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Enzymatic recognition of hydrogen peroxide (H_2O_2) in human plasma samples using HRP immobilized on the surface of poly(arginine-toluidine blue)- Fe_3O_4 nanoparticles modified polydopamine; A novel biosensor

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Abstract

In this study, an innovative strategy was proposed for the electrocatalytical reduction and enzymatic biosensing of hydrogen peroxide (H_2O_2) using chronoamperometry technique. For the first time, immobilization of horseradish peroxidase (HRP) in polydopamine-modified magnetic nanoparticles (PDA-MNPs) was successfully performed. Also, poly(L-arginine/toluidine blue) film–modified glassy carbon electrode was constructed through co-electropolymerization of L-arginine and toluidine blue on the surface of GCE using cyclic voltammetry technique. The engineered hybrid thin film provides strong functionalities for efficient grafting of PDA-MNPs which, in turn, enable the covalent immobilization of HRP. The proposed biosensor was used for the detection of H_2O_2 in the range of 0.5-30 μM with a low limit of quantification 0.23 μM. It also was successfully applied for the investigation of hydrogen peroxide in human plasma samples.

KEYWORDS
biomedical analysis, copolymerization, enzymatic biosensing, horseradish peroxidase, hydrogen peroxide, magnetic nanoparticles

1 | INTRODUCTION

Reactive oxygen species (ROS) are produced as products of oxidative stress factors derived from endogenous sources.[1] The consumption and utilization of oxygen in various physiological processes turn down to the generation of ROS, including hydrogen peroxide (H_2O_2), superoxide (O_2), and hydroxyl radical ($^{\bullet}OH$).[2] All ROSs can be harmful and toxic to organisms at high concentrations.[3] Overproduction of H_2O_2 is a deleterious process, which can be very damaging because it leads to various diseases such as cardiovascular disease, Alzheimer's disease, and cancer.[4] Moreover, it may contribute to tissue damaging, inactivation of some enzymes, and DNA strand breaks or formation of DNA adducts in biological systems.[4-6] Thus, the investigation of trace H_2O_2 is very important and essential in biological fluids.

Various analytical methods have been reported for the determination of H_2O_2 such as spectrophoto- and spectrofluorimetric[7,8] chemiluminescence[9,10] and electrochemical methods.[11] Electrochemical biosensor has gained great attention due to high sensitivity, ease of operation portability, low price, and quick response.[11-13] Enzyme-catalyzed reactions have been extremely carried out for evaluation of H_2O_2. This analyte (H_2O_2) as a classic heme enzyme has been extremely utilized for the determination of H_2O_2 as mimetic enzymes. Immobilized biocatalysts have higher activity and stability compared to free enzymes. Nano-based materials are considered to be an ideal support for enzyme immobilization.[13-17]

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Nanomaterial-based supports, in particular, magnetic nanoparticles (MNPs), endowed with principle functional groups and existence of hydroxyl groups on their surface facilitate surface modification, which leads to their easy functionalization and strong binding of the enzyme molecule.[14,15] MNPs exhibit exceptional characteristics such as small size (<100 nm), high surface area, low toxicity, high magnetization values, and active surface area.[16,17] Individual magnetic particles are susceptible to form a large aggregation, which can negatively influence the magnetic properties,[18] that diminish their efficient magnetic properties, arising rapid biodegradation when they are directly exposed to the biological system.[19]

Polymer-functionalized MNPs provide colloidal stability, which gives rise to improvement repulsive forces, balancing the magnetic and the van der Waals attractive forces acting on the MNPs, leading to prevention of the NPs' agglomeration.[20] Polydopamine (PDA) can be readily self-polymerized under slightly basic conditions (pH = 8.5) by the auto oxidative polymerization of dopamine to form a PDA thin layer.[21,22] PDA-MNPs provide desired functional groups, extremely large surface area to volume ratio, and high mass transfer, which is a useful alternative for surface immobilization.[23,24] A coating of PDA containing hydroxyl- and amine-functional groups with nanomagnetic offers many advantages resulting in promotion of surface properties and capacity of PDA-MNPs,[25] as it allows further binding capacity and high catalytic specificity of the conjugated enzyme. In addition, thiol groups offer an excellent platform for higher binding affinity including attachment of biofunctionalization as well as a stabilizing agent for further MNPs modification.[26-28]

Prepared polymer films via electropolymerization process provide numerous advantages compared to other process including covalent attachment and spin coating.[29,30] It is mainly owing to its ability to control the layer thickness of the polymer film by changing the experimental conditions (eg, scan rate in cyclic voltammetry).[31] The polymerization of these electronically conducting polymers can be done in a straightforward manner with a low cost.[32,33] Amino acids, including amine (—NH_2), carboxyl (—COOH), and hydroxyl (—OH) functional groups have been developing to form a conductive polymer by electropolymerization. Poly-L-arginine (P(Arg)) contains a guanidyl group with multidanate characteristics, which are able to form electrostatic interactions with negatively charged groups as well as hydrogen bonding.[34,35]

Toluidine blue O (Tb), one of the phenoxazine biological dyes with amino-functional group[36] has been physically adsorbed on the surface of P(Arg) through π–π electronic interactions without changing the native structure of L-arginine.[37,38] Moreover, P(Arg)/Tb composite film–modified glassy carbon electrode (GCE) not only induces conductive thin film for amplifying Faraday current but also provides a robust and steady layer for subsequent modification of PDA-MNPs with high surface area. Interaction between poly(amino acid) film and nanoparticles can improve the conductivity of the hybrid systems, enhancing the sensitivity and selectivity performance of the sensor.[39,40]

For the first time, an innovative strategy was proposed for the electrocatalytical reduction and enzymatic biosensing of H_2O_2 using chronoamperometry technique. For the first time, immobilization of horseradish peroxidase (HRP) in PDA-MNPs was successfully performed. Also, poly(L-Arg/Tb) film–modified glassy carbon electrode was constructed through co-electropolymerization of L-Arg and Tb on the surface of GCE using cyclic voltammetry technique. The engineered hybrid thin film provides strong functionalities for efficient grafting of PDA-MNPs, which, in turn, enables the covalent immobilization of HRP.

2 | MATERIALS AND METHODS

2.1 | Chemical and reagents

L-Arginine (L-Arg) was obtained from Sigma-Aldrich, HRP (Type VI, P8375, EC 1.11.1.7) was obtained from Sigma. Tb (C1. 52 040) was purchased from Merck Company, dopamine hydrochloride obtained from Merck. H_2O_2 solutions (from Sigma-Aldrich) were freshly prepared before being used. Phosphate buffer was prepared with sodium phosphate dibasic and sodium monobasic from Sigma-Aldrich, and ferric chloride hexahydrate and ferrous chloride tetrahydrate were purchased from Sigma-Aldrich. Potassium ferrocyanide, potassium ferricyanide, and potassium chloride (KCl) were obtained from Sigma Aldrich (Ontario, Canada). Human plasma samples were obtained from the Iranian Blood Transfusion Research Center (Tabriz, Iran). Ascorbic Acid and uric acid were obtained from Merck Company. All chemicals were used without further purification. Deionized water was used for the preparation of all solutions.

2.2 | Apparatus

Electrochemical measurements were performed in a conventional three-electrode cell (from Metrohm using an electrochemical system comprising of the AUTOLAB system with PGSTAT302N) (Eco Chemie, Utrecht, The Netherlands) using NOVA 1.11 software. A three-electrode system was used, including a GCE (with diameter of 2 mm) as a working electrode, Ag/AgCl-Sat'd KCl (from Metrohm) as a reference electrode, and a platinum wire as a counterelectrode, respectively.

The characteristics of surface morphology of the electrode were investigated by a high-resolution field emission scanning electron microscope (SEM) (Hitachi-SU8020, Czech) with an operating voltage of 3 kV, and energy-dispersive X-ray analysis (EDX) is used to determine the elemental composition and chemical analysis of a specimen in the surface of electrode used in conjunction with SEM.

2.3 | Preparation of the modified electrodes

Prior to modification, the GCE was polished with 0.05 μm α-Al_2O_3 aqueous slurry and was sonicated sequentially in acetone, alcohol, and redistilled water for 10 minutes. The experimental conditions for electro-polymerization of poly(L-Arg/Tb) were carried out according to the following procedure. Briefly, the bare GCE was dipped into 0.1 M phosphate buffer solution (PBS, pH 7.4) containing 0.5 mM Tb and 0.5 mM of L-Arg, and CV was performed in the potential range of

−1.0 to 1.9 V at 50 mV s^{-1} for 10 cycles. Finally, the P(L-Arg/Tb/GCE) was carefully washed with redistilled water for further use. Figure S1 (see supporting information) shows the CVs of electrochemical polymerization of P(L-Arg/Tb) onto GCE. An anodic peak at 1.31 V and a reduction peak at −0.5 V were observed due to the formation of composite of P(L-Arg/Tb). The peak current increased with the progress in the number of cyclic voltammetric scans indicating that an electro-conductive polymer film was formed on the electrode surface successfully.

MNPs were prepared by the conventional co-precipitation process.[41] Briefly, 2.22 g of $FeCl_3$ and 0.86 g of $FeSO_4$ were dissolved in 40 mL of ultrapure water and heated at 70°C. Then, the mixture was centrifuged at 1000 rpm for 30 minutes, at 70°C. After addition of 10 mL of ammonia and 2 mL of citric acid, a black solution was obtained. After that, the mixture was heated at 90°C in continuous stirring for up to 60 minutes. The magnetite precipitates were separated off and washed several times from the solution by a magnet. Finally, the synthesis powder was dried at room temperature. For surface functionalization of MNPs, 10 mg of Fe_3O_4 NPs were dispersed in 10 m dopamine hydrochloride, which was dissolved in 20 mL of PBS (0.1 M, pH = 8.5), and the resulting solution was stirred for 24 hours at the room temperature. After 24 hours, the PDA-MNPs were collected by magnetic decantation and washed three times with deionized water, The resulting precipitate as PDA-MNPs were dried at room temperature for 24 hours for future use.

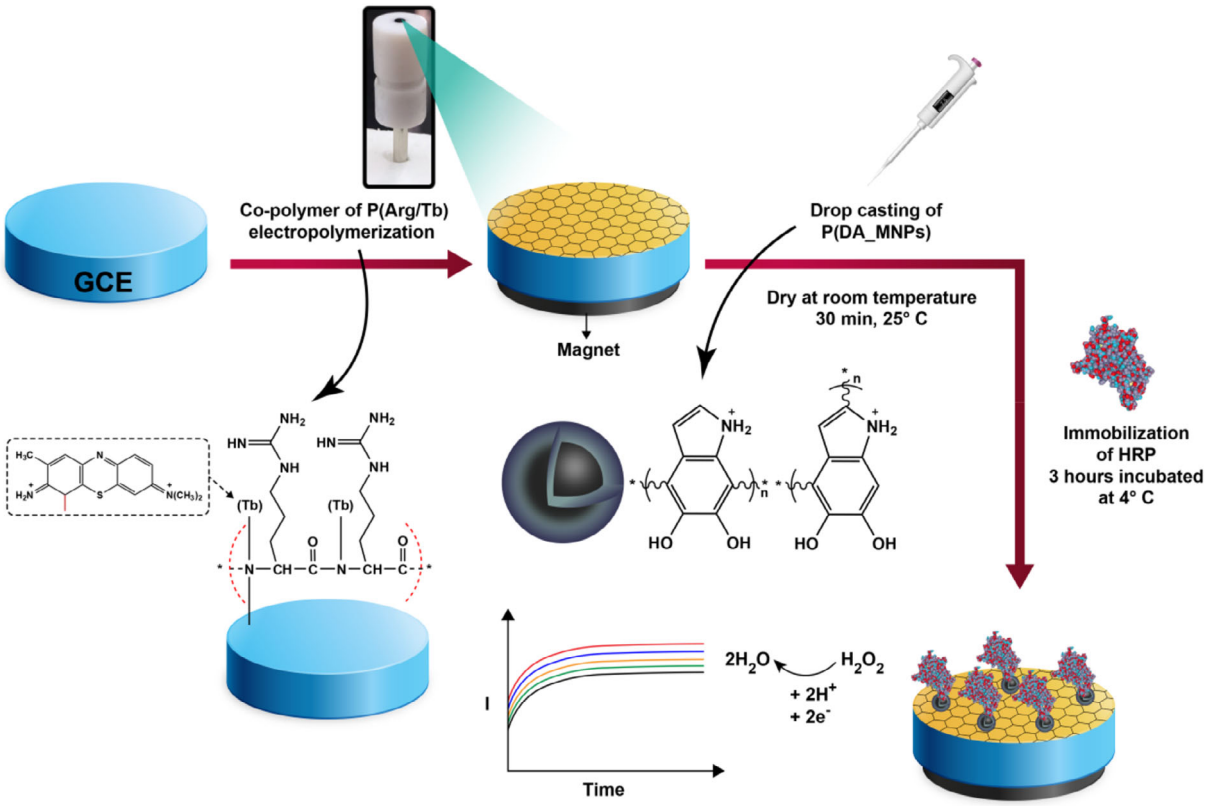
2.4 | Fabrication of enzymatic biosensor

Prepared PD-MNPs bulk was added to 20 mL deionized water and sonicated for 15 minutes to obtain a well-dispersed suspension. For the preparation of electrochemical biosensor, a magnet was placed below the modified electrode of P(L-Arg/Tb). Then, 5 μL PDA conjugated with Fe_3O_4 was cast on to the surface of the P(L-Arg/Tb)/GCE). The magnet underneath the electrode prevents material from spreading the solution onto the surface of the electrode and incubated firmly on the electrode surface, then allowing drying at room temperature for 30 minutes. An aqueous solution of HPR (1 mg/mL) was prepared in sodium phosphate buffer (0.1 M, pH 7.4). Then, 10 μL of enzyme solution was incubated on the surface of modified electrode for approximately 2 hours at the 4°C. Subsequently, the electrochemical sandwich layer–prepared biosensor is ready for the evaluation of H_2O_2. Fabrication procedure of the sandwich-type electrochemical biosensor is schematically illustrated in Scheme 1.

3 | RESULTS AND DISCUSSION

3.1 | Morphology and elementary analysis of biosensors

SEM microscopy was employed to gain insights into characterizing surface morphology of P(L-Arg/Tb)/GCE, PDA-MNPs/(L-Arg/Tb)/GCE,



SCHEME 1 The preparation procedure of HRP/PDA-MNPs/(L-Arg/Tb) biosensor for detection of H_2O_2

and HRP/PDA-MNPs/(L-Arg/Tb)/GCE with different magnification. As can be seen in Figure S2 (see supporting information), P(L-Arg/Tb)/GCE film consists of collection of regular and patterned interconnected net-like structure with a diameter ranging from 18 to 45 nm, which shows the effective modification P(L-Arg/Tb) on the surface of GCE. The EDS detector coupled to the FESEM system indicates their elemental compositions, which are summarized in EDS.

PDA-functionalized MNPs indicate induced partial aggregation of MNP clusters degree with a heterogeneous and quasi-spheres shapes with average diameter particles obtained from 32 to 52 nm, which were well-dispersed on the surface of the electrode (Figures S3 and S4 [see supporting information]). Based on the EDX of PDA-MNPs/(L-Arg/Tb)/GCE, the presence of Fe and an additional O element show that PDA-MNPs are coated on the P(L-Arg/Tb)/GCE surface efficiently. In addition, the decrease of Fe elemental of the modified PDA-MNPs/(L-Arg/Tb)/GCE compared to bulk PDA-MNPs indicates the subsequent coat of PDA-MNPs on to the surface of P(L-Arg/Tb). FESEM images were confirmed by EDS.

HRP immobilized on the surface of MNPs conjugated with PDA is presented in Figure S5 (see supporting information). As can be seen, large coverage of particles is demonstrated compared to PDA-MNPs incubated on the electrode surface. Attaining high percentage of elemental composition of Fe and O rate is due to the heme group of enzyme as well as enzyme retain high percentage of their native structure when immobilized onto the MNP coated with PDA.

3.2 | Electrochemical characterization

Fabrication and operation of the step-by-step proposed biosensor including immobilization of HPR can be pointed out by CV technique. According to Figure 1, a bare GCE indicates a sharp and quasi-reversible redox reaction in $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution containing 0.1 M KCl as an electrochemical probe. When a composite of P(L-Arg/Tb) was modified on the surface of GCE, peak currents emblematically increased compared to GCE. This increase can be attributed to the presence of highly conductive composite layer on the surface, which facilitates electron transfer to the electrode. Furthermore, contrinution of P(Arg) with positive charges of P(Arg) on the electrochemical reaction lead to efficient electron transfer process.[42] After modifying the following electrode with nanocomposite of PDA-MNPs, the response indicated that PDA-MNPs had good magnetic response and gave better performance toward the behavior of reversible redox of $[Fe(CN)_6]^{3-/4-}$. Moreover, it provides high surface area to volume ratio with nanoporous for subsequent enzyme binding on the surface of GCE, leading to sensitivity enhancement of the biosensor. By immobilization of HRP on the surface of developing nanomagnetic composite, the redox peak currents dramatically reduced. This result suggests that HRP coverage makes the less functional group of polymer matrix for the interaction of electrochemical probes, demonstrating the effectiveness of using crosslinking agents for the immobilization of enzymes. It is considered that HRP was immobilized via covalent bond of amino groups on the surface of PDA-MNPs, with carboxyl groups on the enzyme molecules in which resulting binding yields are accessible. The changes in the peak potential and peak current are presented in Table S1 (see supporting information).

The electrocatalytic behavior of different substrates (P(L-Arg/Tb), PDA-MNPs/L-Arg/Tb, and HRP-PDA-MNPs/(L-Arg/Tb)), toward H_2O_2 reduction have been investigated in PBS solution. As can be seen in Figure S6 (see supporting information), clear reduction peak was observed as the definite amount of H_2O_2 at around −0.9 V. This suggests the immobilization of HRP on the surface of PDA-MNPs electrode was successfully established .

More information can be extracted from scan rate investigation of cyclic voltammogram. The key parameter can be obtained based

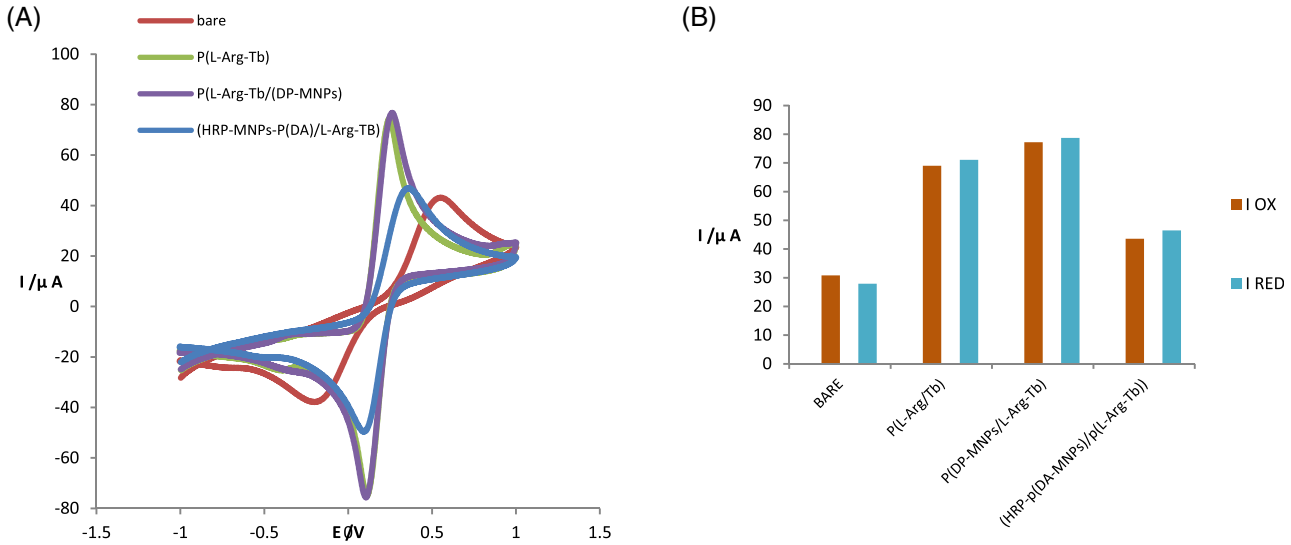


FIGURE 1 (A) Cyclic voltammograms of 10 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M KCl at bare GCE, P(L-Arg/Tb)/GCE, PDA-MNPs/(L-Arg/Tb)/GCE, HRP/PDA-MNPs/(L-Arg/Tb)/GCE. Scan rate: 100 mV s^{-1}. (B) Histogram of redox peak current vs different type of electrode

on the CV technique.[43,44] According to Figures S7 and S8 (see supporting information), anodic and cathodic peak currents of P(L-Arg/Tb) and PDA-MNPs/L-Arg/Tb vary with scan rate, and by progress of scan rate, the peak separation (ΔE) increases, (ΔE_p, is calculated by $E_{pc} - E_{pa}$). As an ideal reversible electrochemical behavior, the peak separation is equal to zero because the redox center is adsorbed onto the electrode, and diffusion does not play a role. According to the negative shift of cathodic and positive shift of anodic peak positions with the progress of scan rate order, the electron transfer is irreversible in both modified electrodes. In addition, the results of anodic peak current Ipa vs square root of the potential sweep rate indicate excellent linearity in anodic peak current with increasing scan rate, which indicates a mass transfer controlling process of oxidation via diffusion. Moreover, the surface coverage, Γ^*, can be obtained from the slope of the line of I_p vs sweep rate following Equation (1)

$$I_p = \left(n^2 F^2/4RT\right) \nu A \Gamma * \quad (1)$$

According to Equation (1), n is the number of electron involved in the reaction and equal 1, F is faraday constant = 96 486 C mol^{-1}; R considers as gas constant = 8.314 J mol^{-1} k^{-1}. T is temperature = 298 K°. A represents geometrical surface area of electrode = 0.0314 cm^2, ν is scan rate. Γ^* (mol cm^{-2}) represents the surface coverage.

The surface coverage of P(L-Arg/Tb) electropolymerized on the surface of GCE was determined to 50 × 10^{-9} mol cm^{-2}, and the surface coverage of PDA-MNPs adhered on P(L-Arg/Tb)/GCE was calculated to 51 × 10^{-9} M cm^{-2} in this study.

A dependence of ln I_p vs ln ν is linear according to the following equation in both modified electrodes:

$$\ln I_p = 0.3238 \ln \nu\left(V s^{-1}\right) - 8.7063 \; R^2 = 0.9978$$

$$\ln I_p = 0.3038 \ln \nu\left(V s^{-1}\right) - 8.6832 \; R^2 = 0.9972$$

Their slop is close to 0.5 and presents diffusion control of both modified electrodes process. A slope close to theoretically value of 0.5 shows that the process is diffusion-controlled and close to 1.0 indicates an adsorption-controlled processes.[45,46]

3.3 | Electrocatalytical study

The electrocatalytical behavior of HRP sensor toward H_2O_2 reduction was evaluated in PBS solution in different concentrations of H_2O_2 by the chronoamperometry technique (Figure 2). E = −0.9 V was selected as the appropriate potential according to cyclic voltammetry study. The current response from the reduction reaction increases in the negative direction indicating the reduction of H_2O_2. The current response was investigated from the concentration of H_2O_2 from 0.5 μM to 30 μM with the regression equation I (μM) = 0.0951C_{H2O2} (μM) + 2.0918. The limit of detection was estimated to be 0.23 μM. Moreover, different concentrations of H_2O_2 were spiked into human plasma samples, and the concentration of H_2O_2 from human plasma was determined by measuring the amperometric response signal (Figure 3). The developed sensor showed its excellent ability to determine H_2O_2 in real samples.[47,48]

Different substrates can be applied for the investigation of H_2O_2, and also variety of supports can be utilized when the macromolecule, in particle, enzyme, is to be immobilized efficiently without the change of nature structure or active functional of enzyme for determination of H_2O_2. To obtain optimal results, the substrate should provide a large specific surface for biocatalyst attachment with stability and less biodegradation. Surface-functionalized MNPs are good candidate since they provide large surface area and the activity of the used model enzymes could be enhanced significantly. The high-achieved activity yields lead to sensitivity of developed biosensor for investigation of H_2O_2. According to Table 1, the proposed bio (sensor), HRP immobilized on the surface of PDA-MNPs displays an acceptable detection limit and better linear range for H_2O_2 determination in comparison to other reported works.[49-55]

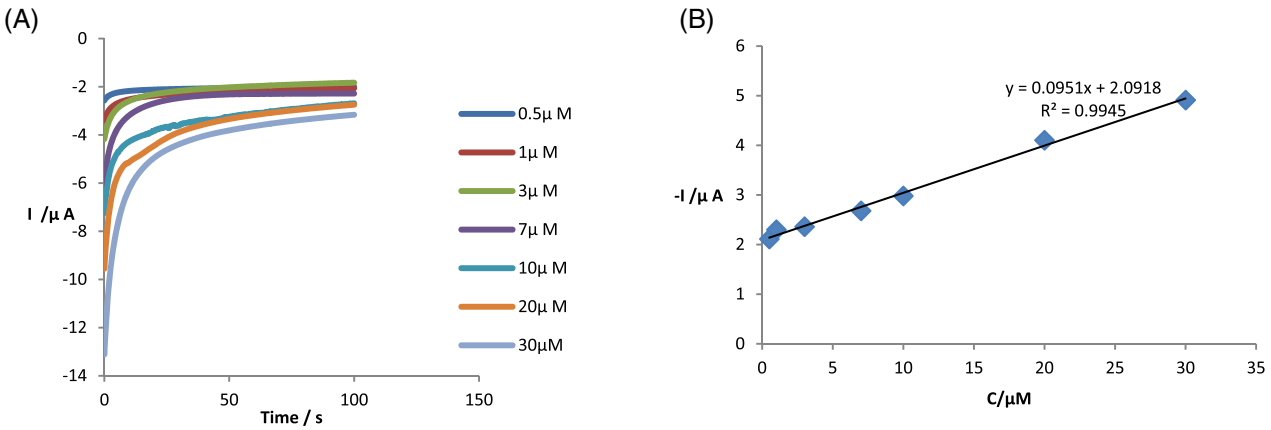


FIGURE 2 (A) Chronoamperometry response of the HRP/PDA-MNPs/(L-Arg/Tb)/GCE to different concentrations of H_2O_2 from 0.5 to 30 μM in 0.1 M PBS solution at physiological pH 7.4. (B) Calibration plot

3.4 | Selectivity of the bio-electrochemical sensor

The selectivity of the biosensor was also investigated in the presence of several interferences like ascorbic acid, dopamine, and uric acid, which are mainly present in the blood to demonstrate a possible bio-electrochemical activity toward H_2O_2. The high detection of H_2O_2 is of crucial importance in diagnosing oxidative stress-related conditions. The interference concentration was kept 10 μM. To do this, the sensor response in the absence and presence of interfering samples was recorded, and the results are shown in Figure S9 (see supporting information). It can be observed that the most and the least interfering substances were uric acid and dopamine, respectively (Table S2 (see supporting information)).

3.5 | Stability and reproducibility

The stability of the P(L-Arg/Tb) composite and PDA-MNPs nanocomposite was studied by CV with different cycle number in 10 mM of $Fe(CN)_6^{3-/4-}$-containing 0.1 M KCl. As can be seen in Figures 4 and 5, there are no significant changes in anodic and

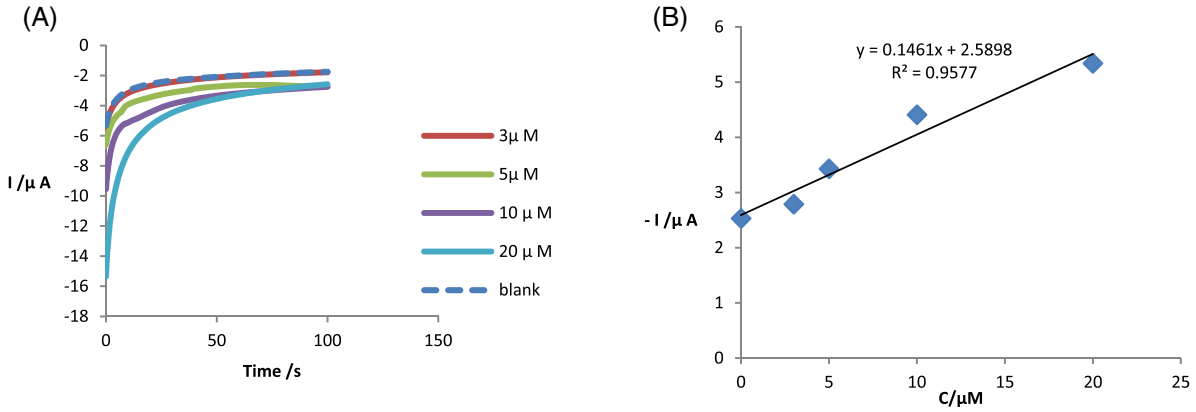


FIGURE 3 (A) Chronoamperometry response of the (bio) sensor toward determination of H_2O_2 in untreated human plasma samples with different concentrations. (B) Calibration curve

TABLE 1 Analytical comparison of present developed bio(sensor) capability for H_2O_2 determination with other reports

Method	Working electrode	Real sample	Linear range	LOD	Reference
Cyclic voltammetry and amperometry	Palladium nanoparticles/GCE	Plasma	10 μM-14 mM	6.79 mM	49
Chronoamperometry	MnO_2/CPE	Groundwater	1.4-65 μg mL	12 mM	50
Amperometry	Pt-MWCNT/CES	Green tea	1-15 mM	10 mM	51
	PAN-PNMThH	—	5 μm-60 mM	3.2 mM	52
Chronoamperometry	NiO/grapheme nanocomposite		0.25-4.75 mM	0.74 μM	53
	HRP/Pß-CD/GCE-based MNP	Plasma	1–15 μM	0.45 μM	54
	HRP/PDA-MNPs/L-Arg/Tb/GCE	Plasma	0.5-30 μM	0.23 μM	Present work

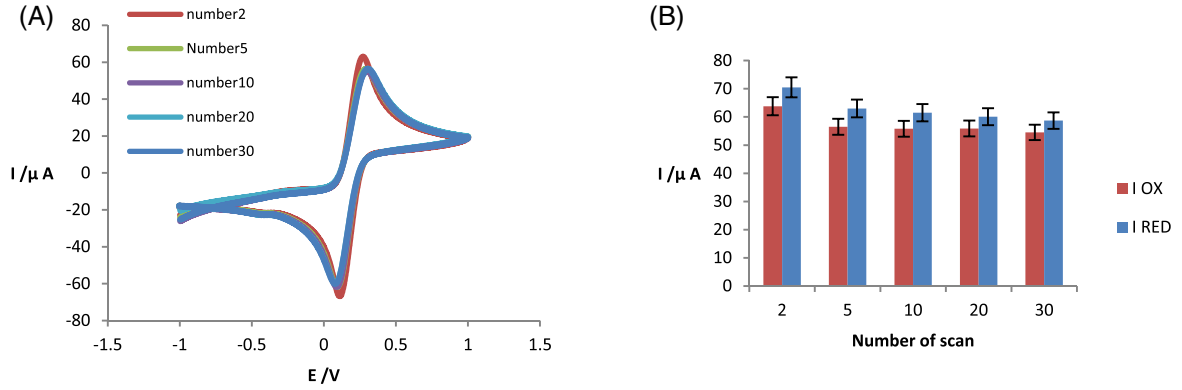


FIGURE 4 (A) Cyclic voltammograms of P(L-Arg/Tb)/GCE in different cycle numbers (2, 5, 10, 20, 30 cycles). (B) Histogram of the redox peak currents of P(L-Arg/Tb)/GCE vs number of cycles. Scan rate: 50 mV s^{-1}

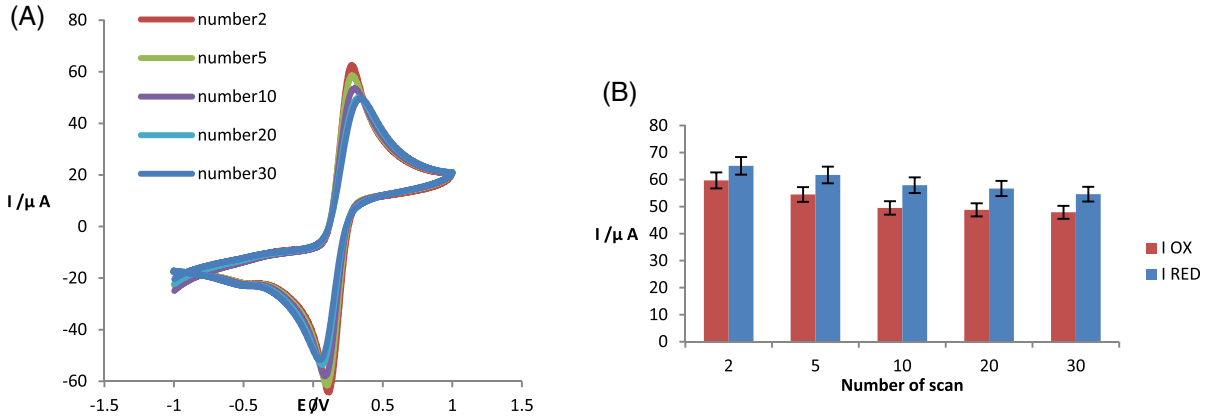


FIGURE 5 (A) Cyclic voltammograms of PDA-MNPs/L-Arg/Tb/GCE in different cycle numbers (2, 5, 10, 20, 30 cycles). (B) Histogram of the redox peak currents of PDA-MNPs/L-Arg/Tb)/GCE vs number of cycles. Scan rate: 50 mV s^{-1}

cathodic peak of P(L-Arg/Tb) composite and PDA-MNPs nanocomposite currents after 30 cycles. The stability of bio-electrochemical sensor was tested by cyclic voltammetry in 3 days storage in 4°C. It was observed that peak currents were decreased only 11% after 3 days, which indicates an appropriate stability. To demonstrate the biosensor performance reproducibility, 10 μM concentrations of H_2O_2 were tested on three different electrodes. The relative SD (RSD) was determined as 3% by chronoamperometry technique. The results indicate that the developed biosensor has a good degree of reproducibility as well as sensitivity and stability (Figures S10 and S11 [see supporting information]).

4 | CONCLUSION

In this study, composite of L-Arg and Tb was electrochemically polymerized onto the GCE with high surface area as well as bring capability for subsequent coating of PDA-MNPs. Surface functionalization of MNPs with PDA substrate increased the surface area and provided active functionalization sites, making them a promising candidate for immobilization of HRP. Proposed biosensor show that excellent electrocatalytical behaviour for the reduction of H_2O_2 with dynamic range of 0.5 to 30 μM which limit of detection is about 0.23 μM. The proposed method exhibited appropriate repeatability and stability toward H_2O_2 investigation. Moreover, it was also feasible for determination of H_2O_2 in real sample of human plasma as well as had essential potential in clinical and biomedical analysis.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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