Research Article

Development of an Electrochemical Biosensor Based on Interaction of Midazolam with Polymer Supported BLM

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Abstract

This paper describes the formation of bilayer lipid membrane (BLM) on polymer support and interaction of midazolam with polymer supported BLM. Aniline was electrochemically polymerized on gold surface, chloroform solution of phospholipid was applied on the dried polymer surface and immediately immersed in 0.1 M NaCl bath solution. The phospholipid molecules self assembled into BLM. The formation of BLM on the polyaniline surface was confirmed by electrochemical impedance spectroscopy (EIS) and cyclic voltammetric methods using 0.1 M NaCl bath solution containing 1:1 ratio 1 mM ferrocyanide and ferricyanide as marking ions. Midazolam concentration induced changes on the electrical properties of BLM was studied and an electrochemical biosensor was developed.

Keywords: Bilayer lipid membranes; Phospholipid molecules; Self assembly; Electropolymerization;Electrochemical Impedance Spectroscopy

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Introduction

Biomembranes are important components of all living organisms forming a boundary between the cells and the cell organelles [1]. The phospholipid molecules, which are *amphiphilic* in nature, are the major components of biomembranes. In biological membranes two phospholipid monolayers are oriented in such way that the two hydrocarbon tails point towards each other and form the core of the biomembranes, while the polar heads turned towards the aqueous solutions on either sides,forming electrified interfaces [2]. The resulting lipid bilayer is a matrix that incorporates different proteins and glycans performing a variety of functions [2].

Biomembranes also form a highly selective barrier between the inside and the outside of the living cells [3]. They are insulators for inorganic ions and maintain a large electrochemical potential across them [4]. The chemical nature of the membrane components and the events that occur at the interface or within the bilayer decide the permeability and structural properties of the biological membranes [5]. For example, biomembranes provide the environmental matrix for proteins that specifically transport certain ions and molecules, receptor proteins and signal transduction molecules. For the last three decades functionalization of interfaces with mimics of biological membranes has been an ongoing effort. Bilayer lipid membranes (BLMs) have been widely accepted as model biological membranes [6].

The model-membrane systems have garnered much attention because they provide a useful and interesting interface between the biological world and man-made materials [7] and thus, have great potential for basic membrane and cell-biology research as well as a variety of biotechnological and biomedical applications.

To study the function of biological membranes, bionic membranes of lipid bilayers are usually used in addition to native membranes [6]. Black lipid membrane (BLM), as an ideal biomimetic model for complex biological membranes, provides a platform for investigating the function of selected protein-embedding biomembrane such as ion channels, ion pumps, and receptors [8]. Traditional suspended BLMs are formed across micrometer sized apertures in Teflon or other plastic septa by using the painting or folding methods. The BLMs allow convenient investigations of the properties of membranes in different surroundings via change of experimental conditions such as

concentration and constituent of solutions [8]. However, one problem encountered with such a configuration is the short-term lifetimes of the BLMs.

Research works to form stable membranes were started before two decades and resulted in supported bilayer lipid membranes on freshly formed hydrophilic surface on metals such as Pt,Au,Ag,Cu,Ni,stainless steel and glassy carbon electrode, which is abbreviated as s-BLM and found wide potential applications in electrochemical biosensors [9]. But its resistance is smaller and specific capacitance is 20-50 times higher than that of a conventional bilayer lipid membranes [9]. A thin, lubricating water layer between the support and the bilayer made the lipids to preserve their lateral mobility, but integral proteins with large hydrophilic domains would interact with the substrate and become immobile, and possibly even denatured. Furthermore, due to the lack of a sufficiently large aqueous compartment on the substrate side of the bilayer, most s-BLM configurations preclude ion translocation. To overcome these drawbacks without compromising the stability provided by the solid support, polymer-supported BLMs were introduced. psBLMs have been formed by separating the lipid bilayer from a solid support by a thin polymer cushion.

Transport phenomena across biomembranes are important processes in cellular biology and they also become significantly important in many medical, pharmaceutical and environmental technologies. For example, permeation of drug is crucial for the effective delivery to the intracellular targets. This is the basis for the technology of liposomal transport systems. The pharmacological actions of many classes of drugs are brought about by their binding to 'specific sites' in membrane bound proteins. However, some liphophilic drugs also exhibit non-specific interaction with the membrane lipid architecture and their concentration in the membrane phase might reach one order greater than that in surrounding medium. The phospholipid and protein molecules of biomembranes are sensitive to the presence of such lipophilic perturbants [10]. For example, Anesthetics, which readily partition into the biomembranes, alter their electrical properties and permeability. The various changes observed in the electrical properties of biomembranes are concentration dependent, usually very rapid and reversible, and frequently voltage-dependent [11].

The understanding of permeation of drugs, drug-like molecules through lipid bilayers and changes brought about by these molecules in the properties of BLM have become an attractive topic in recent drug research. Drug molecules in solution typically form various species due to ionization, complexation, dissociation, ring opening and closing etc. These different forms interact with the bilayer membrane systems in different ways and exhibit different effects on the electrical properties of membrane.

Midazolam (MDZ), a tricyclic benzodiazepine used in anesthetic practice, differs from most of the "traditional benzodiazepines" in having a nitrogen atom in its additional ring structure. This nitrogen is not sufficiently basic to be protonated at physiological pH, but is basic enough to give water soluble salts when treated with strong acids. Its hydrochloride salt ($MDZH^+Cl^-$) displays an important pH dependent ring opening reaction. Only few studies had been made on interaction of benzodiazepines with model membranes and little work has been done on MDZ-BLM interaction. Studies with supported BLMs on non specific interaction of drug molecules are almost scanty.

The long term interaction and changes in the properties of BLMs at the higher MDZ concentrations cannot be studied using conventional lipid bilayer systems due to their short life time. The purpose of this work is to study the interaction between midazolam and polymer supported bilayer lipid membranes systems using electrochemical impedance spectroscopy at various pH and to develop an electrochemical sensor using ps-BLM for detecting MDZ.

Experimental

Midazolam in its hydrochloride form ($MDZH^+Cl^-$), obtained from Neon Laboratories Ltd, India, was used for the studies. A stock solution of egg lecithin containing greater than or equal to 99% L-α-Phosphatidylcholine (procured from Sigma Aldrich) was prepared by dissolving (5mg/mL) in chloroform. Aniline (SRL, India) was distilled under reduced pressure before use. H_2SO_4(Qualigens, India) was used as such without further purification. All the aqueous solutions used in our studies were prepared using Miili-Q water ($R>18M\Omega\ cm^{-1}$). 0.1 M aniline was taken in 0.5 M H_2SO_4 to deposit polyaniline electrochemically on Au electrode surface. The electrochemical studies were carried out using GAMRY REFERENCE 3000, with a three electrodes set up, to deposit polyaniline on Au surface, Au electrode served as working electrode, a Pt foil served as counter electrode while Ag/AgCl electrode served as reference electrode. The electro polymerization of aniline on Au surface was carried out by cyclic voltammetry by scanning potential in the range-0.3 to 1.4 V. After electropolymerization the deposit was washed well with double distilled water and dried under nitrogen atmosphere. 3 µL of the lipid solution was applied on the polymer deposit and immediately immersed into the 0.1M NaCl solution for 30 minutes, where the phospholipid molecules undergo self assembly to form a BLM. Stirring device, vibration isolated platform, faraday cage and Ag/AgCl electrodes were

fabricated following standard procedures [12-16]. In other electrochemical studies BLM formed on the polyaniline (deposited on Au surface) served as working electrode keeping other electrodes same. To study the interaction between MDZ and ps-BLM, after every drug dose, a stabilization period of 30 minutes was allowed for the drug to equilibrate between the two phases. The electrochemical impedance spectra of the bare and drug doped ps-BLMs were recorded in the frequency range 1 MHz to 10 mHz at the open circuit potential by superimposing a sinusoidal AC signal of small amplitude 25 mV. Analysis of the impedance data was done using Gamryechem Analysis software. The cyclic voltammograms of bare and drug doped ps-BLMs were recorded in the potential range 0.0 to 2.0 V at a scan rate of 100 mV/s.

Results and Discussions

Electropolymerization of aniline on Au electrode

Aniline was electrochemically polymerized on Au electrode by cyclic voltammetry. The potentials were swept at the scan rate of 100 mV/s between 1.3 V and -0.3 V. **Figure 1** shows the electrochemical polymerization of aniline on Au electrode. B_1 B_2 B_3

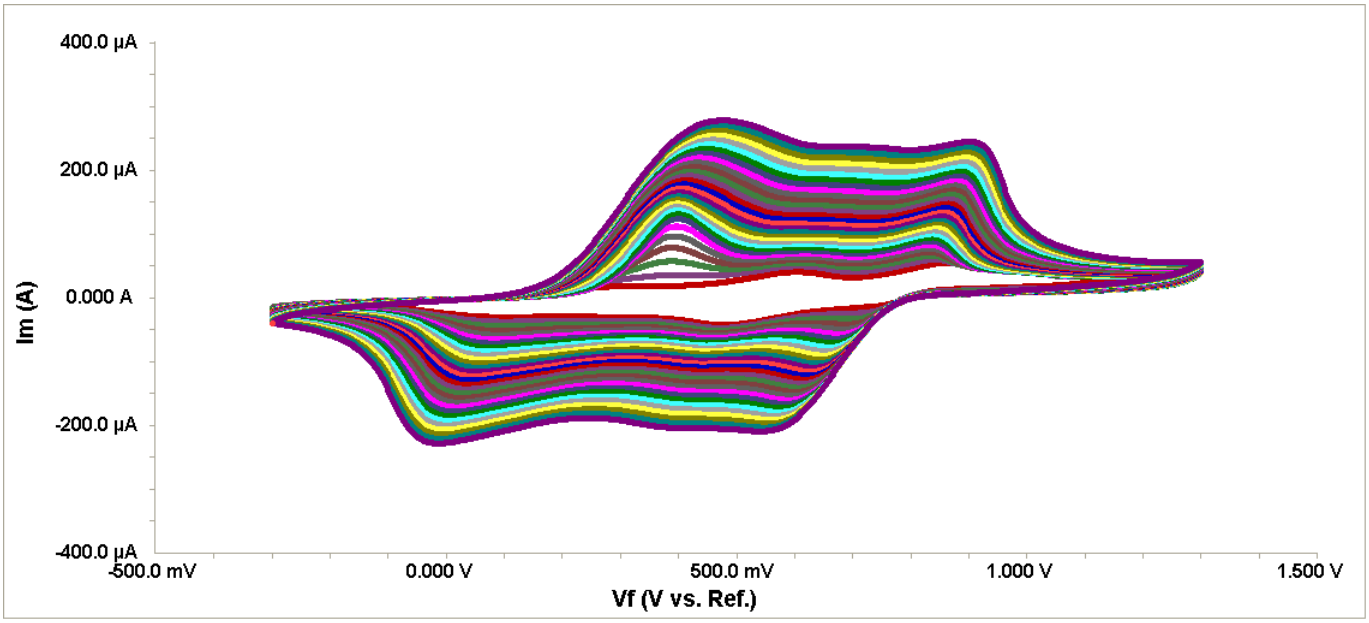

Figure 1 Electropolymerization of aniline on Au Electrode by Cyclic Voltammetry

Various peaks observed during electropolymerization of aniline are shown in **Figure 2**. Three peaks A_1 A_2 and A_3 were observed during positive scanning (oxidation). The first peak A_1 corresponds to leucoemeraldine (le)−emeraldine (em) conversion [17]. The third peak A_3 is related to formation of diradical cation [18]. The peaks B_1 and B_3 observed in the negative scanning are coupled reductive peaks of A_3 and A_1 respectively [18]. The peaks A_2 and B_2 correspond to ortho coupled polymers formed under certain experimental conditions [19] and degradation of polyaniline formed to give soluble species such as benzoquinone (BQ) and hydroquinone (HQ), and/or insoluble fragments containing quinonic functional groups at their ends [20].

Electrochemical Impedance Studies on Formation of BLM on polyaniline surface and its interaction with MDZ

The spontaneous self assembly of phospholipid molecules onto the polyaniline surface, when aniline coated Au surface is immersed in 0.1 M NaCl solution after applying 3 µL of chloroform stock solution of egg lecithin, was monitored by Electrochemical impedance spectroscopy. Electrochemical impedance spectroscopy is a powerful technique to analyze the heterogeneous systems or electrified interfaces interms of an equivalent circuit [21]. The Nyquist plot obtained for BLM formed on polyaniline coated Au electrode is shown in **Figure 3a**. The corresponding bode plot is shown in **Figure 3b**.

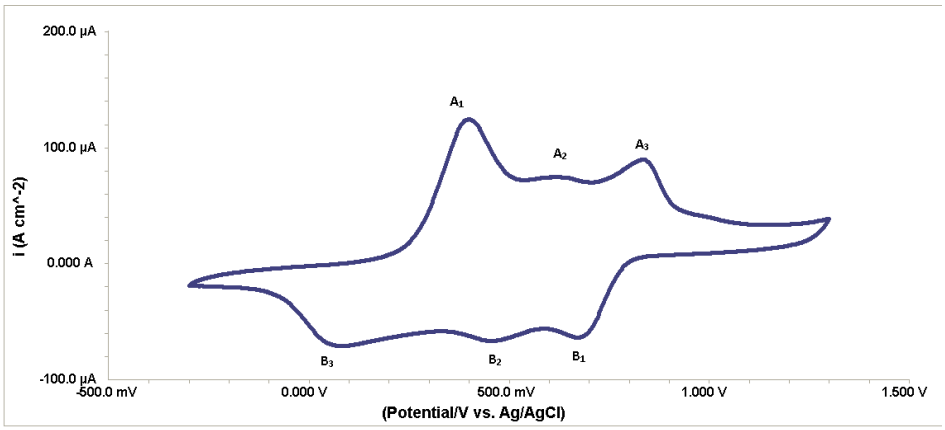

Figure 2 Peaks observed in the electrochemical polymerization of aniline on Au electrode in 0.5 M H_2SO_4 solution.

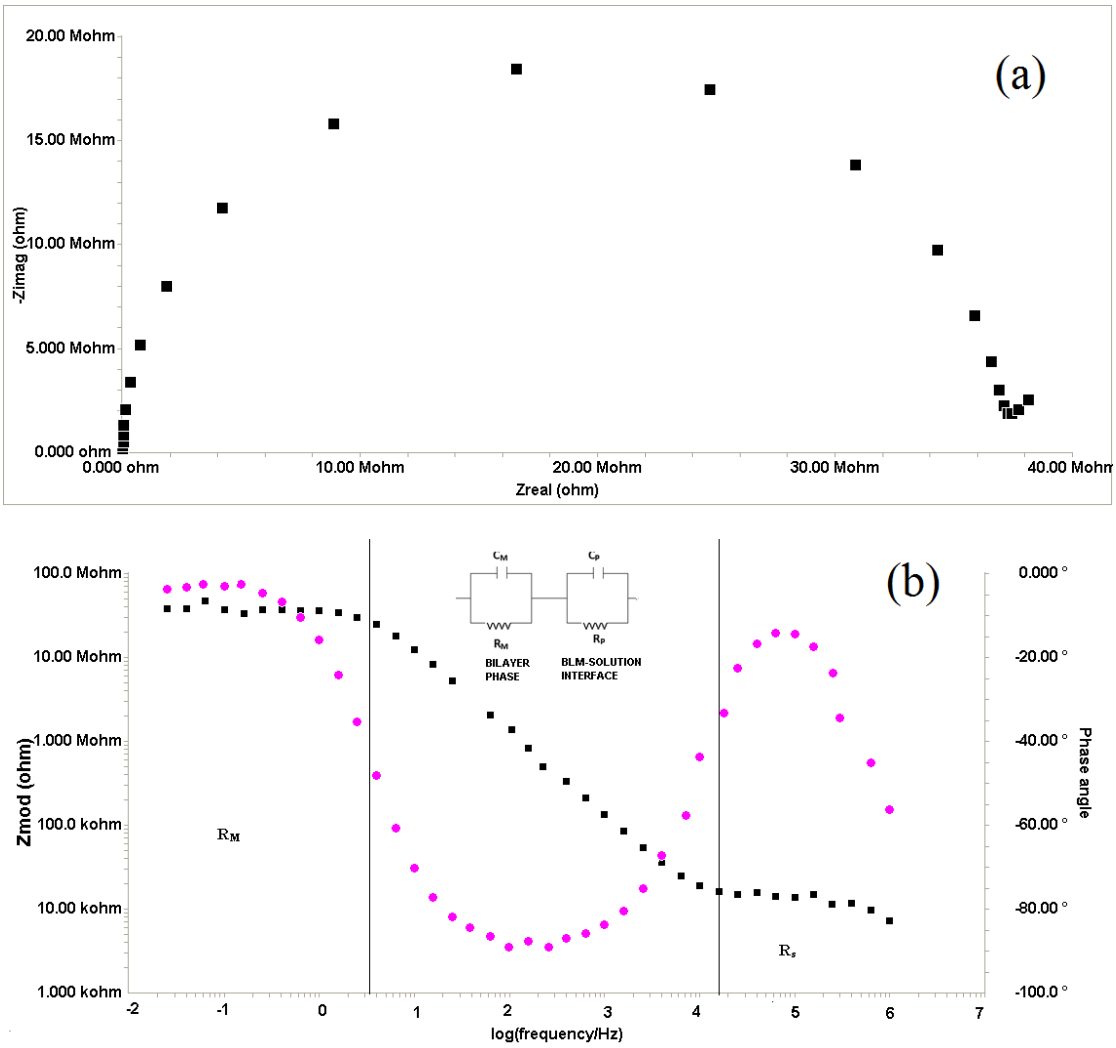

Figure 3 (a) Nyquist plot of BLM formed on polyaniline coated Au surface. (b) Bode plot of BLM formed on polyaniline coated Au surface.

The electrochemical parameters of the BLM formed are evaluated using an equivalent circuit shown in **Figure 4**.

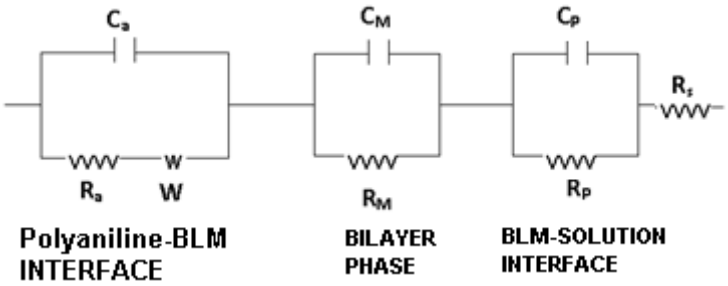

Figure 4 Equivalent circuit of BLM formed on polyaniline coated Au surface.

The self assembly of egg lecithin molecules into a bilayer was confirmed by the calculation thickness of BLM from the capacitance value obtained using above equivalent circuit (Figure 4) employing the following expression [22].

$$C_M = \frac{\varepsilon_0 \varepsilon}{d} \qquad (1)$$

Where ε_0 is the permittivity of free space (ε_0 =8.854*10^{-12} Fm^{-1}], ε is dielectric constant of the lipid bilayer phase ε=2.05 [23].The calculated thickness is 5.4 nm, which is close to the twice the thickness of lecithin monolayer (2.5 nm) [21, 24, 25]. C_a,R_a and W correspond to BLM-Polyaniline interface.

The BLM thus formed in 0.1 M NaCl bath solution on the polyaniline surface is then immersed in 0.1 M NaCl solution containing 1:1 ratio 1 mM ferrocyanide and ferricyanide ions and the impedance was recorded. Generally the BLM formed is impermeable to larger molecules and charged species, but smaller cations like Na^+ and K^+ ions pass through the membrane and offer some conductance [26]. It is interesting to note that the membrane conductance decreases or membrane resistance increases when it is immersed in 0.1 M NaCl solution containing 1:1 ratio 1 mM ferrocyanide and ferricyanide ions at neutral pH (Phosphate buffer solution). This is due to a fact that when BLM is in contact with ferro/ferri cyanide ions they enter into the membrane phase through tiny pores in the membrane surface, which was proved in many BLM based sensors [27, 28], which could attract the positively charged Na^+ ions and slow down them in BLM phase. Due to this the ionic conductance across BLM phase decreases. The impedance spectra obtained for bare and drug doped BLMs in 0.1 M NaCl solution containing 1:1 ratio 1 mM ferrocyanide and ferricyanide ions are shown in **Figure 5**.

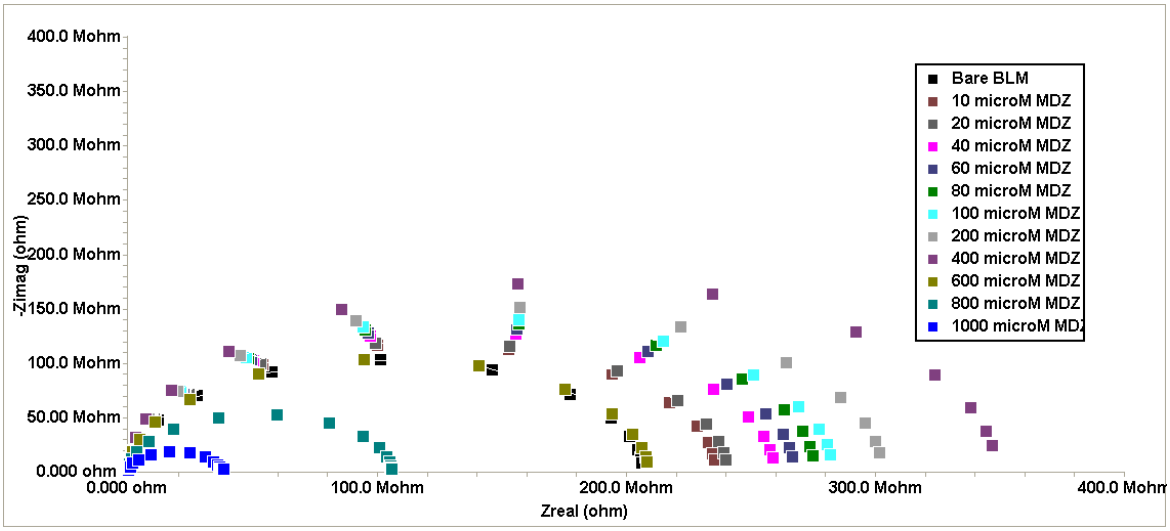

Figure 5 Nyquist plots for bare and drug doped BLMs in 0.1 M NaCl bath solution containing 1:1 ratio 1 mM ferrocyanide and ferricyanide ions.

The obtained Nyquist plots fit well with the equivalent circuit shown in Figure 4. The corresponding electrochemical impedance parameters are shown in Table.1. From Table.1 it is seen that the membrane resistance increases with drug (MDZ) concentration and then decreases from 600 µM. The electrochemical properties and surface charge of BLM is strongly influenced by pH and Cl^- ion concentration in the bath solution [29]. The BLM surface has positive and negative charges due to nitrogen base and phosphate groups. The adsorption coefficient of Cl^- ion is about three orders greater than that of Na^+ ion [29]. Hence most of the positive charges on the BLM surface will be covered by Cl^- ions and the negative charges on the BLM are partially neutralized by Na^+ ions. Therefore there exists a net negative charge on the BLM surface at neutral pH.

Midazolam in its hydrochloride form in solution undergo dissociation and following equilibrium exists among the various species [30].

$$MDZH^+Cl^-_{(solution)} \rightleftharpoons MDZH^+_{(aq)} + Cl^-_{(aq)} \rightleftharpoons [MDZH^+ \ldots Cl^-]_{(ion\text{-}pair)} \quad (2)$$

Due to common ion effect, the above dissociation is strongly affected by Cl^- ion concentration in the bath solution. At low concentration the ionized form of MDZ is present in large amount, from which the positively charged species of MDZ get attached to the negative charges on the BLM surface. From **Table 1** it is also seen that the membrane capacitance increases with MDZ concentration. Figure 6 shows the variation capacitance of BLM phase with drug dose. From this figure it is clear that the increase in capacitance of BLM is biphasic, where the initial increase in capacitance of BLM phase is due to tightening effect shown by ionozed from of MDZ on BLM surface and second phase increase in capacitance is due to the partition of ion pair and neutral forms of MDZ, which are present in large amount at higher doses. The partition of MDZ into BLM phase increases its area which is directly proportional to capacitance of BLM phase.

Table 1 Electrochemical Impedance Parameters of bare and drug doped BLM in 0.1 M NaCl bath solution containing 1:1 ratio 1 mM ferro/ferri cyanide ions.

S.No	Concentration of Midazolam (µM)	Membrane Resistance (R_M) X10^6	Membrane Capacitance (C_M) X10^{-9}F
1	0	206	1.240
2	10	236	1.243
3	20	240	1.247
4	40	256	1.251
5	60	267	1.254
6	80	277	1.256
7	100	283	1.258
8	200	303	1.261
9	400	349	1.264
10	600	209	1.320
11	800	106	1.321
12	1000	37	1.322

Cyclic Voltammetric studies on interaction of MDZ with polyaniline supported BLM

Figure 6 shows the cyclic voltammograms recorded for the interaction of MDZ with polyaniline supported BLM. From this figure it is clear that the current response for le-em conversion increases with drug dose upto 400 µM of MDZ in bath solution and then decreases.

From Impedance studies it is clear that upto 400 µM concentration of MDZ in the bath solution the ionized form of MDZ dominates which interacts at the surface and generates some pores. Through these pores the ferri/ferro cyanide ions can reach the surface and undergo redox reactions favoring the le-em conversion. Beyond 400 µM concentration of MDZ the ion pair and neutral forms of MDZ dominate and get partitioned into the BLM phase, which blocks the passage of ferri/ferro cyanide ions and there is decrease in current response for le-em conversion. The current response shows linear relationship with the concentration MDZ upto 400 µM, which is shown in **Figure 7**. Hence, polyaniline supported BLM can be used to detect the MDZ in solution upto 400 µM.

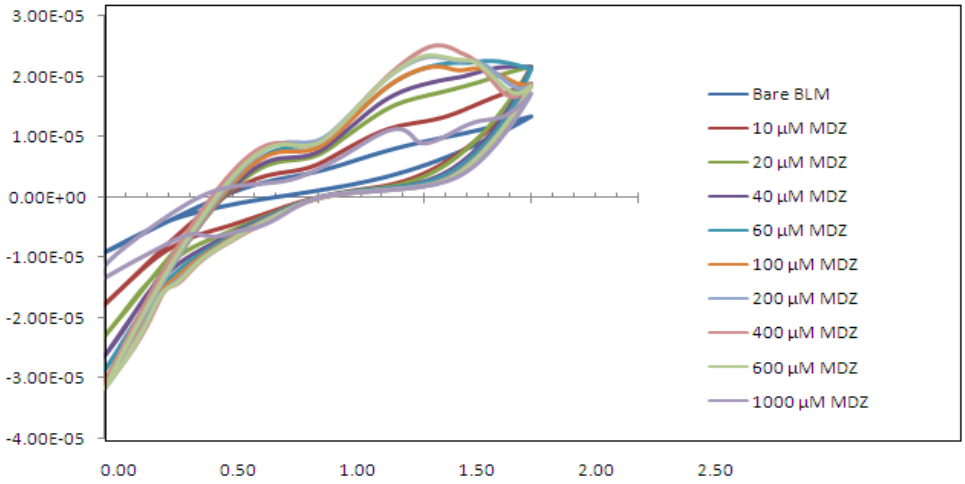

Figure 6 Cyclic Voltammograms of bare and drug doped BLM in 0.1 M NaCl bath solution containing 1:1 ratio 1 mM ferro/ferri cyanide ions.

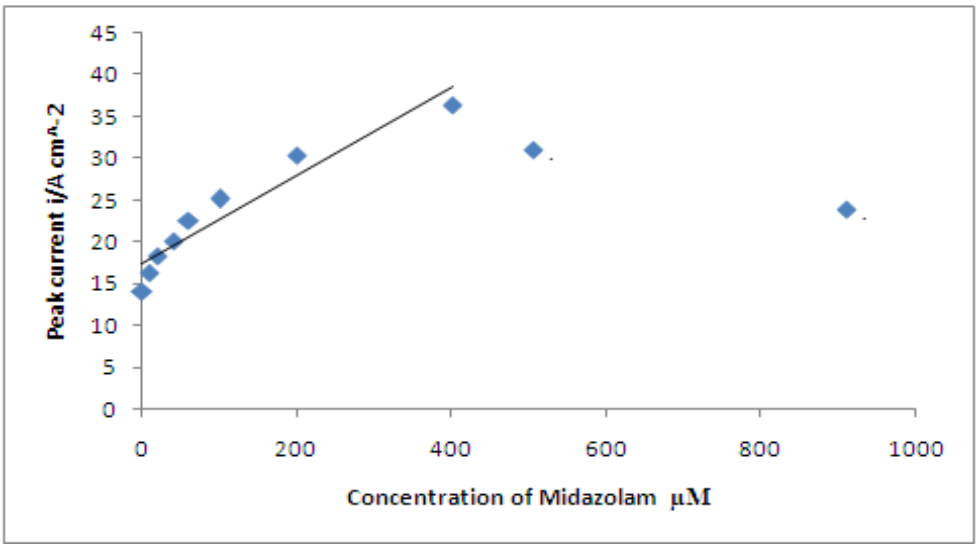

Figure 7 Concentration of MDZ vs Peak Current.

Conclusions

The following are important conclusions drawn from the present studies.

- BLM can be formed on the polyaniline deposited electrochemically on Au surface.
- From EIS studies the formation of BLM was confirmed.
- The conductance of BLM phase decreased when polyaniline supported BLm is immersed in to the 0.1 M NaCl bath solution containing1:1 ratio 1 mM ferrocyanide and ferricyanide ions.
- Cyclic voltammery technique can be used to quantify the MDZ in 0.1 M NaCl bath solution containing 1:1 ratio 1 mM ferrocyanide and ferricyanide ions at neutral pH.

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