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Non-enzymatic electrochemical cholesterol sensor based on strong host-guest interactions with a polymer of intrinsic microporosity (PIM)

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Abstract

Advances in materials science has accelerated the development of diagnostic tools with the last decade witnessing the development of enzyme-free sensors, owing to the improved stability, low cost, and simple fabrication of component materials. However, the specificity of non-enzymatic sensors for certain analytes still represents a challenging task, for example, the determination of cholesterol level in blood is vital due to its medical relevance. In this work, a reagent displacement assay for cholesterol sensing in serum samples was developed. It is based on coating of a glassy carbon electrode with a polymer of intrinsic micro-porosity (PIM) that forms a host-guest complex with methylene blue (MB). In the presence of cholesterol, the MB electroactive probe was displaced due to the stronger association of cholesterol guest to the PIM host. The decrease in the oxidative current was proportional to the cholesterol concentration achieving a detection limit of approximately 0.1 nM.

Keywords: Cholesterol; polymer of intrinsic microporosity (PIM); methylene blue; electrochemical sensor

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1. Introduction

Cholesterol, a well-known fat-like substrate present in food from animal origin such as milk, meat, cheese, egg and seafood,¹ is an essential precursor for the synthesis of vitamin D, steroid hormones and bile acids.²⁻⁴ The normal level of total cholesterol in the blood is < 4.9 mM (1.89 mg mL⁻¹). Levels higher than 6.2 mM (2.4 mg mL⁻¹) are considered as hypercholesterolemia and can lead to several complications such as coronary heart disease, myocardial infarction and atherosclerosis hypertension. The American Heart Association suggests the amount of cholesterol for adults between 40 and 75 years of age to be between 1.81 - 4.90 mM (0.7 - 1.89 mg mL⁻¹).⁵ Too low cholesterol levels (hypocholesterolemia), on the other hand, result in anaemia and hepatopathy.²

Determination of the exact level of cholesterol in serum remains a major diagnostic challenge. Multiple analytical methods have been developed for cholesterol analysis including approaches such as high-performance liquid chromatography (HPLC)⁶ and mass spectrometry.⁷ Fluorometric⁸ and colorimetric enzymatic assays^{9, 10} are the most commonly employed kits for the analysis of serum cholesterol. Chromatographic and mass spectrometric methods are the most accurate and sensitive ones, however they require costly equipment and extensive sample pre-treatment. Enzymatic assays exhibit the highest sensitivity, but they require the use of costly enzymes. These assays are based on conversion of esterified cholesterol to cholesterol by cholesterol esterase. The resulting total cholesterol is then acted upon by cholesterol oxidase to produce cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide (H₂O₂) production is then detected using colorimetric or fluorescence means. Despite of high specificity of these enzyme-based cholesterol assays, which can be implemented in point-of care testing devices,¹¹ the limited stability of the enzymes and the influence of operating parameters such as temperature and pH on their performance are limitations in practice.

Non-enzymatic sensors with the same detection limits and cholesterol specificity are valuable alternatives, but are still not widely developed.^{5, 12, 13} While cholesterol undergoes direct electrochemical oxidation on platinum electrodes,^{14, 15} this approach seems not well-adapted for direct cholesterol sensing. The use of a displacement indicator assay (IDA), by exploiting surface-confined host-guest interactions, represents an elegant approach for enzyme-free electrochemical cholesterol sensing.¹² Yang et al.¹⁶ have applied this concept on calix[6]arene-modified graphene

electrodes. In the presence of cholesterol, the calix[6]arene-methylene blue (MB) host-guest interaction was displaced in favour of cholesterol, leading to a “switch off” electrochemical response. With a linear range of 0.50 to 50.00 μM and a LOD=0.20 μM , this sensor detects low levels of cholesterol, but cannot distinguish between normal and abnormal cholesterol levels, as this situation is situated at around 6.2 mM. The same team proposed an electrochemical detection of cholesterol based on a competitive host-guest recognition using β -cyclodextrin/poly(*N*-acetylaniline)/graphene-modified electrode.¹² A linear response range of 1.00 to 50.00 μM and a low detection limit of 0.50 μM were achieved. Willyam et al. proposed an electrochemical non-enzymatic cholesterol sensor based on β -cyclodextrin/ Fe_3O_4 nanocomposite as a host layer with good linearity up to 150 μM and an estimated limit of detection of 2.88 μM .¹⁷

In this study, we push the displacement indicator assay concept further by introducing a polymer of intrinsic microporosity (PIM) as the electrode surface receptor. PIMs are a unique class of microporous materials,¹⁸ featuring a continuous network of interconnected intermolecular voids of less than 2 nm in width. Depending on the size and chemical nature of the pores, they may be exploited for selective separation,¹⁹ adsorption²⁰ and/or storage of specific molecules: (i) the pores may host catalytically active species or have catalytic activity themselves,^{25, 26} or (ii) they may host probes and act as sensor materials.²¹ The PIM selected in this work, PIM-1 (see structure in **Figure 1**), has a strong affinity to methylene blue (MB), employed as the electroactive probe in our assay. MB molecules enter into the inner cavity of PIM-1 due to the host-guest interactions, resulting in a pronounced electrochemical oxidation peak of MB. In the presence of cholesterol, the MB molecules are displaced by cholesterol due to the stronger affinity of cholesterol to PIM-1, leading to a “switch off” of the electrochemical response in a concentration-dependent manner.

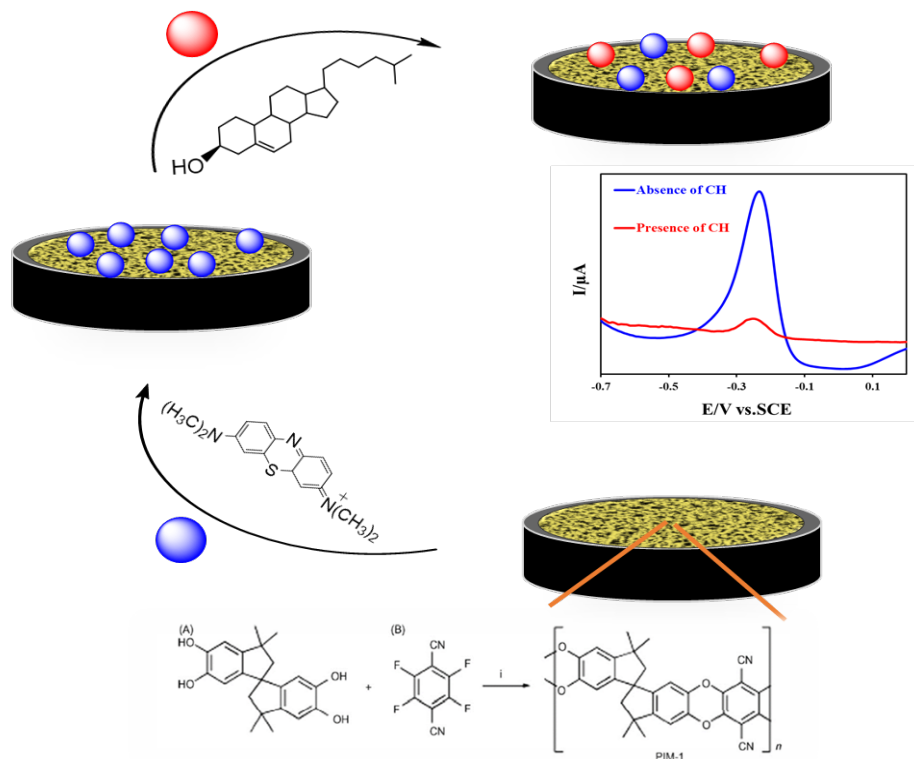


Figure 1. Concept of the cholesterol (CH) sensor based on competitive host-guest molecular recognition of cholesterol by PIM-1 and methylene blue (MB) displacement on a glassy carbon electrode-modified with PIM-1.

2. Materials and Methods

2.1. Chemicals and materials

Methanol, alumina powder, chloroform, sodium chloride, calcium sulphate, potassium acetate, potassium dihydrophosphate, cholesterol, ascorbic acid, uric acid, dopamine, and methylene blue were purchased from Merck Ltd. company. PIM-1 was prepared from 5,5',6,6'-tetrahydroxy-3,3,3',3'-tetramethyl-1,1'-spirobisindane and 2,3,5,6-tetrafluoroterephthalonitrile as described previously.²²²³

2.2. Preparation of PIM-modified glassy carbon electrodes (PIM/GCE)

PIM-1 (5 mg) was dissolved in 5 mL of chloroform to obtain a clear yellow solution. Then 2 μ L of this solution was drop-casted onto a glassy carbon (GC) electrode. The resulting PIM-1/GC electrode was immersed in methylene blue (MB) (1 mM, PBS pH 7.0) for 5 min and used for cholesterol sensing. Since cholesterol is insoluble in water, a stock solution was prepared by

dissolving cholesterol (9.66 mg) in 25 mL of ethanol. Different concentrations of cholesterol were obtained by further dilution in PBS (pH 7.0).

2.3. Instrumentation

Electrochemical measurements were performed on a VA Computrace 797 polarograph (Metrohm, Switzerland) in a three-electrode system including PIM-1/GC working electrode, saturated calomel (SC) reference electrode and platinum auxiliary electrode. A Galvanostat/Potentiostat Micro Autolab III (Metrohm, Switzerland) was used for electrochemical impedance spectroscopic (EIS) measurements. EIS spectra were recorded in the frequency range from 10^{-1} to 10^5 Hz with an amplitude of 5 mV using 5 mM/5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ redox couple (1:1) in 0.1 M KCl as supporting electrolyte. Cyclic voltammograms (CV) were acquired in -0.6 to 0.2 V at a scan rate of 0.1 Vs^{-1} . All measurements were performed at room temperature (approx. $18 \pm 2 \text{ }^\circ\text{C}$).

2.4. Determination of cholesterol in serum samples

The human serum sample was prepared by dissolving 1 mL of human serum in 5 mL of n-hexane and isopropanol (v/v, 2:1). The solution was vortexed for 15 s using a shaker and centrifuged for 5 min at 4,000 rpm; the organic phase was collected in another test tube. This procedure was repeated three times. The collected organic phases were assembled and dissolved in 1 mL of isopropanol. The solution was diluted 10 and 1,000 times with 10 mM phosphate buffer (pH 7.0) and used for electrochemical analyses.²⁴

2.5. Theoretical studies

Geometry optimizations of cholesterol (CH), methylene blue (MB) and polymer of intrinsic microporosity (PIM-1) were performed by using the dispersion including density functional theory with the M06-2X functional associated with the standard 6-311+G** basis set. This hybrid meta exchange-correlation functional was suitable for modeling non-covalent interactions.^{25, 26} The M06-2X/6-311+G** method is suitable for most calculations on medium to large size molecules to deliver reliable and accurate results at minimum cost for a variety of compounds.²⁷ Furthermore, to qualify the interactions between the PIM and cholesterol/methylene blue in the optimized geometries, the binding energy (BE) is evaluated using Eq. 1.

$$BE = E_{\text{PIM/CH or MB}} - (E_{\text{isolated CH or MB}} + E_{\text{isolated PIM}}) + E_{\text{BSSE}} \quad (1)$$

Here $E_{\text{PIM/CH or MB}}$ is the energy of the complex between PIM-1 and cholesterol or PIM-1 and methylene blue, $(E_{\text{isolated CH or MB}} + E_{\text{isolated PIM}})$ is the sum of energies of free polymer and reactant molecule including cholesterol or methylene blue, and E_{BSSE} is the basis set superposition error (BSSE) correction to eliminate the effect of basis set incompleteness by employing Boys–Bernardi counterpoise (CP) technique.²⁸ The complex with the lowest binding energy was considered as the best one. To estimate the zero-point vibrational energies as well as their corresponding thermochemical functions, frequency calculations were performed at the same level of calculations at 298.15 K and 1.0 atmosphere pressure. All reported enthalpies were zero-point (ZPE) corrected with unscaled frequencies.

In addition, the energy gaps (ΔE_g) between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) of cholesterol, PIM-1 and methylene blue were evaluated. Besides, the solvent effect was investigated using the polarized continuum model (PCM)^{29,30} which is a suitable approach to investigate solute-solvent interactions and is usually used in theoretical studies³¹⁻³³ for water solvent with $\epsilon=78.30$. Additionally, to understand the most suitable atom in cholesterol and methylene blue molecules to interact with the polymer, natural bond orbitals (NBO) calculation was carried out. Finally, the solvation energy, $\Delta E_{\text{solvation}}$, defined as the difference between the optimized energies in water and gaseous phases was assessed for the studied compounds, eq.2.

$$\Delta E_{\text{solvation}} = E_{\text{sol}} - E_{\text{gas}} \quad (2)$$

All of the calculations were carried out using the Gaussian 09 program package [10].

3. Results and discussion

3.1. Fabrication of PIM-1 modified glassy carbon electrodes for methylene blue incorporation

Figure 2A depicts the SEM image of the glassy carbon electrode (GCE) modified with PIM-1, revealing a homogenous PIM-1 coating. From the N_2 adsorption-desorption isotherm measurements and Brunauer–Emmett–Teller (BET) calculations (**Figure 2B**), it was found that

PIM-1 exhibits a surface area of $753 \text{ m}^2 \text{ g}^{-1}$ with a pore volume of $0.42 \text{ cm}^3 \text{ g}^{-1}$ and a pore diameter of about 2 nm, in good agreement with literature data.³⁴ **Figure 2C** displays the charge transfer characteristics of the PIM-1/GCE in ferrocenemethanol (1 mM)/KCl (100 mM). While a fully reversible voltammogram was observed on a bare GCE, the charge transfer characteristic was affected by the surface modification due to the insulating, but porous nature of PIM-1.

The applicability of PIM-1/GCE in a competitive binding assay was further investigated upon immersion of PIM-1/GCE into 1 mM methylene blue (MB)/ PBS (100 mM, pH 7.0) for 5 min and thorough rinsing with water. MB, a cationic dye with formal potential between 0.08 and -0.25 V (vs. SCE) in solution with pH 2.0-8.0, is widely used as electrochemical indicator of oligonucleotide strands within a DNA biosensor, but also used as a guest molecule.³⁵ **Figure 2D** displays the cyclic voltammogram of the MB-modified PIM-1/GCE showing the oxidation of the positively charged MB in a $2e^-/H^+$ process into uncharged *leuco*-methylene blue (**Figure 2E**). A surface concentration of $7.5 \times 10^{-11} \text{ mol cm}^{-2}$ of bound MB groups was derived from the integration of the peak area using the following equation:

$$\Gamma = Q/nFA$$

Here, Q is the passed charge, n corresponds to the number of exchanged electrons per molecule (n=2), F is the Faraday constant, and A is the electroactive surface of the electrode (0.11 cm^2) (**Figure S1**).

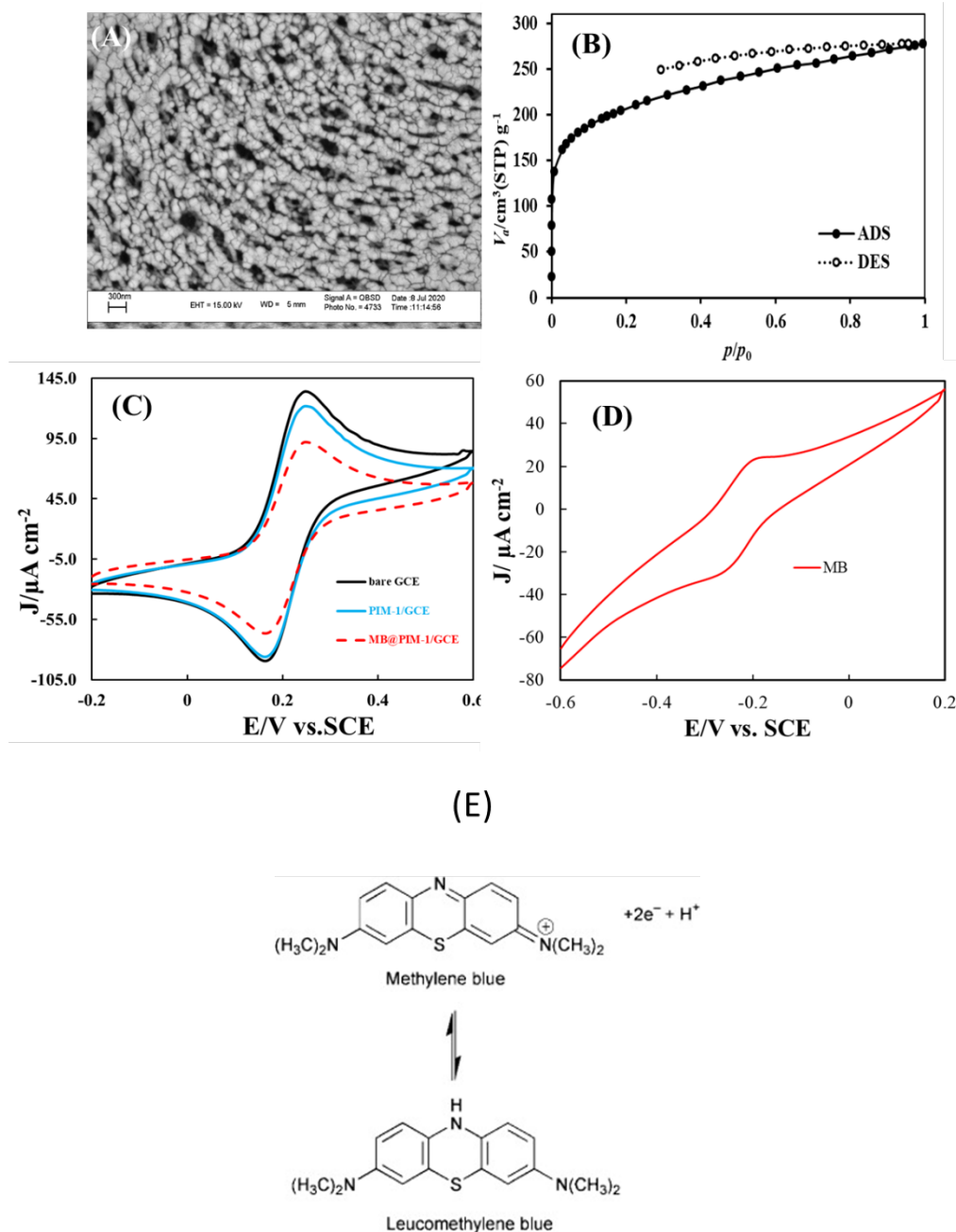


Figure 2. Characterization of the PIM-modified glassy carbon electrode. (A) SEM image of PIM-coated GCE. (B) N₂-adsorption-desorption curves of PIM-1 powder. (C) CVs of GCE, PIM-1/GCE and MB@PIM-1/GCE in ferrocenemethanol (1 mM)/ KCl (10 mM). (D) CV of PIM-1/GCE in PBS (100 mM, pH 7.0) after immersion for 5 min in MB (1.0 mM) followed by washing, scan rate = 0.1 V/s. (E) Process of the electrochemical oxidation of MB⁺ into leuco-methylene blue.

3.2. Cholesterol interaction with PIM-1/GCE

The MB-modified PIM-1/GCE was immersed into an aqueous solution of cholesterol (5.0 μ M) for 20 min. Differential pulse voltammograms (DPVs) were recorded before and after exposure to

cholesterol (**Figure 3A**). A decrease of the MB oxidation peak is in line with the replacement of MB^+ with cholesterol molecules.

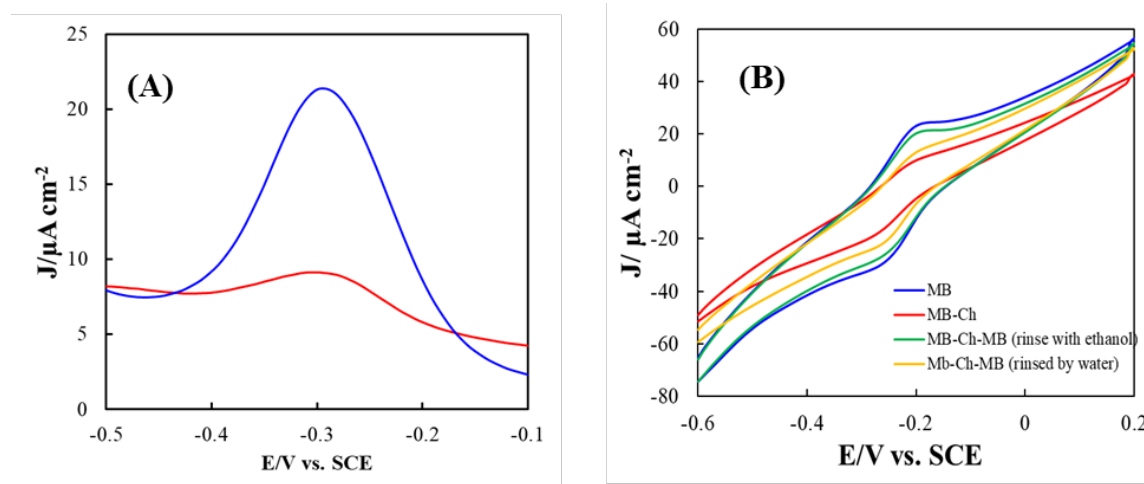


Figure 3. (A) Differential pulse voltammograms of MB-modified PIM-1/GCE in PBS (100 mM, pH 7.0) before (blue) and after (red) immersion of the electrode for 20 min in cholesterol (5.0 μM) followed by washing. (B) CVs of MB-modified PIM-1/GCE in PBS (100 mM, pH 7.0), before (blue) and after (red) immersion for 20 min in cholesterol (5.0 μM) followed by washing with water (yellow), ethanol (green) and re-immersion in MB (1.0 mM), scan rate = 0.1 V/s.

From **Figure 3C**, it seems that the stronger affinity of cholesterol than MB to PIM-1 results in a replacement so that the electrochemical signal is not completely recovered after water rinsing. Ethanol rinsing results in more recovery of the MB loading. The coverages after rinsing with ethanol and water were $6.1 \times 10^{-11} \text{ mol cm}^{-2}$ and $2.1 \times 10^{-11} \text{ mol cm}^{-2}$, respectively.

Figure 4A shows that pH is not influencing strongly the assay and pH 7.0 was used for further studies. The time-dependent replacement of cholesterol with MB is seen in **Figure 4B**. The difference current density before and after replacement (ΔJ) increased by raising adsorption time of cholesterol until 20 min and after that is constant. So, adsorption time of 20 min was chosen for further studies. This graph also confirmed that there is competition and equilibrium between both cholesterol and MB which is discussed by DFT calculations.

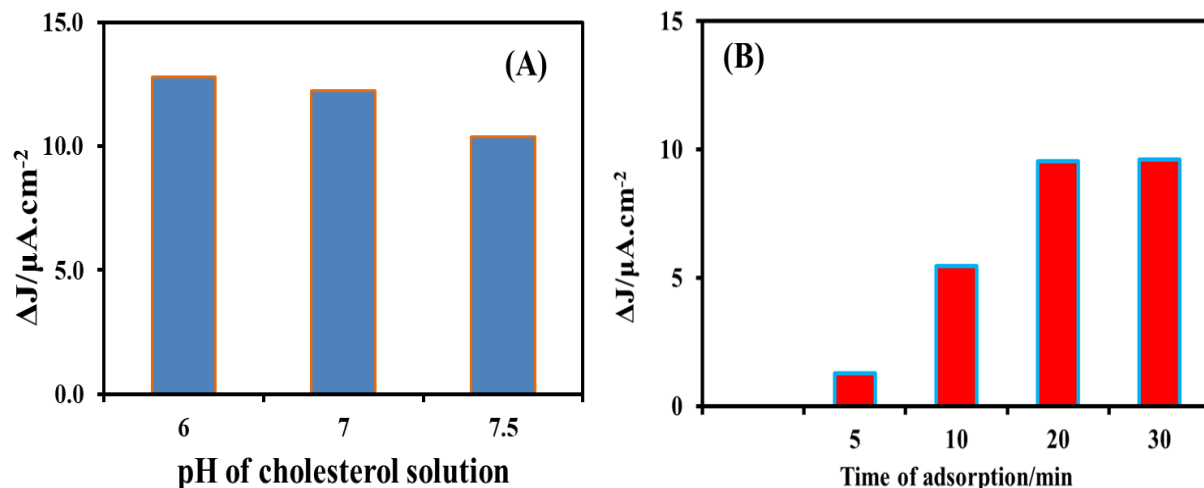


Figure 4. Cholesterol replacement assay: Dependency of replacement on (A) pH of the cholesterol solution and (B) adsorption time.

3.3 Theoretical studies of cholesterol- PIM-1 and MB- PIM-1 interactions

The structures of cholesterol, methylene blue and model of polymer according to **Figure S2** were fully optimized at M06-2X/6-311+G** method. The fully optimized geometries in gas phase were re-optimized by considering the solvent effect using PCM method in water solvent. The calculated results indicate that cholesterol, methylene blue and polymer model are stabilized at about 4.23, 7.56 and 17.67 kcal/mol in water solvent, respectively. **Figure S2** shows the optimized structures of the above-mentioned compounds as well as some structural details in water solvent. Calculation of vibrational frequencies has confirmed stationary point with no negative eigenvalue observed in the force constant matrix. HOMO-LUMO analysis of cholesterol, methylene blue and PIM-1 and molecular electrostatic potential (MEP) and NBO analysis are presented in Supporting Information (**Figure S3-S7**).

According to experimental results, the cholesterol or methylene blue as a reactant is bound to polymer by hydrogen bond and different [PIM/CH] or [PIM/MB)] complexes can be formed. To quantify the interaction between cholesterol, methylene blue and polymer model in the optimized structures, were then approached in order to have the spatial interactions and the binding energies (BE) are evaluated using equation 1 for different complexes.

In order to study the interaction between cholesterol and polymer, the hydroxyl group of cholesterol approached to the polymer molecule from six different directions (including O1, O2,

O3, O4, N1 and N2 atoms), as shown in **Figure S2**. The optimized structures of six different complexes between cholesterol and polymer as well as BE and relative energy according to kcal/mol rather than the most stable complex are displayed in **Figure 5**. Energy comparison of various complexes between polymer and cholesterol reveals that the most stable complex with BE equal -15.78 kcal/mol is [PIMO2/CH], O2 refers to the polymer's atom from which the cholesterol approaches it, two hydrogen bonds with 1.85 and 2.01 Å are formed between hydroxyl hydrogen of cholesterol and hydroxyl oxygen of polymer (O2) and the second one is formed between hydroxyl oxygen of cholesterol and hydroxyl hydrogen of polymer. The increase of O5-H5 bond distance from 0.96 Å in cholesterol to 1.12 Å as well as the bond angle between O5-H5-O2 (175°04) in [PIMO2/CH] complex indicate intermolecular hydrogen bonding between cholesterol and polymer model.

A similar procedure was performed for the interaction between methylene blue and polymer model. Since the nitrogen atoms (N3 and N4 or N5) of methylene blue approached to the polymer molecule from two different directions including H1 and H2 atoms of hydroxyl group to form hydrogen bond. The optimized structures of four different complexes between methylene blue and polymer as well as BE and relative energy of rather the most stable complex are shown in **Figure 6**. It is noticeable that the complexation between polymer model and methylene blue from N4 and N5 atoms are equal. In the most stable complex with BE equal -6.53 kcal/mol ([PLMO1/N3MB], O1 and N3 refer to the polymer's and methylene blue atom, respectively), one hydrogen bond with 2.00 Å is formed between hydroxyl hydrogen of polymer (O1H) and nitrogen atom of methylene blue (N3). Due to the space hinder around N4 or N5 atoms, despite the more negative charge value (-0.369) than N3 (-0.347), the binding energy to form two [PIMO1/N4MB] and [PIMO2/N4MB] complexes is lower than BE from N3 direction i.e. [PIMO1/N3MB] and [PIMO2/N3MB] complexes. So according to our calculations, methylene blue is anchored to the hydroxide ion (H1) by moderate hydrogen bond of 2.00 Å involving nitrogen atom (N3.....H1O1-polymer). The increase of O1-H1 bond distance from 0.97 Å in polymer model to 1.01 Å as well as the bond angle between N3-H1-O1 (170°24) in [PIMO1/N3MB] complex indicate the existence of intermolecular hydrogen bonding between methylene blue and polymer model. **Figure 6** illustrates the different complexes between polymer model and methylene blue as well as BE and relative energy of the most stable complex. From the comparison of the binding energies of the different

complexes between cholesterol and methylene blue, it can be concluded that the polymer has a higher tendency to interact with cholesterol (more negative BE) than methylene blue. Therefore, in the presence of both reactants, a competition is created for binding to the polymer and cholesterol is expected to be favoured.

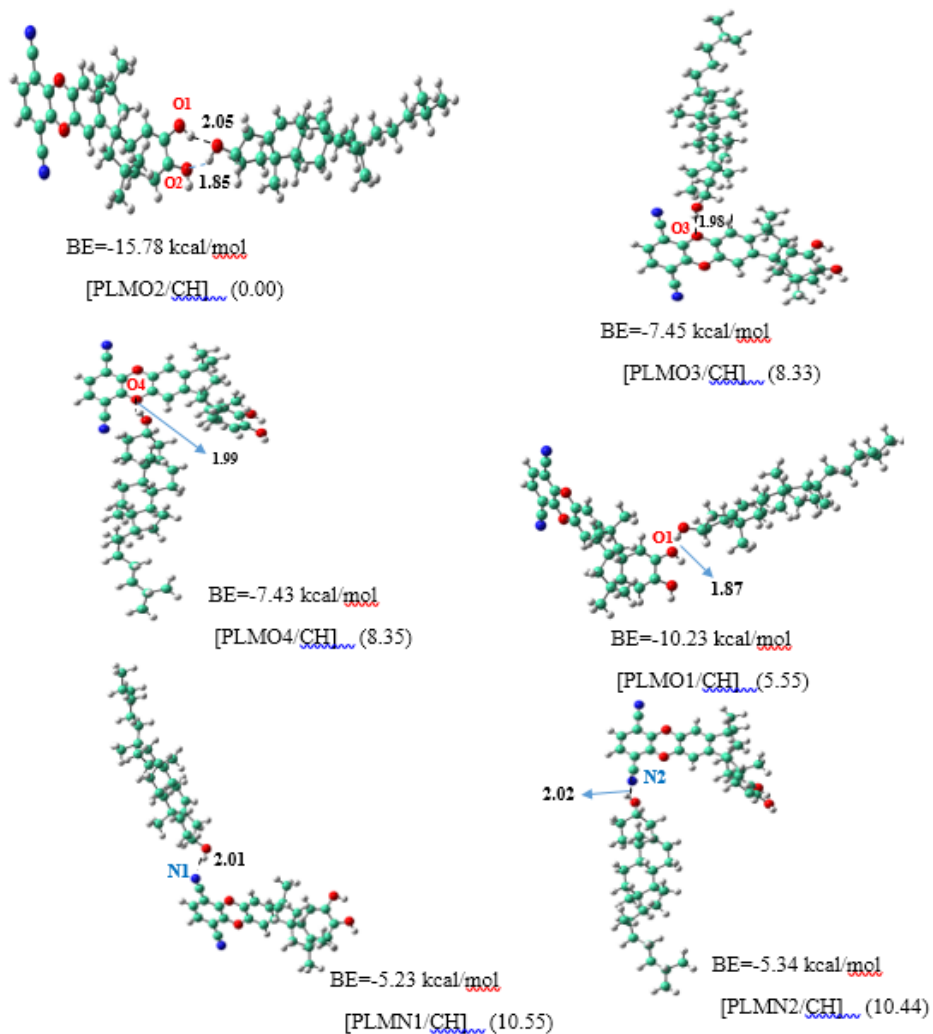


Figure 5. Optimized molecular structures of different complexes from different positions (O1, O2, O3, O4, N1 and N2) between cholesterol and polymer model, BE and relative energies (in the parenthesis) are expressed in kcal/mol and H-bond distances in Å.

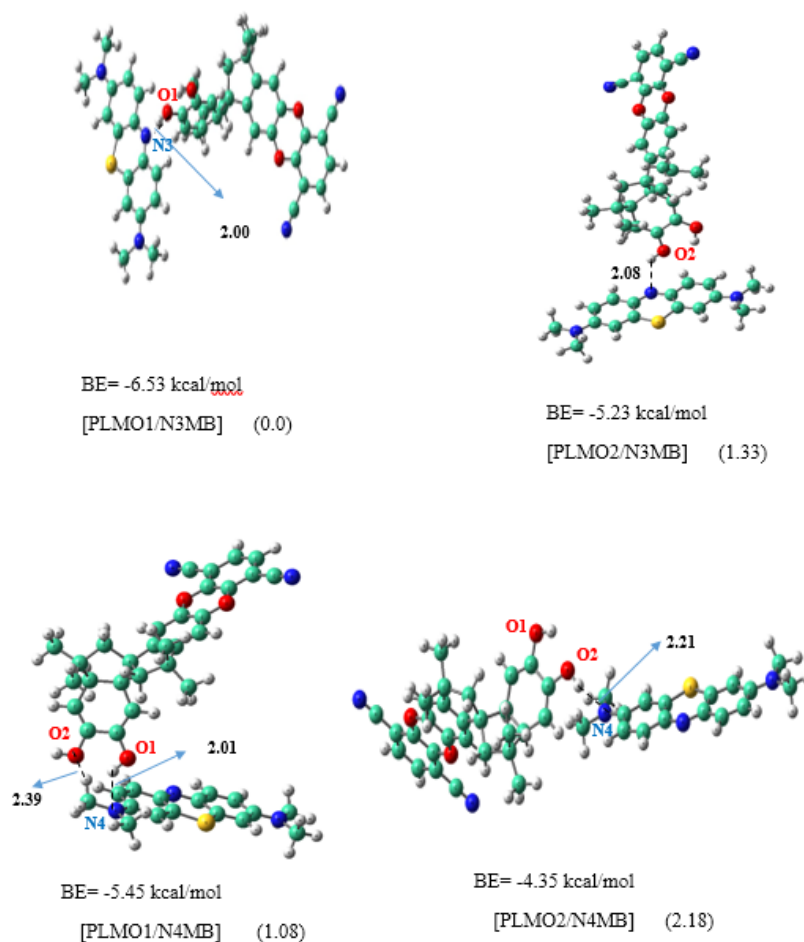


Figure 6. Optimized molecular structures of [PIM/MB] complex between methylene blue and polymer model, BE and relative energies (in the parenthesis) are expressed in kcal/mol and H-bond distances Å.

3.3 Analytical Results

Differential pulse voltammetry (DPV) was applied for quantitative cholesterol determination (**Figure 7A**). The oxidation peak current decreased upon increasing the concentration of cholesterol (**Figure 7B**). The dynamic linear range was 1.0×10^{-9} - 5.0×10^{-8} M and detection limit was determined as 0.1 nM under the optimum conditions (pH of cholesterol solution: 7.0, adsorption time: 20 min and MB concentration = 1 mM), with a sensitivity $J (\mu\text{A}) = 9.0137 + (-75.36) \times [\text{cholesterol}/\mu\text{M}]$ ($R^2=0.9966$).

The reproducibility of this sensor was studied at 1.0×10^{-9} M cholesterol and showed to be 2.72 %. The applicability of MB@PIM-1/GCE for the determination of cholesterol in real samples such as human serum was studied by using the standard addition method. According to the obtained results, $J (\mu\text{A}) = 6.20 + (-73.91)x [\text{cholesterol}/\mu\text{M}]$ ($R^2=0.9801$), a recovery of 101.97 % was recorded.

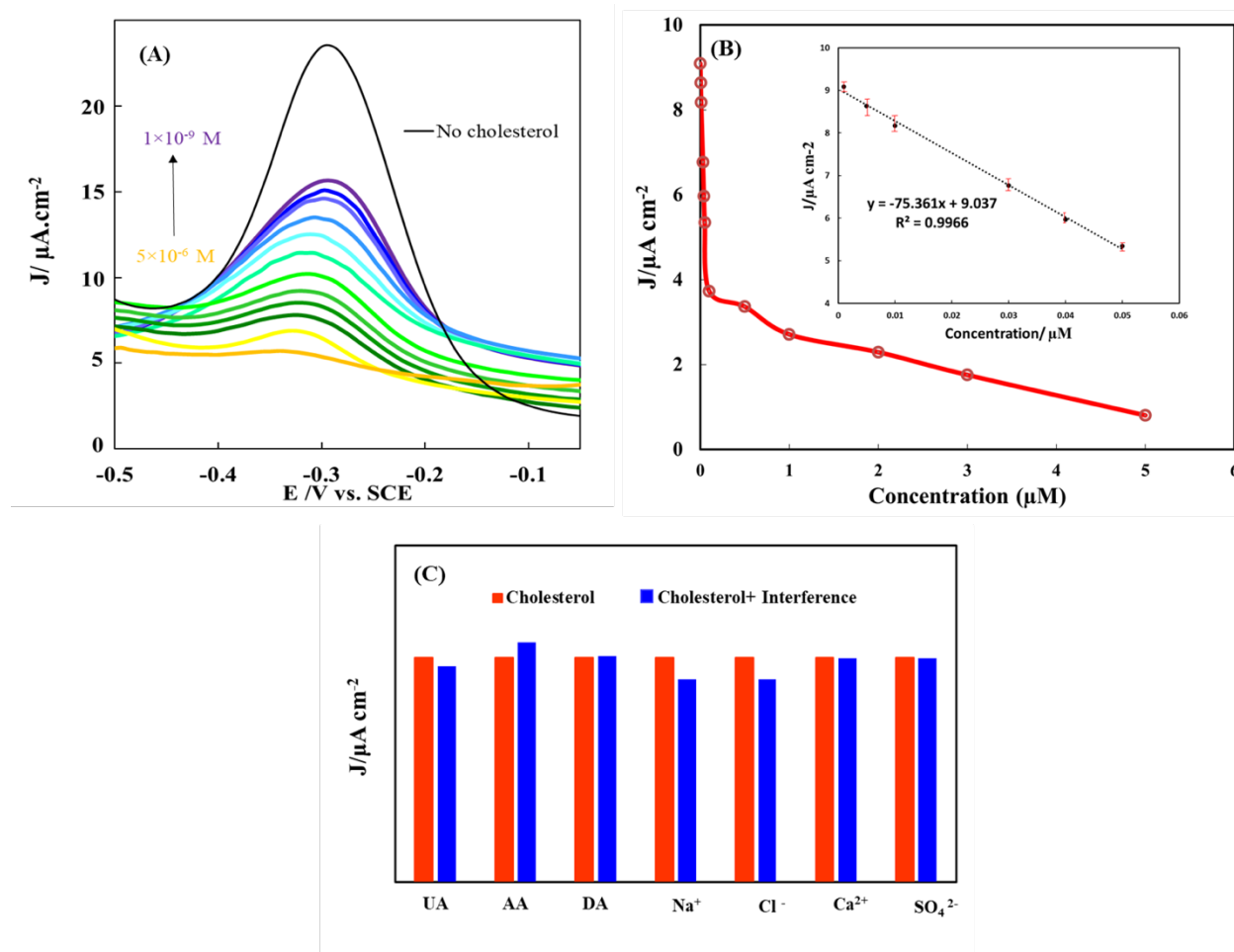


Figure 7. (A) Differential pulse voltammograms of MB-modified PIM-1/GCE acquired at different concentrations of cholesterol in the range of 1.0×10^{-9} to 5.0×10^{-6} M in PBS (100 mM, pH 7.0), (B) Corresponding calibration curve of cholesterol, (C) Interference study.

As the most of cholesterol sensors are enzyme-based sensors, presenting a non-enzymatic cholesterol sensor is valuable. **Table 1** compares the sensing performance of the developed PIM-1/GC electrode with that reported for cholesterol non-enzymatic sensors. The developed sensor

exhibits good selectivity, low detection limit, cost-effective, and easier to use compared to some of literature reports.

Possible interferences on cholesterol determination by the presence of ions (Na^+ , Ca^{2+} , Cl^- and SO_4^{2-}) and biological compounds (Ascorbic acid: AA, Uric acid: UA and dopamine: DA) were investigated (**Figure 7C**). To study the interference of interferences, the interfering specie was spiked to the solution containing cholesterol under optimum conditions (pH of cholesterol solution: 7.0, adsorption time: 20 min and MB concentration = 1 mM). It seems there is no significant interference.

Table 1. Comparison of recent literature data on cholesterol determination with the present work.

Technique	Modifier	LOD/M	DLR	Real sample	Ref
CV	PANI/MWCNTs/Starch	0.01×10^{-3}	$0.032-5 \times 10^{-3}$	cow milk	³⁶
Amperometry	Cu_2O NPs/TNTs	0.05×10^{-6}	$24.4-622 \times 10^{-6}$	Human blood	³⁷
DPV	PVIM-(Co5POM)-MNC composite	1×10^{-15}	$0.5 \times 10^{-5} \times 10^{-3}$ $1 \times 10^{-15} - 200 \times 10^{-9}$	human blood	³⁸
DPV	Composite: Ni-Cubimetal NP/CNF/ACF/PMO (poly methyl orange)	0.002 mg dL ⁻¹	0.04-600 mg dL ⁻¹	human blood	³⁹
DPV	Gr N-doped/ β -CD/FC (in electrolyte)	80×10^{-9}	$0.5 - 100 \times 10^{-6}$	human serum	⁴⁰
DPV, CV	Ru-Pi/ppy	0.54×10^{-10}	$0.16-20.0 \times 10^{-9}$	Human blood	⁴¹
This work	GCE/PIM-1	1×10^{-9}	$1.0 \times 10^{-9} - 5.0 \times 10^{-8}$	Human Serum	

4. Conclusion

A novel sensor for rapid cholesterol detection was prepared by drop-casting PIM-1 at the surface of glassy carbon electrode and using the strong host-guest interaction between PIM-1 and cholesterol as the basis of a methylene blue/displacement assay of as a probe. The fabricated electrochemical sensor exhibited an excellent electrochemical response towards cholesterol. Moreover, the developed electrochemical sensor does not require any antibody or enzyme in the recognition process and still can detect cholesterol efficiently in the nanomolar range with prominent selectivity in the presence of interfering species. Good performance along with simple fabrication and low cost make the developed sensor very promising for medical diagnostics and food industry.

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