rhuEPO/CNTs/GCE, (f) reduced rhuEPO/ Au NPs /GCE and (g) reduced rhuEPO/Au NPs/CNTs/GCE in 0.1 M PBS with pH 7.4 contaning 100 ng/l rhuEPO at a scanning rate of 15 mV/s.

It can form the free sulfhydryl (thiol; -SH) groups on proteins which chemisorb preferentially onto the Au nanostructures of the functionalised substrate through the formation of Au-S bonds [61-63]. In addition, the DPV responses in Figures 3 exhibits that the peak reduced rhuEPO/Au NPs/CNTs/GCE reveals a peak at lower potential and a higher current than that on reduced rhuEPO modified CNTs/GCE and Au NPs /GCE. It is associated with a porous CNTs structure that shows better mechanical and chemical properties, great electrical conductivity and high cell-affinity improving cell attachment, proliferation and differentiation [60, 64-66]. Au NPs is chemically stable, biocompatible, and because of the surface chemistry of Au, the development of decorated Au NPs on CNTs porous structure surface facilitates the functionalization of the nanoparticles with rhuEPO specific antibodies as well as promotes the stability of rhuEPO biosensors [67-69]. Thus, the Au

NPs/CNTs hybrid shows the advantages of the CNTs with their unique electrical and enlarged active surface and the Au NPs with good biocompatibility, conductivity and stability for biosensors. Therefore, the further electrochemical studies were performed using reduced rhuEPO/Au NPs/CNTs/GCE.

The stability effect of the DPV response of reduced rhuEPO/Au NPs/CNTs/GCE was investigated in 0.1 M PBS with pH 7.4 containing 100 ng/l rhuEPO at a scanning rate of 15 mV/s. Figure 4 depicts the 1st and 80th DPV responses of reduced rhuEPO/Au NPs/CNTs/GCE, indicating that after successive 80 scans the peak current decreased 5%. It is demonstrated that the electrochemical response of reduced rhuEPO/Au NPs/CNTs/GCE is highly stable due to successful immobilization of reduced rhuEPO molecules on Au NPs/CNTs hybrid structures, resulting in good biocompatibility for the maintenance of reduced rhuEPO molecules' biological activity [40, 70].
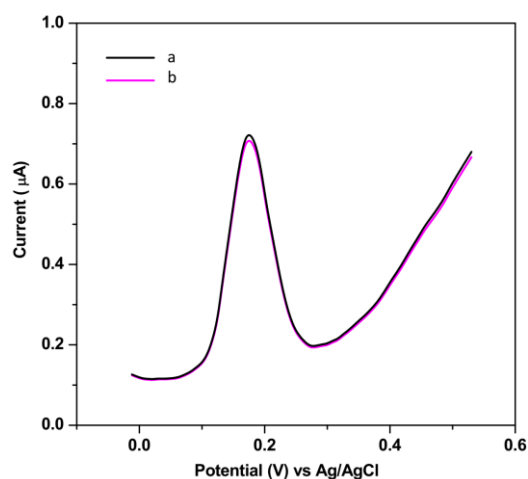

Figure 4. Stability effect of DPV response of reduced rhuEPO/Au NPs/CNTs/GCE in 0.1 M PBS with pH 7.4 contaning 100 ng/l rhuEPO at scanning rate of 15 mV/s; (a) 1st and (b) 80th DPV scans.

The amperometric responses and calibration graph of reduced rhuEPO/Au NPs/CNTs/GCE to successive additions of 1 μgl^{-1} rhuEPO into 0.1M PBS with a potential of 0.16 V are depicted in Figure 5. The obtained responses for each addition 1 μg/l rhuEPO indicate to fast response of reduced rhuEPO/Au NPs/CNTs/GCE as rhuEPO biosensor. As observed, after each injection of rhuEPO solution, the amperometric current intensity increases. The depicted calibration graph in Figure 5b shows that the signal of amperometric responses is linearly increased with a concentration range of 0 to 22000 ng/l, and sensitivity of reduced rhuEPO/Au NPs/CNTs/GCE toward rhuEPO is obtained of 4.3907 μA/μg/l. Additionally, the detection limit can be determined of 1 ng/l. These finding are compared with recent reported rhuEPO sensors and biosensors in Table 1. It is possible to be a novelty of this study that the reduced rhuEPO/Au NPs/CNTs/GCE exhibits a wide linear range or appropriate and lower detection limit value among the reported voltammetric electrochemical sensors [35, 39, 40] due to Au NPs and CNTs incorporation as biocompatibile matrix for protein immobilization, adhesion and susceptibility to chemical modifications [71, 72]. Both Au NPs and CNTs improve the electron conduction path, stability, biocompatibility and electrocatalytic activity of biosensors. CNT-based

modified electrodes can present the excellent electron transfer capabilities for the electrochemical activity of biomolecules. Moreover, antibody-conjugated magnetic beads can enhance the selectivity of the present sensor in comparison the potentiometry, optical sensors and SERS-based sensors [34, 37, 38, 73, 74].
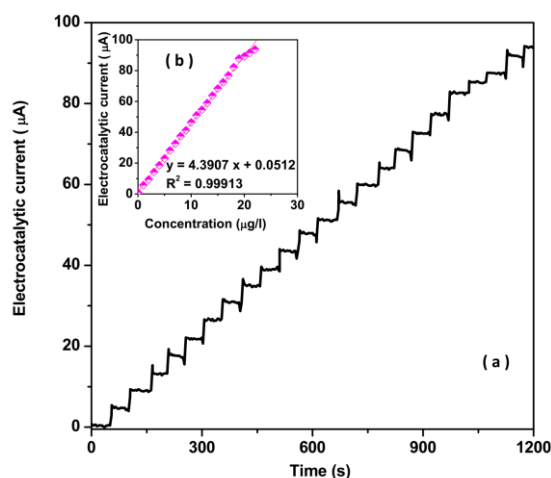

Figure 5. The amperometric responses and calibration graph of reduced rhuEPO/Au NPs/CNTs/GCE to successive addition 1 μgl^{-1} rhuEPO into 0.1M PBS with potential of 0.16 V.

Table 1. Compared the obtained sensing properties of reduced rhuEPO/Au NPs/CNTs/GCE and the recent reported rhuEPO sensors and biosensors

Electrodes	Sensing method	Linear range(ng/l)	Detection limit(ng /l)	Ref.
Reduced rhuEPO/Au NPs/CNTs/GCE	Amperometry	0 to 22000	1	This work
rhuEPO/nanostructured Au electrode	DPV	30 to 3×10^4	30	[39]
4-mercaptophenylboronic acid/biotin/AuNPs	DPV	0.6 to 60	24×10^{-3}	[40]
Mercury electrodes	SWV	40 to 400	17	[35]
3-aminopropyl triethoxysilane and tetraethoxysilane imprinted polymer	Potentiometry	10^4 to 10^6	6500	[37]
Polydopamine imprinted polymer	Potentiometry	10^3 to 10^5	330	[34]
BAN-Hg	Optical sensor	30 to 3×10^6	30	[38]
Anti-EPO/Rod-shaped Au NPs	SERS	0.1 to 10^3	0.1	[73]
rHuEPO/ aptamer-functionalized/ nanostructured Au electrode	SERS	0 to 300	---	[74]

SWV: Square wave voltammetry; SERS: surface enhanced Raman spectroscopy

The applicability and validity of reduced rhuEPO/Au NPs/CNTs/GCE for determining the level of rhuEPO in cyclists' blood plasma specimens were investigated. RhuEPO was extracted by magnetic

beads and reduced on a Au NPs/CNTs/GCE. The electrochemical approach for detection of rhuEPO in human plasma samples by selectively isolating the target rhuEPO using magnetic beads pre-functionalized with capture probes and then directly absorbing the targets onto the nanostructured electrode surface. Thus, proposed rhuEPO extractor magnetic beads with activated primary amine (–NH₂) HN₂ groups on the surface of the beads have been conjugated by EDC coupling reaction which added to the amino groups of the amino functional silane [75, 76]. EDC specifically binds to carboxyl (–COOH) groups of protein, and it can react accurately with NHS to crosslink proteins, which can reduce the occurrence of irregular binding in the system [77, 78]. When NHS reacts with EDC, its form becomes active to bind specific HN₂ functional proteins. Therefore, regular binding from C-terminal to N-terminal on the surface can be assured [77, 79]. Therefore, the antibody-functionalized magnetic beads can specifically bind with rhuEPO and extract rhuEPO from blood plasma. Table 2 exhibits the determination results by amperometry and ELISA analyses which demonstrated the average level of rhuEPO in the cyclists' blood plasma samples were 11.66 ng/l and 11.32 ng/l by amperometry and ELISA analyses, respectively, illustrating the great agreement and validity between amperometry and ELISA analyses. Additionally, the findings of analytical studies obtained from amperometry analysis by the standard addition method are exhibited in Table 2. As seen, the acceptable relative standard deviation (≥4.69%) and relative recovery (≥99.00%) values indcated that the developed method can be utilized for the determination level of rhuEPO in clinical samples.

Table 2. Findings of analytical studies obtained from amperometry and ELISA analysis for determination of rhuEPO in real sample prepared from cyclists's blood plasma.

Amperometry					ELISA	
Content in cyclists's blood plasma sample (ng/l)	Spiked (ng/l)	detected (ng/l)	Recovery (%)	RSD (%)	Content in cyclists's blood plasma sample (ng/l)	RSD (%)
11.66				3.61	11.32	3.29
	2.00	1.94	97.00	3.82		
	3.00	2.97	99.00	4.69		
	4.00	3.90	97.50	4.11		
	5.00	4.95	99.00	3.08		

4. CONCULUSION

In short, the aim of this study was to make an electrochemical biosensor by reducing rhuEPO/Au NPs/CNTs nanohybrid modified GCE for rhuEPO content as a peptide hormone detection in cyclists' blood plasma samples. The GCE surface was modified with an Au NPs/CNTs nanohybrid structure using the electrodeposition method, and rhuEPO was reduced on the nanohybrid structure using chronoamperometry. According to the findings of structural studies, Au NPs were properly distributed on the surface of CNTs and can be regarded as a nanoparticle hybrid nano-disperse

ensemble. Electrochemical investigations revealed that the signal of amperometric responses grew linearly over the concentration range of 0 to 22000ng/l, and a sensitivity of 4.3907 $\mu A/\mu gl^{-1}$ was achieved for reduced rhuEPO/Au NPs/CNTs/GCE toward rhuEPO. The detection threshold was set at 1 ng/l as well. These results demonstrated a wide linear range and an appropriate detection limit value when compared to recently published rhuEPO sensors and biosensors. This was made possible by the incorporation of Au NPs and CNTs as biocompatible matrixs for protein immobilization, adhesion, and chemical modification susceptibility. rhuEPO was extracted using magnetic beads and reduced on Au NPs/CNTs/GCE, and the results showed excellent agreement and validity between amperometry and ELISA analyses. The applicability and validity of reduced rhuEPO/Au NPs/CNTs/GCE were investigated for the determination level of rhuEPO in a cyclists' blood plasma sample. The developed approach can be used to determine the level of rhuEPO in clinical samples thanks to the acceptable relative standard deviation and relative recovery values.

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