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Potentiometric Zn^{2+} Biosensor Based on Bacterial Cells

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Abstract: *Pseudomonas striata* cell mass was immobilized in polyvinylchloride (PVC), a neutral carrier to prepare zinc selective membranes using Dibutyl-phthalate as the plasticizer. Membranes were prepared using 1, 2, 3, 5 and 7% of bacterial biomass, of which the highest response was obtained for 5%. Potentiometric response of the electrode was studied for Zn, Cd and Cu in the concentration range of 10^{-7} to 10^{-1} M. A linear trend between the electrode response and the varying metal concentrations was seen only for Zn^{2+} ions in the range of 10^{-4} to 10^{-1} M. Calibration slope of 22 mV/decade and detection limit of 5×10^{-4} M was obtained for zinc. Electrode showed a sharp response time of 6 sec and pH optima of 3. Thus, the PVC membrane containing the bacterial biomass was found to selectively bind the Zn^{2+} ions and generate the corresponding potential response at different zinc concentrations.

Key words: Zn-metalloenzymes, heavy metal determination, potentiometric biosensor, ionophore

INTRODUCTION

The determination of traces of toxic heavy metals in biological materials, natural waters, soil and air has become very important because the environment is highly vulnerable to this class of pollutants. Heavy metals are accumulated and stored in the living organisms; especially in the marine organisms a very high bioaccumulation of heavy metals can take place (Krawczyk *et al*., 2000). Currently, a huge array of analytical methods for the toxic agents' detection is used. These methods based on spectrophotometry, chromatography, mass spectrometry and various hyphenated techniques; require sophisticated and expensive equipments, highly trained staff and is usually time-consuming (Dzyadevych *et al*., 2005; Sherma and Zweig, 1983). Moreover, these methods can only detect the total amount of heavy metals and not the bioavailable concentrations accessible to the living organisms. Therefore, development of new and inexpensive methods for the detection of bioavailable heavy metals concentrations is highly desirable. Biosensors are useful analytical devices in this respect and several configurations have been described in the past for heavy metal detection. Wide spectrum of biological recognition elements and transducer systems has been used for the fabrication of biosensors (Castillo *et al*., 2004; Amine *et al*., 2006; Bentley *et al*., 2001). The use of metalloenzymes/ metalloproteins as the biological sensing element for heavy metals has several advantages over the other bioreceptors as these are more specific in regard of metal binding (McCall *et al*., 2000). There are certain reports on the use of metal binding proteins/peptides for the fabricaton of heavy metal biosensor (Cherian *et al*., 2003; Chow *et al*., 2005). These biomolecules besides detecting the bioavailable content also allow high selectivity in the recognition of analytes, such as metal ions, in complex natural solutions, e.g., seawater or blood. Combination of this property of biomolecules with

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a suitable transducer system has a great promise as an indicator system that may in the future replace the current techniques of measuring very low concentrations of metal ions (Thompson *et al*., 1996; Kielland, 1937). In the present study, plasticized PVC membranes were used as a support matrix for the entrapment of lyophilized bacterial cells to fabricate a Zn^{2+} selective potentiometric electrode. Al-Hitti *et al*. (1984) demonstrated the immobilization of GOD (Glucose oxidase) within plasticized polyvinylchloride membrane, which was then used for glucose determination. The methodology followed for the preparation of electrode is same as described by Mittal *et al*. (2007). Ligand is that component of the potentiometric sensor which makes the inert support matrix (which is polyvinylchloride in this study) ion-selective in nature. Lyophilized cell mass of *Pseudomonas striata* was chosen as a ligand in the membrane electrode. *Pseudomonas striata* was selected because this strain produces good amount of alkaline phosphatase which is Zn-metalloenzyme and has zinc ligating sites. In addition to this there are many other Zn-metalloenzymes which are present in prokaryotic systems that might be responsible for the zinc selective nature of the electrode. Literature searches have ascertained sequences, zinc content and functional characteristics of the catalytic, cocatalytic and structural zinc sites for families of zinc enzymes. The X-ray structure analyses of 11 enzymes containing a single catalytic zinc atom identify their ligands. This metal forms complexes with any of the nitrogen and oxygen ligands of histidine and glutamate residues with a binding frequency of His>>Glu (Vallee and Auld, 1993). In the present study, the zinc ligating property of *in vivo* alkaline phosphatase and other Zn-metalloenzymes has been explored for the purpose of making a biosensor for Zn^{2+} ions. Lot of ionophore-based chemical sensors have been reported till date which make use of a large number of chemical metal ligands as the ionophore but lack selectivity. This study is a novel attempt to explore the potential of the Zn-metalloenzyme as Zn-ionophore.

MATERIALS AND METHODS

The microbiological study was carried out in Department of Biotechnology and Environmental Studies and the potentiometric studies were done in School of Chemistry and Biochemistry, Thapar University, Patiala (Punjab) from February to April 2008.

Reagents

Reagents like dibutyl phthalate (DBP), o-nitrophenyloctyl ether (o-NPOE) were procured from Sigma-Aldrich. All other chemicals were of analytical reagent grade. Double distilled deionized water was used throughout the experiments.

Ligand Preparation

Pseudomonas striata was cultured on nutrient agar plates for 17 h. The cells were harvested using Tris-HCl buffer pH 8.3. The cell suspension was centrifuged at 8000 rpm for 10 min to obtain a cell pellet which was lyophilized at -50°C under vaccuum using a freeze dryer (Modulyod, ThermoElectron Corporation) to obtain dry cell mass.

Electrode Preparation

Methodology followed for the preparation of PVC membranes and potential measurements were same as described by Mittal *et al*. (2007). Membranes of ~0.2 mm thickness were obtained by pouring a solution of the membrane components of PVC 33%, bio-ligand (lyophilized bacterial cells) 1-7% and dibutyl phthalate/o-nitrophenyloctyl ether 63% dissolved in 2-3 mL of tetrahydrofuran (THF). The viscous solution of the polymer thus obtained was poured in a glass ring of 30 mm diameter placed on a dust free pyrex glass plate. The solvent was allowed to evaporate slowly for about 24 h at room

temperature. To obtain the membrane with similar characteristics, viscosity of the casting solution and rate of solvent evaporation were controlled so that the thickness and morphology of the membranes remained unchanged and the appearance of the film looked pale yellow in colour. The membranes were then removed from glass ring and circular pieces of 1.25 cm diameter were cut and mounted on the ground end of a pyrex glass tube with an adhesive and conditioned with a metal solution ($ZnSO_4/CuSO_4/CdSO_4$) (0.1 M) for 2 h.

EMF Measurements

All the EMF measurements were carried out using the following cell assembly:

Ag/AgCl, KCl (sat.) ||0.1 M $Zn^{2+}/Cd^{2+}/Cu^{2+}$ |membrane| $Zn^{2+}/Cd^{2+}/Cu^{2+}$ test solu|| KCl (sat.), AgCl/Ag

Salt bridges containing KCl were used to provide electricity links between KCl and metal solutions on both sides of the membrane. A digital potentiometer having sensitivity of 0.1 mV (Equiptronics EQ602, India) was used for the potential measurements at 25±0.1°C. Activities were calculated according to the Debye-Huckle equation (Meier *et al*., 1980). Standard metal solutions were obtained by gradual dilution of 0.1 M metal stock solution and their potential measurements were performed.

The membranes were calibrated for the three metal ions viz., Zn Cd and Cu at a concentration range varying from 10^{-7} to 10^{-1} M. Percentage weight of ionophore was also optimized and effect of pH on the EMF response was studied.

RESULTS

Optimization of the Membrane Composition

For optimization of the membrane, effect of the composition on the response characteristics of the electrode like slope of the calibration curve, measurement range and detection limit were studied. The electrode with the ratio PVC:DBP:bacterial cells = 33:62:5%, exhibits the best response with a slope of 22 mV/decade. It was found that DBP is a more effective solvent medium than o-NPOE in preparing the Zn^{2+} ion selective electrode. Amount of the ion carrier/ligand (bacterial cells) affects the sensitivity of the electrode. Sensitivity of the electrode was found to increase with increasing ionophore content until a value of 5% (w/w) was reached. A further increase in the percentage of the ionophore results in decrease of the slope of the electrode.

Calibration Curve, Response Time and Detection Limit

The response time is measured by recording emf of the electrode as a function of time, when it is immersed in the solution to be studied. The estimated time to get stable potential was 6 sec. The electrode shows a linear response towards Zn^{2+} over a wide concentration range of 10^{-4} to 10^{-1} M. The calibration curve has a slope of 22 mV/decade with a detection limit of 5×10^{-4} M, which was obtained from the intersection of two straight-line portions of the curve (Fig. 1). A slow decrease in the EMF is observed beyond 5×10^{-5} M. No particular emf trend was observed for Cd^{2+} and Cu^{2+} ions.

Effect of pH

pH was studied in the range of 2-12 using 2×10^{-2}, 2×10^{-3} and 2×10^{-4} M Zn^{2+} concentration. pH studies were done on membranes with 3 and 5% ionophore concentration. pH was adjusted by the addition of 0.1 N NaOH or HCl as required. It was found that the electrode response was optimum in a very narrow pH range of 3-4 (Fig. 2).

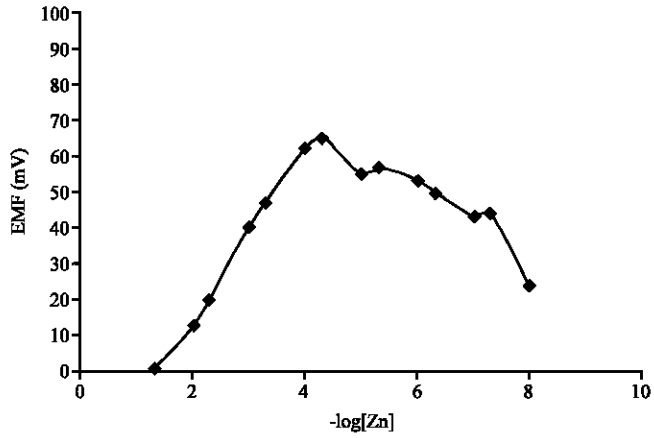

Fig. 1: Response of bioligand based ISE (Ion selective electrode) towards zinc ions

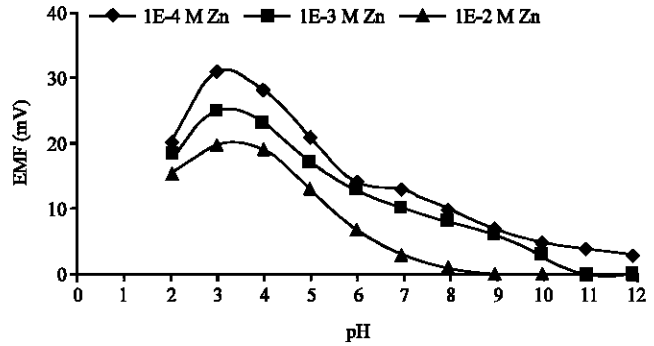

Fig. 2: Effect of pH on potentiometric response of membrane at three different zinc concentrations

DISCUSSION

The membrane material is a plastic, polyvinylchloride (PVC) that is highly hydrophobic and impermeable to any ions. It is plasticized (softened) by addition of a similarly hydrophobic solvent, e.g., DBP (Dibutyl pthalate), o-NPOE (ortho nitrophenyl octyl ether). So far, the membrane is just a flexible piece of plastic, which acts as a near perfect barrier to ions. To make it ion-selective, a neutral ligand which is selective for the analyte and lipophilic in nature is added. Many chemical ionophore based potentiometric biosensors have been reported for the detection of metal ions, but all these suffer from the drawback of low ion selectivity as the ionophores used are not ion specific. The use of bioligands as the ionophore for the construction of potentiometric biosensor is a novel concept. An Ag^+ ion selective electrode using polysulfone matrix embedding metallothioneins as ionophores with the detection limit of about 10^{-5} M was reported by González-Bellavista *et al.* (2009). Since, construction of such biosensors required small amount of proteins they can be dry-stored and have long-lifetimes. In this study, lyophilized cells of *Pseudomonas striata* were used as the source of alkaline phosphatase (Ligand) containing zinc ligating sites. These zinc ligating sites of the enzymes are exposed to the external solution and made available for binding to the metal ions due to the rupturing of the bacterial cell walls by tetrahydrofuran (THF) (solvent used for the preparation of PVC membrane). This was confirmed by observing the THF cell suspension under the microscope.

Zinc binding sites in proteins are often distorted tetrahedral or trigonal bipyramidal geometry, made up of the sulfur of cysteine, the nitrogen of histidine or the oxygen of aspartate and glutamate, or a combination. Zinc in proteins can either participate directly in chemical catalysis or be important for maintaining protein structure and stability. In all catalytic sites, the zinc ion functions as a Lewis acid. An understanding of naturally occurring zinc-binding sites will aid in creating de novo zinc-binding proteins and in designing new metal sites in existing proteins for novel purposes such as to serve as metal ion biosensors (McCall *et al*., 2000). Zinc, always occurring as a divalent cation [zinc(II)] in biological systems is the second most abundant (common) transition metal following iron. Today more than 300 different zinc proteins are known. These include numerous essential enzymes which catalyze the metabolic conversions (synthasis, polymerasis, ligasis, transferasis) or degradation (hydrolasis) of proteins, nucleic acids, lipids, porphyrin precursors and other important bioorganic compounds (Urbanová *et al*., 2008).

Also, it was comfirmed that the activity of alkaline phosphatase which contains the zinc binding sites released due to cell rupture is not lost as was confirmed spectrophotometrically by performing the enzyme assay using para-nitrophenylphosphate (p-NPP) as the substrate at pH 8.3 and 37°C (Barnes and Morris, 1957). The enzymatic reaction leads to the conversion of the substrate (p-NPP) to a yellow colored compound para-nitrophenol (p-NP), whose optical density was measured at 420 nm using a UV-Vis spectrophotometer (Hitachi).

The sensitivity and selectivity of an electrode are significantly affected by the nature of the plasticizer, the composition of ionophore, internal solution (Mi *et al*., 1999; Sokalaski *et al*., 1997; Sokalaski *et al*., 1999), etc. In neutral carrier membranes, plasticizers that are compatible with the ionophore provide a smooth surface to the membrane and hence enhance the response characteristics (Cammann, 1979). The nature of the plasticizer influences the dielectric constant and the mobility of the ions in the membrane. These membrane solvents are seen to strongly influence the working concentration range and the slope of the sensor. It was observed that the electrode with DBP as plasticizer was found to give the best response in terms of the slope and the concentration range. The slopes in the case of the o-NPOE are sub-Nernstian. The potentiometric response of the sensor towards Zn (II) ions is found to be dependent on the concentration of the ionophore used. Different compositions (w/w%) of the ionophore were also tried to obtain the right composition of ionophore that gives the best response characteristics. The maximum sensitivity was observed for 5% (w/w) of the ionophore. On increasing the ionophore content, the slopes are affected; this may be related to the change in the water uptake capacity of the membrane (Kumar *et al*., 2006) and also may be due to the reason that equilibration of the ionophore with the metal ions is maximum at this concentration (Mittal *et al*., 2007).

The linearity observed in the case of Zn^{2+} (Fig. 1) can be attributed to the zinc ligating sites present on Zn-metalloenzymes. These Zn-ligating sites lying at the interface of the internal and test solution are exposed to the concentration gradient across the membrane which leads to the generation of potential difference measured in terms of electromotive force (EMF). This further supports the non-linear trends observed for Cd and Cu, confirming the selective binding of Zinc by the Zinc metalloenzymes.

The results presented as Fig. 2 reveal that the potentials are independent of pH in a very narrow range of 3-4 and this range is taken as the working pH range of the Zn(II) sensor. Variation of potentials above and below these pH values can be related to hydrolysis of Zn(II) (at higher pH) and the competition of H^+ with Zn(II) (at lower pH values).

CONCLUSION

The present study is a novel report on the use of *in vivo* Zn-metalloenzymes as a zinc ligand to fabricate a selective biosensor. Potentiometeric response of Zn-metalloenzymes present in the bacterial

cell mass was quite specific for Zn^{2+} ions with a slope of 22 mV/decade and a sharp response time of 6 sec. The optimum pH for the detection of zinc was 3. The electrode did not show any response towards Cd^{2+} and Cu^{2+}, which indicates towards the zinc selective nature of the electrode. Further work would include testing the selectivity of the electrode which is the ability of an ion electrode to distinguish between different ions in the same solution, to study the effect of different solvent media on the electrode response and to determine the target ion (Zn^{2+}) with probable interfering ions.

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