Metal-Ion Sensors

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A Sensitive Zinc-Activated ^{129}Xe MRI Probe**

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The divalent zinc cation, Zn^{2+}, is an indispensable and ubiquitous element of the body.[1] As the second most abundant transition-metal ion in mammalian tissues, it is involved in many physiological and pathological processes. Zinc plays a vital role not only when bound to metalloproteins, but also in the form of mobile pools. A slight excess or lack of zinc ions can be connected to serious human afflictions, including heart disease, diabetes, cancer, and neurodegeneration such as Alzheimer's disease.[2]

Today, only two noninvasive techniques, optical imaging and magnetic resonance imaging (MRI), have the potential to offer real-time monitoring of the Zn^{2+} distribution in different tissues of the body. However, optical methods suffer from limited penetration depth, which makes them unsuitable for global analysis of relatively large and opaque specimens, such as live animals.[3] On the other hand, MRI is a particularly powerful modality used clinically for anatomic imaging and provides three-dimensional images with excellent resolution. However, conventional molecular MRI techniques that rely on the observation of water protons and require the introduction of contrast agents still suffer from reduced sensitivity and often lack selectivity.[4] A few studies based on gadolinium complexes have been reported for Zn^{2+} imaging.[5] Nevertheless, to our knowledge, the detection threshold of free Zn^{2+} ions is 30 μM, a value slightly above the total Zn^{2+} concentration of 20 μM in blood. Therefore, the development of more sensitive methods is of crucial importance.

Herein we propose the use of hyperpolarized ^{129}Xe nuclear magnetic resonance (NMR) spectroscopy for the sensitive detection of Zn^{2+} ions. To achieve this goal, the noble gas is encapsulated in dedicated host systems bearing a ligand that chelates the Zn^{2+} ions. Cryptophanes, aromatic cage molecules made of cyclotriveratrylene groups,[6] are perfectly suited to this purpose as 1) they can easily be rendered water-soluble,[7] 2) the noble gas has a high affinity for their cavity,[8] 3) when xenon is encapsulated, it takes a specific NMR frequency, and 4) xenon exchange in and out of the cavity insures a continuous refreshment of the Xe@cryptophane environment in hyperpolarization.

Such a ^{129}Xe biosensing approach has already been employed for detection of various biological systems, including enzymes[9–11] and nucleic acids.[12] Also, the first in-cell probing of biological events has been achieved: the endocytosis of transferrin could be detected by using ^{129}Xe NMR spectroscopy.[13] All these NMR spectroscopy studies based on the use of hyperpolarized xenon and molecular hosts are characterized by a high sensitivity. However, metal detection is a difficult challenge, which has never been achieved using such an approach.

We aimed to design a responsive agent in which the chemical shift of encapsulated xenon would significantly vary when Zn^{2+} ions are chelated to it. In this manner, a sensitive spectroscopic imaging based on this resonance-frequency variation can be envisioned. For this purpose, we designed sensor **1**, which is made of three parts (Scheme 1): the cryptophane core hosting xenon, the spacer, and the chelating moiety. A short spacer was chosen to place the chelating moiety near the cryptophane cavity. As a zinc-chelating group we chose nitrilotriacetic acid (NTA), which is easily prepared from L-lysine.[14] Sensor **1** was synthesized from cryptophane **2**, which possesses six carboxylate groups ensuring solubility in water at physiological pH value.[7] We were able to activate only one carboxylate group by esterification with *N*-hydroxysuccinimide. Then, the primary amino group of the enantiopure unit bearing the NTA moiety (L)-**3** was directly coupled to this activated ester to form a chemically stable amide linkage. Thus, the use of host **2** in its racemic form (chirality is due to the helicity of the cryptophane) led to two diastereomers, which will be noted **1P** and **1M** (see the Supporting Information for the nomenclature).

Compound **1** was obtained as a 50:50 diastereomeric mixture (**1P** + **1M**), with a chemical purity higher than 95% after HPLC.

For this sensor, the xenon binding constant is assumed to be in the same range as that of compound **2**, that is, 6000 M^{-1}. As cryptophane **2** has already been shown to behave as a pH sensor,[15] the present ^{129}Xe NMR spectroscopy study was conducted in a phosphate buffer exempt of other ions, at pH 7.4. At this pH value, the affinity of the NTA group for Zn^{2+} ions is well-documented ($\log K_1 > 10$).[16] In the absence

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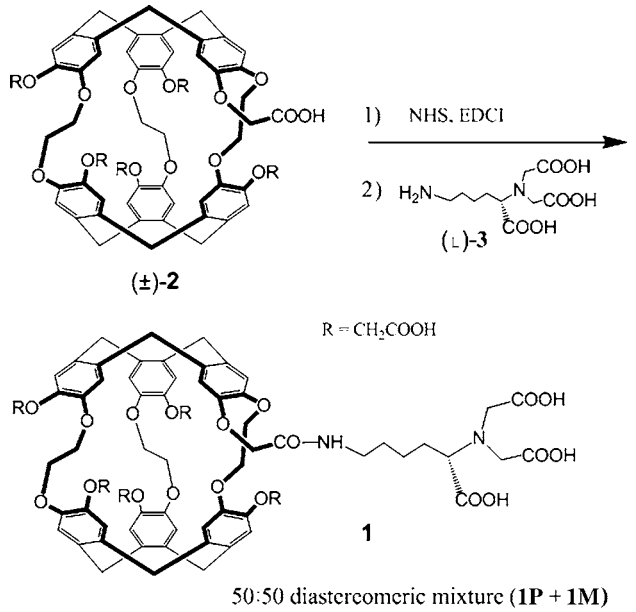

Scheme 1. Synthesis of the Zn-activated ^{129}Xe NMR sensor **1**. For simplicity, only the *PP* isomer of **2** and the corresponding diastereomer of **1** are drawn. For the nomenclature of **2** see the Supporting Information. NHS = *N*-hydroxysuccinimide, EDCI = 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide.

of Zn^{2+} ions, the ^{129}Xe NMR spectrum of **1** at 293 K exhibits two signals: the signal of free xenon in water calibrated at $\delta =$ 196 ppm and at $\delta = 65.6$ ppm the signal of xenon caged in **1** (Figure 1, bottom spectrum). Remarkably, the addition of Zn^{2+} ions splits the latter signal into two new signals with similar intensities located at $\delta = 65.75$ and 67.2 ppm (Figure 1). The emergence of these signals is almost certainly a consequence of the zinc chelation by the NTA moiety, which in turn modifies the electron density experienced by the encapsulated noble gas and consequently its chemical shift. It is likely that Zn^{2+} binding by the NTA probe leads to a decrease in the flexibility of the linker, thus allowing

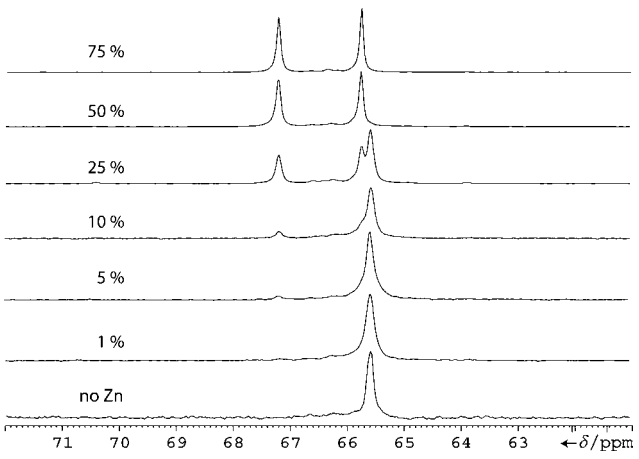

Figure 1. High-field region of the ^{129}Xe NMR spectrum obtained in one scan for **1** (260 μM) in the presence of various proportions of Zn^{2+} ions (w/w%).

^{129}Xe NMR spectroscopy to distinguish between the two diastereomers of **1**.

To check this hypothesis, the two diastereomers **1P** and **1M** were synthesized separately. The two enantiomers of the hexacarboxylate cryptophane derivative **2** were prepared according to a known procedure from enantiopure cryptophanol-A derivatives.[17] The unit bearing the NTA moiety (L)-**3** was then coupled to each enantiomer of **2** by using the same procedure as for the racemic compound. Each compound was independently isolated by reversed-phase HPLC. Figure 2 displays the hyperpolarized ^{129}Xe NMR spectra of the two compounds in the presence of 50% Zn^{2+} ions. The ^{129}Xe NMR spectrum of the diastereomer **1P** shows a single

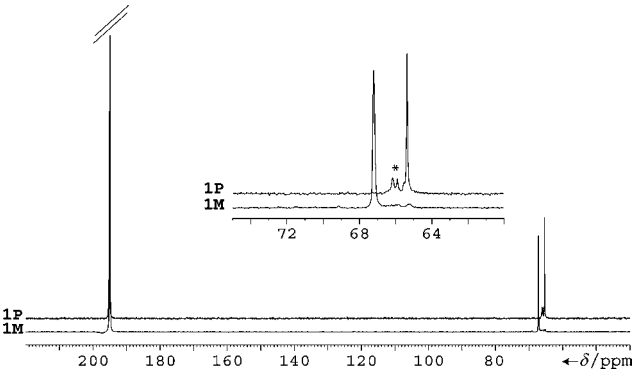

Figure 2. One-scan ^{129}Xe NMR spectra for the two diastereomers **1P** (up) and **1M** (bottom) in the presence of 50% Zn^{2+} ions (w/w). The region around $\delta = 66$ ppm is enlarged. The star denotes minor forms of the cryptophane (see the Supporting Information for the HPLC results).

signal at $\delta = 65.75$ ppm corresponding to the most upfield-shifted signal for encapsulated xenon in the case of the diastereomeric mixture. In a similar way, the ^{129}Xe NMR spectrum of the diastereomer **1M** exhibits a single signal that matches the downfield signal ($\delta = 67.2$ ppm) observed with the diastereomeric mixture. These observations unambiguously confirm our hypothesis and demonstrate that working with enantiopure cryptophane derivatives leads to the appearance of a single new peak when Zn^{2+} is encountered. Large chemical-shift differences for xenon in two cryptophane diastereomers have already been reported.[18] They were explained by the different distortions of the cage for the two diastereomers owing to the interaction of the substituent and the cryptophane core.

It is worth noting (see Figure 1) that saturation occurs around 50% Zn^{2+}, because no further spectral change is observed at higher Zn^{2+} concentrations. This behavior is indicative of a dimerization process, where two molecules of **1** are required to complex one Zn^{2+} ion. This result is confirmed by self-translational diffusion ^{1}H NMR experiments performed with and without Zn^{2+} cations. In the presence of 50% Zn^{2+}, the diffusion coefficient decreased significantly from $(3.87 \pm 0.10) \times 10^{-10}$ to $(2.09 \pm 0.11) \times 10^{-10}$ m^{2}s^{-1} (details in the Supporting Information, Figure S1). This undoubtedly confirms the presence of a 2:1 complex.

Considering this kind of association, it is easily found that three pairs of stereomers are present in solution, when **1** is used in its racemic form. These stereomers can be noted as follows: [Zn(**1P1P**)], [Zn(**1M1M**)], and [Zn(**1P1M**)]. Thus the presence of only two signals for the Zn^{2+} complexes in Figure 2 suggests that xenon in a cryptophane is unable to detect the configuration of the other cryptophane of a Zn complex. As a consequence the chemical shift of xenon present within the cavity of a cryptophane is independent of the absolute configuration of the second cryptophane present on the opposite side of the molecule.

We studied the influence of Ca^{2+} and Mg^{2+} ions on the ^{129}Xe NMR spectrum to test the selectivity of **1** for zinc over other biologically relevant, potentially competing, mobile metal cations. No noticeable effect was observed upon addition of either of these cations. This result was expected given the lower affinity of these metals for the NTA group (three to four orders of magnitude).[16]

Given the sharpness of the signals, the chemical-shift difference of 1.7 ppm (for compound **1M**) between the signals of encapsulated xenon in the absence and in the presence of Zn^{2+} ions is largely sufficient for spectroscopic MRI or in vitro and even in vivo localized spectroscopy. Figure 3 displays images obtained with a dedicated frequency-selective gradient echo sequence. Comparison between images a and b

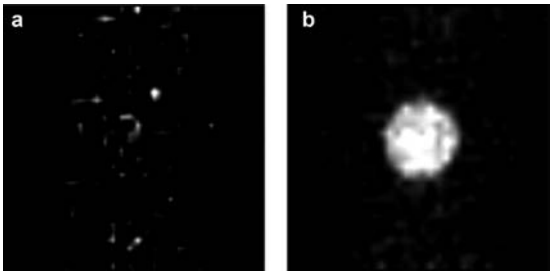

Figure 3. ^{129}Xe axial gradient-echo fast imaging of an 8 mm tube containing sensor **1** (racemic mixture) at 200 μM. In a) the solution contained Ca^{2+} ions at 150 μM. In b) Zn^{2+} ions at a concentration of 100 μM have been added. Images were acquired with the soft pulse centered at $\delta = 67.2$ ppm. Each experiment lasted 13 seconds. The signal-to-noise ratio measured on (b) is approximately 20.

in Figure 3 shows that Zn^{2+} ions can be specifically detected and localized at low concentration in a short time. The sensitivity threshold of our approach for Zn^{2+} detection has been assessed. In a single introduction of 1 atm of hyperpolarized xenon in the NMR tube we were able to detect Zn^{2+} at a concentration of 100 nanomolar (Figure S2 of the Supporting Information). To our knowledge, this is the first time that such a high sensitivity has been obtained, two orders of magnitude better than the other MRI methods. It is likely that the grafting of six NTA groups on an enantiopure version of compound **2** and/or more sophisticated pulse schemes of the HYPERCEST type[19] would further increase the sensitivity of the approach. Indeed recent work of the group of Pines suggests that saturation by multiple inversion pulses can achieve a spectral selectivity that is compatible with the frequency splitting observed herein.[20]

We constructed a powerful ^{129}Xe NMR-based sensor of Zn^{2+} ions, which enables for the first time measurement of Zn^{2+} concentrations as low as 100 nM. This high sensitivity was achieved thanks to a smart ^{129}Xe MRI sensor, where the binding of the ion to the target is accompanied by a change in NMR parameters. Several arguments are in favor of such a sensor based on ^{129}Xe NMR spectroscopy. Given that in–out xenon exchange occurs continuously, repetition of soft pulses at the appropriate frequency can further lower this threshold. Moreover, it is always possible to add hyperpolarized xenon in solution. The protocol thus involves delivery of the sensor and then successive introductions of hyperpolarized xenon. This differs substantially from MRI modalities that use contrast agents.

Herein, we have also demonstrated the importance of working with enantiopure cryptophane derivatives. The counterpart of a more challenging chemistry is the preparation of potent sensors, where a net change in frequency for encapsulated xenon, when the ion binds to the target, gives a further gain in sensitivity, even in low or inhomogeneous magnetic fields, such as those encountered in in vivo MRI.

The capacities of these types of sensors to detect intracellular zinc will now be assessed. Our previous experience shows that cryptophane derivatives are likely to penetrate cells.[13] To check this, the design of bimodal probes that are detectable through fluorescence and hyperpolarized ^{129}Xe NMR spectroscopy is underway.

Experimental Section

86%-enriched ^{129}Xe from Cortecnet was polarized by spin-exchange optical pumping of rubidium[21] by using a recently installed home-built setup based on laser diodes. The photons exiting from a duo-FAP system (2×30W) and circular polarizer from Coherent illuminated a Pyrex cell that was placed at the center of a 100G magnetic field. Because the bandwidth of the laser diodes was about 2 nm, pressure-broadening was necessary. Therefore, the pressure in the cell rose 3 atm (measured at room temperature) with a mixture of 2% Xe/10% N_2/88% He. The pumping cell was heated for two minutes to 410 K by using a flow of hot N_2 in an external envelope, in a fashion similar to what was developed for our previous experimental setup.[22] Then xenon was condensed in a cold finger inside a 3kG solenoid immersed in liquid nitrogen, and thus it was separated from helium and nitrogen. The average polarization value with this experimental setup was 15%, measured for the gaseous phase in the NMR spectrometer.

For the experiments with the biosensor, hyperpolarized xenon was introduced on top of the solution in the upper part of the screwed NMR tube by using a vacuum line in the fringe field of the NMR magnet. Then a vigorous shaking of the tube followed by a 10 s delay ensures complete dissolution and equilibration of both gaseous and dissolved phases.

Each NMR tube received about 1 atm of hyperpolarized xenon on top of the solution. The 32×32 point images (781×781 μm per pixel) were obtained by using a gradient echo sequence employing a Gaussian pulse of 8 ms without slice selection, with an echo time of 4.7 ms and a repetition time of 25 ms.

The ^{129}Xe spectra and the images shown were recorded at 11.7 T and 293 K with HNX-TBI and dual ^{129}Xe-^{1}H 5 mm Bruker probe heads for the former and a micro-5 Bruker probe head for the latter.

The signals are referenced with respect to xenon gas extrapolated to zero pressure.

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