

## Sensor based on polyvinyl chloride immobilized *Pseudomonas striata* cells as metal-ionophore

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**Abstract:** PVC membrane containing lyophilized cell mass of *Pseudomonas striata* was prepared using dibutyl-phthalate as the plasticizer. Anionic interferences were excluded by use of potassium salt of tetrakis (4-chlorophenyl) borate (KTCIPB). The electrode was found to be fairly selective and sensitive for the zinc ions. It exhibited a linearity range of  $10^{-1}$  to  $10^{-5}$  M with near nernstian slope of 26.2 mV per decade. The membrane electrode showed a sharp response time of 6-10 sec and detection limit of  $5 \times 10^{-5}$  M at  $25 \pm 1^\circ\text{C}$  in the pH optima of 3-5. The interference was found to arise only from few transition metals such as  $\text{Hg}^{+2}$ ,  $\text{Ag}^+$  and  $\text{Pb}^{+2}$ .

**Keywords:** Zn-metalloenzymes, Heavy metal determination, Potentiometric biosensor, Ionophore.

### Introduction

The toxic nature of the heavy metals necessitates the need of their determination in biological materials, natural waters, soils and air even at trace levels. Bioaccumulation of the heavy metals has been reported to be higher in the upper trophic levels at concentrations surpassing those found in water supplies (Krawczyk *et al.*, 2000). The conventional methods used for the determination of the heavy metals based on spectrophotometry, chromatography, mass spectrometry and various hyphenated techniques require sophisticated and expensive equipments, highly trained staff: besides they are usually time-consuming (Sherma & Zweig 1983; Dzyadevych *et al.*, 2005). Also these conventional methods give the estimate of the total heavy metals present in the environment which is different from the bioavailable concentration that actually affects the living organisms. Thus, need arises for the fast and inexpensive methods to detect bioavailable heavy metals. 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 (Bentley *et al.*, 2001; Castillo *et al.*, 2004; Amine *et al.*, 2006). Of the different bioreceptors used for the fabrication of the heavy metal sensor, metalloenzymes/metalloproteins are potentially most promising because of their specificity for metal binding (McCall *et al.*, 2000). Different metalloproteins/peptides have been used for developing heavy metal sensors (Cherian *et al.*, 2003; Chow *et al.*, 2005). The high selectivity of these metal binding molecules even in complex natural solutions like sea water or blood when combined with a suitable transducer 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 (Kielland, 1937; Thompson *et al.*, 1996). In the present work plasticized PVC membranes were used as a support matrix for the entrapment of lyophilized bacterial cells to fabricate a  $\text{Zn}^{2+}$  selective potentiometric electrode. *Pseudomonas striata* was selected because this strain produces sufficient amount of alkaline phosphatase which is Zn-metalloenzyme and has zinc ligating sites. In addition, 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 complex with any of the nitrogen and oxygen ligands of histidine and glutamate residues with a binding frequency of  $\text{His} \gg \text{Glu}$  (Vallee & Auld, 1993).

In the present work the zinc ligating property of *in-vivo* alkaline phosphatase and other Zn-metalloenzymes has been explored for the purpose of making a biosensor for  $\text{Zn}^{2+}$  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. 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).

### Materials and methods

#### 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  $-50^\circ\text{C}$  under vacuum using a freeze dryer (Modulyod, ThermoElectron Corporation) to obtain dry cell mass.

#### Electrode preparation



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%, potassium salt of tetrakis(4-chloro-phenyl) borate (KTCIPB) 1-3% and dibutyl phthalate/ o-nitrophenyloctyl ether 59-65%, dissolved in 2-3 ml of tetrahydrofuran (THF). The viscous solution of the polymer thus obtained

*Potentiometric selectivity coefficients*

Selectivity coefficients were evaluated by the fixed interference method (FIM) (interfering ion concentration fixed at  $1 \times 10^{-3}M$ ) and matched potential method (MPM), a specified amount of primary ions is added to a reference solution and the membrane potential is measured. In a separate experiment, interfering ions are successively added to an identical reference solution until the membrane potential matches with that one obtained before with the primary ions (Umezawa *et al.*, 1995).

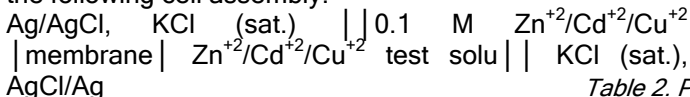
Table 1. Optimization of membrane ingredients

PVC (wt%)	Plasticizers (wt%)	Ligand (wt%)	KTCIP B (wt%)	Slope (mV/decade)	Detection limit (M)
33	65 (DBP)	2	-	15	$1 \times 10^{-4}$
33	64 (DBP)	3	-	18	$1 \times 10^{-4}$
33	63 (DBP)	4	-	20	$5 \times 10^{-4}$
33	62 (DBP)	5	-	22	$1 \times 10^{-5}$
33	61 (DBP)	6	-	21	$6 \times 10^{-4}$
33	60 (DBP)	7	-	21	$5 \times 10^{-4}$
33	62(2-NPOE)	5	-	19	$1 \times 10^{-4}$
33	61 (DBP)	5	1	25	$1 \times 10^{-5}$
33	60 (DBP)	5	2	26.2	$5 \times 10^{-5}$
33	59 (DBP)	5	3	21	$1 \times 10^{-4}$

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 hrs 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:



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  $25 \pm 0.1^\circ C$ . Activities were calculated according to the Debye-Huckel equation (Kielland, 1937). 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 and discussion**

The membrane material is a plastic, polyvinyl chloride (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 phthalate), o-NPOE (ortho nitrophenyl octyl ether). 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.

*Optimization of the membrane composition*

The sensitivity and selectivity of an electrode are significantly affected by the nature of the plasticizer, the composition of ionophore and internal solution (Mi *et al.*, 1999; Sokalaski *et al.*, 1997; Sokalaski *et al.*, 1999). Hence, 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 (Table 1). The electrode with the ratio PVC:DBP:bacterial cells:KTCIPB = 33%:60%:5%:2%, exhibits the best response with a slope of 26.2mV/decade. It was found that DBP is a more effective solvent medium than o-NPOE in the preparing the  $Zn^{+2}$  ion selective electrode. Amount of the ion carrier (Bacterial cells) affects the sensitivity. Sensitivity of the electrode increases with increasing ionophore content until a value of 5% (w/w) is reached. A further increase in the percentage of the ionophore results in decrease of the slope of the electrode. This may be due to the reason that equilibration of the ionophore with the metal ions is maximum at this concentration. Addition of potassium salt of tetrakis (4-chloro-phenyl) borate (KTCIPB) is known to increase the sensitivity of the membrane as it reduces the anionic interference. It is observed that the addition of this lipophilic cation improved the working electrode sensitivity (Linear range:  $10^{-1}$  to  $10^{-5}$  M, Slope: 26.2mV/decade) and detection limit  $5 \times 10^{-5}M$ .

*Zinc ligating sites*

Tetrahydrofuran (THF) used as a solvent for the preparation of PVC

Table 2. Potentiometric selectivity coefficients

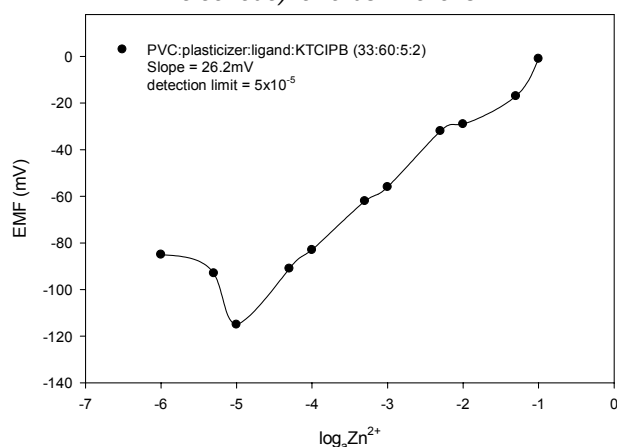
$(K_{Zn^{2+},B}^{Pot})$  for interfering ions

Interfering ions (B)	$-\log K_{Ag^+,B}^{pot}$	
	FIM	MPM
$Co^{2+}$	-2.2	-2.6
$Mn^{2+}$	-3.1	-2.8
$Cd^{2+}$	-3.2	-2.9
$Ni^{2+}$	-2.8	-2.1
$Cu^{2+}$	-3.2	-3.3
$K^+$	-3.5	-3.2
$Fe^{3+}$	-3.1	-2.9
$Mg^{2+}$	-3.5	-3.1
$Pb^{2+}$	+0.2	+0.5
$Hg^+$	+1.4	+0.9
$Ag^+$	+1.2	+1.0

membrane leads to the rupture of the bacterial cell walls as was confirmed by observing the THF cell suspension under the microscope, releasing the cell content and hence exposing the Zn-ligating sites of the enzyme. Also, it was observed that the activity of alkaline phosphatase 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 & 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).

Fig. 1. Response of Bio-ligand based ISE (Ion selective electrode) towards zinc ions



Calibration curve, response time shelf life and detection limit

The electrode shows a linear response towards  $Zn^{2+}$  over a wide concentration range of  $10^{-5}$  to  $10^{-1}$  M. The calibration curve has a near Nernstian slope of 26.2mV/decade with a detection limit of  $5 \times 10^{-5}$  M which was obtained from the intersection of two straight-line portions of the curve (Fig.1). The slow decrease in the emf beyond  $10^{-5}$  M may be due to the release of  $Zn^{2+}$  ions from membrane in to the solution. No particular emf trend was observed for  $Cd^{2+}$  and  $Cu^{2+}$  ions (data not shown). The reason for the linearity observed in the case of  $Zn^{2+}$  can be attributed to the Zinc ligating sites present 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). 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 Gonzalez-Bellavista *et al.*, 2009. Since construction of such biosensors required

small amount of proteins they can be dry-stored and have long-lifetimes.

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 s. Although always kept at 4-5°C, the response of the electrodes stored in dry is much better than that of the electrode stored in 0.1 M  $Zn^{2+}$  solution. The soaked electrodes showed a 20%, 70% and 100% sensitivity decrease after 2, 4 and 6 days of storage, respectively (data corresponding to 5 days of storage is shown in Fig. 2).

The longer life span of the electrodes which were stored under dry conditions could be attributed to the prevention of the oxidation of the cysteine residues present in the metalloproteins (Gonzalez-Bellavista *et al.*, 2009).

#### Effect of pH

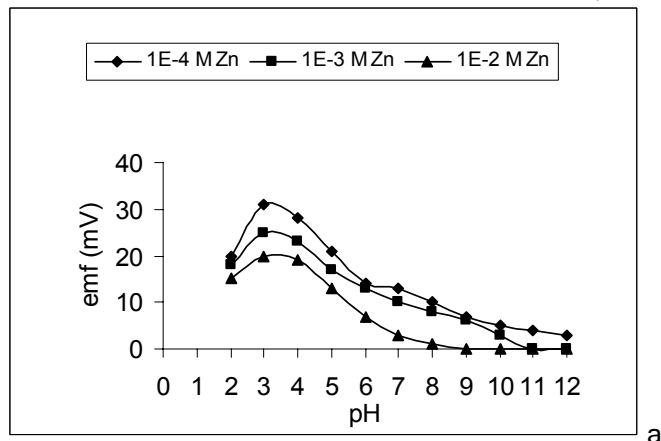
pH was studied in the range of 2-12 using  $1 \times 10^{-2}$ ,  $1 \times 10^{-3}$  and  $1 \times 10^{-4}$  M  $Zn^{2+}$  concentration. pH studies were done on membranes with 3 % and 5 % ionophore concentration. pH was adjusted by the addition of 0.1 N NaOH or  $HNO_3$  as required. It was found that the electrode response was optimum in a very narrow pH range of 3-4 (Fig.3). At pH above and below this range a sharp decrease in the emf value was observed

#### Potentiometric selectivity coefficient

One of the main features of any ion-selective electrode is its response to the primary ion in presence of other ions. Ion selective electrodes are rarely ion specific. The ability of an ion selective electrode to distinguish between different ions in the same solution is expressed as the selectivity coefficient  $-\log K_{Ag^+,B}^{pot}$ . The selectivity coefficient is not always constant and depends on several factors including the concentrations of both ions, the total ionic strength of the solution and the temperature. All electrodes are sensitive to some or other ions to some extent. For many applications these interferences are insignificant and can often be ignored. In some extreme cases, however, the electrode is far more sensitive to the interfering ions than the primary ions and can be used if the interfering ions are present only in trace quantities or completely absent. In our case, some interferences between  $Zn^{2+}$  and other metal ions could also be envisaged due to the well-known order of affinity of heavy metal ions for thiolates ( $Hg(II) \gg Cu(I) \approx Ag(I) \gg Cd(II) > Pb(II) > Zn(II)$ ) which is very close to that of Metallothionein (Vasak, 1991). The observed values of the selectivity coefficients are presented in Table 2. For alkali and alkaline earth metal ions transition metal ions, the  $-\log K_{Ag^+,B}^{pot}$  values lie in the range of 3, 2 except for  $Pb^{2+}$  ions. Also the membrane electrode did not show any serious interference from  $Cu^{2+}$  ions. It is important to note that the selectivity coefficients for the  $Zn^{2+}$  electrode, with reference to most of the alkali and alkaline-earth metal ions, are quite small. This means that this membrane

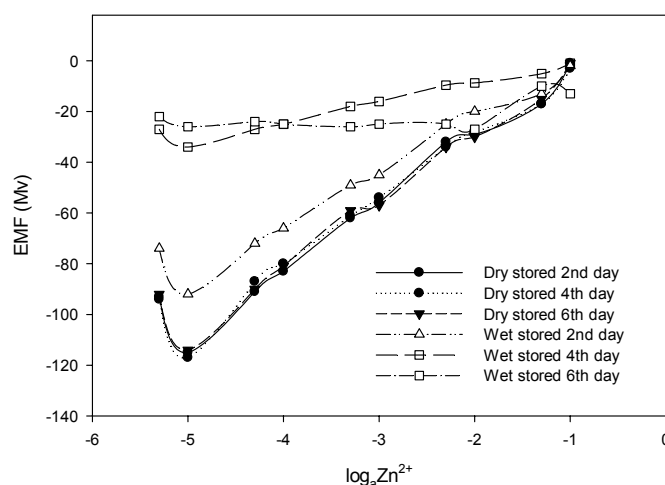
electrode will be highly efficient for determination of trace

Fig. 2. Potentiometric response of the electrode stored in different conditions over the period of 6 days



mounts of zinc in the presence of a large excess of alkali and alkaline-earth metal ions. The zinc response is seriously interfered with by small amounts of  $Hg^{2+}$  and  $Ag^+$  ions; so these two ions must be removed before the analysis of zinc from the samples. This is in good correspondence with literature data regarding electrodes of different nature (Wroblewski & Brzozka, 1995; Siswanta *et al.*, 1996; Chen *et al.*, 2000) and with the higher affinity of this metal ion for the Cys residues if compared with that of the other metal ions studied ( $Pb^{2+}$ ,  $Zn^{2+}$ ,  $Cd^{2+}$  and  $Cu^{2+}$ ).

Fig. 3. Effect of pH on potentiometric response of membrane at three different zinc concentrations



## Conclusions

The present study reports the use of *in vivo* Zn-metalloenzymes as a zinc ligand to fabricate a selective biosensor. Potentiometric response of Zn-metalloenzymes present in the bacterial cell mass was quite specific for  $Zn^{2+}$  ions with a near Nernstian slope of 26.2mV/decade and a sharp response time of 6-10 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. The electrode was found to be quite selective for zinc ions except for the interference shown by  $Hg^{+2}$ ,  $Ag^+$  and  $Pb^{+2}$ .

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