



Rational design of hyperpolarized xenon NMR molecular sensor for the selective and sensitive determination of zinc ions

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ABSTRACT

Although Zn^{2+} ions are involved in large numbers of physiopathological processes, non-invasive detection of Zn^{2+} ions in opaque biological samples remains a huge challenge. Here, we developed a novel zinc-responsive hyperpolarized (HP) ^{129}Xe -based NMR molecular sensor. This HP ^{129}Xe -based NMR molecular sensor was synthesized by attaching 2-(diphenylphosphino) benzenamine as ligand for zinc ions to the xenon-binding supramolecular cage, cryptophane. The ^{129}Xe NMR spectroscopy of such molecular sensor was shifted up to 6.4 ppm in the presence of Zn^{2+} ions, which was nearly four times larger than that of the reported similar sensor. The application of the sensor would benefit low concentration detection by using indirect NMR/MRI method. The response exhibited high sensitivity and selectivity as discriminated from other six potentially competing metal ions. The application of this sensor in the analysis of zinc ions in the rat serum samples was also evaluated. The strategy is generally applicable in developing sensitive and selective sensors for quantitative determination of zinc ions.

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1. Introduction

Zinc is an essential trace element in the human body [1] and is the second most abundant transition metal in the living organisms [2]. Zn^{2+} ions not only constitute many enzymes [2] and proteins [3], but also play a key role in various physiological processes [4]. Either a deficiency or excess of Zn^{2+} ions will cause a physiological dysfunction of the organism. Thus, it is of great significance to develop a highly sensitive and specific method for the detection of Zn^{2+} ions.

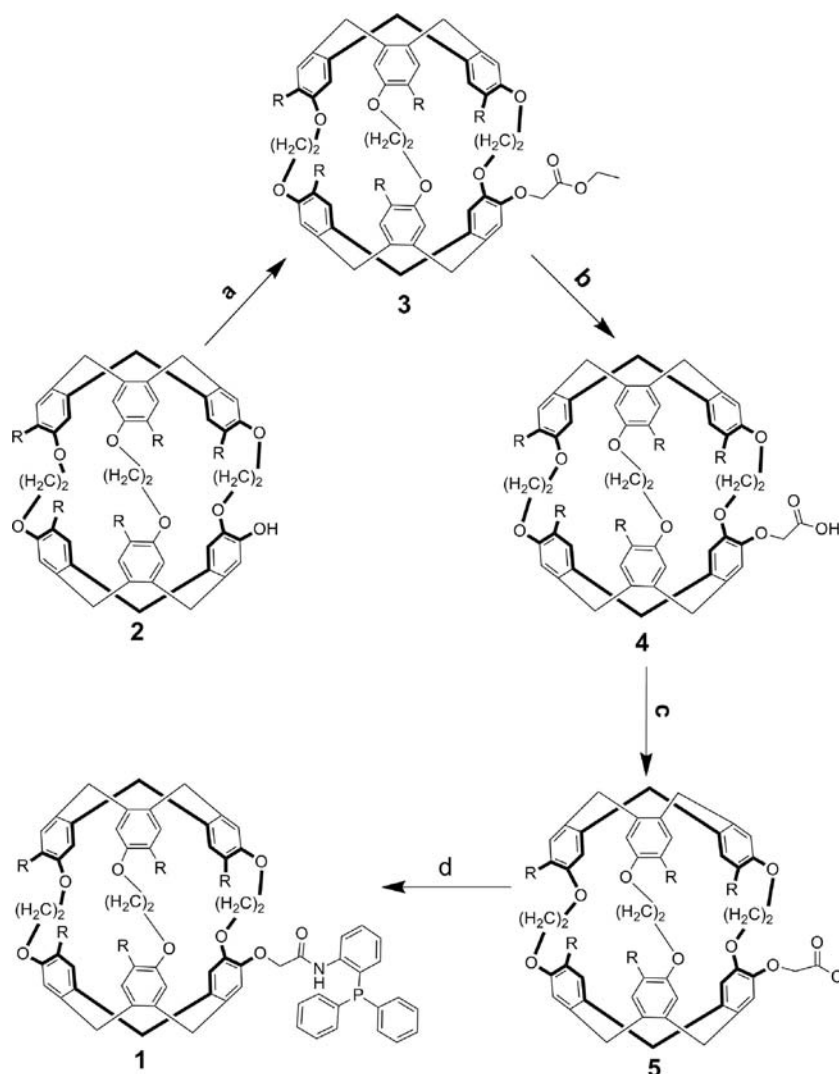
There are many chemosensors for detection of Zn^{2+} , which are mostly based on quinoline, benzazole or fluorophores. However, the major chemosensors are unsuitable to offer real-time monitoring of the Zn^{2+} distribution in different tissues of the body. Moreover, NMR molecular sensors could provide a potential tool for detecting the Zn^{2+} ions in the living organisms because of its non-invasive and non-destructive their characters. However, the conventional NMR sensors are not as sensitive as most of their peers such as fluorescent sensors [5,6]. The application of NMR molecular sensors has been therefore limited by their intrinsic low sensitivity at thermal equilibrium. In contrast, the use of hyperpolarized ^{129}Xe based NMR molecular sensor suggests a possible solution [7]. The nuclear spin of the xenon atom can be hyperpolarized by a spin-exchange optical pumping (SEOP) technique [8–10], and the

resulted nuclear spin polarization could be enhanced by four orders of magnitude in comparison to the Boltzmann polarization [11], leading to an amplification of the NMR sensitivity by a factor of 10,000 [12].

Cryptophanes are an important class of supramolecular hosts that can form stable inclusion complexes with small molecules [13–16], and cryptophane-A is considered as one of the most suitable host molecules for xenon [17]. A Xe-cryptophane complex can be formed by self-assembly of xenon with a cryptophane, and the ^{129}Xe NMR spectra display two dramatically different chemical shifts for the encapsulated Xe and free Xe in solution [16]. As Xe has large, polarizable electron cloud, the chemical shift of encapsulated Xe was extremely susceptible to the surrounding environment. A Xe-based sensor is usually formed by the combination of the cryptophane and a ligand via a molecular linker, and when the ligand interacts with the targeting molecule, it will affect the electron density experienced by the encapsulated Xe, resulting in the change of chemical shift. Therefore, hyperpolarized ^{129}Xe -based NMR molecular sensor could be an exceptional tool for the sensitive detection of targeting molecules. Such Xe-based sensor strategy has already been used for the detection of a variety of biological systems, including proteins [7], enzymes [18], nucleic acids [19], metal ions [20] and in-cell biological targets [21]. 2-(diphenylphosphino) benzenamine and its derivatives are a group of ligands [22–24], which are able to chelate with zinc ions [22]. It is widely used as a highly efficient and selective ligand, due to its feasible preparation, stability in air, and great selectivity.

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Scheme 1. Synthesis of HP ¹²⁹Xe-based NMR molecular sensor 1. R=OCH₃. Conditions and reagents: (a) Br-CH₂COOC₂H₅, K₂CO₃, acetone, 56 °C, 5 h, 81%; (b) NaOH, THF, 66 °C, overnight, 95%; (c) SOCl₂, Et₃N, DCM, N₂, 39 °C, overnight; (d) C₁₈H₁₆NP, N₂, 25 °C, 12 h, 44%.

In this study, a HP ¹²⁹Xe-based NMR molecular sensor exploiting 2-(diphenylphosphino) benzenamine as a ligand for the capture of zinc ions was synthesized. The synthesized sensor was composed of a cryptophane moiety as a Xe-based NMR signal reporting part, 2-(diphenylphosphino)benzenamine as the chelating moiety, and a molecular linker (Scheme 1). The detection of Zn²⁺ ions by the new HP ¹²⁹Xe-based NMR molecular sensor was conducted, and the HP ¹²⁹Xe-based NMR molecular sensor responded to Zn²⁺ ions with a high selectivity. It is worth noting that the chemical shift difference of the encapsulated xenon is nearly four times larger than that of the reported similar sensor, which is important for the low concentration detection by using indirect NMR/MRI method, such as Hyper-CEST [25], and the feasibility of the application of the indirect NMR/MRI method has also been improved because of the largely enhanced chemical shift difference.

2. Experimental

2.1. Materials

2-(Diphenylphosphino) benzenamine was purchased from VsciChemTM Technology (Beijing) CO., Ltd. Thionyl chloride was

purchased from Shanghai Jin Shan Ting XinTM Chemical Reagent Factory. Deuterated toluene-d₈ was obtained from LandisvilleTM NJ Norell Inc. USA, and 1,1,2,2-tetrachloroethane was commercially available from SinopharmTM Chemical Reagent Beijing Co., Ltd.

2.2. Apparatus

A tailor-designed xenon hyperpolarizer was used in this study [26]. The high performance liquid chromatographs (HPLC) were produced by the Scientific Software International, Inc. The ¹H NMR and ¹²⁹Xe NMR spectra were obtained with a BrukerTM AVANCE 500 spectrometer and BrukerTM AVANCE III 400 spectrometer, respectively, and the mass spectra were produced by a BrukerTM micrOTOFQ spectrometer.

2.3. Synthesis of sensor 1

2.3.1. Synthesis of compound 3

To conjugate the alkyl linker to Cryptophane-Cage 2, 881 mg (1 mmol) compound 2 was dissolved in 30 mL acetone. Excess amounts of anhydrous potassium carbonate and ethyl bromoacetate were added to the solution, and the reaction was allowed to initiate and sustained with stirring and refluxing for 5 h, followed by a series of processes including cooling down to room temperature, filtering,

and solvent evaporation. The recovered substance was purified by silica gel column chromatography to yield product **3**, which was a white solid substance, and the eluent used was chloroform: hexane=5:1(v/v), resulting a ~81% yield. The synthesis was performed under the guiding of Ref. [27].

2.3.2. Synthesis of compound **4**

Compound **3** was dissolved in 30 mL tetrahydrofuran, followed by the addition of 4 M sodium hydroxide aqueous. The reaction was allowed to stir overnight with refluxing. The solution was then cooled down to room temperature, extracted by chloroform, and evaporated under vacuum. The white recovered substance was subject to a silica gel column chromatography to produce a pure product **4**, which was a white solid substance. The eluent used was chloroform: hexane=4:1 (v/v), resulting ~95% yield of product **4**. The preparation of compound **4** was carried out by referring to the procedure described in ref [27].

2.3.3. Synthesis of cryptophane-2-(diphenylphosphino)benzenamide (sensor **1**)

Compound **4** (30 mg, 0.032 mmol) was dissolved in 20 mL of dry dichloromethane in a dry flask, where 2 mL of dry thionyl chloride was added. The reaction was stirred overnight with reflux under a nitrogen atmosphere. The solution was then evaporated under vacuum to obtain compound **5**.

9 mg (≈ 0.03 mmol) of 2-(diphenylphosphino)benzenamine was dissolved in 10 mL dry dichloromethane, to which 12 mg dry triethylamine was added afterwards. The compound **5** was dissolved in 10 mL dry dichloromethane and added to a ligand-contained solution in an ice bath under nitrogen atmosphere. The reaction was then allowed to stir for 12 h, followed by an addition of water and extraction of reaction product by using dichloromethane. The combined organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated to a small volume. The recovered substance was subsequently purified by silica gel column chromatography to generate white solid product **1** in ~44% yield, where an eluent of dichloromethane: methanol=50:1 (v/v) was used.

^1H NMR (CDCl_3 , 20 °C, 500 MHz) chemical shifts referenced to $(\text{CH}_3)_4\text{Si}$: δ =8.21(s, 1H; NH), 7.81(q, 2H; Ar), 7.76(t, 4H; Ar, J=9.8 Hz), 7.69(q, 3H; Ar), 7.64(m, 2H; Ar), 7.59(d, 3H; Ar, J=8.6 Hz), 7.08(s, 1H; Ar), 7.00(s, 1H; Ar), 6.89(m, 4H; Ar), 6.85(d, 2H; Ar, J=6.0 Hz), 6.78(s, 3H; Ar), 6.75(s, 1H; Ar), 4.72(m, 24H; CH_2), 3.89(m, 12H; CH_3), 3.78(s, 3H; CH_3).

MS (ESI) m/z : calcd for $\text{C}_{73}\text{H}_{68}\text{NO}_{13}\text{P}$: 1236.4428 $[\text{M}+\text{K}]^+$; found: 1236.4167.

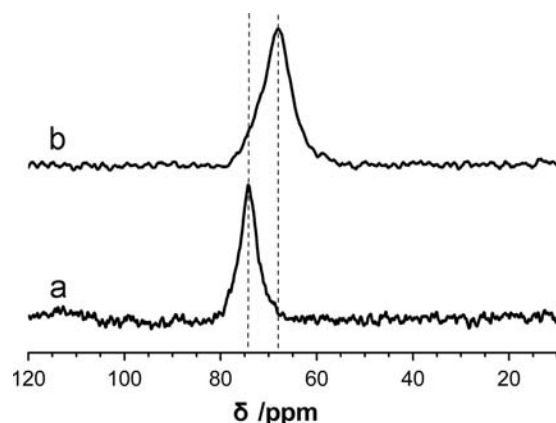


Fig. 1. High-field region of the ^{129}Xe NMR spectra obtained for sensor **1** (100 μM) in the presence of Zn^{2+} . (a) $C_{\text{Zn}(\text{II})} = 0 \mu\text{M}$; (b) $C_{\text{Zn}(\text{II})} = 1680 \mu\text{M}$.

2.4. Preparation of hyperpolarized xenon

The hyperpolarized xenon was prepared via the spin-exchange optical pumping method [8,9,28] by using an in-house-made xenon polarizer, which was developed based on our on-site equipment and techniques [11,26,29]. The gas mixture comprised 2% Xe, 10% N_2 , and 88% He; the temperature in the pumping cell was 438 K and the pressure was 74 psi. The flow rate of xenon gas mixture was 0.1 standard litre per minute with an average xenon spin polarization of ~20%. After the polarization process, the hyperpolarized xenon was transferred into a 10-mm NMR tube containing the sample for NMR spectrometry.

2.5. ^{129}Xe NMR experiments

All the ^{129}Xe NMR experiments were conducted on a BrukerTM AVANCE III 400 spectrometer equipped with a 10-mm double tuned liquid probe. The Larmor frequency of ^1H and ^{129}Xe was 400.17 MHz and 110.69 MHz, respectively. The amplitude of radio frequency-B1 field was set at 8.9 kHz for ^{129}Xe NMR. The sample was placed into a tailor-made NMR tube for the transferring of hyperpolarized Xe gas from the polarizer. All the xenon chemical shifts in solution were evaluated by referring to the xenon gas signal, which was calibrated at 0 ppm.

3. Results and discussion

The ^{129}Xe NMR experiments clearly distinguished sensor **1** and its chelate with Zn^{2+} ions (Fig. 1). In the absence of Zn^{2+} ions, there were two signals presenting in ^{129}Xe NMR spectroscopy, which were assigned to dissolved free xenon at $\delta = 212.9$ ppm and xenon caged in **1** (Xe@**1**) at $\delta = 74.2$ ppm. The addition of Zn^{2+} ions resulted a change of chemical shift of Xe@**1** from $\delta = 74.2$ ppm to $\delta = 67.8$ ppm, which demonstrated that the Zn^{2+} ions were chelated with the ligand, and this in turn modified the electron density experienced by the encapsulated Xe and consequently its chemical shift.

Since the chemical shift of xenon is extremely sensitive to the chemical environment, to confirm these results, it is necessary to examine the direct influence of the free Zn^{2+} ions on Xe@**1**.

To investigate the influence of free Zn^{2+} ions in solution, 1680 μM Zn^{2+} ions were added into 100 μM compound **4**, which had cryptophane cage without the modification of chelating moiety (Fig. 2b). The chemical shift of the encapsulated xenon did not have an obvious change with the addition of Zn^{2+} ions (Fig. 2), indicating that Zn^{2+} ions could not directly affect the chemical shift of the caged xenon. This result also proved that the chelating moiety modified cryptophane cage was chelated to Zn^{2+} ions through a chelating reaction, which was the reason for the chemical shift change of Xe@**1**.

Furthermore, the affinity of the sensor to Zn^{2+} ions was evaluated by adding Zn^{2+} ions at four different concentrations into 100 μM sensor **1** (Fig. 3). No significant change in the chemical shift of Xe@**1** was observed for the sample containing 1.68 equiv (Fig. 3a) Zn^{2+} ions. Whereas, in the case of the sample added with 3 equiv Zn^{2+} ions, an altering was observed for the signal of Xe@**1** at the signal bottom (Fig. 3b). As the Zn^{2+} ions concentration increased to 8 equiv (Fig. 3c), the peak of Xe@**1** was split into two, which were assigned to Xe@**1** with and without chelated Zn^{2+} ions. When the Zn^{2+} ions at the concentration of 16 equiv were used (Fig. 3d), the signal shifted up-field by 6.4 ppm as compared with the signal of the none Zn^{2+} sample. Through the deconvolution of spectra, the dependence of the chelated sensor on the concentration of the added Zn^{2+} ions was evaluated. Through the simulation of the stability constant equation, the stability constant

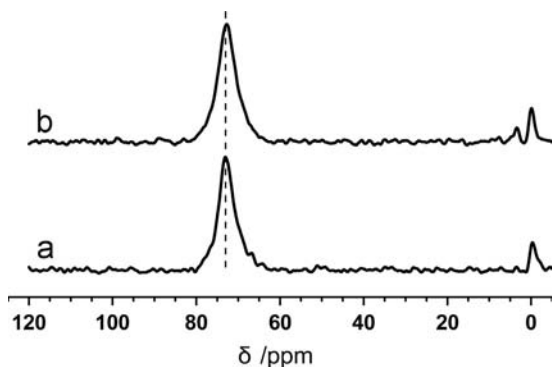


Fig. 2. High-field region of the ^{129}Xe NMR spectra obtained for compound **4** ($100\ \mu\text{M}$) in the absence and in the presence of Zn^{2+} . (a) $C_{\text{Zn(II)}}=0\ \mu\text{M}$; (b) $C_{\text{Zn(II)}}=1680\ \mu\text{M}$.

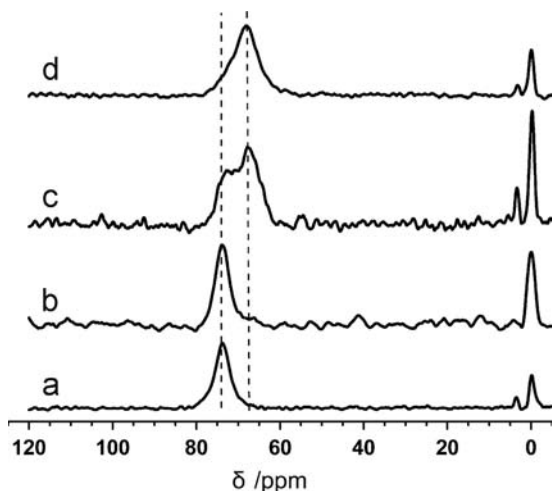


Fig. 3. High-field region of the ^{129}Xe NMR spectra obtained for sensor **1** ($100\ \mu\text{M}$) in the presence of four concentrations of Zn^{2+} . (a) $C_{\text{Zn(II)}}=168\ \mu\text{M}$; (b) $C_{\text{Zn(II)}}=300\ \mu\text{M}$; (c) $C_{\text{Zn(II)}}=800\ \mu\text{M}$; (d) $C_{\text{Zn(II)}}=1680\ \mu\text{M}$.

was determined $K=8.27 \times 10^6\ \text{M}^{-2}$. Normally, a high affinity is preferable during the development of the molecular sensor to increase the detection sensitivity. The low affinity caused by a relatively high Zn^{2+} ions concentration in contrast to the case of sensor **1** was inevitable to result in a distinct chemical shift change of the caged Xe. Fortunately, this potential problem of suppressed sensitivity had been overcome by the ultrahigh sensitivity because of hyperpolarization, allowing a greatly enhanced sensitivity more than 10,000 times higher than that of the traditional NMR.

Further, to demonstrate the specificity of the sensor to Zn^{2+} ions, we investigated the response of sensor **1** to calcium(II), magnesium(II), mercury(II), palladium(II), copper(I), and silver(I) ions, with the ^{129}Xe NMR spectra shown in Fig. 4. No noticeable influence of the added metal ions on the NMR signal was observed, except for Zn^{2+} . The obtained results indicated that the sensor developed in this study was highly specific to the detection of Zn^{2+} ions among potentially competing metal ions.

The magnitude of the chemical shift difference of $\text{Xe}@\mathbf{1}$ after the chelating with the metal ions demonstrated the sensitivity of sensor to the targeted ions. A large chemical shift difference would alleviate the resolution requirement of the NMR/MRI, as the resolution for the detection of the complex biological samples could be very low due to the field-shimming problem. Furthermore, a relatively large chemical shift would be extremely important for some high sensitivity indirect measurement methods, e.g. Hyper-CEST [25] and MT. Hyper-CEST possesses the highest sensitivity among the NMR/MRI methods known so far, and

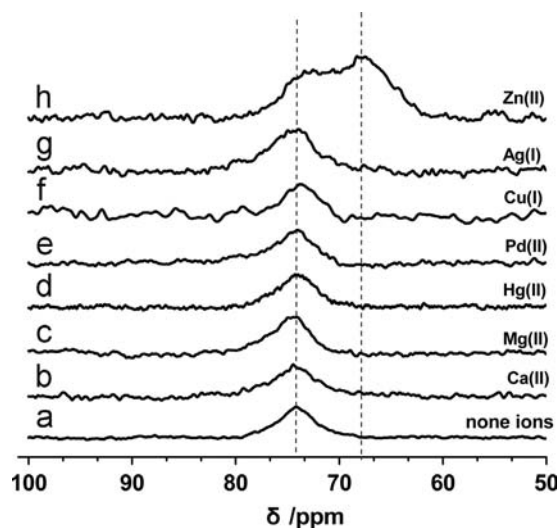


Fig. 4. High-field region of the ^{129}Xe NMR spectra obtained for sensor **1** ($100\ \mu\text{M}$) in the presence of various metal ions in concentration of $800\ \mu\text{M}$. (a) None ions; (b) Ca^{2+} ; (c) Mg^{2+} ; (d) Hg^{2+} ; (e) Pd^{2+} ; (f) Cu^{+} ; (g) Ag^{+} ; (h) Zn^{2+} .

Table 1
Recovery studies of zinc ions in rat serum samples.

Sample	Taken (μM)	Found (μM)	Recovery (%)	RSD (%)
1	800	774	96.7	6.4
2	1600	1657	103.5	5.6
3	3200	3106	97.1	7.0
4	6400	6321	98.7	7.3

sensitivity is of vital importance for its application in low concentration detection [25]. However, because of its intrinsic indirect measuring method, its frequency resolution was much lower than that of the conventional methods, and this leads to a high requirement for the chemical shift sensitivity of the sensor. In this work, the ^{129}Xe NMR based sensor **1** showed a 6.4 ppm chemical shift change in respect to that of the Zn^{2+} ions, which was much larger than 1.7 ppm of the similar sensor as reported [20].

In order to investigate the reproducibility of this method, the inter-assay precision was estimated by determining the response of $100\ \mu\text{M}$ sensor **1** for six times, which were immersed in $400\ \mu\text{M}$ zinc ions. The coefficients of variation (CV) were calculated to be 1.7%, indicating acceptable fabrication reproducibility. The intra-assay precision of the sensors was evaluated by assaying one sensor **1** for six replicate determinations, and the CV was 0.87% at the zinc ions concentration of $400\ \mu\text{M}$. When the sensor **1** was not in use, it was stored at $4\ ^\circ\text{C}$ in dry condition. No obvious variation in the response to zinc ions was observed in the first 10-day storage. After a 30-day storage period, the sensor retained 90% of its initial response, indicating an acceptable stability.

To further demonstrate the practicality of the proposed sensor, the recovery test was studied by adding different amounts of zinc ions into rat serum samples. The recoveries were from 96.7% to 103.5%. The average precision was $\pm 6.0\%$ (Table 1). The results indicated that the proposed method was highly accurate, precise and reproducible. It can be used for direct analysis of practical samples.

4. Conclusion

In this article, we have shown the rational design of the HP ^{129}Xe -based NMR molecular sensor and the specific determination

of Zn^{2+} . More importantly, this ^{129}Xe -based NMR molecular sensor possesses excellent chemical shift sensitivity, which is desirable for the low concentration indirect NMR/MRI measurement. By taking the advantages of the magnetic resonance molecular imaging, this zinc-activated HP ^{129}Xe -based NMR molecular sensor suggests a great potential to be used in the monitoring of Zn^{2+} ions and investigating Zn^{2+} ions related physiopathological processes in biological organisms in the foreseeable future.

Acknowledgments

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