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Sol-gel Immobilized Horseradish Peroxidase Modified Carbon Paste Electrode towards the Determination of Hydroquinone in Non-Aqueous Solvents: A Voltammetric Study

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Abstract- A new type of biosensor for hydroquinone (H_2Q) has been synthesized based on the carbon paste electrode (CPE) immobilized with Horseradish Peroxidase (HRP), through silica sol-gel (SiSG) entrapment. The electrochemical properties of biosensor are characterized by employing the electrochemical methods like cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The proposed biosensor show high sensitivity in determination and rapid response towards H_2Q under optimum conditions. The electrocatalytic response of H_2Q was detected in methanol, ethanol, 2-propoanol, 1-butanol and acetone. The good results were obtained in ethanol as a solvent and acetate buffer solution (ABS) as supporting electrolyte, the experiments were carried out in combination of these two media. The anodic peak current of H_2Q shows a linear relation with concentration range of 5×10^{-6} M to 10×10^{-4} M. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 1.5×10^{-6} M and 5.0×10^{-6} M respectively. The electrochemical impedance spectroscopy (EIS) result confirmed the occurrence of rapid electron transfer at HRP-SiSG/CPE. Moreover the stability, reproducibility, and repeatability of the biosensor were also studied. The proposed sensor was successfully applied for the determination of H_2Q in real samples and the result were found to be commensurate.

Key words- Horseradish Peroxidase, Silica Sol-gel, Hydroquinone, Carbon Paste Electrode, Cyclic Voltammetry, Differential Pulse Voltammetry, Electrochemical Impedance Spectroscopy

1. INTRODUCTION

Hydroquinone (H_2Q) is derivative of phenolic compounds which has a assortment of uses in many fields such as reducing agents, anti oxidants, polymerization inhibitor, photographic developer, dyes, azo dyes stuffs and other chemical intermediates [1,2]. H_2Q is a very important pollutants in food, medical and environmental matrices [3]. Accredited analytical procedures are required for the determination of H_2Q in various matrices with high sensitivity. So for many numerous methods have been developed for their determination including liquid chromatography [4,5], synchronous florescence [6], chemiluminescence [7,8], spectrophotometry [10], pH based flow injection analysis [11], electrochemical methods [12-15],etc. However, most of the above methods have some disadvantages, such as requiring more time and complex, high cost, low sensitivity and complicate pretreatment procedures etc. In recent years electrochemists has paid more attention in the direction of designing and development of novel sensors, due to its easy preparation, fast detection, low consume and high sensitivity [16-18].

Horseradish Peroxidase (HRP) is an important metalloenzyme, (it contains two metal centers i.e., iron heme group and calcium atom), and it is always used as an electron acceptor. Among the peroxidase, HRP has been one of the most widely studied enzymes in the development of enzyme based biosensor. The peroxidases have a property of impelling phenolic compounds and coupling between them entitles the direct electron oxidation/reduction relation with a covalent binding. Because of the deep implant of the HRP active site which is in unfavorable assimilation [19], it is a challenge to obtain the direct electrochemistry of HRP. According to Marcus theory the electron transfer mechanism is absolute factor for the direct electrochemistry of redox enzyme, which depends on the overall distance between electrode surface, redox site of enzyme and location of the enzyme electrode [20]. In order to adapt a good biosensor many materials such as conducting polymers, biomolecules, nanoparticles, dyes, ionic liquids, were employed to improve the ideal environment around the enzyme to provide favorable orientation and to hasten the electron transfer between enzyme and the surface of the electrode [21].

Many of the properties of enzymes have been examined such as HRP, esterase and lipases in non-aqueous solvents [22]. In contrast HRP has been comparatively and extensively studied in organic solvents. The active heme site of HRP losses its native conformation and increases the co-ordination number of heme group from 5 to 6 [23]. The enzyme electrode was used for the determination of H_2Q in non-aqueous solvents like methanol, ethanol 2-propanol, 1-butanol, acetone and 0.1 M acetate buffer solution (ABS) (pH 5.0) solution as supporting electrolyte, a linear response was obtained over the H_2Q concentration and its correlation coefficient of both anodic and cathodic current was almost equal to unity [24].

In this present work we have concentrated on embedding HRP in SiSG film on carbon paste electrode. The HRP-SiSG/CPE can promote the direct electron transfer of the enzyme

immobilized on the electrode surface. The resulting HRP-SiSG/CPE biosensor exhibited a high sensitivity and better stability for the determination of H_2Q.

2. EXPERIMENTAL SECTION

2.1. Reagents

All chemicals were obtained from commercial sources and used without further purification. Horseradish Peroxidase (E.C. 1.11.1.7 type –VI-A - S/5 mg, Amoracia rusticana source, 1840 U/mg), Hydroquinone were purchased from Sigma–Aldrich chemicals Co.USA.Tetraethyl orthosilicate (TEOS), Triton–X–100 were obtained from Sigma–Aldrich chemicals Co. USA. Ethanol was purchased from Changshu Yangyuan chemical from China. Methanol, 2-propanol, 1-butanol were purchased from Merck Specialties pvt limited, Mumbai. Acetone was purchased from fisher scientific pvt limited, Mumbai. The graphite fine powder was procured from Lobo Chemie and silicon oil from Himedia. Acetate buffer solution (ABS) was prepared by mixing 0.1 M sodium acetate and 0.1M acetic acid. All the aqueous solutions were prepared with double distilled water. The enzyme stock solution and working solutions of chemicals were stored in cool place.

2.2. Material

The electrochemical measurements were conducted in a three electrodes cell at the room temperature 25±2 ^{o}C. The working electrode was the enzyme immobilized carbon paste electrode (HRP–SiSG/CPE). The reference electrode was a saturated calomel electrode system and glassy carbon rod electrode was used as an auxiliary electrode. Measurements were carried out using CH–Electrochemical Analyzer (Model CHI–660D, CH Instruments, USA). The pH values were measured with Elico U 120 pH meter and a combined pH CL 51 B electrode.

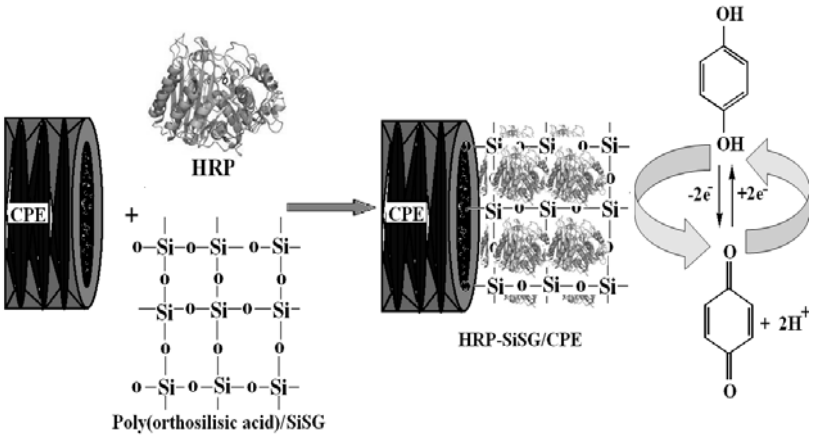
2.3. Preparation of carbon paste electrode

The carbon paste electrode was prepared by hand grinding of 85% graphite power and 15% of silicon oil using agate mortar for 40-45 min to get homogeneous paste. This carbon paste was incorporated in to 2 mm diameter of Teflon tube and electrical contact was made with a copper wire through the tube. The modified electrode was smoothed on the piece of soft filter paper before measurement [25, 26].

2.4. Fabrication of HRP–SiSG/CPE

A homogenous TEOS silica sol gel was prepared by mixing 2 ml of TEOS, 1 ml of H_2O, 50 µl of 0.1 M HCl, 25 µl of 10% Triton-X-100. The mixture was stirred for 1 h until a clear sol is formed. The sol can be stored for one month when it is kept in refrigerator. The 5 µl of 5 mg/ml enzyme stock solution was added to the mixture of 5 µl of stock SiSG solution,

40 µl of ABS. A drop of this dispersion with the volume of 5 µl was casted onto the surface of the CPE, then it was allowed to polymerize at room temperature for 3-5 min. The electrode was gently washed with ABS and is used for further experimental procedure [27]. The 0.5 U of enzyme was immobilized on the electrode surface. The fabrication procedure of the biosensor was illustrated in scheme 1.



Scheme 1. A schematic diagram showing the steps involved in the fabrication of HRP–SiSG/CPE

3. RESULTS AND DISCUSSION

3.1. Investigation of electrochemical behavior of HRP-SiSG/CPE biosensor using cyclic voltammetric studies.

Electrochemical behavior of H_2Q at bare CPE, HRP-SiSG/CPE in 40% of ethanol (EtOH)/0.1 M ABS (pH 5.0) were studied using CV and DPV. Fig. 1 shows the electrochemical responses obtained with modified CPE in 40% EtOH/0.1 M ABS (pH 5.0), as shown in the figure no peak was observed at bare CPE without H_2Q (curve a), when 0.2 ml of 10 mM H_2Q solution was added, the anodic and cathodic peak of H_2Q was observed at 367 mV and 28 mV at bare CPE (curve b) respectively. The peak potential separation (ΔE_P) was about 339 mV. This indicates that the H_2Q exhibit a reversible electrochemical behavior at bare CPE. The CV behavior of H_2Q was also studied at HRP-SiSG/CPE (curve 'c'), as shown in Fig. 1, under the same conditions, the anodic and cathodic peak potentials of H_2Q appears at 203 mV and 28 mV, respectively, with a great reduce in ΔE_p value to 173 mV, in comparison with bare CPE respectively. In addition, the anodic and cathodic peak currents also increased greatly.

These results indicates that the modifier provide a favorable environment for the functioning of proteins, in which native conformation of the proteins are retained and electron transfer rate are greatly enhanced compare with those involving protein alone at bare

CPE [26]. The surface concentration of electroactive HRP (Γ) in HRP–SiSG/CPE surface could be estimated according to following equation [29].

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

Where, 'I_p' is the peak current, 'A' is the electrode active surface area, 'ν' is the scan rate, 'n' is the number of electrons, R, T and F has their usual meanings. By considering the above values, 'Γ' was calculated as 7.89×10^{-8} mol cm^{-2}, which was found to be about 40 times more than the monolayer coverage of HRP (4.96×10^{-11} mol cm^{-2}) on 3-mercaptopropionic acid modified gold electrode [30]. These results indicated that the multiple layers of active HRP were coated on the electrode surface.

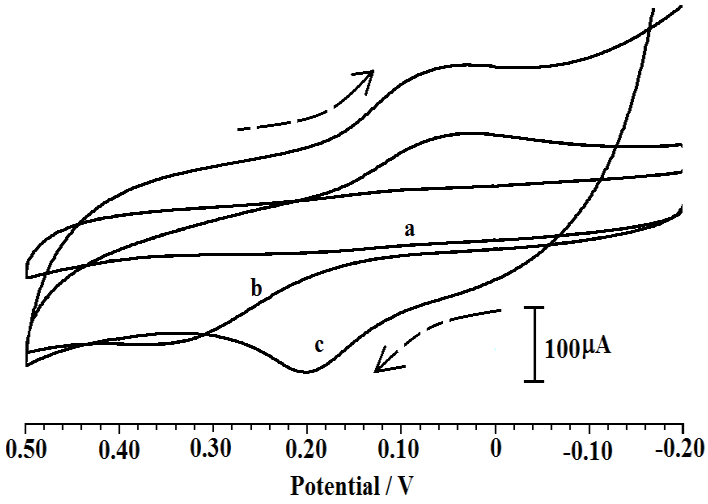


Fig. 1. Cyclic voltammograms for the electrochemical response of 2×10^{-4} M H_2Q (a) Blank (b) Bare CPE (c) HRP-SiSG/CPE in 40% EtOH/ 0.1 M ABS at the scan rate10 mV/S

3.2. Electrocatalytic response of HRP-SiSG/CPE in organic solvents.

The electrocatalytic response of HRP-SiSG modified sensor surface towards H_2Q was examined in different organic solvents at methanol, ethanol, 2-propanol, 1-butanol and acetone, 0.1 M ABS (pH 5.0) as supporting electrolyte by using both CV and DPV techniques. The electrochemical response of HRP-SiSG/CPE towards the lower concentrations of H_2Q in methanol, 2-propanol, 1-butanol and acetone was found to be less in comparison with EtOH. A favorable electrocatalytic activity of HRP-SiSG/CPE surface towards H_2Q in EtOH expressed a good redox system and this was shown in Fig. 2A&B respectively.

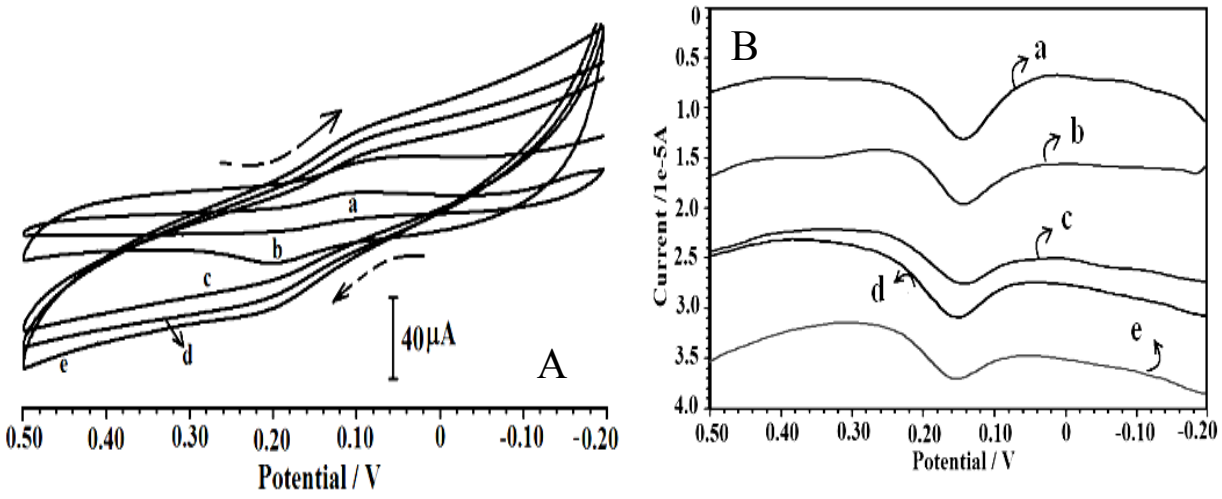


Fig. 2. A) Cyclic voltammograms for the electrochemical response of 2×10^{-4} M H_2Q at HRP-SiSG/CPE (a) 40% methanol/ 0.1 M ABS pH (5.0) (b) 40% ethanol/ 0.1 M ABS pH(5.0) (c) 40% 2-propanol/ 0.1 M ABS pH (5.0) (d) 40% acetone/ 0.1 M ABS pH (5.0) (e) 40% 1-butanol/ 0.1 M ABS pH (5.0) **B)** Differential pulse voltammograms for the electrochemical response of 2×10^{-4} M H_2Q at HRP- SiSG/CPE (a) 40% methanol/ 0.1 M ABS pH (5.0) (b) 40% ethanol/ 0.1 M ABS pH (5.0) (c) 40% 2-propanol/ 0.1 M ABS pH (5.0) (d) 40% acetone 0.1 M ABS pH (5.0) (e) 40% 1-butanol/ 0.1 M ABS pH (5.0)

3.3. Electrochemical response of biosensor in EtOH

The immobilized HRP-SiSG/CPE biosensor towards H_2Q was examined in organic solvent of EtOH in a range of 10% to 60%, in the combination with 0.1 M ABS buffer of pH 5.0. A typical CV and DPV with a good electrocatalytic activity of surface modified electrode were shown in Fig. 3A&B respectively. It was observed that as the percentage of EtOH increases from 10%, there was gradual increase in the peak currents. The maximum peak currents observed at 40% of EtOH. Beyond 40% there was a decrease in the peak currents. Hence, a 40% of EtOH was chosen for further experiments. The surface modified biosensor showed a good electron transfer rate and favorable peak currents for the redox reaction of HRP towards H_2Q in organic solvents [24].

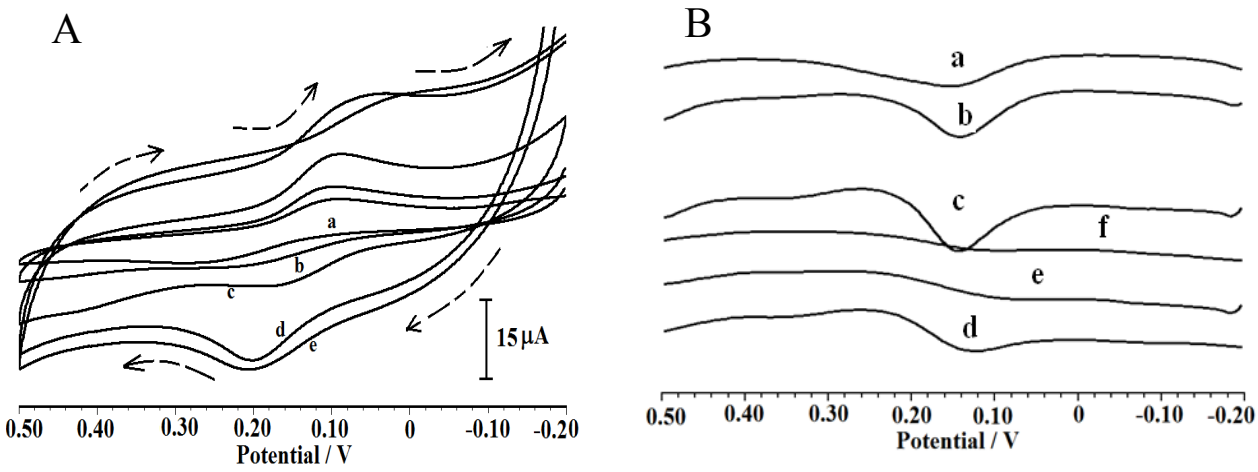


Fig. 3. A) Cyclic voltammograms for the electrochemical response of 2×10^{-4} M H_2Q at HRP-SiSG/CPE in different ratio of EtOH/0.1 M ABS pH (5.0) (a) 10% EtOH/ 0.1 M ABS (b) 20% EtOH/ 0.1 M ABS (c) 30% EtOH/ 0.1M ABS (d) 40% EtOH/ 0.1 M ABS 50% EtOH/ 0.1 M ABS (f) 60% EtOH/ 0.1 M ABS, **B)** Differential pulse voltammograms for the electrochemical response of 2×10^{-4} M H_2Q at HRP- SiSG/CPE in different ratio of EtOH/0.1 M ABS pH(5.0) (a) 10% EtOH/ 0.1 M ABS (b) 20% EtOH/ 0.1 M ABS (c) 30% EtOH/ 0.1 M ABS (d) 40% EtOH/ 0.1 M ABS (e) 50% EtOH/ 0.1 M ABS (f) 60% EtOH/ 0.1 M ABS

3.4. Effect of scan rate

The effect of scan rate on the peak currents of H_2Q at various scan rates were investigated by cyclic voltammetric process. Both anodic and cathodic peak currents were increases with increasing potential scan rate, a well assign redox peaks were obtained for H_2Q and showed in Fig. 4A. The investigation exhibited that the peak currents increased linearly with square root of scan rate ($\nu^{1/2}$), suggesting that the electron transfer reaction was a diffusion controlled process. The relationship of the redox peak current with square root of scan rate was constructed and shown in Fig. 4B. The electrocatalytic process of HRP-SiSG/CPE towards H_2Q was shown below [31].

$$\text{HRP } (Fe)^{II} + C_6H_4O_2 \longrightarrow \text{Compound I } ([Fe^{IV}=O]^+) + C_6H_4O_2 \quad \text{(R1)}$$

$$\text{Compound I} + ([Fe^{IV}=O]^+) + e^- + H^+ \longrightarrow \text{Compound II } ([Fe^{IV}=O] \quad \text{(R2)}$$

$$\text{Compound II} + ([Fe^{IV}=O]) + e^- + H^+ \longrightarrow \text{HRP } (Fe^{III}) + H_2O \quad \text{(R3)}$$

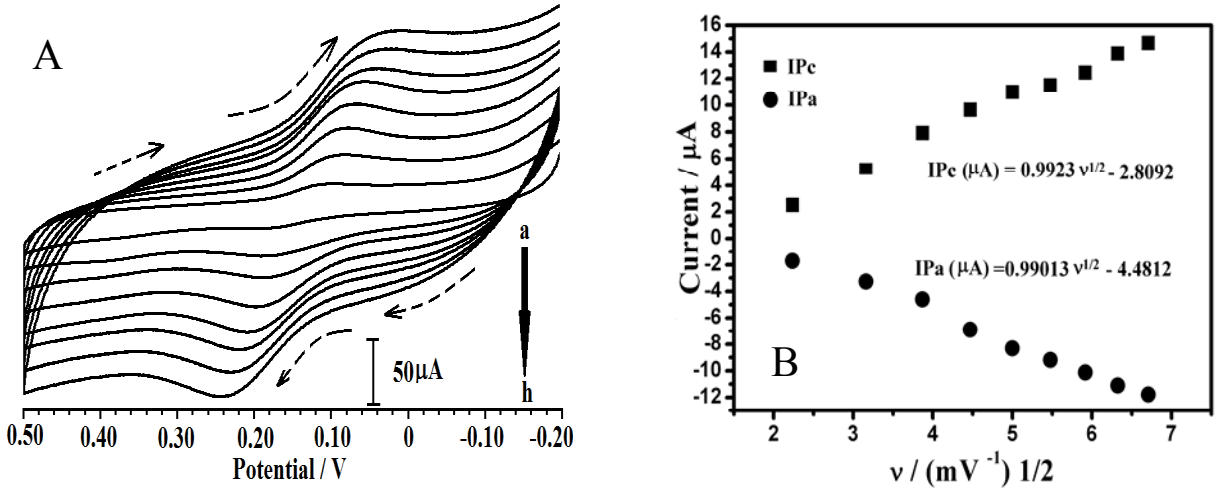


Fig. 4. A) Cyclic voltammograms of variation of scan rate for H_2Q at HRP-SiSG/ CPE.(a to h, 5–40 mV/S) in 40% EtOH/ 0.1 M ABS (pH 5.0) solution, **B)** Calibration plots for the redox peak currents *vs*. the square root of scan rate

3.5. Effect of concentration of H_2Q at fabricated biosensor

The electrocatalytic activity of HRP-SiSG/CPE towards H_2Q have been studied by employing DPV by varying from 4×10^{-5} M to 1×10^{-3} M and was shown in Fig. 5A. A graph between I_{pa} *vs*. concentration of H_2Q showed two linearity's from the concentration of 4×10^{-5}M to 1×10^{-4}M and 2×10^{-4}M to 1×10^{-3}M with linear regression equation of I_{pa} (µA)=0.693.C µML+0.488 µA and I_{pa} (µA)=0.6335 .C µML+7.2104 µA respectively. The correlation coefficient of both linearity's was 0.99087 and 0.98254. The sensitivity of second linearity curve range was decreased due to the kinetic limitations [32, 33]. The LOD and LOQ of this biosensor were estimated as 1.5 µM and 5.0 µM. The detection limit and quantification limit was calculated by using following expressions [34, 35].

$$LOD = \frac{3S}{M}$$

$$LOQ = \frac{10S}{M}$$

Where, S is the standard deviation of mean value of differential pulse voltammograms and M is the slop of the calibration plots.

The operant Michaelis-Menten constant gives an indication towards the enzyme-substrate kinetics for biosensor and it was calculated from the electrochemical version of Line weaver-Burk equation [36].

$$\mathbf{1/i_{ss} = 1/i_{max} + K_m^{app}/i_{max}C}$$

Where, '$\mathbf{i_{ss}}$' is the steady state current after the addition of substrate and 'i_{max}' is the maximum current measured under saturated substrate condition. The K_m^{app}, i_{max} values of the biosensor towards H_2Q was found to be 0.10 mM, 9.5 μA respectively.

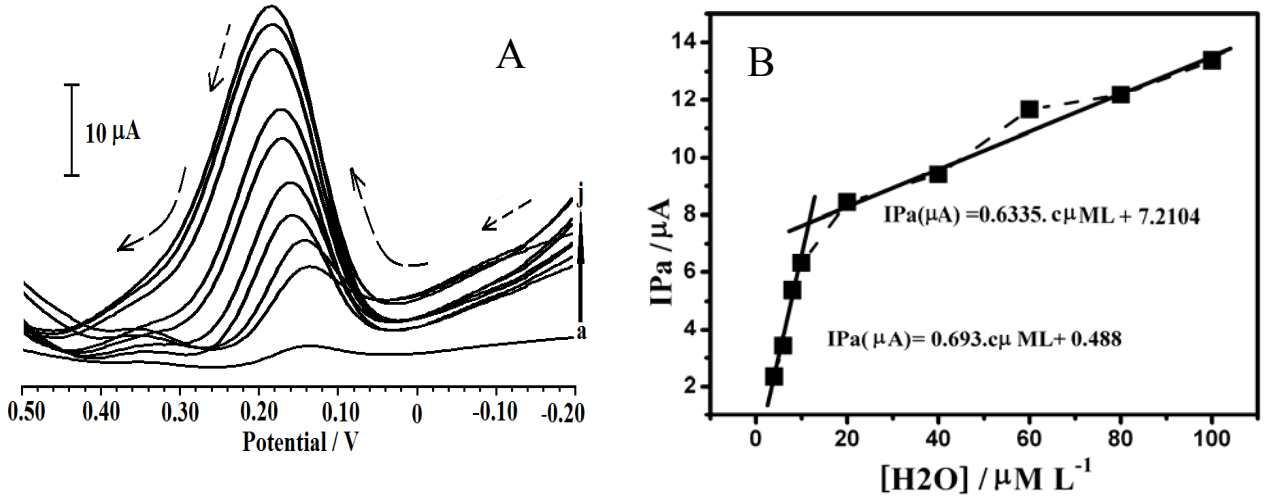


Fig. 5. A) A differential pulse voltammograms of H_2Q for different concentrations. (a) 4×10^{-5} M (b) 6×10^{-5} M (c) 8×10^{-5} M (d) 1×10^{-4} M (e) 2×10^{-5} M (f) 4×10^{-4} M (g) 6×10^{-4} M (h) 8×10^{-4} M (i) 1×10^{-3} M , **B)** Calibration plot of H_2Q concentration

3.6. Investigation of electrochemical behavior of H_2Q and influence of mediator

In electrocatalytic electrochemical process unfavorable direct electron transfer were observed between HRP-SiSG/CPE towards substrate (H_2Q) in all organic solvents [24, 37]. Pyrogallol (PG) was used as a mediator to facilitate the electron transfer effectively. Both mediator and substrate PG/H_2Q expressed reversible electrochemical behavior in organic solvent media. Fig.6. depicts CV for H_2Q, with a mediator (PG) of constant concentration, sub sequentially on gradual increase of H_2Q concentration, both anodic and cathodic peak currents and electron transfer rate was found to be increased. The linearity curve was shown in inset of Fig. 6.

3.7. Investigation of stability and repeatability of HRP-SiSG/CPE sensor towards H_2Q

The manufactured biosensor was gently tested for 40 cycles in the potential range from -0.2 to 0.5 V at scan rate of 25 mV s^{-1} in EtOH/0.1 M ABS buffer solution (pH 5.0) containing 2×10^{-4} M H_2Q. We noticed that after 40 cycles there was no disturbance in the peak currents of the system and showed in Fig.7A. From this result the HRP-SiSG/CPE was stable and measurement was repetitive and reproducible, hence it can be carried out for the selective determination of H_2Q. A plot was drawn between repetitive measurements against peak currents, the graph showed that the measurements were stable, confirming the electrode stability Fig.7B. The durable stability of HRP-SiSG/CPE biosensor was investigate under the

storage conditions (-4°C). It was observed that activity of immobilized HRP was stable up to 30 days.

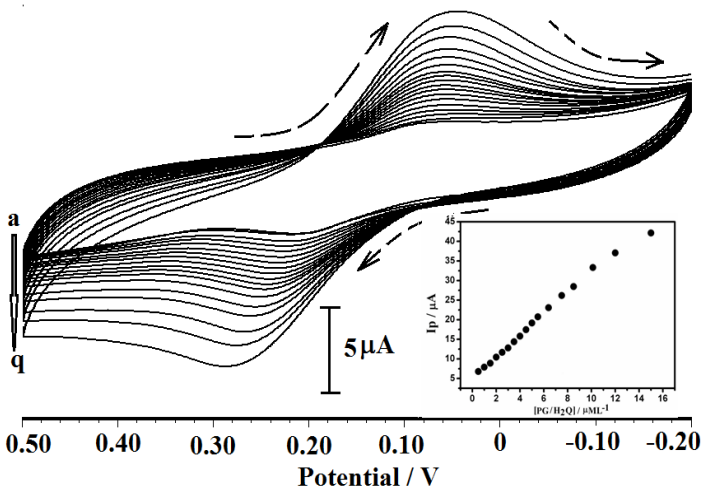


Fig. 6. Cyclic voltammograms for electrochemical response of mediator studies for PG (2×10^{-4} M constant) /H_2Qat HRP-SiSG/ CPE by sequential addition of H_2Q (a) 0.5×10^{-6} M (b) 1×10^{-6} M (c) 1.5×10^{-6} M (d) 2×10^{-6} M (e) 2.5×10^{-6} M (f) 3×10^{-6} M (g) 3.5×10^{-6} M (h) 4×10^{-6} M (i) 4.5×10^{-6} M (j) 5×10^{-6}M (k) 5.5×10^{-6} M (l) 6.5×10^{-6} M (m) 7.5×10^{-6} M (n) 8.5×10^{-6} M (o) 10×10^{-6} M (p) 12×10^{-6} M (q) 15×10^{-6} M . Inset calibration plot of H_2Q

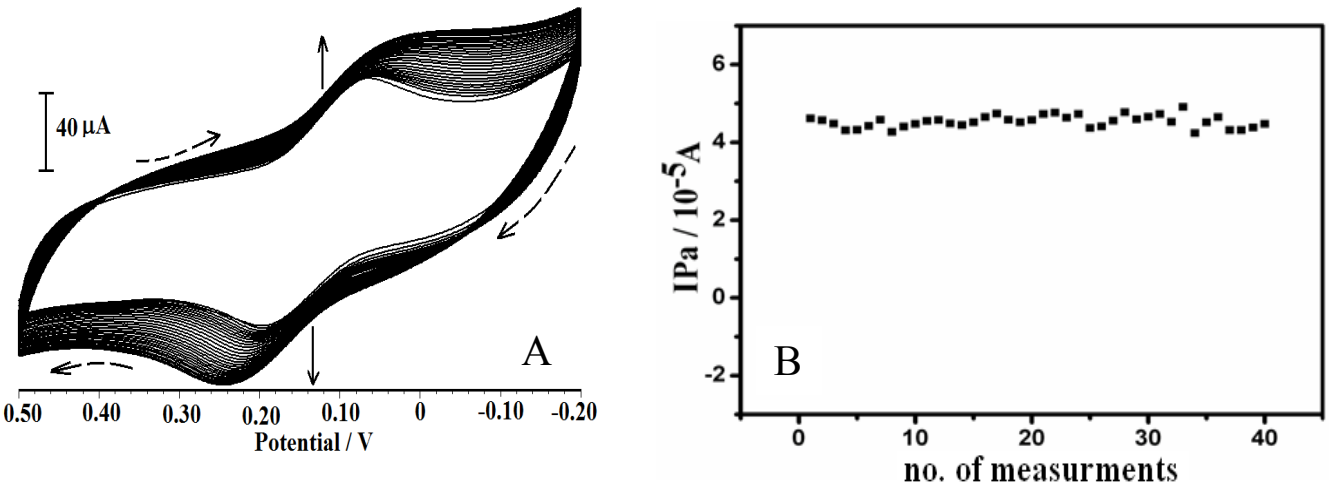


Fig.7. A) Cyclic voltammograms for 40 multiple cycle of 2.0×10^{-4} M H_2Q in 40% EtOH/0.1 M ABS solution of pH 5.0 at a scan rate of 25 mV s^{-1}, **B)** A plot of number of repetitive cycles *vs.* I_p

3.8. Analytical applications.

The proposed analytical procedure was applied to evaluate the reliability of the biosensor under optimized conditions by the determination of H_2Q in local tap water and mineralized water samples. The spike and recovery studies were achieved through DPV. The amount of H_2Q in the tap water and mineralized water sample were determined by the calibration method and was tabulated in the Table 1. The recovery was in the range 98-103%, which clearly indicates the applicability and reliability of the proposed method.

Table 1. Analytical performance of spiked samples studied of H_2Q

S.No	SampleMatrix	Added(μM)	Found [a](μM)	Recovery%	S.D (%)	RSD	Bias
1	Mineralized Water	40	39.5	95.5	0.06	1.74	-1.25
		60	59	98.3	0.04	0.98	-1.7
		80	82	102.5	0.07	2.04	+2.5
2	Tap water	60	61	101	0.06	1.43	+1
		80	79	98	0.08	1.60	-2
		100	103	103	0.02	0.34	+3

3.9. Electrochemical impedance spectroscopy (EIS) characterization of biosensor /chemical sensor

Electrochemical alternating current impedance technique was a power full tool to characterize the electrochemical process occurring at the electrode interface [38, 39]. The curve of the EIS includes a semicircle and linear portions, with the former at higher frequencies corresponding to less electron transfer and later at lower frequencies corresponding to higher electron transfer. The surface electron transfer resistant (Ret) of impedance spectroscopy controls the interfacial electron transfer rate of the redox peak, between the solution and electrode. EIS studies for the CPE and HRP-SiSG/CPE was investigated by the alternating current frequencies between 1 Hz to 100 KHz, with equilibrium potential of 0.289 V. Fig. 8, reveals the results of EIS represented in the form of Nyquist plots (Z" Vs Z') for the CPE and HRP-SiSG/CPE. The electrochemical impedance process was carried out in EtOH/0.1 M ABS (pH 5.0) solution. Bare CPE (a) exhibited a

straight line with large probe in comparison with HRP-SiSG/CPE (b). Which indicates the modified biosensor show less impedance and more electron transfer rate. The Randles equivalence circuit was constructed for the data obtained and showed in inset of Fig.8. Where Rs represent the resistant of solution and Q-CPE indicates constant phase current, Ret is expressed charge transfer electron resistance, R_p represent polarization resistance, and Cd1 represent double layer capacitance.

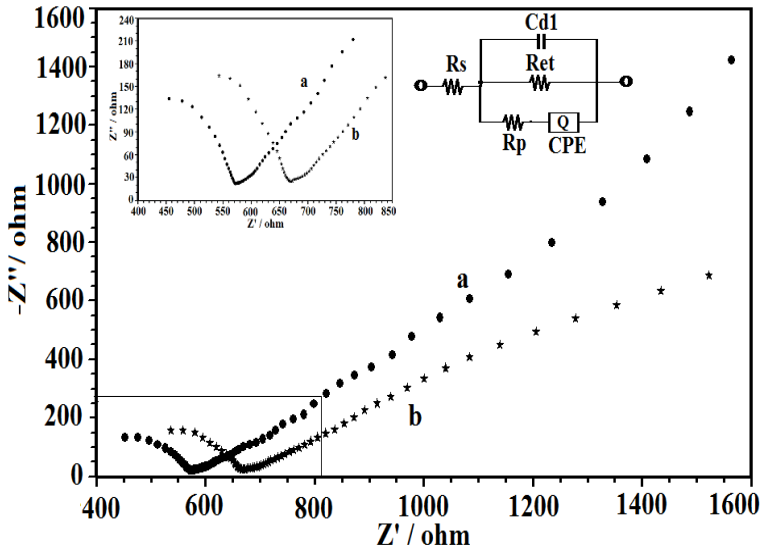


Fig. 8. EIS spectrum of H_2Q (a) CPE (b) HRP-SiSG/ CPE, inset equivalent circuit at HRP- SiSG/ CPE Amplitude: 5 mV, Frequency: 1 Hz to 100 KHz

4. CONCLUSION

The result of the present investigation showed a novel method for the determination of H_2Q by using CV and DPV at HRP-SiSG/CPE. The proposed biosensor has shown many advantages, such as effectiveness, novel electrocatalytic activity, and long-term durability. The biosensor gives a fast response, broad linearity range, low detection limit with satisfactory stability, repeatability and good potential applications towards the determination of H_2Q in real samples.

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