Chapter 4

Electroactive Prussian Blue encapsulated Iron Oxide Nanostructures for Mediator-free Detection of Free Cholesterol

4.1. Introduction

In the previous chapter, the two different phases of nanostructured iron oxide (Fe$_3$O$_4$ and α-Fe$_2$O$_3$) have been utilized for the fabrication of electrochemical biosensors for detection of cholesterol. The biosensors employed the use of an artificial redox mediator and thus resulted in the fabrication of 2$^{nd}$-generation biosensors. As discussed in section 3.4, the encapsulation of Fe$_3$O$_4$ NPs with prussian blue (PB), an electroactive material, can prevent oxidation and agglomeration of NPs along with enhancing their electrochemical activity and can thus eliminate the use of artificial mediator and result in 3$^{rd}$-generation electrochemical biosensor. PB is a prototype of metal hexacyanoferrates with well-known electrochromic, electrochemical, photophysical, and magnetic properties and potential analytical applications[257]. Due to its excellent electrocatalysis and its analogy with peroxidase enzymes, PB has been widely used as an electron-transfer mediator in the amperometric biosensors[258]. Several H$_2$O$_2$ sensors and enzymatic biosensors have been fabricated using bulk form of PB and its composites[259, 260]. However, very few reports on the utilization of nanosized PB for application in biosensors are available. Zhang et al. have reported the fabrication of glucose biosensor based on direct assembly of PB modified electrode with the Ionic Liquid-Chitosan matrix assisted glucose oxidase immobilization[261]. Zhu et al. have reported the fabrication of amperometric glucose biosensor using nanosized PB-CNTs composite and excellent biosensing characteristics were obtained due to improved
electrocatalytic activity towards the reduction of H₂O₂[262]. Thus, encapsulation of PB onto Fe₃O₄ NPs may prove significant for the fabrication of an electrochemical biosensor which enroutes direct electron transfer across the interface due to its extraordinary electrocatalytic activity.

Electrical communication of redox enzymes to the electrode has been a subject of extensive research and an essential prerequisite for the development of effective electrochemical biosensors[263, 264]. Direct exchange of electrons from enzymes to the electrode is prohibited due to the insulating protein barrier which surrounds the redox centres of enzyme molecules. This limits the performance of a biosensor[265]. Diffusional redox mediators, the tethering of redox relay units to the enzymes, incorporation of enzymes into redox polymers and redox hydrogels are the common practices to establish electrical contact between redox group of enzymes with the electrode and shuttle electrons from the enzymes[266, 267]. The incorporation of NPs with coating of redox active material with appropriate dimensions and functionalization onto the electrode surface can act as a current collector and an electron relay to the immobilized enzyme[268]. This shortens the electron transfer distance and establishes an electronic pathway for efficient electron transfer.

For biosensing application, a precise control over the spatial arrangement and distribution of the NPs onto the substrate is of utmost importance[269]. Among the various techniques, electrophoretic deposition provides tunable control over the degree of deposition of the charged colloidal particles onto an electrode surface under the influence of an applied electric field. It offers rapid deposition of desired materials and their composites with a simple laboratory setup and uniform films can be obtained by
optimizing parameters such as solution concentration, applied potential, pH etc[222]. The utilization of the orderly arranged PB-Fe₃O₄ NPs on the ITO electrode for the immobilization of the enzyme molecules may result in improved biosensing performance due to the facilitation of direct electron transfer from redox centres of biomolecules to the electrode.

This chapter reports the fabrication of 3rd-generation cholesterol biosensor based on the PB-modified Fe₃O₄ NPs. Kinetic parameters and biosensing characteristics of the ChOx/PB-Fe₃O₄ NPs/ITO bioelectrode have been investigated using CV in the absence of any artificial redox mediator. The excellent sensitivity, low value of $K_{m}^{app}$ and high charge transfer rate constant ($k_c$) have been obtained for the bioelectrode due to facile and direct electron transfer properties of PB-Fe₃O₄ NPs.

4.2. Experimental Section

4.2.1. Synthesis of Prussian Blue encapsulated Fe₃O₄ NPs

The Fe₃O₄ NPs have been prepared via hydrolytic reaction based on chemical co-precipitation of metal salts with an alkali as reported in chapter 3. To encapsulate Fe₃O₄ NPs with PB, 1 mmol of K₃[Fe(CN)₆] and 0.5 mmol of citric acid was mixed with 30 ml of Fe₃O₄ NPs solution (0.5 mg ml⁻¹) while stirring for 30 mins. Solution containing 1 mmol of FeCl₂ and 0.5 mmol of citric acid was then dropwise added to the mixture. The resultant mixture was stirred for additional 30 mins. Immediate appearance of blue colour indicated the formation of PB-Fe₃O₄ NPs[270]. Citrate ions induced carboxyl groups at the surface of NPs which were utilized for covalent attachment of ChOx molecules.

The synthesized Fe₃O₄ NPs carry positive charge in 0.1 M HCl solution (zeta potential ~ 38.5 mV) [Fig. 4.1(A)]. Addition of K₃[Fe(CN)₆] (zeta potential ~ -27.3 mV)
[Fig. 4.1(B)] results in the electrostatic adsorption of $[\text{Fe(CN)}_6]^{3-}$ ions onto the positive surface of Fe$_3$O$_4$ NPs. Also, CN$^-$ ions can form complex with Fe$^{3+}$ ions onto the surface of NPs resulting in adsorption of $[\text{Fe(CN)}_6]^{3-}$. Further, addition of Fe$^{2+}$ ions into the solution result in the formation of PB shell on the surface of Fe$_3$O$_4$ NPs [216]. The pH of the suspension was recorded as 2.0.

![Zeta Potential Graphs](image)

**Figure 4.1:** Zeta potential graphs of Fe$_3$O$_4$ NPs (a); $K_3[\text{Fe(CN)}_6]$ (b).

**4.2.2. Electrophoretic Deposition of PB encapsulated Fe$_3$O$_4$ NPs**

The nanocrystalline films of PB encapsulated Fe$_3$O$_4$ NPs have been obtained using two-electrodes system with platinum plate as auxiliary electrode and ITO substrate as the deposition electrode. The PB-Fe$_3$O$_4$ NPs carry positive charge at pH 2.0. Thus, ITO substrate was connected to cathode terminal while platinum electrode was made anode. Concentration of PB encapsulated Fe$_3$O$_4$ NPs was adjusted to obtain current of 1 mA and uniform films of PB-Fe$_3$O$_4$ NPs were obtained on ITO surface by applying 5 V for 60 s.

**4.2.3. Enzyme Immobilization**
Prior to enzyme immobilization, the PB-Fe$_3$O$_4$ NPs/ITO electrode was dipped in PBS containing 0.4 M 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.1 M N-hydroxysuccinimide (NHS) for 4 h at 30 °C. The NHS acts as an activator for the carboxyl groups present at the surface of PB-Fe$_3$O$_4$ NPs/ITO electrode and EDC acts as the coupling agent. The modified electrode was then incubated with 20 ml of ChOx solution (1 mg ml$^{-1}$) at 4 °C for overnight. The NH$_2$ groups of the enzymes form covalent amide bond (CO-NH) with the activated carboxyl groups at the surface of PB-Fe$_3$O$_4$ NPs/ITO electrode and result in the formation of ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode [Fig. 4.2]. The loosely bound enzymes were washed off using 100 mM PBS containing 0.05% tween-20.

Figure 4.2: Schematic illustration for the preparation of nanostructured PB-Fe$_3$O$_4$ electrodes and mechanism for the fabricated cholesterol biosensor.

4.3. Results and Discussion

4.3.1. TEM Studies

Figure 4.3(a) shows TEM micrograph of Fe$_3$O$_4$ NPs indicating the average size of 10 nm.

The lattice fringe of ~2.60 Å obtained from fringe pattern (Inset) match with d-value (2.56 Å) corresponding to (311) hkl plane of Fe$_3$O$_4$ nanocrystals (JCPDS file: 890951). On encapsulation of Fe$_3$O$_4$ NPs with PB [Fig. 4.3(b)], the average size of NPs increases
to 18 nm, suggesting the shell thickness of four nm. Due to the formation of thick shell of PB, magnetic interaction among NPs has been considerably reduced resulting in highly dispersed and stable NPs. However, formation of dimers and trimers on capping of PB over Fe₃O₄ NPs can be observed.

![Figure 4.3: TEM micrographs of (a) Fe₃O₄ NPs; (b) Prussian blue modified Fe₃O₄ NPs.](image)

4.3.2. UV-visible Studies

Figure 4.4(A) shows the optical absorption spectra from Fe₃O₄ NPs and PB encapsulated Fe₃O₄ NPs. The absorption onset of Fe₃O₄ NPs is at ~ 600 nm [Figure 4.4(A)(a)]. Because of the quantum size effect, this onset value is blue-shifted by 100 nm as compared to that of the bulk Fe₃O₄. The band near 300 nm corresponds to ligand field transitions of Fe³⁺ and shoulder peak around 480 nm corresponds to excitation of Fe-Fe pair[271].

On addition of K₃[Fe(CN)₆] to Fe₃O₄ NPs, absorption from Fe₃O₄ NPs is masked by the characteristic peaks of K₃[Fe(CN)₆] at 260 nm, 301 nm and 412 nm[272] [Fig. 4.4(A)(b)]. On further addition of FeCl₂, sharp absorption occurs around 700 nm which marks the formation of PB over Fe₃O₄ NPs[270] [Fig.4.4(A)(c)]. Absorption band starts
at 500 nm and extends to 900 nm with the maximum absorption at 700 nm which is ascribed to the mixed-valence charge transfer of the polymeric complex [Fe(II)-C-N-Fe(III)] confirming the formation of PB on the surface of Fe₃O₄ NPs[216].

**Figure 4.4:** (A) UV- vis absorption spectra of (a) Fe₃O₄ NPs; (b) Fe₃O₄ NPs + K₃[Fe(CN)₆]; (c) PB modified Fe₃O₄ NPs. (B) FTIR spectra of (a) Fe₃O₄ NPs; (b) PB modified Fe₃O₄ NPs.

### 4.3.3. FTIR Studies

Figure 4.4(B) shows the transmittance spectra of iron oxide NPs and PB encapsulated iron oxide NPs. Peak at 618 cm⁻¹ corresponds to the vibrations of Fe-O from Fe₃O₄ NPs[273] [Fig. 4.4(B)(a)]. The peak at 1587 cm⁻¹ corresponding to the H-O-H bending mode and the band at 3400 cm⁻¹ corresponding to the O-H stretching vibrations indicate the presence of hydroxyl groups at the surface of Fe₃O₄ NPs.

In case of PB encapsulated Fe₃O₄ NPs [Fig. 4.4(B)(b)], the peak at 609 cm⁻¹ corresponds to Fe-O vibrations of Fe₃O₄ NPs. The sharp peak at 2082 cm⁻¹ is the characteristic of PB and its analogues and corresponds to C-N stretching vibrations. The peak at 498 cm⁻¹ corresponds to the formation of [Fe(II)-C-N-Fe(III)] complex confirming encapsulation of PB onto Fe₃O₄ NPs[125]. The band at 3323 cm⁻¹ and peak at
1602 cm\(^{-1}\) corresponding to O-H stretching and H-O-H bending modes, respectively, indicates presence of water molecules in the NPs.

**Figure 4.5:** XRD spectra of (a) Fe\(_3\)O\(_4\) NPs; (b) PB modified Fe\(_3\)O\(_4\) NPs; (c) PB-Fe\(_3\)O\(_4\) film on ITO.

### 4.3.4. X-ray Diffraction Studies

Figure 4.5(a) shows XRD pattern from Fe\(_3\)O\(_4\) NPs. The peaks match with those of magnetite (Fe\(_3\)O\(_4\)) suggesting NPs have cubic spinel structure. The peaks obtained at 2\(\theta\) = 30.07°, 35.67°, 43.35°, 52.65°, 57.35°, 62.86° corresponds to (hkl) planes of (220), (311), (400), (422), (511) and (440), respectively (JCPDS file: 890599). Except for the broadening of the peaks, all the position and relative intensity of diffraction peaks are identical with the standard spectrum for bulk magnetite. The average size of NPs has been calculated using Debye Scherrer formula and is found to be \(\sim\) 6 nm from the most intense peak at 2\(\theta\) = 35.50°.

In fig. 4.5(b), the additional peaks at 2\(\theta\) = 24.73°, 39.42°, 50.69°, 65.98°, 68.97° match with those of PB and correspond to (hkl) planes (220), (420), (440), (640) and (642), respectively[274]. The average size of PB encapsulated Fe\(_3\)O\(_4\) NPs (calculated
from the diffraction peak at $2\theta = 35.25^\circ$ is $\sim$10 nm. The increase in average size of NPs from 6 nm to 10 nm confirms capping of Fe$_3$O$_4$ NPs with PB. Figure 4.5(c) shows XRD pattern from the nanocrystalline film of PB encapsulated Fe$_3$O$_4$ NPs onto ITO substrate where peaks from ITO at $2\theta = 32.95^\circ$ and 45.48$^\circ$ along with peaks from PB-Fe$_3$O$_4$ NPs also contribute to the spectrum. The decrease in the broadening of peaks is indicative of the aggregation of NPs while deposition.

![SEM images of (a) PB-Fe$_3$O$_4$ NPs/ITO electrode; (b) ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode.](a) (b)

**4.3.5. SEM Studies**

Figure 4.6 shows the morphological changes of nanocrystalline film of PB-Fe$_3$O$_4$ after enzyme immobilization. SEM micrograph reveals dense packing of PB-Fe$_3$O$_4$ NPs onto the ITO substrate [Fig. 4.6(a)]. However, the irregular size of the NPs reveals agglomeration during deposition. Densely packed globular structure in the nanoscale provides conducive environment for immobilization of the biomolecules. Figure 4.6(b) shows the uniform and complete coverage of ChOx molecules onto the surface of PB-Fe$_3$O$_4$ NPs/ITO electrode. The covered and packed granular structure indicates
immobilization of ChOx molecules onto the nanocrystalline film of PB-Fe$_3$O$_4$ resulting in the formation of ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode.

4.3.6. Electrochemistry and Kinetic Analysis

To investigate the interfacial kinetics, CV studies have been conducted before and after ChOx immobilization on PB-Fe$_3$O$_4$ NPs/ITO electrode as a function of scan rate (10-100 mVs$^{-1}$). The anodic peak potential shows a slight shift towards the positive potential whereas the cathodic potential shows slight shift in the reverse direction with the increasing scan rate suggesting a quasi-reversible redox process. The enhancement in peak current with increasing scan rate is indicative of facile charge transfer of the redox moieties embedded in enzymes with PB-Fe$_3$O$_4$ NPs/ITO electrode. The peak potentials vary linearly with the logarithm of scan rate in accordance with Laviron’s theory [Fig. 4.7(A)] and may be given as:

\[
E_{pa}\text{ (PB-Fe}_3\text{O}_4\text{ NPs/ITO)} = 0.1238 + 0.0248 \ln \nu; \quad R=0.99; \quad SD=0.0023 \quad \ldots(4.1)
\]

\[
E_{pc}\text{ (PB-Fe}_3\text{O}_4\text{ NPs/ITO)} = 0.1506 - 0.0401 \ln \nu; \quad R= -0.99; \quad SD= 0.0031 \quad \ldots(4.2)
\]

\[
E_{pa}\text{ (ChOx/PB-Fe}_3\text{O}_4\text{ NPs/ITO)} = 0.1024 + 0.0271 \ln \nu; \quad R= 0.99; \quad SD= 0.0023 \quad \ldots(4.3)
\]

\[
E_{pc}\text{ (ChOx/PB-Fe}_3\text{O}_4\text{ NPs/ITO)} = 0.1206 - 0.0401 \ln \nu; \quad R= -0.99; \quad SD= 0.0031 \quad \ldots(4.4)
\]

The plot of ln $\nu$ versus anodic peak potential ($E_{pa}$) and cathodic peak potential ($E_{pc}$) yields two straight lines with slopes of $X = RT/(1-\alpha)nF$ and $Y = RT/\alpha nF$, respectively. The values of $\alpha$ and $k_s$ have been found to increase from 0.38 and 17.12 to 0.45 and 45.15 s$^{-1}$, respectively, on immobilization of the enzyme molecules [Table 1]. This shows that the redox centres (FAD) of ChOx molecules are in close proximity of the nanostructured PB-Fe$_3$O$_4$ surface, resulting in facile electron transport across the interface.
Figure 4.7: (A) Anodic current and cathodic current as a function of square root of the scan rate for the PB-Fe$_3$O$_4$ NPs/ITO electrode and ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode. (B) Anodic and cathodic peak potential as a function of logarithm of scan rate for the PB-Fe$_3$O$_4$ NPs/ITO electrode and ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode.

The anodic and cathodic current for the PB-Fe$_3$O$_4$ NPs/ITO electrode and the ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode are proportional to square rate of the scan rate [Fig. 4.7(B)]. This indicates that the ionic transport from the bulk solution to the electrode occurs exclusively by diffusion[115]. Diffusion coefficient (D) is determined by the concentration gradient of ionic species between the bulk and the interface and has been calculated using Randles-Sevcik equation. The electroactive surface area of the electrode has further been obtained by using the calculated diffusion coefficient. The surface concentration of ionic species for the PB-Fe$_3$O$_4$ NPs/ITO electrode has been calculated as $3.68 \times 10^{-13}$ mol cm$^{-2}$ which increases to $1.23 \times 10^{-12}$ mol cm$^{-2}$ for the ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode. The diffusion coefficient increases from $0.23 \times 10^8$ cm$^2$s$^{-1}$ to $0.97 \times 10^8$ cm$^2$s$^{-1}$ on enzyme immobilization. In addition, on immobilization of ChOx molecules onto PB-Fe$_3$O$_4$ NPs/ITO electrode, the electrochemical surface area increases from 0.22 cm$^2$ to 0.31 cm$^2$ [Table 4.1]. The higher
electroactive surface area and higher surface concentration is in accordance with the enhanced redox currents on immobilization of ChOx molecules.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>$\alpha$</th>
<th>$k_s$</th>
<th>$\Gamma$ (mol cm$^{-2}$)</th>
<th>D (cm$^2$s$^{-1}$)</th>
<th>A (cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB-Fe$_3$O$_4$ NPs/ITO</td>
<td>0.38</td>
<td>17.12</td>
<td>$3.68 \times 10^{13}$</td>
<td>$0.23 \times 10^8$</td>
<td>0.22</td>
</tr>
<tr>
<td>ChOx/ PB-Fe$_3$O$_4$ NPs/ITO</td>
<td>0.45</td>
<td>45.15</td>
<td>$1.23 \times 10^{12}$</td>
<td>$0.97 \times 10^8$</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Table 4.1: Values of $\alpha$, $k_s$, $\Gamma$, D and A for the PB-Fe$_3$O$_4$ NPs/ITO electrode and ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode.

The fabrication of the ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode has been investigated electrochemically at each modification step in PBS (pH 6.5) without any redox mediator at scan rate of 50 mV s$^{-1}$. Figure 4.8(A) shows the CV curves for ITO electrode, PB-Fe$_3$O$_4$ NPs/ITO electrode and ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode. The ITO electrode shows an oxidation peak at 0.76 V in slight acidic conditions. The ITO electrode modified with PB-Fe$_3$O$_4$ NPs shows additional set of redox peaks at +0.2 V and +0.1 V corresponding to the transition of PB to prussian white and prussian white to PB, respectively[257]. Thus, PB-Fe$_3$O$_4$ NPs/ ITO electrode acts as a self mediated electrode due to the facile charge propagation inside the film. The immobilization of ChOx molecules onto PB-Fe$_3$O$_4$ NPs/ITO electrode results in enhanced oxidation current due to the direct transfer of electrons between the enzyme and the electrode. The enzyme molecules are attached to the electrode surface in such a way that their redox centres are in close proximity, this may result in direct electron transfer between the enzyme and the electrode.

4.3.7. Cholesterol Detection

To show the potential application of enhanced electrochemical properties of Fe$_3$O$_4$ NPs on incorporation of the electroactive PB, CV response studies of ChOx/PB-Fe$_3$O$_4$
NPs/ITO bioelectrode towards different concentrations of cholesterol have been carried out in PBS at scan rate of 50 mVs⁻¹. Anodic peak current at +0.20 V has been found to increase with the increasing concentrations of cholesterol [Fig. 4.8(B)]. ChOx catalyzes the oxidation of cholesterol (3β-hydroxysteroids) to the intermediate product Δ5-6-ene-3β-ketosteroid (cholest-5-en-3-one) and then isomerization of the intermediate yields Δ3-4-ene-3β-ketosteroid (cholest-4-en-3-one). Key to this conversion is the FAD cofactor, which gets reduced to FADH₂ and H₂O₂ is liberated[275]. The PB-Fe₃O₄ NPs catalyzes the reduction of H₂O₂ resulting in the generation of electrons. The reduced cofactor of ChOx molecules undergoes direct exchange of electrons with the nanostructured PB-Fe₃O₄ electrode to get reoxidized and thus results in increase in peak current with the increasing concentrations of cholesterol.

The ChOx/PB-Fe₃O₄ NPs/ITO bioelectrode exhibits linear increase in current in the range of 10-500 mg dl⁻¹ (0.25-12.5 mM) [Fig. 4.8(C)] and achieves steady-state current in less than 15 s indicating fast and direct electron transfer from enzyme to the electrode. The value of sensitivity has been calculated from the linear region of the calibration plot and is found to be as 53.94 nA mg⁻¹ dl cm⁻² (2.15 mAM⁻¹ cm⁻²) with regression coefficient of 0.99 and standard deviation of 0.65 µA. The detection limit of the bioelectrode (DL = 3*SD/Sensitivity) is calculated to be as 36.78 mg dl⁻¹ (0.92 mM). The high sensitivity, low response time and low detection limit of the fabricated mediator-free cholesterol biosensor reveals the enhanced bioelectrocatalytic activity of the enzyme onto PB-Fe₃O₄ NPs/ITO electrode and facile direct charge transport properties of PB-Fe₃O₄ NPs. The Kₘ^app value has been calculated using Lineweaver Burk plot and is estimated to be as 2.82 mg dl⁻¹ (0.07 mM). The low value of Kₘ^app signifies
the high catalytic efficiency of enzyme onto nanostructured PB-Fe$_3$O$_4$ electrode resulting in better interaction of ChOx with cholesterol.

**Figure 4.8:** (A) Cyclic voltammograms of ITO electrode, PB-Fe$_3$O$_4$ NPs/ITO electrode and ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode in PBS (100 mM) at scan rate of 50 mVs$^{-1}$. (B) Cyclic voltammetric response of ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode for varying concentrations of cholesterol (10-400 mgdl$^{-1}$) in PBS (100 mM) at scan rate of 50 mVs$^{-1}$. (C) Anodic peak current as a function of cholesterol concentration. (D) Current response of ChOx/PB-Fe$_3$O$_4$ NPs/ITO bioelectrode towards cholesterol (100 mgdl$^{-1}$) in presence of different interferents.
The effect of potential interferents on the cholesterol measurements has been investigated by taking the solution containing 1:1 ratio of cholesterol (100 mg dl\(^{-1}\)) and interferents such as glucose (5 mM), urea (1 mM), uric acid (0.1 mM) and ascorbic acid (0.05 mM) [Fig. 4.8(D)]. In the presence of interferents, 1-2% change of current has been observed from the CV response of the ChOx/PB-Fe\(_3\)O\(_4\) NPs/ITO bioelectrode which has been calculated using following equation:

\[
\% \text{inter} = \frac{I_{\text{chol}} - I_{\text{int}}}{I_{\text{chol}}},
\]

where \(I_{\text{chol}}\) and \(I_{\text{int}}\) are the changes in current corresponding to the cholesterol and the 1:1 mixture of cholesterol with interferent. The low detection potential (+0.15 V) of the biosensor circumvents the effect of other analytes present in blood, which may easily get oxidized at higher potential and interfere with signal from the cholesterol. Thus, the proposed biosensor with low detection potential would be an efficient platform for point-of-care diagnostic device.

**Figure 4.9:** Shelf-life curve of ChOx/PB-Fe\(_3\)O\(_4\) NPs/ITO bioelectrode showing reduction in peak current with time.

The reproducibility of the ChOx/PB-Fe\(_3\)O\(_4\) NPs/ITO bioelectrode has also been investigated with cholesterol concentration of 100 mgdl\(^{-1}\). The bioelectrode can be used
upto 15 times without appreciable loss of the signal which is evident by the low relative standard deviation of 3.5%. In addition, the ChOx/PB-Fe₃O₄ NPs/ITO bioelectrode retained 97% of the current response after 9-10 weeks (when stored at 4°C) indicating good stability [Fig. 4.9]. Comparison of the response characteristics of present mediator-free cholesterol biosensor with those reported in literature shows that electroactive prussian blue encapsulated iron oxide nanostructures results in high sensitivity and specificity towards cholesterol detection and low $K_{m}^{\text{app}}$ value shows the high enzymatic activity of ChOx molecules towards cholesterol onto electroactive prussian blue encapsulated iron oxide nanostructures[113, 121, 128, 276, 277] [Table 4.2].

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Sensitivity (AM⁻¹)</th>
<th>Detection Limit (μM)</th>
<th>Linear Range (μM)</th>
<th>$K_{m}^{\text{app}}$ (mM)</th>
<th>Shelf life (Days)</th>
<th>Response time (s)</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Pt/SAM/PB/PPy-ChOx/Nf</td>
<td>8.50×10⁻³</td>
<td>8.00</td>
<td>50-300</td>
<td>0.09</td>
<td>25</td>
<td>---</td>
<td>[113]</td>
</tr>
<tr>
<td>PB Silicic sol-gel/ GCE</td>
<td>0.33</td>
<td>0.12</td>
<td>1-80</td>
<td>---</td>
<td>35</td>
<td>---</td>
<td>[121]</td>
</tr>
<tr>
<td>PANI-CdS/ITO</td>
<td>1.02×10⁻³</td>
<td>1190.00</td>
<td>1250-12500</td>
<td>0.82</td>
<td>---</td>
<td>20</td>
<td>[276]</td>
</tr>
<tr>
<td>PANI-PB/GCE</td>
<td>---</td>
<td>0.18</td>
<td>1-80</td>
<td>0.54</td>
<td>30</td>
<td>20-30</td>
<td>[128]</td>
</tr>
<tr>
<td>PPy-PB/GCE</td>
<td>0.04×10⁻³</td>
<td>0.60</td>
<td>10-100</td>
<td>---</td>
<td>30</td>
<td>30</td>
<td>[277]</td>
</tr>
<tr>
<td>PB-Fe₃O₄ NPs/ ITO</td>
<td>2.15×10⁻³</td>
<td>920.00</td>
<td>620-12500</td>
<td>0.07</td>
<td>65</td>
<td>15</td>
<td>Present work</td>
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</table>

Table 4.2: Comparison table summarizing response characteristics of present mediator free cholesterol biosensors alongwith those reported in literature.

4.4. Conclusions

The Fe₃O₄ NPs with average diameter of 10 nm were encapsulated with four nm thick shell of PB. The capping of PB onto Fe₃O₄ NPs reduces magnetic interaction among Fe₃O₄ NPs and imparts monodispersity and stability to the NPs. The electrophoretically deposited PB-Fe₃O₄ NPs onto the ITO substrate exhibits excellent electrocatalytic activity and enroutes direct electron transfer from the immobilized enzyme molecules to the nanostructured PB-Fe₃O₄ film resulting in the fabrication of mediator-free 3rd-generation cholesterol biosensor. The proposed cholesterol biosensor exhibits low
detection limit and low \( K_{m}^{app} \) value due to facile charge transport through the PB-Fe\(_3\)O\(_4\) film. The low operating potential (+0.15V) of the biosensor circumvents the effect of potential interferents, rendering the biosensor to be highly selective for cholesterol detection. Also, the biosensor shows good reproducibility and stability. However, the sensitivity of the biosensor has been compromised due to the absence of mediator which lowers the electrochemical current. The metal nanostructures such as gold, silver etc are known to exhibit excellent electrocatalytic properties. Thus, it is anticipated that metal nanostructures can perhaps permit the mediator-free detection of cholesterol while improving the sensitivity and other biosensing parameters.