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Fluorescence-based ion sensing in lipid membranes: a simple method of sensing in aqueous medium with enhanced efficiency

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Detection of ions in chemical, biological and environmental samples has gathered tremendous momentum considering the beneficial as well as adverse effects of the ions. Generally, most of the ions are beneficial up to an optimum concentration, beyond which they are toxic to human health. However, most of the fluorescence-based ion sensors are only active in non-aqueous solution because of the low solubility of the sensor molecules in aqueous buffer medium. In the present work, we have demonstrated that encapsulation of an aqueous insoluble thiocarbonohydrazone-locked salicylidene-based macrocyclic ligand in 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphocholine (POPC) membranes allows the selective detection of Zn^{2+} in aqueous medium with approximately 3-fold enhanced efficiency compared to its efficiency in DMSO medium. We have further modulated the charge of the membrane surface by adding various concentrations of a negatively charged lipid, 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphoglycerol (POPG), and showed that negative surface charge further enhances the Zn^{2+} sensing efficiency up to approximately 6-fold. This strategy opens up a new avenue of utilizing organic sensors to detect vital ions in aqueous medium.

Introduction

Detection of ions has gathered significant momentum in the last couple of decades owing to their toxic effects.[1] While plants and animals are known to require ions as macro and micro nutrients, their concentration beyond an optimal level can be lethal. Therefore, a significant amount of effort has been applied to the detection and removal of excess and unnecessary ions. Fluorescence-based ion sensors have gained popularity over other counterparts due to their high sensitivity, tunability, efficacy in cellular environments, and cost effectiveness.[2] However, the major challenge of fluorescence-based sensors is their poor solubility in aqueous medium. From biological perspective, application of these probes in the aqueous medium of cellular milieu would be extremely important, considering the high aqueous solubility of ions. Therefore, strategies need to be designed to enable non-aqueous fluorescence-based sensor molecules to be active in aqueous medium without undergoing any synthetic modification. Zinc is an extremely important ion, since its deficiency leads to neurological disorders like Alzheimer's disease, Parkinson's disease, epilepsy, and ischemic stroke while its excess cellular concentration contributes to hypoglycaemia-induced neuronal death.[3–6]

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Lipid membranes provide an inimitable environment for the solubilization of polar, nonpolar, and amphipathic molecules. The unique blending of polar headgroup and hydrophobic fatty acyl tail creates a nano-environment of graded polarity, mobility, and heterogeneity, which facilitates the solubilization of macrocyclic ligands of varied polarity.[7–9] A recent report has shown that a thiocarbonohydrazone-locked salicylidene-based macrocycle ligand (Fig. 1) senses Zn^{2+} ions in dimethylsulfoxide (DMSO) medium with a very high affinity (K_d of 6 μM); however, the probe is insoluble in aqueous medium.[10] In this work, we aimed to utilize membrane incorporation of the thiocarbonohydrazone-locked salicylidene-based macrocyclic ligand to make it an efficient Zn^{2+} sensor in aqueous buffered medium. We incorporated the macrocyclic probe (Fig. 1) in 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphocholine (POPC) vesicles and evaluated its Zn^{2+} sensing ability in phosphate buffer at pH 7.4.

Material and methods

Materials

The thiocarbonohydrazone-locked salicylidene-based macrocyclic ligand has been synthesized as described earlier.[10] 1-Palmitoyl-2-oleoyl-*sn-glycero*-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphoglycerol (POPG) were obtained from Avanti polar Lipids (Alabaster, AL). Spectroscopic grade DMSO was purchased from Spectrochem (India), and was

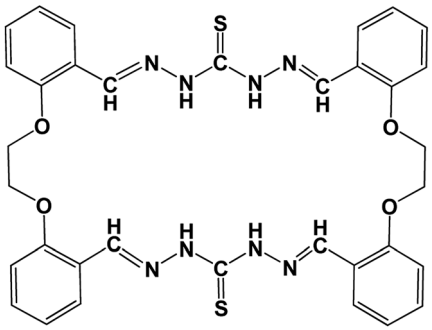

Fig. 1 Chemical structure of thiocarbonohydrazone-locked salicylidene-based macrocyclic ligand (Probe).

used for the preparation of probe stock solution. All other chemicals used in the work were of the highest available purity. Water was purified through Millipore (Bedford, MA) Milli-Q water purification system.

Sample preparation

We have used small unilamellar vesicles (SUVs) of POPC and mixture of POPC/POPG of different mole fractions as the membrane systems for measurements. The concentrations of lipid and probe were kept constant at 500 μM and 5 μM, respectively in all experiments. The lipid was dissolved in chloroform and air dried to make a thin film. The film was kept in vacuum desiccator for complete removal of chloroform. The DMSO solution of the probe was added to the dried lipid and mixed well with the lipid before buffer was added to the lipid. The mixture of the lipid and probe mixture was hydrated (swelled) by adding 10 mM phosphate buffer of pH 7.4. The sample was vortexed for 1 h for uniform dispersion of lipids. The vesicles have been prepared by sonication method and the average hydrodynamic radii of the vesicles are 40–50 nm with a polydispersity index less than 0.2.[11,12] Background samples were prepared the same way except that the probe was omitted.

Steady state fluorescence measurements

Steady state fluorescence measurements were performed with Hitachi (Japan), model F-7000 spectrofluorometer, using quartz cuvette with 1 cm pathlength. Excitation and emission slits with a nominal band pass of 5 nm were used for all measurements. Fluorescence measurements were performed keeping excitation wavelength at 340 nm.

Results and discussion

Experimentally, 5 μM probe was reconstituted in 500 μM POPC and its fluorescence spectrum was recorded after excitation at 340 nm. Fluorescence spectrum of the membrane-incorporated probe is shown in Fig. 2A. Although the probe does not show much fluorescence in homogeneous solution (like DMSO) in absence of Zn^{2+} ion, it has decent fluorescence intensity in membrane environment. This could be attributed to the rotational deactivation pathways of the probe being restricted in the membrane environment.[13] The relatively broad fluorescence spectrum of the probe in the membrane system could be owing to emissions from multiple solvated states. The heterogeneity in the solvated states might be originated from the rotation of various single bonds present in the probe molecule. Addition of Zn^{2+} ions to the probe-incorporated membrane in buffer at pH 7.4 further enhances the fluorescence intensity by ~2-fold with a red shift of approximately 15 nm (Fig. 2A). This change in fluorescence intensity upon Zn^{2+} addition is highly consistent and reproducible. The observed red-shift in emission maximum could be attributed to the ground state complex formation between the probe and Zn^{2+} ion, which has already been confirmed by UV-visible spectroscopic measurements.[10]

The enhancement of fluorescence intensity with significant red shift in emission maximum clearly indicates complex formation between the probe and Zn^{2+} ion. Fluorescence intensity of the probe as a function of Zn^{2+} ion concentration has been shown in Fig. 2B and the data were analyzed using classical Langmuir model for adsorption of ligand to multiple, equivalent, and non-interacting surface sites, as described

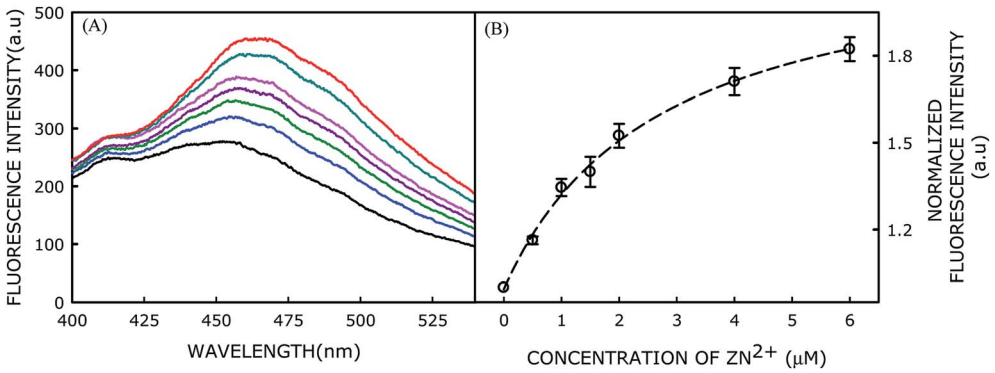

Fig. 2 (A) Fluorescence spectra of the probe in POPC membranes in presence of 0 μM (black), 0.5 μM (blue), 1 μM (green), 1.5 μM (yellow), 2 μM (pink), 4 μM (cyan), and 6 μM (red) Zn^{2+}; (B) plot of normalised fluorescence intensity of the probe as a function of Zn^{2+} ion concentration (POPC was kept constant at 500 μM). The probe concentration was kept constant at 5 μM; it was excited at 340 nm and emission intensity was measured at respective emission maximum. All measurements were carried out in 10 mM phosphate buffer, pH 7.4. Data points shown are mean ± SE of at least three independent measurements.

earlier, to obtain the dissociation constant (K_d) utilizing the following equation:[14]

$$\left(\frac{F}{F_0}\right) = 1 + \frac{[\text{Lipid}]}{[\text{Lipid}] + K_d}$$

The dissociation constant (K_d) of Zn^{2+} with respect to the probe in POPC membranes was found to be 2.68 μM, which clearly implies that its affinity for Zn^{2+} is approximately 3-fold higher in lipid membranes compared to that in DMSO solution. Moreover, the Zn^{2+} ion-dependent change in fluorescence intensity of the probe suggests the interfacial localization of the probe as the ions do not permeate in the hydrophobic region of the membrane. In membranes, the sensing activity occurs in a 2-dimensional surface rather than in a 3-dimensional solution (DMSO or any homogeneous solution). Thus, the effective concentrations of both probe and Zn^{2+} ion increase in 2-dimensional surface with increasing probability of productive collisions between a probe molecule and Zn^{2+} ion,[15] and thereby enhancing the probability of probe-ion interaction. Therefore, the higher affinity could be due to the complex formation between the probe and Zn^{2+} at the membrane surface.

We have further evaluated the selectivity of the lipid-incorporated probe toward Zn^{2+} ion by measuring the fluorescence intensity in presence of other metal ions (6 μM). Interestingly, we have observed an enhancement of fluorescence intensity only in presence of Zn^{2+} ion (Fig. 3).

Moreover, we have modulated the membrane surface charge by adding various proportions of negatively charged lipid 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphoglycerol (POPG) to POPC, and evaluated Zn^{2+} affinity of the probe in different POPC/POPG lipid membranes. Fluorescence intensities of the probe in presence of different concentrations of Zn^{2+} ions in POPC/POPG (90/10 mol%), POPC/POPG (80/20 mol%), and POPC/POPG (70/30 mol%) membranes are shown in Fig. 4(A–C).

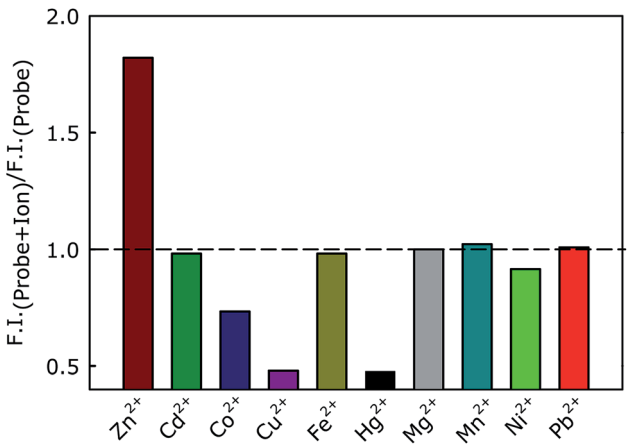

Fig. 3 Relative fluorescence of probe incorporated membrane in presence of various metal ions. The probe and ion concentrations were kept constant at 5 μM and 6 μM, respectively and was excited at 340 nm and emission intensity was measured at respective emission maximum. All measurements were carried out in 10 mM phosphate buffer, pH 7.4. Data points shown are mean ± SE of at least three independent measurements.

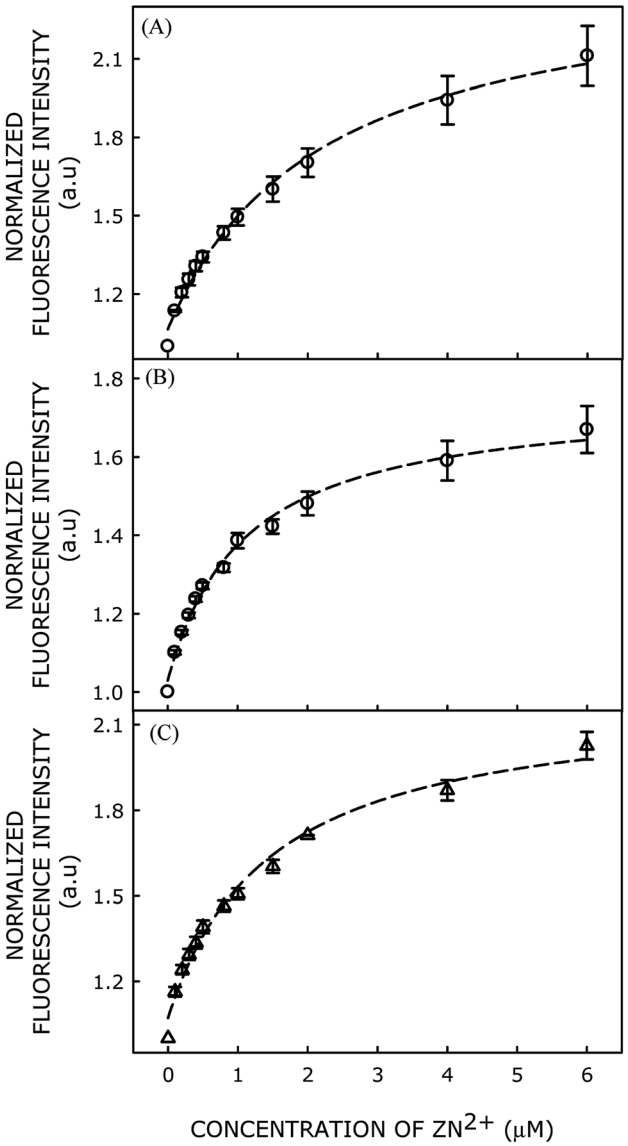

Fig. 4 Plot of normalised fluorescence intensity of the probe as a function of Zn^{2+} ion concentration. Approximately 500 μM of a mixture of (A) POPC/POPG (90/10 mol%), (B) POPC/POPG (80/20 mol%), and (C) POPC/POPG (70/30 mol%) were used at room temperature for this experiment. The probe concentration was kept constant at 5 μM, and was excited at 340 nm to measure emission intensity at respective emission maxima. All measurements were performed in 10 mM phosphate buffer, pH 7.4. Data points shown are mean ± SE of at least three independent measurements.

Dissociation constant of Zn^{2+} binding to the probe in various membranes has been calculated using classical Langmuir model for adsorption of ligand to multiple, equivalent, and non-interacting surface sites, and subsequently plotted as a function of mol% of POPG in Fig. 5. Interestingly, the K_d value decreases (*i.e.*, affinity for Zn^{2+} increases) with increasing mol fraction of negatively charged POPG in the membrane (Fig. 4), eventually saturating beyond 20 mol% of POPG. Our results clearly

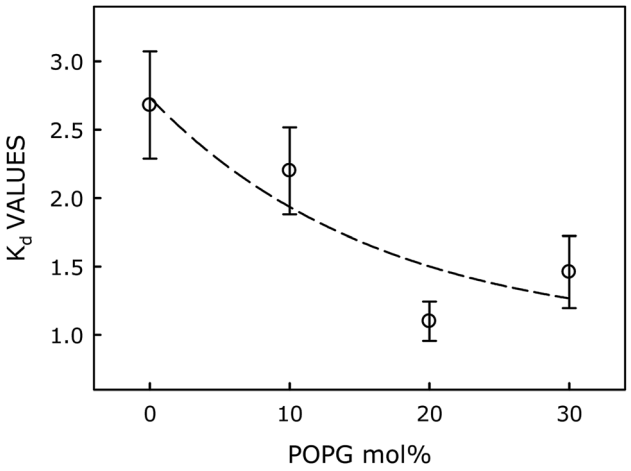

Fig. 5 Plot of K_d as a function of mol% of the negatively charged lipid (POPG). Lines joining data points are provided as viewing guides.

demonstrate that binding affinity of the probe toward Zn^{2+} ion increases significantly with increasing negative charge on the membrane surface. This implies that the negative charge of the membrane augments Zn^{2+} ion concentration near the membrane surface, which increases affinity of the probe for binding with Zn^{2+} ion.

Taken together, our results suggest that membrane encapsulation of the macrocyclic probe (insoluble in aqueous medium) enables it to sense nanomolar concentration (detection limit is in nanomolar) of Zn^{2+} in phosphate buffer at pH 7.4 (schematic representation in Fig. 6). Moreover, Zn^{2+} sensitivity of the probe can be fine-tuned by manipulating the proportion of negative charge on the membrane surface. Successful application of the membrane-incorporated probe as an ion sensor in aqueous solution opens up the possibility to utilize an organic solvent-soluble probe in aqueous medium, without undergoing any synthetic modification.

Conclusion

In conclusion, this study proposes lipid encapsulation of a thiocarbonohydrazone-locked salicylidene-based macrocyclic probe as a strategy to selectively detect Zn^{2+} ions in aqueous medium with approximately 3-fold higher efficiency. Further, introduction of negative surface charge on the membrane enhances its ion-sensing efficiency dramatically. Our findings can open up novel possibilities of ion sensing in aqueous medium by using hydrophobic probes, without undergoing any synthetic modifications.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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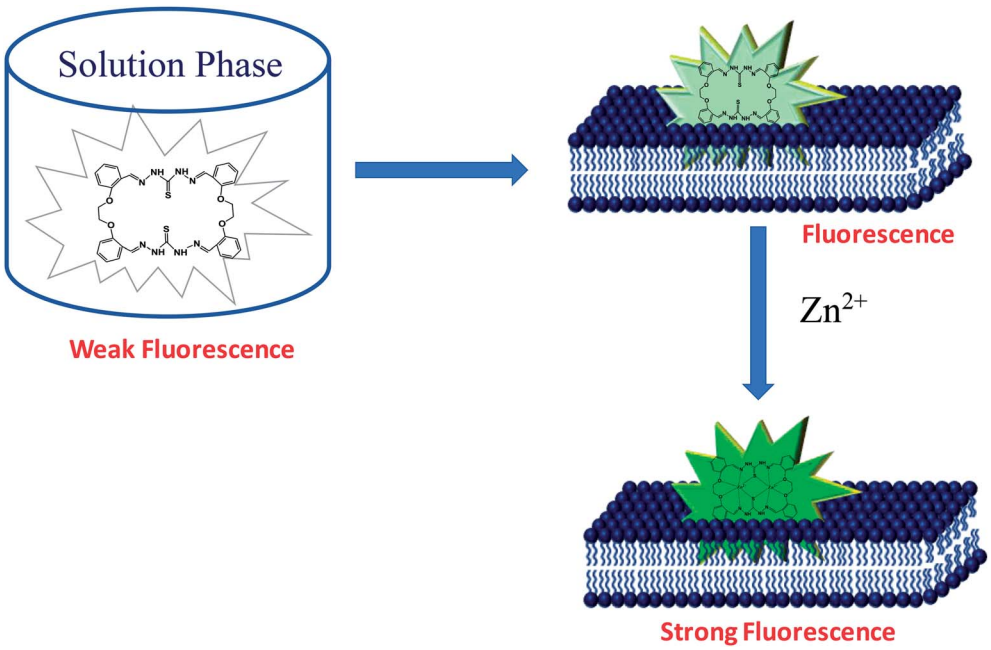

Fig. 6 Schematic representation of membrane incorporation of the ion sensor for ion detection in aqueous solution.

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