Imaging of Extracellular pH Using Hyperpolarized Molecules

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Abstract: Many diseases can overrule natural pH regulatory mechanisms and alter the extracellular pH (pHₑ). A non-invasive method that resolves pHₑ in vivo with high spatial and temporal resolution could therefore improve diagnosis and monitoring of diseases, contributing to the concept of precision medicine. During the last decades, several techniques have been proposed to image pHₑ non-invasively. The majority of these methods rely on magnetic resonance because of its good spatial resolution, high penetration depth, non-ionizing radiation and excellent complimentary soft tissue contrast. Dissolution dynamic nuclear polarization (DNP) is an emerging concept to enhance nuclear magnetic resonance (NMR) signals by more than four orders of magnitude, making it possible to observe in vivo metabolic processes in real-time. Here, we summarize and review recent developments in pHₑ imaging techniques based on hyperpolarization methods and give an overview of recently discovered hyperpolarized pH sensor molecules that have been applied in vitro and in vivo.

Keywords: extracellular pH · hyperpolarization · imaging agents · NMR spectroscopy · pH imaging

1. Introduction

Pathologies such as infections, inflammation, ischemia, renal failure, pulmonary disease or cancer are characterized by abnormal metabolism, which can disturb pH regulatory mechanisms and lead to changes of the extracellular pH (pHₑ).[1–3] Because pHₑ can be critical for disease progression[4] and drug efficiency[5], imaging of pHₑ could prove valuable for diagnosis, drug development, treatment selection and response to treatment monitoring,[1,6,7] thereby contributing to the concept of precision medicine.[8,9] However, currently no method for imaging of pHₑ is available in the clinic.

To this day, a multitude of approaches and modalities have been proposed to map pHₑ non-invasively in vivo. Positron emission tomography (PET) tracers like 11C-labelled dimethyl-oxazolidinone (DMO)[10], 11CO,[11] and [18F]FDG glycosylamines[12] show a pH-dependent tracer distribution. The pH low insertion peptide (pHLIP), conjugated with 18F,[13] 64Cu[14] or 99Tc,[15], inserts into cell membranes in regions of acidic pHₑ.

Optical methods exploit the fluorescence signal of a tracer and measure pHₑ either based on a ratiometric approach (fluorescence ratio imaging microscopy, FRIM), or relying on a pH-dependent fluorescence lifetime (fluorescence lifetime imaging microscopy, FLIM).[16] The versatile chemical structure of many fluorescence biosensors facilitates coupling to molecules that target specific disease markers. pH-sensitive dyes were recently attached to pHLIP and used for tumor pHₑ imaging in mice.[17,18] Although fluorescence based pHₑ imaging has a tissue penetration depth of only a few millimeters,[19] it offers excellent spatial and temporal resolution. Such penetration depth limitations of optical imaging can be partially overcome by using optoacoustic techniques for which a pH-sensitive albumin-based probe has recently been introduced.[20]

High penetration depth, excellent soft tissue contrast and good spatial resolution is possible by magnetic resonance imaging (MRI). Standard proton MRI can be combined with imaging of pHₑ by utilizing exogenous molecules with pHₑ-dependent relaxivities or exchange properties. For instance, gadolinium (Gd)-loaded single- [21,22] or poly- [23] ion complexes show altered hydrogen bonding or water accessibility at increased proton concentrations, directly affecting the longitudinal spin lattice relaxation constant (T₁) of water. For an absolute pHₑ determination, the in vivo concentration of these complexes needs to be determined, which was achieved by subsequent or simultaneous injection of pH-sensitive and pH-insensitive Gd-complexes.[24]. Frullano et al. developed a bimodal agent that measures pHₑ by MRI and, at the same time, agent concentration by PET.[25]

Exogenous paramagnetic and diamagnetic complexes with dissociable protons have been designed to image pHₑ in vivo based on chemical exchange saturation transfer (CEST) and have been termed PARCEST and DIACEST, respectively. In

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CEST approaches, the resonance signal of bound protons is selectively saturated by a radiofrequency pulse leading to a decrease of the bulk magnetic resonance (MR) signal because of exchange between the bound and the bulk water pool. pH can then be determined from a calibration curve relating pH to the CEST contrast, e.g. an acidic pH relates to a slower exchange compared to the exchange at physiological pH. Several chelators loaded with paramagnetic lanthanides have been suggested as pH-sensitive PARACEST or DIACEST agents, respectively. Three FDA-approved CT/X-ray contrast agents, whose pH-sensitivity was recently investigated, are the DIACEST molecules iohexol, iopamidol, and iopromide (acidicEST).

Chemical shift based sensors with pH-sensitive resonance signals of alternative nuclei, such as $^1$H, $^3$F and $^3$P, have been developed to estimate pH$_v$ in vivo using magnetic resonance spectroscopy (MRS). The two membrane-impermeable and non-toxic imidazole derivatives IEPA and ISUCA contain pH-sensitive protons which show a change in chemical shift as a function of pH. Fluorinated derivatives of vitamin B$_6$ accumulate in the intra- and extracellular space and the respective pH values can be obtained from differences in chemical shift with respect to a chemical shift standard. 3-aminoxypropylphosphonate (3-APP) has been widely used to measure pH$_v$ in vivo using animal models and as a gold standard to validate new pH$_v$ sensors (e.g. ZK-150471). Nevertheless, these MRS-based $^1$H, $^3$F and $^3$P pH$_v$ sensors suffer from the intrinsic low sensitivity of NMR for the detection of molecules at low concentration. Therefore, their application for imaging of pH$_v$ in vivo remains challenging.

In addition, a variety of biocompatible nanoparticles, nanogels and quantum dots using a multitude of modalities have been presented for pH imaging. They can be designed both to target disease specific surface structures and to detect pH. One approach, for example, uses magnetic discs spaced by swellable hydrogels that change their shape with pH, thus generating detectable pH-dependent magnetic fields. Recently, the first multifunctional theranostic pH sensor for bimodal pH imaging and photodynamic therapy was developed.

2. Hyperpolarized pH Sensor Molecules

Hyperpolarization is an effective approach to increase NMR signal amplitudes by up to five orders of magnitude and to overcome the sensitivity limitations of traditional magnetic resonance spectroscopic imaging (MRSI). To this day, several hyperpolarized molecules belonging to different molecular classes that are sensitive to pH at physiological and pathological pH values (pH 6.4–pH 7.4) have been presented. These molecules are summarized in Figure 1. Their NMR properties as well as the respective references are given in Table 1.

$^{13}$C-labelled [1,5-$^{13}$C$_2$]zymonic acid, (1) and $^{13}$C-bicarbonate (2) are the only two molecules that have been applied to image pH$_v$ in preclinical studies in vivo. Hyperpolarized $^{13}$C-bicarbonate was presented in 2008 as the first hyperpolarized pH$_v$ imaging sensor. In the following years, molecules such as $^{13}$C[pyruvate] and $^{13}$C-1,2-glycerol carbonate (GLC, 4) were investigated to be used as precursors of bicarbonate. Zymonic acid was recently presented as a novel pH$_v$ sensor. It is a cyclic dimer of pyruvate, non-toxic, most likely accumulates in the vascular and extracellular space, and allows pH$_v$ determination based on the pH-dependent chemical shifts of its two $^{13}$C atoms with respect to a chemical shift reference.

$^{13}$C-labelled dicarboxylic acids and molecules of the Good’s buffers family have been shown to be pH-sensitive in vitro. [2-$^{13}$C]diethylmalonic acid (5) and [1-$^{13}$C]N-(2-acetamido)-2-aminoethanesulfonic acid (ACES, 6) were used by...


2.1 Hyperpolarization of pH Sensor Molecules

So far, three hyperpolarization techniques have been applied to increase the NMR signal intensity of molecules that are pH-sensitive in the physiologically and pathologically relevant range: 1) spin exchange optical pumping (SEOP), 2) signal amplification by reversible exchange in shield enables alignment transfer to heteronuclei (SABRE-SHEATH) and 3) dissolution dynamic nuclear polarization (DNP).

SEOP is a polarization method that can be applied to noble gasses. First, circularly polarized laser light is used to selectively excite a D1 transition of an alkali metal vapor (e.g. rubidium). Then, spin exchange through collisions of the noble gas atoms with the polarized alkali metal atoms transfers the electron-polarization to the noble gas nuclei. Hyperpolarized \(^{129}\)Xe bound to a cryptophane cage which is coated with carboxylic acids shows a pH-dependent chemical shift at pH 3.5–5. Similar properties at physiological pH were reported by Riggle et al. who have synthesized the xenon-cryptophane pH sensor complex (10) that inserts into membranes of cancer cells at acidic pH.

SABRE transfers the polarization of parahydrogen via an activated catalyst (iridium) to the compound of interest. As this transfer is most efficient at a magnetic field strength smaller than that of the Earth’s magnetic field, the apparatus is “shielded” by μ-tesla metals (SABRE-SHEATH). A major limitation of SABRE is the need of transient metal binding to the target compound, which restricts its application to a few molecule classes. Nevertheless, the method was already used to enhance the NMR signal of several \(^{15}\)N-heterocycles like imidazole (8). Although direct parahydrogen induced polarization (PHIP) was not yet used for hyperpolarization of molecules that are pH-sensitive in the physiologically and pathologically relevant range, it might be possible to find an unsaturated precursor molecule, that, after addition of parahydrogen, yields a pH-sensitive molecule relevant for in vivo applications. Both SABRE and PHIP do not require the use of expensive cryogens and could offer a low-cost approach to generate hyperpolarized pH-sensitive molecules.

DNP is by far the most applied hyperpolarization technique for in vivo imaging and is already used in clinical studies. Preclinical (HyperSense, Oxford Instruments) and clinical (SPINlab, GE Healthcare) polarizers are commercially available and can increase the NMR sensitivity of several nuclei such as \(^{1}C\), \(^{15}N\) or \(^{89}Y\) by up to five orders of magnitude. During DNP, polarization from electrons provided by free stable radicals (most commonly the trityl radical OX063) that exhibit \(\sim 95\%\) electron polarization at a temperature of \(\sim 1.2\) K and a magnetic field strength of 3.35 T (HyperSense) is transferred to the nucleus of interest in a frozen solid state. Clinical and several home-built polarizers operate at a higher magnetic field strength (up to 7.0 T) and are able to achieve more than 2-fold higher polarization levels than at 3.35 T.

After a sufficient polarization build up (usually \(\sim 0.5–2\) h), the compound is dissolved by a pressurized, heated and pH-

Figure 1. Hyperpolarized pH sensor molecules. Overview of pH-sensitive molecules labelled with \(^{13}C\), \(^{15}N\), \(^{89}Y\) and \(^{129}Xe\) (red, blue, violet, yellow, respectively) which have successfully been hyperpolarized. References and NMR properties of the molecules can be found in Table 1.
buffered solution. The SPINlab hyperpolarizer is designed to filter potentially toxic radicals and includes a quality control mechanism to ensure that only physiologically tolerable solutions are injected. Further approaches to remove the radicals used during hyperpolarization exploit the low solubility of modified BDPA[70], organosilica[71] or agarose[72] bound radicals in aqueous solution, or the usage of immiscible mixtures of organic and aqueous solvent vapors that dissolve the radical and the hyperpolarized compound separately.[73] A radical free hyperpolarization technique relying on UV light induced polarization was recently proposed.[80]

So far, 13C-pyridine derivatives (7), members of the Good’s buffers family (6) and 13C-dicarboxylic acids (5) have been polarized with DNP to sense pH changes in vitro. The only hyperpolarized molecules that have successfully been used to image pH in vivo are 13C-bicarbonate (either hyperpolarized directly (2) or derived from 13C-labelled and hyperpolarized precursor molecules (3, 4)) and 13C-zymonic acid (1).

### 2.2 NMR-Mechanisms of pH-Sensitivity

The acid dissociation constant (pKₐ) determines the center of the range of pH-sensitivity of an NMR-active nucleus. It is derived from the chemical equilibrium of two species – the acid (HA) and its conjugate base (A⁻) – that are in pH-dependent exchange with the exchange rate k. If k is much smaller than the difference (Δν) in NMR resonance frequencies (in Hz) of HA and A⁻ (k ≪ Δν), the two species are in slow exchange and two distinct peaks are observed (Figure 2).

In this case, the pH can be determined by a ratiometric approach from either the signal amplitudes or integrals (I, equation 1). A prominent example is the equilibrium of bicarbonate (HCO₃⁻) and carbon dioxide (CO₂), which has a moderate exchange rate of 0.1 Hz and a relatively large chemical shift difference of 35 ppm (≈ 1.1 kHz at 3 T).[3]

\[
pH(\delta) = pK_a + \log_{10}\left(\frac{I(A^-)}{I(HA)}\right) \tag{1}
\]

If the two species are in fast exchange, i.e. if k is much larger than Δν (k ≫ Δν), only a single peak will appear in the NMR spectrum. The observed chemical shift (δ) is the mean of the chemical shifts of HA and A⁻ weighted by the signal integrals. A modified Henderson-Hasselbalch equation can be used to determine the pH from the observed chemical shift with the sign of the second summand being positive for downfield shifts and negative for upfield shifts with increasing pH values (equation 2). δₘᵢₙ and δₘₐₓ are the lower and upper limits of the observed chemical shift and correspond to the chemical shift of the fully protonated and deprotonated form of the molecule or vice versa.

\[
pH(\delta) = pK_a \pm \log_{10}\left(\frac{\delta - \delta_{\text{min}}}{\delta_{\text{max}} - \delta}\right) \tag{2}
\]

With the exception of bicarbonate (2, 3, 4) and the xenon-cryptophane pH sensor (10), all hyperpolarized nuclei in the molecules shown in Figure 1 are in fast exchange. Their chemical shift differences range from 3 ppm to 100 ppm, which equals Δν ≈ 10⁻²–10² Hz at 3 T. Exchange rates of hydroxyl and amino protons are on the order of 10⁻³–10⁴ Hz.[81]
**In vivo** exchange rates of the mentioned pH sensors are dependent on several factors like proton diffusion, temperature and the pH itself. This could lead to a so-called intermediate exchange regime \((k \approx \Delta \nu)\) for which the proton exchange rate is approximately equal to the frequency separation. The resulting resonance signal is characterized by a broad line-width which is unfavorable for an accurate pH determination. Measuring at a different external magnetic field strength (ideally at a clinically relevant field strength) might be a possible solution to circumvent this problem.

### 2.3 Sensitivity, Signal Lifetime, pH-Sensitivity and Biocompatibility of pH Sensor Molecules

The pH biosensors shown in Figure 1 bear different isotopically enriched \((>99\%)\) spin-\(^{15/2}\) nuclei (X). Their NMR sensitivity at thermal equilibrium with respect to hydrogen is defined by the gyromagnetic ratio \(\gamma(X)/\gamma(^1H)\). Hyperpolarization increases the NMR sensitivity, which can be quantified by the polarization enhancement or the polarization level. The polarization enhancement \((\varepsilon)\) is the ratio of the hyperpolarized signal \((S_{\text{hyper}})\) to the thermal signal \((S_{\text{thermal}})\) measured under the same experimental conditions. The polarization level \((P_{\text{hyper}})\) can be determined by multiplying the thermal polarization level \(P_{\text{thermal}}\) with \(\varepsilon\) (equation 3):\

\[P_{\text{hyper}} = S_{\text{hyper}} / S_{\text{thermal}} \cdot \tanh \left( \frac{\gamma(X)hB_0}{2k_B T} \right) = \varepsilon \cdot P_{\text{thermal}} \quad (3)\]

with \(h\) being the reduced Planck constant and \(k_B\) the Boltzmann constant.

The relative sensitivities of \(^{13}\text{C}\) and \(^{129}\text{Xe}\) nuclei are similar to each other while the ones of \(^{15}\text{N}\) and \(^{98}\text{Y}\) nuclei are more than 10-fold or 100-fold smaller, respectively. Achievable polarization levels for \(^{13}\text{C}\), \(^{129}\text{Xe}\), \(^{15}\text{N}\), and \(^{98}\text{Y}\) are 15–70%, 1–64%, 2–10% and 1–8%, respectively. To our best knowledge, \(^{15}\text{N}\) and \(^{98}\text{Y}\) nuclei have only been polarized using preclinical polarizers and their polarization levels can probably be enhanced by a factor of two if high-field polarization techniques are used.

For **in vivo** applications, hyperpolarized substances need to be transported from the polarizer to the scanner and prepared for injection. After administration, further time is required for the molecules to circulate in the blood and accumulate in the region of interest before signal acquisition is possible. Although these procedures can be accelerated and standardized, a high initial polarization level and a long spin lattice relaxation time \((T_1, both in vitro and in vivo)\) are critical for hyperpolarized experiments.

Hyperpolarized signals decay with the spin lattice relaxation time \((T_1)\), which is mainly governed by dipolar coupling with neighbouring protons. Dipolar coupling scales with \(\gamma^2\) and the distance between the interacting nuclei \((d^{-6})\). Heteronuclei with directly attached protons have a relative short \(T_1\), which for instance makes \(^{13}\text{C}\)-carbonyls favourable for hyper-

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**Figure 2.** pH-dependent fast and slow chemical exchange of molecules with NMR resonance signals. (a) In slow exchange \((k \ll \Delta \nu)\), two resonance signals at a distinct chemical shift are observed. Their signal amplitudes or integrals change with respect to pH (left column). A single resonance signal shifts with respect to pH when HA and A\(^{-}\) are in fast exchange \((k \gg \Delta \nu)\). The theoretically observable fractions of HA and A\(^{-}\) are indicated in dashed lines (right column). (b) A modified Henderson-Hasselbalch equation allows the pH calculation from resonance signals that are in slow (equation 1) or fast (equation 2) exchange.

In the case of the cryptophane-bound \(^{129}\text{Xe}\) (10), its chemical shift is affected by a combination of pH-dependent peptide conformational changes and membrane insertion.
polarization. If the molecule of interest is not symmetric, chemical shift anisotropy effects scaling with $B_0^2$ need to be taken into account as well.\cite{55} In case dipolar coupling between high-$\gamma$ nuclei (H) and heteronuclei is inevitable, deuteron enrichment is a feasible strategy to prolong $T_1$, which was demonstrated for hyperpolarized molecules, such as choline\cite{86}, glucose\cite{87} and pyruvate\cite{88}. So far, pH-sensitive dicarboxylic acids\cite{55}, e.g. (5), have been deuterted and thus exhibit long $T_1$ values of $>1$ min even at high field strength ($B_0=11$ T)\cite{55} The same strategy should be beneficial for hyperpolarized ACES (6) and zymonic acid (1), which contain replaceable protons as well.\cite{50,56}

Radical removal after dissolution is not only important to guarantee physiologically tolerable solutions for injections, but also to reduce paramagnetic relaxation between the pH sensor and the radical in solution. Techniques for radical filtering or radical free polarization for DNP were summarized in section 2.1.

In special cases where isotopically enriched nuclei of interest are directly attached to spin-1 nuclei (i.e. $^{13}$C,$^{14}$N), isotope exchange (i.e. $^{14}$N vs. $^{15}$N) is a feasible way to avoid fast scalar coupling relaxation to quadrupolar nuclei at Earth’s magnetic field (i.e. ACES, 6). Alternatively, magnetic transfer lines\cite{89} as well as hand-held permanent magnets\cite{90} or specifically designed electromagnetic transport boxes\cite{91} have been proposed to reduce this unfavorable relaxation process during transport of the samples and to increase $T_1$\cite{92}.

Ji et al. showed that metabolites (or pH sensors) can be trapped in microparticles that physically separate them from paramagnetic polarization agents, which allows long time storage (up to 16 h) and facilitates a convenient transport of the hyperpolarized substances even to a remote MR scanner.\cite{93} SAMBADENA (Synthesis Amid the Magnet Bore, A Dramatically Enhanced Nuclear Alignment) – a recent polarization approach based on PHIP – constantly produces the hyperpolarized substance directly in the MR scanner and avoids polarization losses during transport.\cite{94}.

Chemistry-based approaches to increase the polarization level and $T_1$ use well polarizable precursor molecules that yield the desired hyperpolarized pH sensor molecule at high concentrations after chemical decomposition. The most commonly used molecule used for hyperpolarization, [1-$^{13}$C]pyruvate, yields $^{13}$C-bicarbonate in presence of hydrogen peroxide (3).\cite{52,55} Korenchan et al. have recently presented 13C-enriched carbonates which decompose to sodium bicarbonate and biologically tolerable side products (4).\cite{94} These precursors have up to three-fold longer $T_1$ times than bicarbonate, which saves magnetization during transport and sample preparation.\cite{93,95}

Another elegant approach to prolong hyperpolarized signal lifetime exploits the phenomenon of so-called long-lived states, which are prevalent in molecules bearing a pair of magnetically equivalent spin-1/2 nuclei.\cite{96-98} They form either a triplet or singlet nuclear spin state, with the lifetime of the singlet state being potentially much longer than $T_1$, since it is not affected by dipolar relaxation mechanisms. Although long-lived states cannot be directly observed by NMR, magnetization can be transferred from the triplet to the singlet state for storage and later on be recovered for the experiment by chemical reactions\cite{89}, specific pulse sequences\cite{100-102} or at low μ-tesla magnetic fields.\cite{103,104} This concept has not yet been applied to pH-sensitive molecules, but it was already used in vivo\cite{103} and could potentially increase the available signal intensity of hyperpolarized pH imaging molecules as well.

Although the signal enhancement is highest for $^{13}$C-nuclei, their $T_1$ is shorter compared to $^{15}$N and $^{89}$Y. The $T_1$ of 129Xe is in a similar range as the one of $^{13}$C. Nonetheless, DNP polarization techniques combined with efficient transportation and fast acquisition techniques (see section 2.5) allow for the acquisition of a pH image within a few seconds.

In general, the highest pH-sensitivity of a pH biosensor is around its $pK_a \pm 1$. For ratiometric approaches, an accurate pH determination requires a high SNR, which minimizes potential errors of the quantification of the signal amplitudes (or integrals). Hyperpolarized bicarbonate (2) was the first and so far only ratiometric sensor applied both in vitro and in vivo.\cite{13} A main drawback of the molecule is its short $T_1$ (in vivo $\sim 10$ s at both 3 T and 9.4 T\cite{95}) and a small SNR of $^{13}$CO$_2$ at physiological pH due to the low p$K_a$\cite{95,103}.

The pH sensitivity of chemical shift based sensors is given by $\Delta \delta$, the difference of the maximum and minimum observed chemical shift. The bigger $\Delta \delta$, the higher the pH-sensitivity of the sensor. Table 1 gives an overview of the pH-sensitivity of chemical shift based sensors. Proton binding and dissociation at the nitrogen of $^{15}$N-heterocycles changes the chemical environment of the NMR-active nucleus leading to the largest chemical shift changes, compared to $^{89}$Y-DOTP (9), $^{129}$Xe cryptophane (10) and $^{13}$C-labelled pH biosensors (1, 5, 6). However, the suggested $^{15}$N-labelled pH sensors (7, 8) are likely to be toxic and would therefore not be transferable to the clinic. DOTP (9) forms high affinity complexes with yttrium\cite{96} and has a relatively large $\Delta \delta$ of $\sim 10$ ppm, but toxicity and low sensitivity of $^{89}$Y also preclude this sensor from in vivo use. The xenon-cryptophane sensor (10) and $^{13}$C-labelled chemical shift pH sensors (1, 5, 6) have a similar pH-sensitivity, ranging from $\Delta \delta \approx 2.5$ ppm to $\approx 8$ ppm, and cryptophanes have also already been shown to be non-toxic when applied in cell experiments.\cite{107-109} The pH determination using the $^{129}$Xe sensor in HYPER-CEST experiments allows a targeted detection of aberrant pH at picomolar concentrations in vitro. The two advantages of the chemical shift based $^{13}$C pH sensors (1, 5) are a high polarization level and – except for ACES (6) – a long $T_1$. For diethylmalonic acid (5), the toxicopathological profile needs to be evaluated for in vitro compatibility. Zymonic acid (1) is used as a food additive and flavouring agent\cite{110} and does not show toxicity against cells and rodents.\cite{50} The molecule is synthesized from [1-$^{13}$C]pyruvate and exhibits two pH-sensitive carbon atoms. It is the first hyperpolarized chemical shift based pH sensor that was successfully applied in vivo (see section 2.6).
2.4 Screening Strategies for New Hyperpolarizeable $^{13}$C pH$_v$ Biosensors

Hyperpolarized $^{13}$C pH sensors need to have an exchangeable proton, e.g. in a carboxylic acid-, stable enol- or amine-group, that is close to a $^{13}$C nucleus with long $T_1$. For in vivo use, the pK$_a$ of this proton should be between 6.4 < pK$_a$ < 7.6 in order to detect aberrant changes compared to the physiological pH$_v$.

Small molecules are in general favorable, as they are easy to synthesize and to label and since they tend to have a long $T_1$, which decreases as a function of the molecule size and correlation time ($T_C$). Because dipolar coupling with high-$\gamma$ nuclei determine the magnetization loss and $T_1$ shortening to a major extent, systems without directly neighboring protons to the hyperpolarized nucleus are favorable. Fortunately, most pH-sensitive functional groups like carbonyls and carboxylic acids are devoid of directly attached protons. For an efficient hyperpolarization based on DNP, the molecule should be self-glassing or soluble at high concentrations (>4 M) in common glassing agents such as dimethyl-sulfoxide, glycerol or N,N-dimethylacetamide. Furthermore, non-toxicity and sufficient chemical stability during the dissolution process and in aqueous solution is necessary.

Before being applied in vivo, the pH-sensitivity (pK$_a$) of potential pH sensor candidates should be proven to be independent of the following five parameters: 1) sensor concentration, 2) temperature, 3) ionic strength, 4) bivalent metal ions and 5) proteins. This is because sensor concentration, temperature and ionic strength may vary slightly in different tissues, and because bivalent metal ions and proteins are present in vivo. Both ions and proteins can complex or unspecifically bind to the sensor molecule, which potentially alters the pH-sensitive mechanism and might lead to an erroneous pH determination.

2.5 MR Acquisition Strategies for Hyperpolarized pH-Sensitive Nuclei

The requirements for MRI sequences with regard to spectral resolution depend on the underlying mechanism of the pH sensor molecule: For molecules in the slow exchange regime, such as $^{13}$C-bicarbonate (2, 3, 4), two distinct NMR resonances need to be detected, whereas for molecules in the fast exchange regime, such as zymonic acid (1), one varying, pH-dependent chemical shift of a pH-sensitive nucleus with respect to a reference peak needs to be resolved.

The most straightforward method for spatial imaging of pH using hyperpolarized compounds either in fast or slow exchange is a 2D chemical shift imaging (CSI) sequence, as shown in Figure 3 in an in vitro phantom experiment.

CSI has been applied with different gradient encoding strategies such as Cartesian, spiral, or radial readouts and can be combined with variable flip angle schemes to increase the encoding efficiency. A strategy to speed up the inherently slow phase-encoded CSI sequence is the use of an echo-planar spectroscopic imaging (EPSI) readout, which allows to acquire spectroscopic images within a couple of seconds. EPSI increases the gradient hardware and magnetic field homogeneity requirements but reduces the time needed to acquire an image, thereby improving SNR and real resolution.

For the case of $^{13}$C-bicarbonate (2, 3, 4), three alternative acquisition schemes were applied for pH imaging: first, a Dixon-type IDEAL spiral CSI, second a gradient-echo sequence which utilized a resulting spatial shift in the frequency encoding direction between the bicarbonate and CO$_2$ resonances for separation of the respective signals and third, a 3D double spin-echo pulse sequence with a flyback...
Upon treatment with bicarbonate and ammonium chloride, bicarbonate showed pH alterations, which were confirmed by spectroscopic $^{13}$P pH measurements with 3-APP[11] Other studies detected an acidic tumor microenvironment in transgenic adenocarcinoma of mouse prostate (TRAMP),[54,116] Scholz et al. induced an acute inflammation with Concanavalin A and measured an acidic pH 7.0 compared to systemic blood pH 7.4.[59]

Zymonic acid is a cyclic dimer of pyruvic acid and can be synthesized in one step and in reasonable quantities. Physiological parameters that could influence the pH sensitivity (see section 2.4) do not affect its pK$_a$ Diewel et al. co-polarized ZA with $^{13}$C-urea to back-calculate the in vivo pH$_e$ from a calibration curve (Figure 3). Zymonic acid was demonstrated to image the pH$_e$ of three kidney compartments indicating the renal blood filtration process (Figure 5). Furthermore, an acidification of the tumor microenvironment of subcutaneous

2.6 In vivo pH$_e$ Imaging with Hyperpolarized $^{13}$C Nuclei

So far, hyperpolarized $^{13}$C-bicarbonate (2, 3, 4) and [1,5-$^{13}$C]zymonic acid (1) have been used to image pH$_e$ in vivo in preclinical studies.

Brindle et al. pioneered the field of hyperpolarized pH imaging by introducing $^{13}$C-bicarbonate as the first hyperpolarized in vivo pH$_e$ imaging sensor. They demonstrated the feasibility of hyperpolarized $^{13}$C pH imaging by probing the acidic tumor microenvironment of EL4 lymphoma (Figure 4).

![Figure 4](image-url). In vivo pH$_e$ imaging with hyperpolarized $^{13}$C-labelled bicarbonate. (a) Axial proton image of a mouse bearing a subcutaneous EL4 tumor. (b) pH$_e$ image calculated from the ratio of the intensity images of bicarbonate in (c) and carbon dioxide in (d). The tumor in (a) is outlined in red, in (b–d) in white. Adapted and with permission from [5].
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mammary adenocarcinoma (MAT B III) was imaged and confirmed with 3-APP and invasive measurements with an optical electrode.

Both bicarbonate (2) and zymonic acid (1) have advantages and disadvantages. Bicarbonate has a relatively short $T_1$ and CO$_2$ freely diffuses through membranes. The measured pH is therefore a mean of the extra- and intracellular pH although the value seems to be rather weighted towards the extracellular fraction.$^{[51]}$ In disease states, carbonic anhydrase, which speeds up the process of reaching a steady state in the bicarbonate/CO$_2$ equilibrium, could partially be inhibited, which could impair the accuracy of the measurement. Nonetheless, the use of highly polarizable precursors makes this endogenous compound promising for further in vivo applications.

Zymonic acid has a longer $T_1$ in vivo than bicarbonate and distributes in vascular and extravascular (interstitial/extracellular) compartments. The signal stemming from the vascular compartment after intravenous injection of zymonic acid is dominant because of the large blood pool. The detection of other compartments is challenging because it requires a sufficient chemical shift separation compared to the signal in blood and a sufficient signal-to-noise ratio. However, it has been demonstrated both in tumor and kidney that the interstitial/extracellular pH$_i$ can be detected simultaneously together with the vascular pH within the same voxels, rendering this molecule interesting for further in vivo applications.$^{[50]}$

3. Summary

Many efforts have been undertaken to develop imaging methods for quantitative, non-invasive pH$_i$ imaging. However, so far, none of these techniques has been applicable in the clinic. In patients, local deviations from the systemic pH are often caused by pathologies, such as cancer, inflammation, infection, ischemia, renal failure or pulmonary disease, making pH a potentially useful clinical imaging biomarker.

During the last decade, several hyperpolarized pH$_i$ sensor molecules using various polarization techniques have been developed (Figure 1 and Table 1). The underlying mechanism of pH-sensitivity for all of these techniques is a $pK_a$-dependent protonation or deprotonation in the vicinity of the hyperpolarized nucleus. This leads to a pH-dependent chemical shift for nuclei in fast exchange and to a change in the signal ratio for nuclei in slow exchange. Efficient, robust and fast pH imaging approaches for hyperpolarized nuclei are available. In addition, prolongation of $T_1$ by deuteration or long-lived spin states could be exploited in the future as a strategy to enhance NMR-sensitivity.

Two hyperpolarized pH$_i$ sensors have so far been applied in vivo: hyperpolarized $^{13}$C-bicarbonate and [1.5.$^{13}$C]zymonic acid. Using both sensors, it was possible to measure extracellular tissue acidification in tumors. Bicarbonate was used to detect a decrease in pH in acute inflammation and zymonic acid resolved three different pH compartments within the same renal voxel, where the ability to resolve more than one pH compartment per voxel is a unique feature of fast exchanging pH-sensitive molecules. Therefore, both $^{13}$C-bicarbonate and zymonic acid have demonstrated promising results for pH$_i$ imaging in preclinical animal models. Besides increasing NMR-sensitivity and spatial resolution of the imaging approaches, future work needs to evaluate the benefit of pH imaging techniques for specific diagnosis and monitoring of disease as well as for specific selection and prediction of therapies. Since intravenous injections of both bicarbonate and zymonic acid have shown no toxic side-effects and since hyperpolarized pyruvate is already being used in clinical studies, the translation of hyperpolarized pH$_i$ sensors to the clinic seems promising.

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