REVIEW PAPER

Xenon for NMR biosensing – Inert but alert

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Abstract NMR studies with hyperpolarized xenon as functionalized sensor or contrast agent recently made notable progress in developing a new approach for detecting molecular markers and parameters of biomedical interest. Combining spin polarization enhancement with novel indirect detection schemes easily enables a 10^7 -fold signal gain, thus having promising potential to solve the NMR sensitivity problem in many applications. Though an inert element, ^{129}Xe has exquisite NMR properties to sense molecular environments. This review summarizes recent developments in the production of hyperpolarized xenon and the design and detection schemes of xenon biosensors.

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Introduction

Magnetic resonance research on condensed matter has found many important applications since its beginning in the 1940s. Hence it is not surprising that several significant milestones were contributions from different fields of science, some of them honored with Nobel Prizes in Physics (Bloch and Purcell in 1952, Kastler in 1966), Chemistry (Ernst in 1991, Wüthrich in 2002) and Medicine (Lauterbur and Mansfield in 2003). Nuclear magnetic resonance (NMR) is of particular interest because of its broad range of applications that makes it such a valuable tool for scientists in many disciplines. Today, this is especially the case in the life sciences [1,2] and progress in the emerging field of biomedical imaging is therefore also linked to new NMR-based detection schemes.

Many approaches originate from NMR spectroscopy applications such as structural analysis and are now transferred to imaging techniques. Due to its inherent low sensitivity, conventional nuclear magnetic resonance imaging (MRI) relies on the detection of protons in abundant substances like water and fat in biological tissue. The increasing interest in understanding diseases on the molecular level requires new molecular probes (or contrast agents in a more general way) that can visualize pathologic alterations at an early stage through molecular markers at rather low concentrations. This field of diagnostic imaging, also referred to as molecular imaging [3,4], requires to overcome the MRI sensitivity problem in order to combine its well known advantages (unlimited penetration depth, use of non-ionizing radiation, good spatial and temporal resolution, intrinsic spectral information) with molecular specificity.

The classes of currently developed sensitive molecular probes for MRI are either based on indirect detection schemes, i.e., transferring information onto the abundant

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and easily detectable water signal [5], or on the application of so-called hyperpolarization techniques that artificially increase the spin polarization of a very dilute agent before being delivered to the object of interest [6,7]. Molecular specificity of the detection scheme is an important prerequisite that can easily be accomplished in NMR due to the inherent chemical shift information of the employed radio signal. This effect, caused by the molecular-specific electronic shielding or de-shielding of the detected nuclei is a valuable feature in this context.

Surprisingly, one of the most potential candidates for molecular recognition is one of the least obvious nuclei in terms of biomedical sensing: xenon. As a noble gas, it is quite inert and only a few compounds are known, none of them representing an endogenous substance or being of any physiological relevance. Xenon exists in two NMR-active isotopes, the spin-1/2 nucleus ^{129}Xe (26.4% natural abundance) and the spin-3/2 nucleus ^{131}Xe (21.2%). The former one has exquisite NMR properties since it can easily be hyperpolarized (see below) and has an extremely large chemical shift range as well as favorable relaxation characteristics. The NMR signal of ^{129}Xe is extremely sensitive to its molecular environment due to the high polarizability of its large electron cloud [8]. It can therefore be a probe for various interactions with molecules even though it is not involved in forming covalent bonds. Since it is quite lipophilic, the investigation of hydrophobic binding pockets in proteins is one of the applications of Xe NMR [9]. Interaction with various other biological materials have been studied, also including experiments in aqueous solution since the solubility in this polar solvent is still sufficient to identify several signals of ^{129}Xe in different aqueous environments [8,10].

While the initial MRI application of hyperpolarized noble gases like xenon and helium is imaging of the lung [11] (an organ where the limited proton density makes it otherwise difficult to achieve sufficient image quality), recent developments focus on the design of contrast agents in solution that incorporate the advantages of ^{129}Xe . These so-called xenon biosensors use the nucleus as a spy that is temporarily trapped in a macromolecular cage [12]. Such cages can be functionalized to bind to a specific analyte, thereby conferring biochemical specificity to the NMR signal of the otherwise inert element. The aim of this review is to summarize the concept of such biosensors and highlight recent developments and future potential for this rather young member of MR contrast agents. Section 2 will give a short introduction of the NMR properties of xenon and the possibilities to enhance spin polarization. Sections 3 and 4 will focus on different examples of molecular sensing and the required detection technique to further increase sensitivity, respectively. The sensing of physico-chemical parameters like temperature and micro-environment is finally briefly discussed in Section 5.

Xenon nuclear magnetism

Comparison with protons

Since xenon has to be introduced into the object of interest as an exogenous agent, it is required to be detectable at

relative low concentrations. Its limited solubility for aqueous solutions (and therefore also in many biological tissues) is given by an Ostwald solubility coefficient corresponding to ca. 3.3 mM/atm [13] (i.e., the amount of gas dissolved per liter liquid and at 1 atmosphere pressure). Compared to the detection of ^1H in conventional imaging with a proton concentration of ca. 110 M in pure water, the xenon spin density will be relatively low. Moreover, the gyromagnetic ratio γ that determines the NMR properties of the nucleus is ca. 3.6 times lower than that of protons (Table 1 compares the properties of ^1H and ^{129}Xe). This has two consequences that both lead to a decreased sensitivity:

1. The population ratio R of the two spin states that can be approximated at high temperatures in a given external field B_0 by the Boltzmann factor by $R = \exp(-\gamma B_0/kT)$ is reduced by ca. 73% compared to ^1H (k is the Boltzmann constant, T the absolute temperature). Consequently, both spin states are almost equally populated (Fig. 1a) at room temperature. At a field strength of $B_0 = 9.4\text{ T}$, only ~ 9 spins in a million contribute to the macroscopic magnetization M that is detected in a radio frequency coil (110 MHz in this case) through Faraday induction.
2. The reduced induction frequency is also linked to a further loss in sensitivity since the rate dM/dt determines the recorded voltage signal for classical RF Faraday detection.

Both effects yield a relative receptivity of 2.16×10^{-2} compared to ^1H and make it impossible to perform xenon NMR with low spin densities under conventional circumstances. Hence, some special conditions have to be applied to significantly boost the sensitivity. The resonance frequency is a parameter that can only be changed by the external field and is therefore linked to a limited range of improvement. The population difference, however, is a quantity that can be influenced in a manner that results in a spin polarization exceeding the thermal polarization by several orders of magnitude. Such a condition is called hyperpolarization and the method to achieve this will be described in the following section.

Hyperpolarization of ^{129}Xe

The polarization P of a spin system is defined as the population excess of one spin state over the other, normalized by the sum of the populations:

$$P = \frac{N_+ - N_-}{N_+ + N_-}$$

Table 1 NMR properties of the spin-1/2 nuclei of hydrogen and xenon.

	^1H	^{129}Xe
Natural abundance [%]	99.99	26.40
γ [MHz/T]	42.576	11.841
Molar receptivity [%]	100.00	2.16
Spin concentration in water [M]	111.1	0.0033/atm

Figure 1 (a) Population ratio of upper spin level vs. lower spin level for thermally polarized ^{129}Xe and ^1H at different field strengths. Because of the small Boltzmann factor for nuclear magnetic moments, there exists almost equal population for temperatures around 300 K. (b) Corresponding spin polarization in thermal equilibrium. (c) Sketch illustrating the difference between thermal polarization with almost no net magnetization and hyperpolarization with significant excess of one spin orientation.

For thermal equilibrium at room temperature, this quantity is given by $P = (1 - R)/(1 + R)$ (Fig. 1b). When generating non-equilibrium conditions (Fig. 1c), the enhancement over thermal polarization (given by the Boltzmann factor as described above) can theoretically reach values as high as 10^5 . The manipulation of the xenon spin angular momentum is achieved in a three-step process that is called spin exchange optical pumping (SEOP) [14].

In terms of controlling angular momentum, photons represent a system that can easily be manipulated. The initial step is therefore the production of circularly

polarized light which is then used to pump a certain transition in an effective one-electron system represented by alkali metal vapor. Most polarizer setups work with rubidium vapor for which a laser emitting at 795 nm can drive the D_1 transition to achieve high Rb electron spin polarization in the second step. For this purpose, a droplet of Rb is heated inside a glass cell to generate a saturated vapor atmosphere and is exposed to a magnetic field of ca. 2.5 mT. The circularly polarized σ^+ light traveling along the field axis selectively pumps the transition $5^2S_{1/2} \rightarrow 5^2P_{1/2}$ with a spin flip $m_s = +1/2 \rightarrow m_J = \pm 1/2$ (see Fig. 2a).

Figure 2 (a) Atomic fine and hyperfine structure for rubidium used as polarization precursor in spin exchange optical pumping. The resolved hyperfine structure allows driving transitions between the ground state and the first excited state $2P_{1/2}$. Circularly polarized light in combination with a magnetic field enables selective depopulation of one of the magnetic sub-levels in combination with a spin flip. Mixing and relaxation processes cause equal de-excitation into the ground state sub-levels. Assuming sufficient photon density, the selective pumping is efficient enough to transfer half a spin angular momentum per photon. (b) Overview of the spin exchange optical pumping setup. Laser light from a diode array passes a circular polarizer to selectively pump one rubidium transition in the pumping cell that is exposed to a static magnetic field. Collision processes enable spin–spin interactions between the Rb electrons and Xe nuclei to enhance nuclear spin polarization of the noble gas.

While collisional mixing equalizes populations of the two sub-levels of the excited state, radiation-free relaxation (due to optical quenching with N_2 gas in the glass cell) populates both sub-levels of the ground state. Eventually, there will be a significant over-population of the spin-up level since the other one is continuously depleted by the laser. This Rb electron spin polarization is then converted into Xe nuclear spin polarization in the third step (Fig. 2b): dipolar interactions between two spins that come close enough to each other lead to flip-flop processes that convert many of the xenon spin-down states into the spin-up condition. For a detailed description of the physics behind the SEOP method, the reader is referred to [14].

Nevertheless, a recent development should be mentioned in this context that has significantly improved this technique. The popular type of lasers used for emission at 795 nm is based on diode arrays that come with a relative high power output (up to 10^2 W) at reasonable production cost. These lasers emit a relative broad spectrum of up to a few nm bandwidth. Consequently, many polarization setups run at increased pressure to broaden the D_1 transition for better photon absorption (the line broadening is ca. 18.6 GHz/amg [15]). The density of photons with matching wavelength is critical to maintain the Rb electron spin polarization as high as possible. For this purpose, a gas mixture is used that contains a large fraction of He (up to 90%) to increase the pressure and the percentage of Xe is only $\sim 2\%$ (the remaining fraction is N_2 for quenching the optical transition). A high percentage of the noble gas would depolarize the Rb electron spins too much and result eventually in less detectable magnetization. Pressure broadening, however, can only partially compensate for a broad laser emission and a relative large fraction of the emitted power would be wasted. New systems therefore incorporate volume Bragg gratings (VBGs) to achieve a line-narrowed laser emission [16,17]. Such gratings reflect a small bandwidth of the initial spectrum back onto the diode and force it to emit at this very frequency. Laser profiles of less than 1 nm bandwidth can be achieved (Fig. 3) with this technique and ensure excellent pumping conditions of the Rb vapor, therefore yielding polarizations of $>50\%$ for ^{129}Xe [17,18]. Such a signal enhancement of four orders of magnitude means that the relative receptivity compared to ^1H is boosted to $>2 \times 10^2$. A photon flux for ca. 120 W of pumping power corresponds to $\sim 5 \times 10^{20}/\text{s}$. For comparison, the Rb saturation vapor density at a temperature of 150°C is roughly $1 \times 10^{14}/\text{mL}$ according to the Smithells formula [19]. Such lasers are therefore usually strong enough to polarize a Rb atmosphere at these average temperatures (local temperatures in the cell can be much hotter [20]) within a few ms [21].

Although a high photon density is beneficial for keeping the Rb atmosphere polarized, it has been reported that increasing laser powers do not necessarily improve Xe polarization [17,22]. Due to the quenching gas, most of the photon energy (~ 1.5 eV) is eventually converted into heat, leading to temperatures that can significantly exceed that of the outside heating mechanism [20]. If the droplet of Rb is located directly inside the pumping cell or at least in areas that are affected by this additional heating, further Rb vaporization starts with subsequent light absorption. This chain reaction is called Rb runaway and leads to inhomogeneous cell illumination with a decrease in Xe

Figure 3 Emission profiles of a line-narrowed diode laser for SEOP. The setup consist of six diode bars with slightly different emission wave lengths, yielding a profile with two main maxima and a total FWHM of <1 nm. No absorption is observed when the pumping cell is cold (dashed line). Heating the cell generates rubidium vapor that absorbs a substantial fraction of the photon flux (black solid line). The relative absorption is plotted in red, showing that the D_1 line profile at 4.5 bar absolute pressure is already broad enough to absorb light over almost the entire emission spectrum. This ensures efficient use of the polarized photons to achieve high xenon spin polarization.

polarization. In order to prevent this deleterious side effect, the Rb vaporization can be separated spatially from the pumping cell to keep the Rb density in the illuminated volume constant [23].

Delivery of hp Xe

Delivery of hyperpolarized xenon can be done in two different ways: some polarizer setups contain a cold trap that allows separating the noble gas from the rest of the gas mixture by immersing the trap into liquid nitrogen while flowing the gas mixture through the polarizer outlet (a permanent magnet around the trap is usually used to conserve the xenon ice polarization by keeping the spin states separated). In this mode, the cell is either loaded once with a batch of xenon gas mixture and pumping maintains for, e.g., 15 min or the gas mixture flows continuously through the cell before xenon ice collection is stopped. Sublimation of the ice with hot water around the trap then produces a volume of hyperpolarized gas to be used in the NMR magnet. This technique requires some time to accumulate a batch of highly concentrated hyperpolarized xenon and is the method of choice whenever a high signal is required for a few acquisitions.

Alternatively, the mixture can be used as is without condensation of xenon. This method obviously works with lower concentration of hyperpolarized nuclei but has the advantage that it can be used in a continuous flow setup where xenon is bubbled repetitively into test solutions and allows signal averaging over a long time [24,25]. The sample can stay inside the NMR magnet without being repositioned for repetitive gas delivery which is convenient if whole series of spectra or images are acquired.

Various carrier agents have been tested for xenon delivery into objects of biomedical interest. When working with life animals, inhalation through the lung is always one option but also some alternatives like injection of saline solution, lipid emulsion or plasma expanders have been tested [8,26]. When choosing a carrier agent for a specific application, the required travel time to the region of interest is one parameter to be considered. Some solutions might have a higher solubility for xenon than water does but the relaxation of the hyperpolarization might also occur faster [26].

Sensor constructs for molecular specificity

Since xenon is not part of any biologically relevant molecules, the biochemical specificity of the NMR signal has to be generated in an indirect way. This is achieved by trapping the noble gas in a functionalized molecular host that specifically alters the resonance frequency of the hyperpolarized nuclei.

Cryptophane hosts

As an non-polar agent, xenon binds in hydrophobic cavities of host molecules like cryptophanes [27–29] with exchange rates k_{ex} that correspond to residence times in the millisecond regime [30]. The resonance frequency changes significantly when xenon from the solution pool is trapped inside such cavities (see Fig. 4): Aromatic ring structures of the cyclotrimeratrylene (CTV) units cause a change $\Delta\omega$ in chemical shift of more than 100 ppm [31]. This value is large enough to acquire spectra in the slow exchange regime, i.e., $\Delta\omega > k_{\text{ex}}$ with two distinct peaks, one for free ^{129}Xe in solution and one for caged xenon. The extend of $\Delta\omega$ and the binding properties depend on the size of the host [32,33], potential decoration of the cage [34], and the solvent (unmodified cryptophanes are highly hydrophobic). The host–guest interaction is dominated by van der Waals

forces, therefore the size of the cavity relative to the size of the noble gas ($V_{\text{vdW}} \approx 42 \text{ \AA}^3$ for Xe) is important. The cavity size can be adjusted by the spacers between the CTV caps (see Table 2 for different cage sizes). Various combinations of either three identical or different spacers have been realized so far [32,35]. Recent studies report evidence for an ‘induced fit’ where the host adapts its conformation to the guest [34,36]. There is a wide range of xenon binding constants for unmodified cryptophane from $K = 5\text{--}10 \text{ M}^{-1}$ for cryptophane-333 (also called cryptophane-E; cavity volume $V_c \approx 121 \text{ \AA}^3$) to $K = 28\,000 \text{ M}^{-1}$ for cryptophane-111 ($V_c \approx 81 \text{ \AA}^3$) [33] in organic solvent at 278 K (the three digits refer to the length of the $-\text{O}(\text{CH}_2)_n\text{O}-$ spacer between the CTV caps). Temperature has a strong impact on the binding effect since the value for the latter cage in the same organic solvent (1,1,2,2-tetrachloroethane- d_2) drops to $10,000 \text{ M}^{-1}$ when temperature is raised to 293 K [37].

The situation in aqueous solution is more difficult due to the strong hydrophobicity. One of the most commonly used cages, cryptophane-A (i.e., cryptophane-222, $V_c \approx 95 \text{ \AA}^3$ [32]), is characterized by $K = 3900 \text{ M}^{-1}$ in organic solvent at 278 K [33]. The affinity of the lipophilic xenon for the cavity is assumed to be higher in a polar solvent but no quantification has been published so far because of the extremely low solubility of unmodified cryptophane-A in water. Replacing its methoxy groups by $-\text{OCH}_2\text{COOH}$ moieties improves the solubility significantly and also yields convenient values of $K = 6800 \text{ M}^{-1}$ at 293 K [38] in aqueous solution. This was topped recently by a water-soluble triacetic acid cryptophane-A derivative with $K = 33\,000 \text{ M}^{-1}$ [39] and $K = 42\,000 \text{ M}^{-1}$ for a tris(triazole ethylamine) cryptophane [40]. A cryptophane-A functionalized with tris(triazole propionic acid) showed a K of only $\approx 17\,000 \text{ M}^{-1}$ in [39]. The authors hypothesized that the introduction of ionizable groups creating a dipole moment inside the cavity improves the binding affinity. This argument would explain the observations made for the hexacarboxylic acids [38]. Contrary to the negative effect of increased temperature on the binding constant in organic solvent (see above), a water soluble cage can show increased binding capabilities at elevated temperature in buffer solution [41].

It should be mentioned that cryptophanes also bind other guests, e.g. water, methanol, xenon or chloroform [28,36]. The corresponding affinities turn out to be quite different. In aqueous solution, water does not seem to be a strong competitor for xenon, but chloroform, e.g., competes with the noble gas [31] and turns out to be an improper solvent when testing hydrophobic cages for xenon chemical shifts. Apart from the solvent being a potential

Figure 4 ^{129}Xe NMR spectrum of xenon dissolved in water with 5% DMSO and $\sim 200 \mu\text{M}$ of the molecular cage cryptophane-A monoacid. Xenon in gas phase on top of the solution is used as reference at 0 ppm. Free Xe in solution resonates at ca. 191 ppm whereas atoms trapped in the cage cavity yield a signal at ca. 61.8 ppm. Due to high mobility of the free cage, the line width is <0.5 ppm.

Table 2 Comparison of volumes according to van der Waals radii of xenon and different cryptophane hosts. See text for details on cryptophane nomenclature.

	Volume [$\text{\AA}^3$]
Xenon	42
Cryptophane-111	81
Cryptophane-222	95
Cryptophane-333	121

guest molecule, other small gases have been tested for their interactions with cryptophanes [42] in order to analyze the potential for size- and geometry-selective sensors targeted for various gas molecules. Potential competitors for xenon therefore have to be considered when designing experiments.

The chemical shift range of xenon in various cryptophanes allows the simultaneous use of different cages for sensing different target molecules, thereby providing a multiplexing option. This enables comprehensive studies of correlated biological events. Due to excellent spectral resolution, the conditions are even better than in optical imaging where fluorescent dyes sometimes show spectral overlap. Cryptophanes-222, -223, -233 and -333 and their hexacarboxylic acid derivatives have been investigated in a systematic study [38] and the stepwise modification of the cage size causes a chemical shift change of 7–17 ppm with the larger cages showing a high-field shift. These properties have been used in a study with cryptophane-111 and a water soluble version of cryptophane-222 to achieve selective ^{129}Xe MR imaging of two separate phases (water floating on top of tetrachloroethane) [43]. Recently, the range of chemical shifts to be exploited has been increased significantly by a new cryptophane-111 functionalized with six $[(\eta^5\text{-C}_5\text{Me}_5)\text{Ru}^{\text{II}}]^+$ ($[\text{Cp}^*\text{Ru}]^+$) moieties [34]. The potential of building an entire biosensor library based on various host molecules combined with different targeting units as already proposed in [12] will undoubtedly be of major importance for future applications of biomedical relevance.

For the sake of completeness it should be mentioned that cryptophanes are not the only hosts that have been tested for xenon encapsulation. A cucurbit[6]uril derivative and cucurbit[5]uril have been proposed as potential structure for further biosensor development [44,45]. The xenon affinity of this larger construct is ca. 1300 M^{-1} in aqueous solution. However, no experiments have been reported to date with a functionalized version of this host. This aspect has been investigated in multiple studies for cryptophanes and will be discussed in the next section. Another host structure that has been studied are functionalized zeolites [46].

Functionalization

In order to achieve detection of a specific biomolecular marker, the cage structure is functionalized with a so-called targeting unit. The prototype of this idea was implemented as a biotinylated construct [12] that is able to bind to avidin via its strong interaction characterized by $K = 10^{15}\text{ M}^{-1}$. In principle, any antibody or ligand can serve as an affinity tag to achieve biochemical specificity. Hence, a variety of different systems have been demonstrated over the last years: enzyme-specific or receptor-specific sensors for certain cancer markers [47–50] belong to this group as well as constructs revealing DNA hybridization [51] or peptide–antigen interactions [52].

In the case of enzyme-specific constructs, sensing can either be enabled through a binding event that causes the cage molecule to interact with the enzyme surface. The example of a cryptophane-A derivative with an affinity tag for human carbonic anhydrase II (CAII, it catalyzes reversible hydration of carbon dioxide and is thought to play

a role in the formation of certain tumors and polycystic kidney disease) revealed several van der Waals contacts of the cage [48,53], thereby causing a chemical shift change of 3–7 ppm compared to the free sensor. The length of the linker between the xenon host and the benzenesulfoamide moiety coordinating to the active site Zn^{2+} ion in CAII is an important aspect in sensor design. A flexible linker that is long enough to keep the cage outside the 15 Å deep cleft of the zinc ion will ensure rapid re-orientation of the cage that guarantees sufficiently narrow NMR lines. However, an excessive linker length limits the observable chemical shift changes [54] and thereby the specificity to discriminate between bound and unbound sensors.

An alternative for enzyme detection, especially if access for an affinity tag turns out to be difficult, is the attachment of a substrate molecule to a cryptophane. This has been demonstrated with a sensor revealing activity of matrix metalloproteinase-7 (MMP-7, an enzyme secreted from many human tumors) [47] through the peptide RPLALWRS that is cleaved into RPLA + LWRS. The ^{129}Xe NMR signal of the cage construct shows a change in chemical shift of 0.5 ppm after proteolysis. The advantage of this approach over stoichiometric protein binding is an amplification through the catalytic efficiency of the enzyme that allows detection of MMP-7 in the picomolar range.

The affinities between sensor and target are very diverse in intensity. The enzyme-specific constructs for MMP-7 ($K_M \approx 4 \times 10^{-5}\text{ M}$) and the sensor for the detection of DNA hybridization ($K_d \approx 10^{-6}\text{ M}$) [51] are relatively weak interactions compared to the biotin-avidin affinity ($K_d \approx 10^{-15}\text{ M}$). The CAII sensor is characterized by a somewhat intermediate affinity ($K_d \approx 2\text{--}11 \times 10^{-8}\text{ M}$; one approach implemented in this specific context is the use of a tri-functionalized cryptophane to introduce several targeting moieties per xenon carrier [48]). These prototypes of xenon biosensors provide important information with promising perspectives for affinities of biomedical relevance that will be addressed in future studies.

Strategies for amplification and cellular uptake

Similar to other MRI contrast agents like gadolinium-based relaxation enhancers where novel designs aim for increasing the load of paramagnetic ions per nanocarrier [55], the sensitivity of xenon biosensors can be increased when attaching several NMR-active units to each targeting moiety. The first approach in this direction was based on water-soluble dendrimers that can carry several cryptophanes through electrostatic interactions. Generation 4 and 5 polyamidoamine (PAMAM) dendrimers can encapsulate 7 and 11 cages, respectively [56]. However, a biotinylated version of the latter one carried a maximum load of three cages with further release of one cryptophane upon binding of the biotin to avidin. Loose fixation of the cages obviously lacks the stability of a covalent bond but comes with the advantage of circumventing elaborate synthesis work and helps to improve sensitivity by two means. The overall sensitivity enhancement compared to a conventional biotinylated cryptophane [54] was ca. 8-fold: first, the signal-to-noise ratio is doubled because two cages per biotin unit are present. Second, the lack of a covalent bond avoids formation of diastereomeric peaks

of the peaks of the chiral cage [57], thereby concentrating the signal in a single peak. Finally, the dynamic nature of the linkage between the carboxylic acid and the multiple amine groups of the dendrimer also reduces the ^{129}Xe NMR line width by 50%.

A second amplification approach is based on a strategy that was previously developed for paramagnetic contrast agents [58,59]. Viral capsids represent a carrier system that can be labeled on both the exterior and interior with NMR-detectable units. The MS2 capsid is a nearly spherical structure with icosahedral symmetry and 32 pores of ca. 2 nm diameter each. It is composed of 180 monomers that spontaneously assemble into empty, noninfectious carriers. Decoration of such scaffolds with cryptophanes can be achieved in a much more efficient way than in the case of the PAMAM dendrimer: ca. 125 cages can be incorporated per MS2 capsid [60]. This effect increases significantly the sensitivity for detection of caged xenon and has a promising potential for the further development of biosensors since such capsids can also be functionalized with nucleotide aptamers to bind to specific cell receptors [61]. In this approach, the MS2 does not only carry a large load of xenon hosts but also helps to increase the solubility and biocompatibility of the otherwise hydrophobic cages. A negative side effect is substantial line broadening of the NMR signal due to reduced molecular mobility of the cryptophane. Future studies will show how much this effect can be reduced by optimized coupling to the capsid.

Another aspect of biosensor design is the attachment of functional units that allow for cellular uptake and thereby accumulation of contrast agent in cells that over-express certain molecular disease markers like transmembrane receptors etc. One advantage of the xenon biosensor approach is the separated delivery of the functionalized host structure and the hyperpolarized nuclei (similar to a pre-targeting approach). Other hyperpolarization techniques like para-hydrogen induced polarization (PHIP, [62,63]) or dynamic nuclear polarization (DNP, [64]) increase the polarization of nuclei that are covalently bound in the detected molecule and therefore require rapid delivery of the entire construct to the site of interest. Xenon biosensors can be administered first, followed by an appropriate time for clearance at sites of unspecific binding and accumulation in areas receptive for the tailored affinity tag. For this purpose, different peptido-cryptophanes have been developed in initial studies [50,65].

Polycationic peptides rich in arginine can be used to solubilize cargo molecules and transport them through cellular membranes [66,67]. Hence, a (D-Arg)₉-cryptophane-A conjugate was studied for unspecific uptake into CAPAN-2 cells [50]. The same study included an alternative conjugate for non-specific cell uptake, namely a cage attached to the residues 48–60 of the HIV-1 TAT peptide. This transcriptional activator has been used in previous studies for cellular uptake of superparamagnetic particles in ^1H MRI [68]. Moreover, specific uptake was also investigated by targeting an integrin receptor on the cell surface. This type of receptor is over-expressed in various cancer cell lines. Especially the $\alpha_v\beta_3$ integrin receptor (ABIR) has been in the focus of many studies since it is upregulated in many tumors but shows minimum expression in most normal tissues [69]. Linear multimeric (RGD)_n sequences can be

used to target ABIR for cargo uptake into cells [70]. Therefore, an (RGD)₄ dodecamer attached to cryptophane-A was studied as a third peptido-cage construct for cellular uptake [50]. All constructs were labeled with the water soluble fluorescent dye Cy3 for verification of membrane translocation and subsequent quantification.

Incubation for ca. 4 h turned out to be sufficient to achieve intracellular concentrations of 100–160 μM in CAPAN-2 cells for these three conjugates, thus representing a 10–16-fold enrichment compared to the cage concentration in the provided medium. Experiments show that the unspecific uptake of the first two constructs occurred as energy-dependent endocytosis [50]. For the receptor-mediated uptake, data suggest that especially the α_v subunit is critical for the specific recognition of the (RGD)₄ motif.

A later version of an integrin-targeted biosensor in terms of cryptophane functionalized with cyclic RGDyK peptide and Alexa Fluor 488 dye showed increased water solubility and allowed more detailed analytical and cell characterization studies [65]. It was demonstrated that cryptophane was only moderately toxic to normal lung fibroblasts (50% viability was reached in the cancer cell lines studied at concentrations of 75–100 mM). The first NMR data revealed a shift of 4 ppm downfield upon binding of the sensor to the $\alpha_{\text{IIb}}\beta_3$ integrin receptor. Altogether, these initial results are very promising for future developments since the observed sensor uptake yields concentrations within the detection limits of ^{129}Xe NMR experiments (see below).

Internalized sensors are still accessible for hyperpolarized xenon since it crosses the cell membrane relatively fast without substantial loss of its polarization [71]. This is an important result for interpreting data obtained in a recent study demonstrating a transferrin receptor-targeted biosensor [49]. Using a hydrophobic version of a cryptophane cage, significant unspecific binding of the transferrin-labeled sensor to the membrane can be observed as demonstrated by an additional NMR signal ca. 10 ppm left of the normal solution signal. However, specific uptake into human erythroleukemic K562 cells (exhibiting 1.6×10^5 sensors per cell) was demonstrated.

Xenon NMR encoding and indirect detection

Since the state of hyperpolarization deviates significantly from thermal equilibrium, some special aspects of the behavior of the detected magnetization have to be considered for the spatial encoding of ^{129}Xe MRI. Any longitudinal component that has been converted into detectable transverse magnetization by a radio frequency (RF) excitation pulse is 'lost' after the corresponding T_2 decay if the spin ensemble is an enclosed system without any re-delivery of fresh hyperpolarization. Hence, either fast pulse sequences are employed that work with weak excitation pulses and divide the available reservoir of longitudinal magnetization for the required acquisition steps of the raw data set [72] or the acquisition scheme is repeatedly interrupted by xenon delivery with nuclei from a reservoir outside the RF coil.

The large chemical shift range of ^{129}Xe NMR signal and the tremendous polarization enhancement would make it dispensable to work at high magnetic field strength.

However, the low gyromagnetic ratio compared to protons requires the use of relatively strong gradients to achieve sufficient spatial resolution, and such micro-imaging systems paired with powerful gradient systems usually come with fields $B_0 > 7$ T. Bandwidths of acquired xenon spectra therefore easily extend over several 10^4 Hz when they include a gas peak for reference and peak from xenon in solution, an aspect that has to be considered when using shaped pulses to excite various xenon resonances.

Challenges of direct detection

Although the use of hyperpolarization techniques comes with a significant boost in sensitivity, the spin density in xenon biosensor studies is low for two reasons, thus making direct detection a challenging issue in many applications: First, the xenon concentration in tissue is low (e.g., ca. $1\ \mu\text{M}$ in human brain [73], higher in more fatty environments like the liver). Second, the sensor concentration for interesting targets that this type of detection aims to compete for with, e.g., PET and optical imaging is $<10^{-6}$ M. Compared to tissue with ca. $100\ \text{M}$ water proton concentration, this represents a significantly reduced magnetization, even if the hyperpolarization can compensate for a factor $\sim 10^4$. Hence, direct detection of encapsulated xenon requires signal averaging, especially when operating the polarizer in a continuous flow mode where the partial pressure of hyperpolarized gas is relatively low. Assuming a mixture with 2% isotopically non-enriched Xe and a pressure of 5 bar, the concentration of detectable ^{129}Xe in water is ca. $86\ \mu\text{M}$. Only a small fraction of these atoms will finally be bound to the host structure [24]. The problem aggravates when the sensor molecule is bound to a microscopic structure (e.g., cells) that reduces its molecular tumbling and therefore causes substantial line broadening [24,74] (see Fig. 5).

Initial imaging studies of xenon biosensors suffered from this low signal-to-noise ratio and required extensive measurement times of several hours while achieving only very limited spatial resolution [25]. Expected signal improvements by using cryogenically cooled NMR probes or isotopically ^{129}Xe -enriched gas would only yield a factor of 3–4 (additionally, the price of enriched xenon rises much faster than its percentage of ^{129}Xe). The need for an alternative detection method to reduce the acquisition time by 1–2 orders of magnitude was therefore obvious. All of the following approaches are based on the fact that the caged xenon is only temporarily bound to the host structure. Although the exchange dynamics are slow on the chemical shift scale, the replenishment of hyperpolarized xenon inside the cryptophane for a selective saturation-recovery experiment appears on the millisecond timescale [30]. The first approach was therefore based on the idea to perform a selective, repetitive readout of the caged magnetization only and saving the bulk free xenon as a reservoir that regenerates sensor-related signal after an exchange interval of several ms [24,30]. This method – later termed exchange signal averaging – still detects a signal that is very noisy for each individual acquisition. Other approaches therefore switch the roles of the two xenon pools and use the encapsulated nuclei only for

Figure 5 Influence on microscopic objects on xenon biosensor spectra. Agarose beads (grey) carrying avidin labels (green) can bind a biotinylated biosensor (blue). The ^{129}Xe NMR spectrum of such a mixture shows three resonances of dissolved xenon: xenon in contact with the agarose surface (1), xenon in more or less pure water compartments (2) and a broad signal (line width > 2.5 ppm) from xenon encapsulated in the bound sensor (3).

encoding while the bulk pool is the one that is detected at much better signal-to-noise ratio. These indirect detection schemes will be discussed in the following section.

Indirect detection schemes

The underlying idea of indirect detection schemes has been applied under various circumstances in NMR experiments. Sensitivity enhancement is achieved through encoding spins at the original site while detecting them under much better conditions at a different site. This concept can either be conceived on a molecular level, i.e., spins bound in one molecule at low concentration undergo chemical exchange and swap to a different molecular environment at high concentration that is easier to detect [75]. Alternatively, it is applied on a microscopic scale where spins are transferred from an encoding region to a detection region while still being embedded in the same molecule [76]. A comparison of different applications is summarized in [77].

Encoding for the first method is usually implemented in terms of saturation transfer from one type of magnetization onto the detection pool and was therefore termed chemical exchange saturation transfer (CEST) [75]. Spectral

information is included by applying the selective saturation pulse to different parts of the spectrum and displaying the signal intensity of the detection pool as a function of the saturation pulse carrier frequency (so-called z-spectra [78]). The second method works with a pair of selective 90° excitation pulses, separated by an evolution interval that allows various resonances to evolve as transverse magnetization before being stored as longitudinal magnetization and detected either as the same molecule in a different location [76,79] or at the same location but after chemical exchange [80] (see Fig. 6 for comparison of the two methods). The spectral information is contained in the intensity modulation of the detected signal that is achieved via increasing the evolution time τ_{evol} and performing a Fourier transformation with respect to this dimension. Unlike for CEST (which allows to reveal the presence of a sensor signal in just two acquisitions), a whole series of data points must be acquired in order to perform the Fourier transformation. A recent application of this principle to ^1H NMR has been termed frequency-labeled exchange (FLEX) [81].

The saturation transfer technique has been coined Hyper-CEST [82] when used in the context of hyperpolarized nuclei — as an analog to the Para-, Dia- and Lipo-CEST techniques that are used in ^1H NMR with paramagnetic, diamagnetic or liposomal contrast agents [83–85]. A special case where the exchange between xenon in gas phase and in solution state has been applied for a similar approach was called xenon transfer contrast (XTC, [86]). Xenon is an ideal candidate for such saturation transfer experiments because the large chemical shift range allows to selectively saturating the weak resonance of the biosensor while detecting the strong resonance of free xenon in solution with negligible direct saturation. Complete saturation can be achieved under certain circumstances, especially since longitudinal relaxation of the hyperpolarization often is slow compared to the saturation period. Hundreds to thousands of Xe atoms pass through each cage while applying the saturation pulse. This is a big advantage compared to direct detection which corresponds more to a snapshot technique where each occupied host can only contribute with a single Xe atom. All

Figure 6 Indirect detection schemes for xenon biosensor NMR spectroscopy and imaging. The aim is to amplify the weak signal intensity I_c of caged xenon (at resonance frequency ω_c) by transferring information onto the strong solution peak $I_s \gg I_c$ at ω_s . (a) For CEST detection, a series of datasets with saturation pulses at different frequencies $\omega_1 \dots \omega_i$ around ω_c is collected. Spectral information is encoded in this frequency sweep and happens therefore at the same time the spins exchange from the cage into the solution pool. Integration of the acquired solution signals gives a saturation profile or so-called z-spectrum. The amplitude of the indirectly detected peak at ω_c is proportional to the maximum saturation intensity I_{sat} . A minimum of two acquisitions (at ω_c and far off-resonant) is required to reveal the presence of a single sensor. (b) For exchange magnetization transfer (MT) detection, the signal transfer happens in smaller chunks and it is timewise separated from the spectral encoding. Selective excitation of the cage signal generates a magnetization that evolves during τ_{evol} . It is then stored as longitudinal magnetization and immediately transfers onto the solution signal. Repeating this n times yields a measurable change in the subsequently generated solution signal. This turns into a modulation I_{mod} of the intense signal when performed with different evolution times $\tau_1 \dots \tau_j$. Fourier transformation with respect to τ_{evol} turns this modulation into the spectral signature of the caged xenon. However, the FT algorithm requires a whole series of data points τ_{evol} to reconstruct even a single signal.

the observed signal change is attributed to a very efficient CEST effect, thereby amplifying the signal from the molecule of interest by several orders of magnitude and reducing acquisition time often by a factor 10^7 [87].

Hyper-CEST advantages

CEST experiments with thermally polarized protons suffer from the fact that the ^1H chemical shift range is relative small and the exchange of labile protons is often too fast to observe two separate resonances. Moreover, the saturation is partially masked by the counteracting relaxation that drives the system back to the equilibrium magnetization. Hence, CEST experiments often require high magnetic fields to achieve improved conditions for both chemical shift separation and unwanted fast longitudinal relaxation. This is not the case for saturation transfer experiments with ^{129}Xe since its chemical shift range is large and the above mentioned favorable relaxation conditions apply.

Hyper-CEST can therefore be much more efficient than ^1H CEST with diamagnetic substances (also called DIACEST [85]) that often has to work with weak saturation pulses to avoid direct saturation of the observed water peak (so-called spillover effect). The first demonstration of Hyper-CEST was already able to image biosensor distributions at micromolar concentrations [82] since the saturation transfer caused a signal decrease of ca. 50% of the free Xe in solution. The NMR-active sensor was present at $\sim 1.3\ \mu\text{M}$ only and the dissolved, detectable xenon (i.e. hyperpolarized ^{129}Xe) at $\sim 2.1\ \mu\text{M}$. DIACEST agents usually require significantly higher concentrations in the millimolar range to cause noticeable signal decrease (concentrations 0.25–250 mM were studied in [75]). They are also based on significantly higher concentrations of detectable nuclei (factor 2600 or 4400 for protons at 7 or 11 T, respectively, compared to xenon in [82]).

To improve the sensitivity of ^1H CEST, compounds with high numbers of exchangeable protons can be used. Cationic polymers at concentrations of $\sim 10^{-4}\ \text{M}$ were reported to cause water signal changes of 40–50% [88]. Poly-L-lysine (PLL), for example, involves more than 7000 exchangeable sites, thus yielding an enhancement factor of more than 486,000. Polyuridylic acid, a polymer of 2000 uridine units, was observed at $10\ \mu\text{M}$ with an enhancement factor of $\sim 10.8 \times 10^6$ via its imino protons [89]. A similar and very efficient application of this principle is the type of LIPOCEST agents that can be detected in concentrations of ca. 90 pM [90]. The amplification strategy presented with the above mentioned capsid approach [60] is also related to these efforts and yields a detection threshold that is even lower: 700 fM for such a construct with ~ 100 xenon hosts is the current sensitivity limit for Hyper-CEST.

However, proton CEST agents with fewer active sites have been further improved over the last years and it is worthwhile to compare them to xenon CEST experiments. Achieving efficient saturation of one resonance while minimizing spillover effects onto other resonances (both the detected one or neighboring exchangeable signals) is one of the main concerns in all CEST approaches. The following example shows how the classic CEST effect depends on the experimental parameters. Spillover effects are of minor importance as long as the amplitude of the

saturation pulse (given in Hz) is negligible compared to the chemical shift difference (in Hz) between the saturated and the observed resonance [91]. Whereas Hyper-CEST can easily achieve signal changes of $\sim 50\%$ in many applications, CEST with protons require relative strong saturation pulses to induce a comparable effect. At 4.7 T, the frequency separation of the saturated protons of PLL from the detected water signal is approximately +700 Hz and a saturation pulse amplitude of $3\ \mu\text{T}$ ($\sim 128\ \text{Hz}$) is required to yield a CEST decrease of $\sim 30\%$. Unfortunately, the off-resonance saturation at $-700\ \text{Hz}$ is also related to a signal change of $\sim 25\%$. Performing such studies at 11.7 T is one loophole (albeit an expensive one) to increase the chemical shift difference to approximately 1.8 kHz. Then, saturation pulses up to 250 Hz (i.e., $\sim 6\ \mu\text{T}$) can be used, as the spillover-based signal decrease is only 3% [92].

The small intrinsic chemical shift range of DIACEST compounds has therefore been tackled by introducing agents with a larger chemical shift separation to allow for higher saturation power and faster chemical exchange. This is achieved by incorporation of paramagnetic shift reagents close to the exchangeable water molecules and the corresponding compounds are called PARACEST agents. Introduced by Zhang et al. [93], the first example was based on saturation of the bound water signal of a Eu^{3+} complex formed by a DOTA tetra(amide) derivative. With a chemical shift separation of 49.7 ppm (9.8 kHz at 4.7 T) relative to free water, a saturation of 61% was achieved, whereas the reference experiment with saturation at $-9.8\ \text{kHz}$ showed no spillover effect. Some PARACEST agents are used with saturation powers up to $250\ \mu\text{T}$ ($\sim 10.6\ \text{kHz}$) at 7 T [94]. However, compared to Hyper-CEST, the shorter lifetimes of the coordinated water causes broad saturation dips in the z-spectra (see Fig. 7) and decrease the selectivity of the PARACEST agents. A comparison of different complexes showed that only those with Eu^{3+} , Tb^{3+} , Dy^{3+} , and Ho^{3+} have the right combination of water-exchange time and induced chemical shift to be potential candidates for $B_0 < \sim 10\ \text{T}$ [95].

All these numbers show that ^1H CEST is usually much less efficient than Hyper-CEST where the strongest pulses reported so far do not exceed ca. $30\ \mu\text{T}$ [87]. A recent study also addressed the question of using improved saturation schemes for efficient Hyper-CEST encoding without cw power deposition [96]. In summary, the main improvements compared to thermally polarized nuclei are as follows:

1. Signals of xenon associated with other molecules show an intrinsically huge chemical shift range compared to free dissolved xenon in solution. Hence, no paramagnetic agents are required to reduce spillover effects, even if experiments would be performed at field strengths of $\sim 1\ \text{T}$. Unlike the protons of PARACEST agents, however, the exchange rates of atomic xenon are relatively slow and yield more selective saturation dips in z-spectra (Fig. 7) and can be used for multiplexing with different sensors in the same experiment.
2. At the same time, the slow exchange causes no problems in terms of transfer efficiency because T_1 relaxation for hyperpolarized xenon in solution is slow compared to the saturation time. Therefore, the depolarization is stored much more effectively in the

Figure 7 Illustration of Hyper-CEST advantages (blue z-spectrum) compared to CEST with protons (red z-spectrum). All frequencies are referenced at 0 Hz to the detected bulk solution pool of either water or free xenon. Classical CEST experiments need relative strong saturation pulses to achieve significant signal reduction in z-spectra. However, exchangeable protons used for CEST resonate relative close to the water peak. The saturation dip in the z-spectrum at $+\delta$ therefore suffers from overlap with the response from direct saturation at 0 Hz and saturation at $-\delta$ is not negligible. PARACEST agents solve the overlap problem by inducing a large chemical shift to coordinated water molecules that mediate the CEST effect to the bulk pool. The dips are relative broad due to fast chemical exchange of the water. Hyper-CEST, however, is characterized by saturating signals that are also far off resonant from the solution peak, but the chemical exchange of caged Xe is relative slow. Even for small spin pools, the CEST effect is very efficient and leads to very pronounced and narrow saturation responses. Direct saturation effects do not occur in this case.

bulk pool than in the case of thermally polarized protons that underlie competing relaxation effects. Complete saturation can be easily achieved, and CEST with hyperpolarized xenon benefits from the full dynamic range of the bulk signal.

These advantages allow to keep the concentration of the detected protein in the molecular imaging application rather low ($\sim 10^2$ nM) while reducing the acquisition time by 99.97% compared to conventional biosensor detection [25,82]. Keeping in mind that SEOP-induced hyperpolarization already yields a 10^4 -fold signal enhancement, the combination with CEST techniques has been demonstrated to provide a sensitivity gain of seven orders of magnitude [87] to detect nanomolar concentrations of constructs with one active cage site. When combined with the previously mentioned approach of high cage loads per carrier, the detection limit could be improved to ~ 700 fM in the case of cage-capsid conjugates [60]. Further improvement might be expected from a novel detection scheme called Hyper-SAGE where the hyperpolarized Xe is extracted from solution and detected in gas phase at high spin density while still carrying information encoded during its dissolved state [97].

Sensing of physico–chemical parameters

Instead of using xenon biosensors just for revealing the presence of a specific target, they can also be used to sense

physico-chemical parameters of the micro-environment. The highly sensitive chemical shift of encapsulated xenon has been shown to reveal changes in pH [98] and temperature [28,87]. Due to a pronounced shift of ~ 0.3 ppm/K, the latter effect has been used for implementing very sensitive MRI thermometry [99]. Absolute temperature mapping is possible when acquiring spectra that include the peak of free Xe in solution as an internal reference.

The cage also renders the noble gas in some cases more sensitive to its molecular environment than free xenon: Aqueous solutions with dilute lipid membranes concentrations or cell suspensions only show moderate chemical shift differences between free Xe in and outside the membranes [71] (except for the case of red blood cells). However, caged xenon shows a shift of 10 ppm when changing between the two environments [100]. This makes it easy to identify membrane-bound sensors that have an unspecific affinity to the membrane because of a hydrophobic cryptophane [49].

Indirect detection schemes like Hyper-CEST incorporate the ability to sense for parameters that influence the exchange dynamics. Increasing temperature is known to accelerate chemical exchange significantly for $T > 25$ °C [74] and therefore enable to enhance the CEST-related contrast. This can also be used for temperature mapping at low sensors concentrations [99] where direct detection would be too time consuming. It is also an effect that can be exploited to detect very small concentrations of functionalized Xe [87]. Although the solubility of Xe is lower at

elevated temperature, the benefits from seriously increased CEST amplification are dominant to make the method more sensitive at increased temperature.

Conclusion

Xenon biosensors provide a promising approach to combine molecular specificity of NMR signals with high sensitivity of hyperpolarization techniques. Though still a relative new field of research, significant progress has been achieved in terms of demonstrating systems for various biochemical targets and implementing encoding techniques for further sensitivity enhancement. Many aspects still need to be studied before mature applications as an *in vivo* contrast agent will become reality but simple animal studies might be implemented in the near future. As with many other NMR techniques, *in vitro* applications may also provide a lot of information related to biological systems. Future studies will show how much this approach can contribute to making MRI a valuable tool in molecular imaging by reducing the sensitivity gap to other nuclear methods like PET and SPECT.

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