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RESEARCH ARTICLE

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Chemical binding of horseradish peroxidase enzyme with poly beta-cyclodextrin and its application as molecularly imprinted polymer for the monitoring of H_2O_2 in human plasma samples

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Abstract

In this study, a selective and sensitive molecular imprinting-based electrochemical sensors, for horseradish peroxidase (HRP) entrapment was fabricated using electro polymerization of ß-Cyclodextrin (ß-CD) on the surface of glassy carbon electrode. Poly beta-cyclodextrin P(ß-CD) provide efficient surface area for self-immobilization of HRP as well as improve imprinting efficiency. The proposed imprinted biosensor successfully utilized for detection of HRP with excellent analytical results which linear range is 0.1 mg/mL to 10 ng/mL with LOD of 2.23 ng/mL. Furthermore, electrocatalytical activity of the prepared biosensor toward the reduction of hydrogen peroxide was investigated in the ranges of 1 to 15 μM with a detection limit of 0.4 μM by using chronoamperometry technique. The developed biosensor was used for the detection of hydrogen peroxide in unprocessed human plasma sample.

KEYWORDS
biomedical analysis, bioreceptor, biotechnology, heme protein imprinting, polymer

1 | INTRODUCTION

Molecularly imprinted polymers (MIPs) are synthetic materials, which are able to mimic molecular target of antibodies, enzymes, and other biological and chemical receptor. This technique is based on the "lock-and-key" principle, often described as a devising target molecular lock to match target molecular key.[1,2] The process of molecular imprinting polymer is done by the polymerization of a functional monomer, initiator, and cross-linker with molecular template.[3] Protein imprinting and rebinding considers as challenging issues due to fragile structures, large molecules of biomacromolecules, and *poor rebinding efficiency*. Hence, it brings about some difficulties such as low binding capacity, weak affinities, small *accessible site for loading of* macromolecules, inhibition of *target-binding kinetics*, *poor selectivity*, *and difficulty in template removal*.[4,5] Surface imprinting technique is popular and most applicable for imprinting of biomolecules including proteins,[6] which diffusion problem could be solved to the some extent between template and polymer interaction. Therefore, *high affinity recognition sites* can be achieved on the surface of substrate.[2] Polymeric materials are usually organic materials, suffering from a low binding *capacity* and *slow binding kinetics* to the target species.[7,8] The use of imprinted thin films and nanoparticles can overcome abovementioned problems.[9] Various methods involving the preparation of MIP films have been suggested according to chemical polymerization, while the implementation of the polymerization of electroactive functional monomers, as novel concepts, serves as a new alternative strategy for the preparation of MIP.[10,11] In contrast to in situ chemical polymerization, *preparation procedure in* molecular imprinting-based electrochemical (EC) sensors can be done without the use of cross-linkers, initiators, or catalysts,[12] which are necessary functional elements in chemical polymerization, *therefore* a facile and economical process emerge as advantageous approach for MIP formation. Electrosynthesized polymers as imprinted polymer allows to *regulate* the *thickness* and the *density* of the *polymer layer with* EC control of the *polymerization* process (CV scan cycles). Also, a pre-polymerized layer is directly formed onto an electrode surface, with high stability has been developed to provide a large surface area and excellent conductivity with high affinity as an efficient substrate for biomolecules immobilization. The biomolecules can be facily immobilized on the electrode surface and electrons flow is established *between* the *electrodes*, thus, the electron

transfer between the active sites of the biomolecules and the electrode is enhanced which can effectively improve sensitivity and selectivity of the sensors the biomolecules and the electrode is enhanced which can efficiently improve selectivity and sensitivity of the constructed (bio)sensors.[13-16]

Horseradish peroxidase (HRP), a glycoprotein obtained from plant root with a molecular weight of 42 000 Da,[17,18] is an important heme-containing enzyme with two different types of metal center, iron (III) protoporphyrin IX located at the active site. HRP is considered as a member of the hydrogen peroxide group, which are defined as oxidoreductases enzymes that use H_2O_2 as the electron acceptor for the reduction of peroxides and oxidize a wide variety of organic and inorganic compounds.[19,20] HRP enzyme can be used extensively in various fields including sensing of biogenic amine,[21,22] nitric oxide,[23] DNA,[24-28] detoxification of organic compounds like substituted phenols, oil removal from wastewater[29-31] and so on.

For engineering efficient MIP, conducting polymers as novel supporting material led to improve the sensitivity of the EC biosensor and provide efficient surface for binding the of imprinting target molecules to the surface of electrode.[16,32-35] β-cyclodextrin, a series of cyclic oligosaccharides, with seven glucose units, linked together in *the shape* of a *truncated cone*.[36] *Molecular structure of* β-cyclodextrin possesses some unique advantages in the MIP formation. *Appropriate* spatial *orientation of* β-cyclodextrin is popularly applied in the cooperative binding of the large templates through different types of intermolecular interactions such as *van der Waals forces*, *hydrogen bonds*, *and* dipole-dipole interaction, *which* are be assumed to be responsible for the polymer-template interactions.[37,38] Furthermore, the presence of β-CD in MIP as functional monomer provides high *specific surface area*, large *pore volume*, *and average pore diameter* which contribute the binding capacity of MIPs.[39] The different strategies involving preparation of molecularly imprinted β-CD polymer via chemical polymerization have been suggested[40-43] while the synthesized poly *beta-cyclodextrin P*ß-(CD) by the mean s of electropolymerization, as presynthesis of a polymer film, consider as novel concept in MIP. A prepolymerized layer of electroactive poly β-cyclodextrin *P* ß-(CD) emerges as rich site accessibility for immobilization of HRP via self-assembled on anchored polymer film which can be greated and binded through non-covalent bonds. Electropolymerization of *beta-cyclodextrin* ß-(CD) is appropriate candidate for the immobilization of HRP due to electron conductivity, facilitation electron transfer, and providing large surface area for the effective immobilization.[44-46]

For the first time, an innovative bioimprinted sensor was designed for the by the combination of electropolymerization of ß-cyclodextrin and self-bioassay of horseradish peroxide surface molecular imprinted technique.

2 | MATERIALS AND METHODS

2.1 | Materials and chemicals

Beta-cyclodextrin ß-CD and HRP were purchased from Sigma-Aldrich company, hydrogen peroxide (30%) was purchased from Sigma-Aldrich Chemical Co, Human Cancer Antigen 15 (CA 15-3), Human Cancer Antigen 125 (CA 125), *prostate-specific antigen* (PSA) and Human Carcino Embryonic Antigen(CEA) ELISA kits were purchased from CanAg Diognastic AB technology Co., (Gotenberg, Sweden). Bovine serum albumin (BSA), potassium ferrocyanide $K_4Fe(CN)_6$, potassium ferricyanide $K_3Fe(CN)_6$, and potassium chloride KCl were obtained from Sigma Aldrich (Ontario, Canada). Phosphate buffer saline solution (PBS) (0.1 M) was prepared by solving of Na_2HPO_4 (0.1 M) and NaH_2PO_4 (0.1 M) in deionized water. Human plasma samples were obtained from the Iranian Blood Transfusion Research Center (Tabriz, Iran).

2.2 | Instruments

EC measurements were conducted in a conventional three-electrode cell (from Metrohm) powered by an EC system comprising of the AUTOLAB system with PGSTAT302N (Eco Chemie, Utrecht, The Netherlands) using NOVA 1.11 software. A glassy carbon electrode (GCE) as working electrode, Ag/AgCl-Sat'd KCl (from Metrohm) as reference electrode and a platinum wire as counter electrode was used.

Electroanalytical measurements, including cyclic voltammetry (CV), differential pulse voltammetry (DPV), and chronoamperometry were used for biosensor fabrication and *characterization*. $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ was selected as the EC *probe solution* because it can pass through the MIP and has capability *to* enter the imprint cavity. Evaluation and determination of H_2O_2 by bio-imprinted polymer have been investigated by chronoamprometry technique in 0.1 M PBS solution (pH 7.4). *Field emission scanning electron microscopy* FE-SEM, Hitachi-SU8020, Czech) with an operating voltage of 3 kV provides topographical and elemental information of electrode surface by different magnifications. The elements identification of the electrode were analyzed by an energy dispersive spectroscopy (EDS) coupled with the FE-SEM equipment.

2.3 | Electropolymerization of *β-cyclodextrin* on the surface of GCE

Electro-synthesis of β-CD was carried out based on following: 6.0 mmol L−1 of β-CD was prepared in phosphate buffer 0.05 M (pH = 5) and sonicated for 15 minutes. Before the electropolymerization, GCE was pre-treated by 0.3 and 0.05 μm of alumina aqueous slurry and thoroughly rinsed with, alcohol and redistilled water, respectively. The electro-polymerization of *β*-CD was performed using CV technique by sweeping the potential from −2.0 to 2.2 V (20 repetitive cycles), which scan rate was 100 $mV.s^{-1}$. So, modified electrode was rinsed with distilled water (DW) to remove physically absorbed materials on the surface of electrode. Based on the experimental results (Figure 1), the peak currents increased with the increasing successive cycles. In the *first cycle*, *oxidation* of monomer only can *take place then by subsequent cycles*, *the polymer network slightly grow in each cycle*, *indicating the electropolymerization process*

occurs during consecutive *scan on* the electrode surface.[47,48] This is an evidence to formation of P(*β*-CD) films where it thicknesses can be easily controlled.

2.4 | The possible mechanisms of the electropolymerization of *β*-CD

In general, the electro-polymerization begins with the monomer oxidation to form a radical cations. When *monomer produces a radical cations, it can combine with* the successive addition of radical cation or with another monomer to produce a oligomer, which in next steps, it can couple with another oligomer, thus by subsequent steps *Chain-Growth Polymers are formed on the various electrode surface.*[49-51] *The proposed mechanism of electropolymerization of β*-CD is shown in Figure 2.

Two mechanisms should contribute *simultaneously* to formation of the polymer film. In the first stage, *by applying oxidation potential* polarization of *β*-CD initiate which is responsible to produce radical cation on the carbon containing hydroxyl group. One approach of this active site is based on connecting to the surface of GCE electrode *through covalent bonding, leading to a ketone.*[52] In the second stage, *carboxylic acids and/or aldehydes* are commonly *generated* by the oxidation of the primary hydroxyls. In this pathway, the *esterification reaction happens between carboxylic acids* and primary hydroxyl (—OH), leading to dimer formation. Therefore, monomer molecules are *successively* combined to each other and the P(β-CD) was synthesized on the surface of electrode.

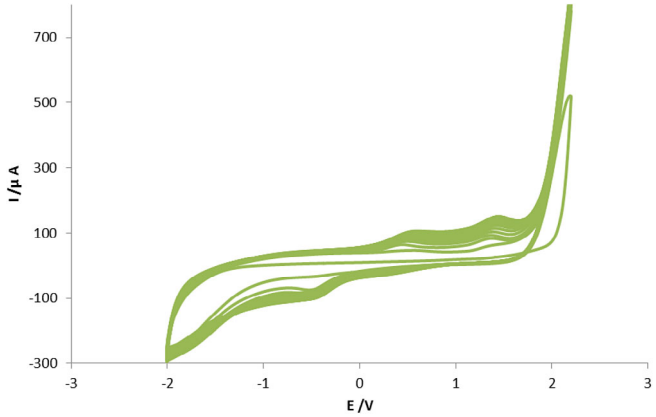

FIGURE 1 CVs of P(*β*-CD) on the surface of GCE in the presence of 0.05 M *PBS* (pH = 5) containing 6 mM *β*-CD at scan rate of 100 mV/s. Number of cycle is 20

2.5 | Biosensor fabrication based on imprinted polymer method

Protein imprinted of HRP grown in P(β-CD), as supporting material, was carried out by combination of electropolymerizing of β-CD and self-assembly surface molecular imprinting technique. The obtained P(β-CD)/GCE was immersed in buffer phosphate solution pH (6.98) in the presence of 23.8 μM of HRP. HRP template molecules were facially *trapped* on the P(*β-CD*) matrix by hydrogen bonding interaction of OH which take part in binding protein. The elution of

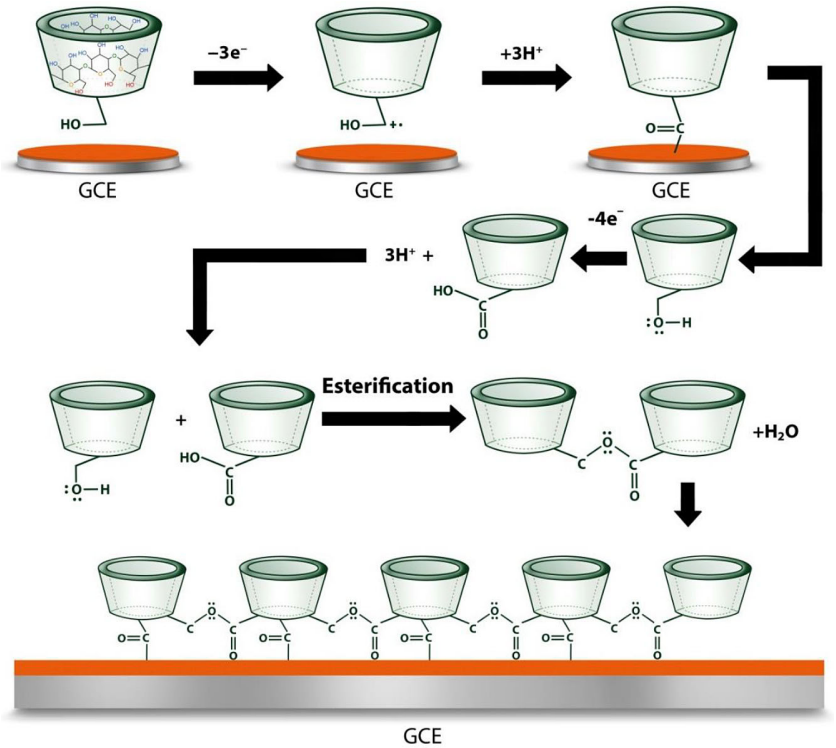

FIGURE 2 *Electropolymerization procedure of β*-CD on the glassy carbon electrode[12]

template from the matrix of polymer was done in 1.0 M hydrochloric acid for 20 minutes and the removal of binding protein has been investigated by EC technique. The obtained electrode is named MIP/P(β-CD)/GCE. Similar protocol was used by other reports (Figure 3).[38]

2.6 | Morphological and structural characterization of prepared bio imprinted sensor

For this purpose, FESEM was used to the evaluation of electrodes morphology and structure of prepared films. Also, elementary analysis was done by EDS method. As shown in Figure 4A, the morphological of P(β-CD) provides a homogenous polymer film with shape like monodispersed spherical, which are best suited for macromolecular loading efficiency. The spherical beads of such polymer enhance pore volume and surface area,[53] which yield high imprinting efficiencies with increasing interaction strength for non-covalent bonding.

Figure 4B demonstrates the binding of the protein on to the surface of P(β-CD)/GC which made the total surface of polymer matrix covered and impact. Also, existence of new elements like phosphor and iron confirmed protein immobilization using proposed procedure. Therefore, EDS analysis confirmed FE-SEM results. Figure 4C indicates removal of template from the imprinted polymer with different morphology and irregular structure after the removal of protein is totally clear in the film polymer.

3 | RESULTS AND DISCUSSION

3.1 | EC characterization

Preparation steps of the bio-imprinted sensor were characterized by CV in a $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ + 0.1 M KCl as supporting electrolyte. As can be seen in Figure 5, when GCE was modified by p(β-CD) the redox peaks obviously increased compared to the bare GCE, indicating that the P(β-CD)/GCE with high electrical *conductivity and* unique property can *accelerate electron flow of probe including* $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$. When the HRP template molecules were immobilized on the P(β-CD) matrix by hydrogen bonding interaction the peak current of the obtained electrode dramatically reduced, exhibiting successful assemble of HRP which make it possible to retard electron transfer due to poor conductivity. After elution of template agent from the 3D polymer, the imprinting cavity is re-formed which facilitate the diffusion of the probe to the cavity of imprinted polymer. Therefore, the peak current was increased, indicating successful removal of HRP biomacromolecules.

The elimination of HRP targets have been investigated in HCL (1.0 M) for *several minutes. By following time increasing trends, it is observed that the imprinted sites were constructed which facilitate probe diffusion* in polymer matrixes on the electrode surface (Figure 6).

For the kinetic study, CVs of P(β-CD) modified GCE were recorded at various sweep rates (10-500 $mV.s^{-1}$) in $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ solution. As can be seen in Figure 7, the electron transfer is

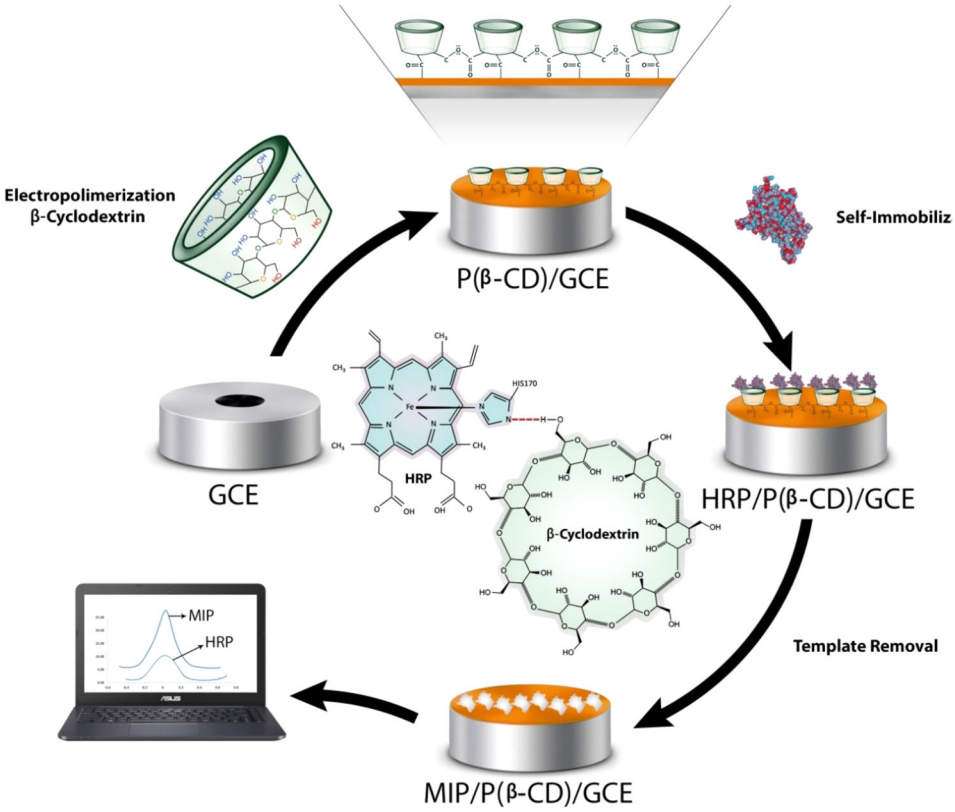

FIGURE 3 MIP/P(β-CD)/GCE (biosensor) preparation and supplication to detection of H_2O_2

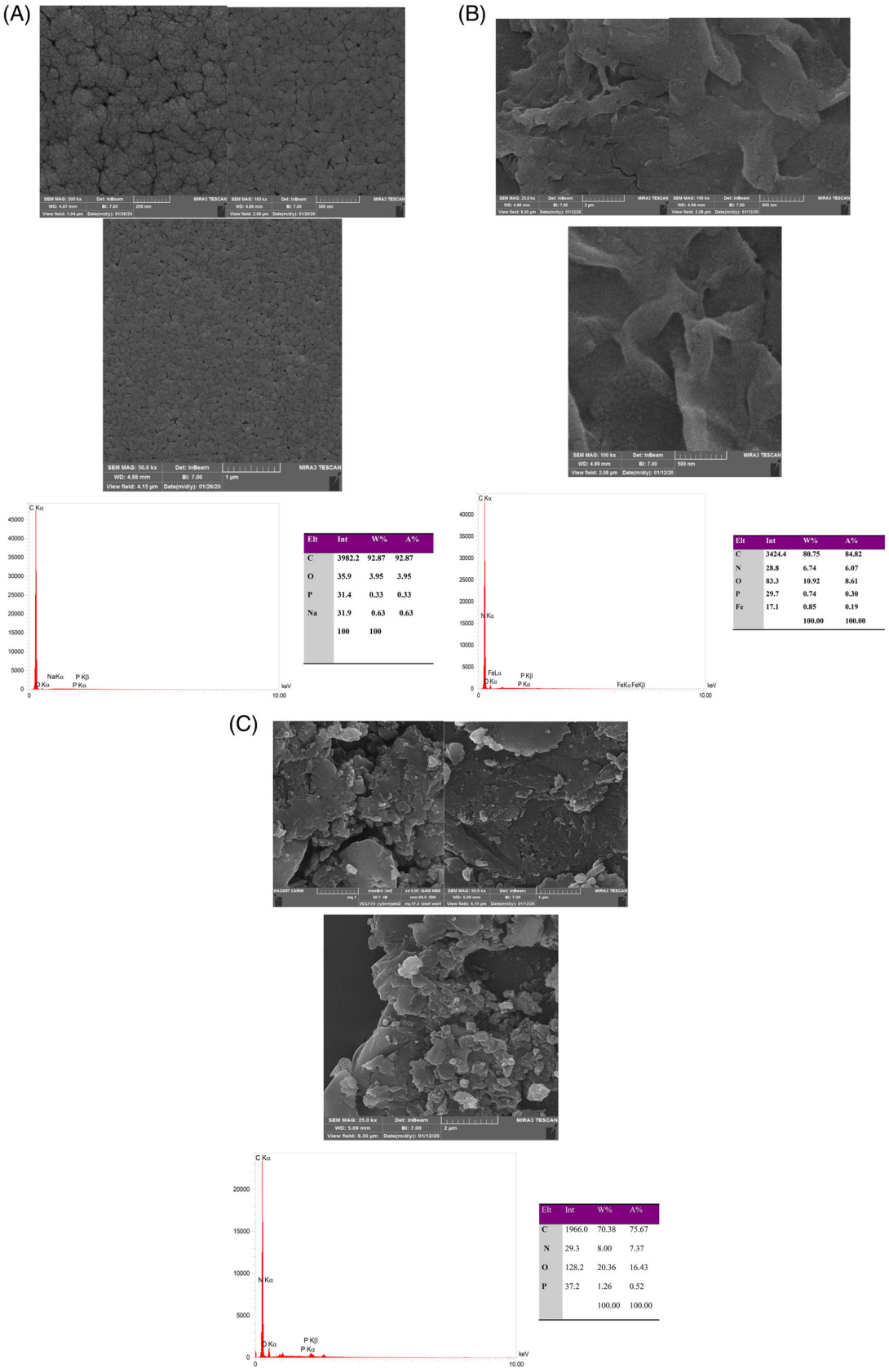

FIGURE 4 A, Field emission scanning electron microscopy of P(β-CD)/GCE in various magnifications along with EDXs. B, Field emission scanning electron microscopy of HRP/P(β-CD)/GCE in various magnifications along with EDXs. C, Field emission scanning electron microscopy MIP/P(β-CD)/GCE in various magnifications along with EDXs

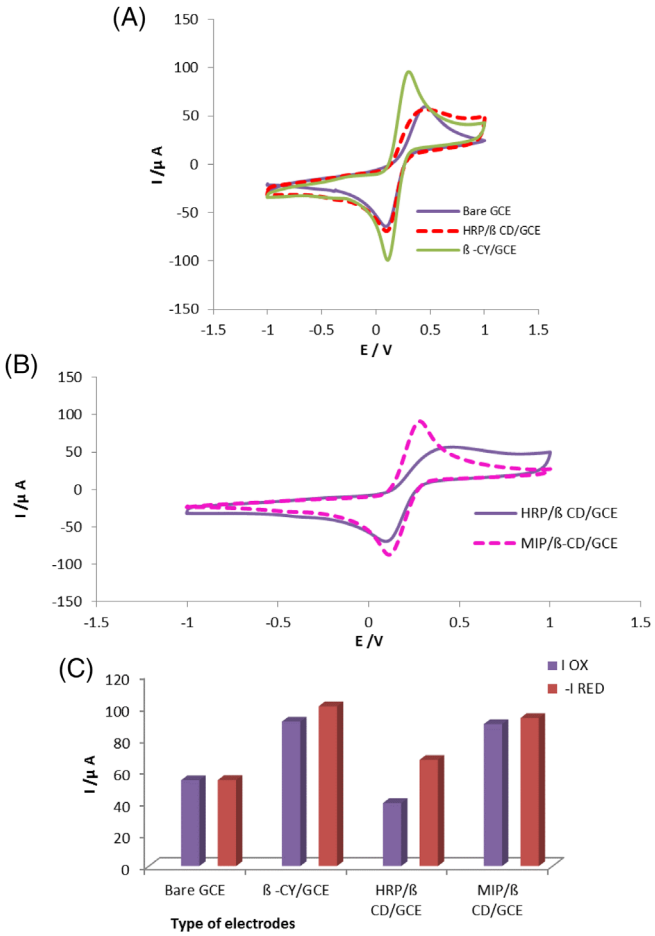

FIGURE 5 A, CVs of preparation steps of bio-imprinted polymer in $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ + 0.1 M KCl (0.01 M) at a sweep rate of 0.1 V/s. B, Comparison CVs HRP/ß CD/GCE and MIP/ß CD/GCE. C, Peak currents variation vs type of modified electrodes

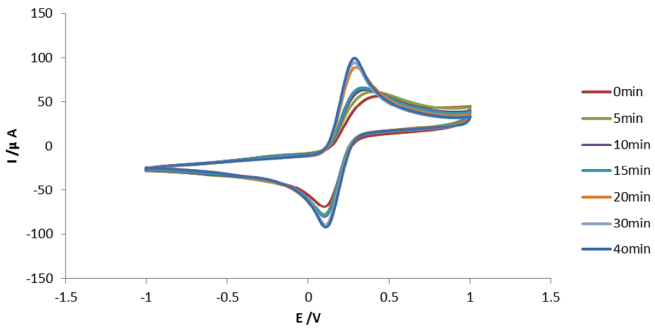

FIGURE 6 CVs of HRP/β-CD/GCE imprinted polymer at different elution time (0.5, 10, 15, 20, 30, and 40 minutes)

irreversible according to the negative shift of cathodic and anodic peak positions.

Also, the value of Γ^* as surface coverage of polymer film was obtained using the slope of the I_p vs ν. This value is often can be obtained by Equation (1).[54]

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

which was 66×10^{-9} mol.cm^{-2} in this study. The order of the increase of peak current with respect to scan rate was evaluated from the slope of the plot of lnI_p vs lnν as indicated in Figure 7C a slop is close to 0.5. Therefore, *mass transfer* was *considered* to be controlled by diffusion.

3.2 | Analytical approach

3.2.1 | EC determination of HRP by the MIP/β-CD/GCE

The most important feature of MIPs is their capability of selectively binding target and sensitive target detection. In this study, different concentrations of HRP have been evaluated by using DPV technique by 0.01 M $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ as EC prob. As seen in Figure 8, peak currents of the bio-imprinted polymer as receptor of HRP increased by the decreasing of HRP concentration, which can be described as blocking the imprinted site by the macromolecule of HRP. So, retard electron transfer to the formed cavity and inhibit probe diffusion through imprinted films. According to Figure 8B, liner range was obtained from 1.0×10^{-8} mg/mL to 0.1 mg/mL with which regression equation is I (μA) = 1.6793 C_{HRP} + 6.8029 (R^2 = 0.9955) and ΔI (μA) = 1.6646 C_{HRP} + 15.981 with the LOD of 2.23×10^{-9} mg/mL.

3.2.2 | Bioelectrocatalytic *activity* of HRP for the reduction of H_2O_2

The catalytic scheme of H_2O_2 reduction by HRP was indicated in Figure 9.[55,56]

The following bioelectrocatalytic *activity* of HRP is based on anaerobic oxidase by the mean of ferric-ferrous cycle.[57] Hydrogen peroxide (H_2O_2) is widely used as a reduction substrate for the reaction of HRP. During the redox reactions, the hydrogen peroxide bonds to the vacant octahedral position on the iron and initiate the oxidation of the heme moiety to an intermediate compound I, leading to free radical via de-protonation by electron transfer. In the next steps, the reduction of compound I to compound II occurs which the reduction of compound I to compound II and compound II to the rest state is often achieved by phenolics or aromatic amines.[58] According to CV curves of reduction of current response increased with increment of H_2O_2 concentration which prove successful immobilization of HRP on the matrix of polymer (Figure 10).

The electrocatalytical activity was studied using chronoamperometry technique at −0.55 V (reduction potential). According to Figure 11, the current signals increased by H_2O_2 concentration increment. There was a dynamic liner range from 1 to 15 μA with a liner regression Equation I (μA) = 0.0493$C_{(H2O2)}$ + 0.9366, which the detection limit LOD is 0.45 μ M.

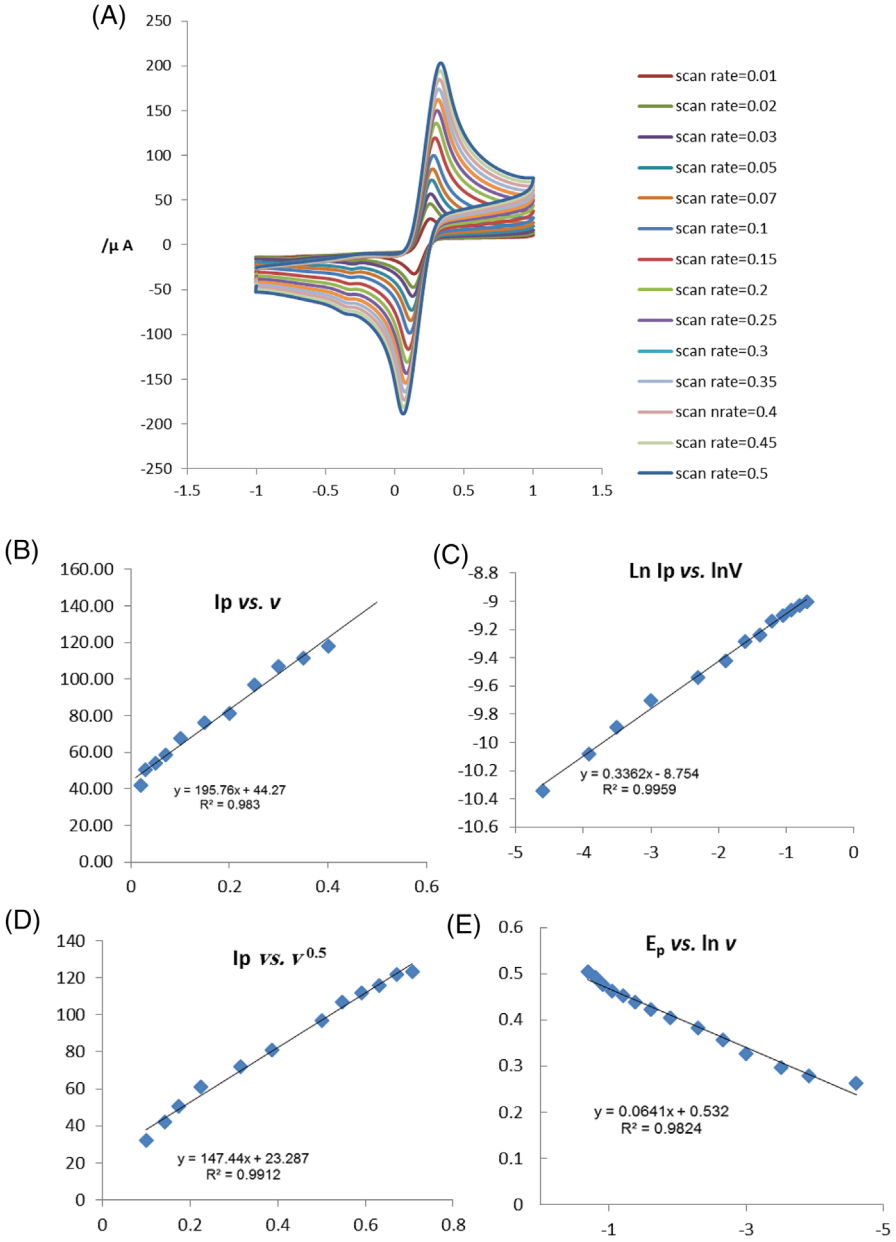

FIGURE 7 A, CVs of P(β-CD)/GCE in $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (0.01 M) + 0.1 M KCl at various sweep rates (10-20-30-50-70-100-150-200-250-300-350-400-450-500 mV/s). B, The dependency of I_p vs *v*. C, Variation of lnIp vs Lnv. D, The variation of I_p vs $v^{0.5}$. E, Variation of E_p vs Lnv

The optimization of pH on the response of HRP imprinted sensor toward reduction of hydrogen peroxide was investigated by CV technique from pH range of 4 to 8 in the presence of H_2O_2 (Figure 12A). According to Figure 12B, the EC signals increased with increment of pH from 4 to 6.98. But, further increase causes reduction of the peak currents, suggesting pH 6.98 is optimal pH value, which are in agreement with previously developed biosensor of HRP.[59] So, *enzyme activity* of HRP *reaches its maximum*. Any pH value higher than neutral *pH alters* the basic *protein structure can be caused protein denaturation*. *These changes can be induced in* weak function of HRP enzyme[60,61] . Also, the polymer matrix does not alter *optimum pH* for the electrocatalytical activity of the imprinting binding of HRP toward reduction of H_2O_2[20] .

Also, the curve of E_p vs pH is indicated in Figure 12C. As can be seen, the peak potential of the H_2O_2 using proposed sensor is pH dependent, with a linear relationship. According to this Figure, Ep was shifted to more positive potentials by increment of PBS solution pH. Moreover, the slope of E_p vs pH plots is 0.02, so it was concluded

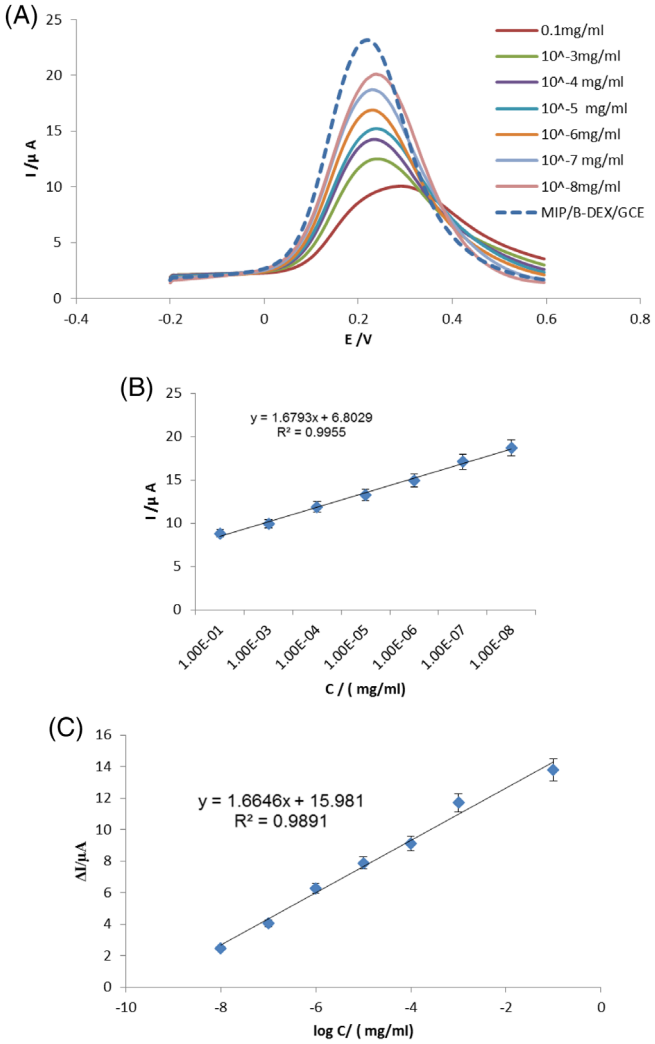

FIGURE 8 A, DPV of MIP/P(β-CD)/GCE in different concentrations of HRP for 5 seconds in 0.01 M $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$. B, Dependency of the corresponding peak currents of MIP/P(β-CD)/GCE vs concentration of HRP. C, Calibration curve

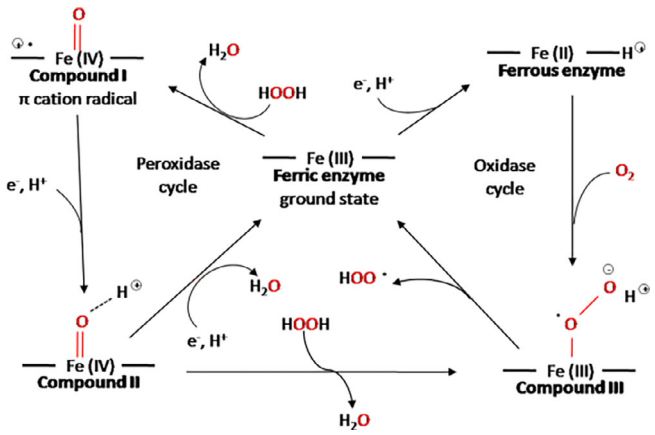

FIGURE 9 H_2O_2 reduction process by HRP in real sample

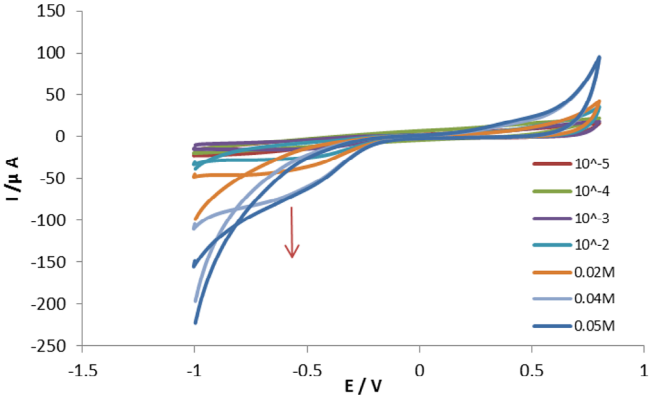

FIGURE 10 CVs of HRP/P(β-CD)/GCE in H_2O_2 solution (pH = 7.4) including of PBS

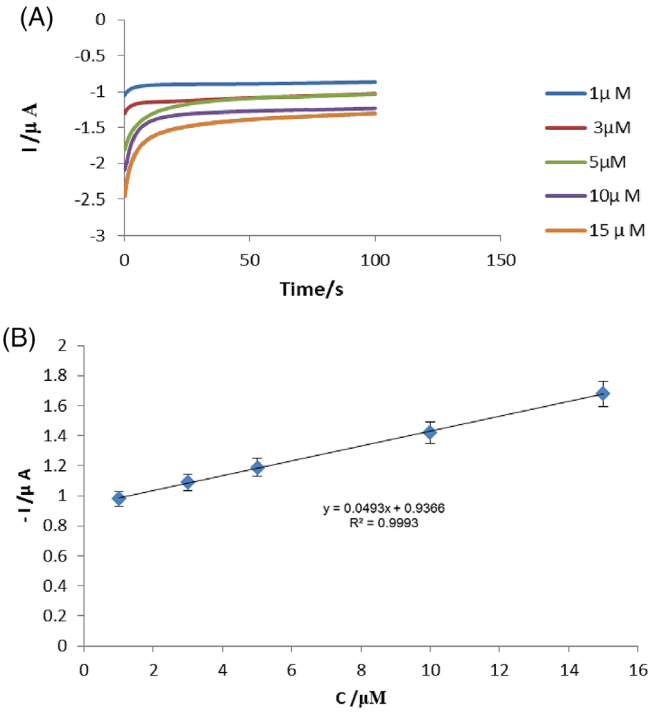

FIGURE 11 A, Chronoamperometry response of the HRP/β-CD/GCE to different concentrations of H_2O_2 from 1 to 15 μM in PBS solution at physiological pH. B, Calibration plot

that reduction of peroxidase reaction of HRP proceeded by involving two electrons and one proton transfer.[62]

The developed bio imprinted polymer was employed toward detection and sensitive determination of hydrogen peroxide in unprocessed human plasma samples at optimum pH (6.98).

The concentration of H_2O_2 in unprocessed human plasma sample was measured through additional standard of H_2O_2 by chronoamperometry technique (Figure 13). The determined amount from unprocessed human plasma was calculated as 9 μM (S/N = 3).

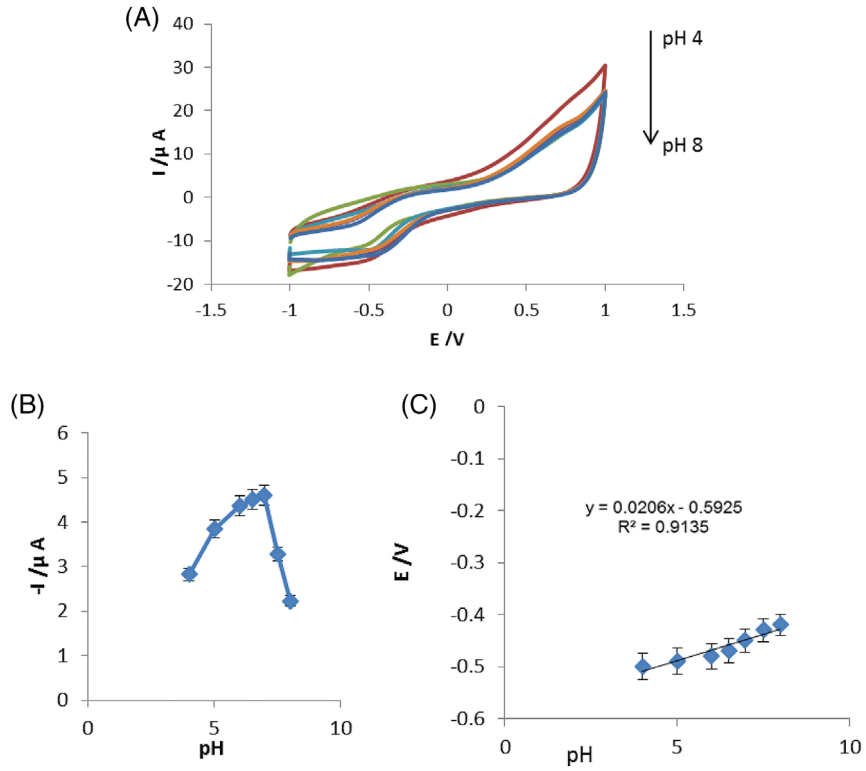

FIGURE 12 A, CVs of bio-sensor toward H_2O_2 reduction in different pH. B, Variation of I_p of H_2O_2 reduction vs pH. C, Dependency of peak potential vs pH (4-8)

3.2.3 | Selectivity of engineered molecular imprinted bio sensor

In MIP, the target molecule can re-bind with imprinted matrix and recognize the receptor not only by size but also by the type of target molecule functionality and arrangement in space. To further evaluate the selectivity of MIP/P(β-CD)/GCE, other proteins like PSA, BSA, CA 125, and CA 15-3, and CEA, were selected as interfering substances with concentration of 0.1 mg/mL. The selectivity was investigated by current response of each protein measured by MIP/P(β-CD)/GCE separately with DPV technique in the solution of $Fe(CN)_6^{3-/4-}$. As can be seen in Figure 14, the peak current of HRP is higher than interfering agents of BSA, CEA, and PSA which indicate that the obtained MIP has better recognition selectivity for HRP template and the BSA, CEA, and PSA do not interfere with imprinted cavity because their big size and arrangement are not proportional to the cavity of imprinted polymer. So, retarded probe diffusion from matrix imprinted polymer. However, CA 125 and CA15-3 with higher current response could consider as interference because of greatly smaller molecular size, which would enable quick diffusion to the imprinting layer.

3.2.4 | Stability and repeatability of constructed bio imprinted polymer based biosensor

For the evaluation of polymer film stability on the surface of GCE, CVs of P(b-CD)-GCE were recorded in the standard solution at various cycles (2, 5, 10, and 50). As can be seen in Figure 15, excellent stability was obtained after 50 cycles, which shows appropriate stability of proposed polymer film. The results indicate that the peak current of P(*β*-CD)/GCE retained nearly stable after 50 subsequent number of cycles.

The stability of constructed HRP/P(*β*-CD)/GCE sensor was detected by measuring the peak currents after storage of the biosensor in 5°C for 15 days. It is observed that the biosensor peak currents decreased only15.1% after 15 days which indicate good stability and function of bio-imprinted polymer. The repeatability of MIP/P(β-CD)/GCE was tested by 0.1 mg/mL HRP by CV technique in 6 cycles of elution-rebinding test of modified protein electrode, and found that the relative SD (RSD) was evaluated as 3.46%.

4 | CONCLUSION

In summary, a facile, selective, sensitive, and *repeatable* bio-imprinted polymer was engineered based on P(ß-CD) film and self-assemble of horseradish peroxide surface molecular imprinted technique. The obtained bioimprinted sensor shows a catalytic behavior toward reduction of H_2O_2 in real samples. P(ß-CD) film was used as the supporting substrate through electro-polymerization which provide simple and efficient matrix film with the large surface area and coverage toward subsequent re-binding of HRP targets. Also, excellent electro-conductivity of ß-cyclodextrin facilitate electron transfer of P(ß-CD). The proposed imprinted biosensor successfully utilized for detection of HRP with excellent analytical results which linear range is

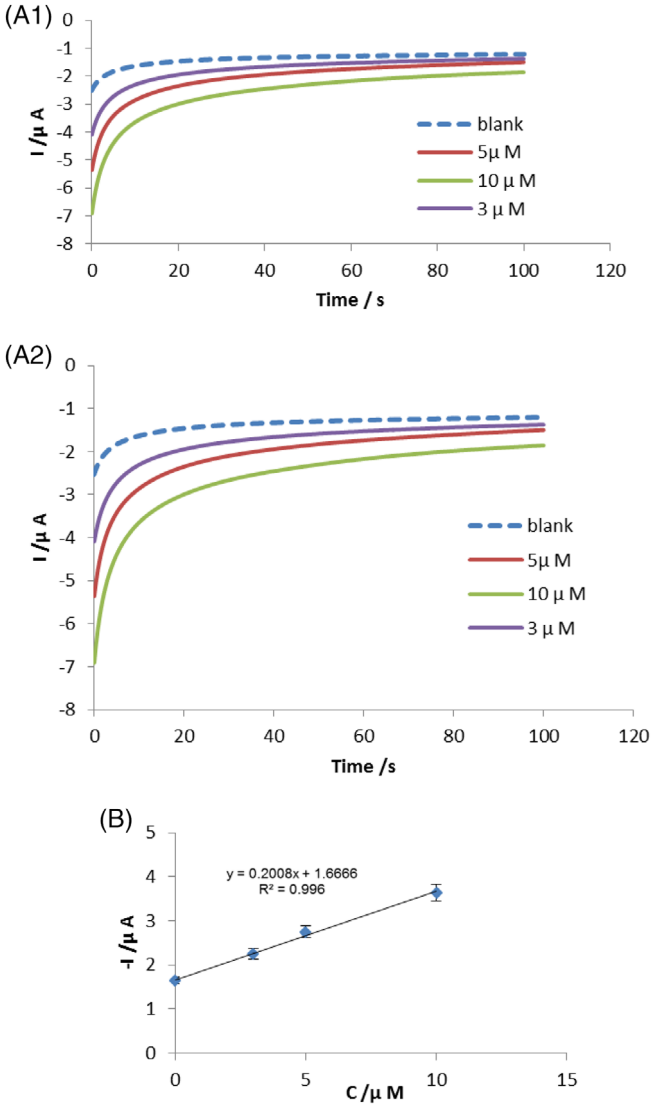

FIGURE 13 A1 andA2, chronoamperometry response of (bio) sensor toward sensitive determination of H_2O_2 in untreated human plasma samples with different concentrations (3, 5, and 10 µM). B, Calibration curve

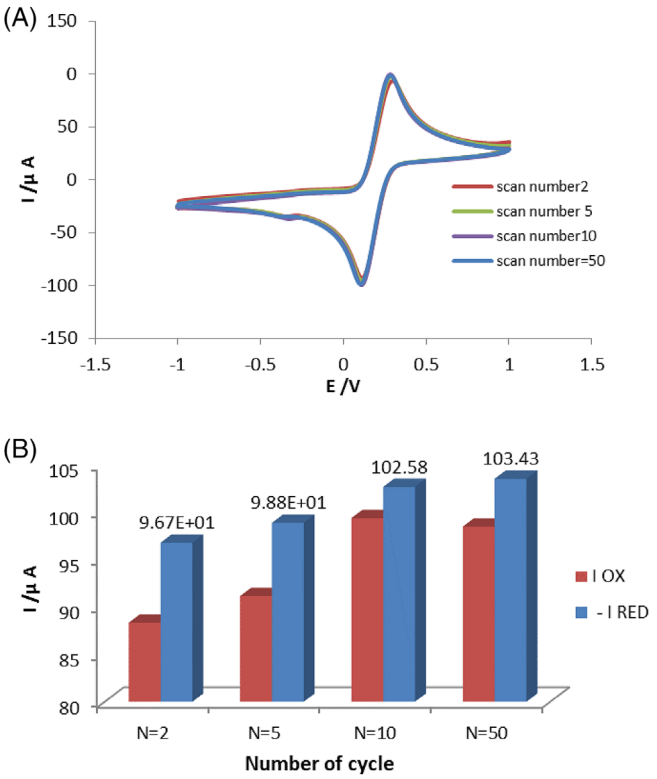

FIGURE 15 A, CVs of P(b-CD)-GCE were recorded in the standard solution at variation cycles (2, 5, 10, and 50) in the standard solution. B, variation of I_{pox} vs cycle No

0.1 mg/mL to 10 ng/mL with LOD of 2.23 ng/mL. Also, MIP-based biosensor was applied for the measurement of hydrogen peroxide in un-processed human plasma. Proposed method shows an appropriated platform toward biomedical analysis of H_2O_2 in real samples/ clinical studies.

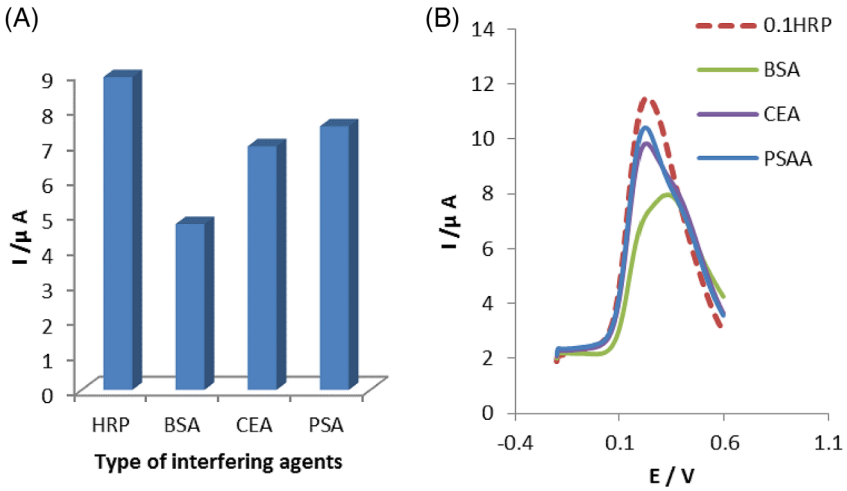

FIGURE 14 A, DPVs of MIP/P(β-CD)/GCE in $Fe(CN)_6^{3-/4-}$ (0.01 M) + KCl (0.1 M) in the presence of BSA, CEA, and PSA as interfering agents. B, Variation of I_p vs type of interfering agents

DATA AVAILABILITY STATEMENT

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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