Leibniz-Gemeinschaft

Final report

Development of Novel NMR Probes: Improving Cell Profiling for Early Diagnosis

Leibniz-Institute: Leibniz-Institut für Molekulare Pharmakologie (FMP) im Forschungsverbund Berlin e.V.
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Contents

Executive summary

Knowledge in molecular biology concerning the onset and progression of diseases is constantly growing. However, the abilities for non-invasive diagnostic-imaging seriously lack behind because this knowledge is challenging to translate into detectable signals that can be used for tomographic image reconstruction. There are nonetheless serious efforts to implement molecular targeting strategies by small molecules or biological macromolecules into modern imaging applications for diagnosis and therapy of disease. Molecular recognition events can thereby be combined with certain detection techniques that are based for example on fluorescence, radionuclides or magnetic resonance (MR) phenomena. Purpose of this project was the development of new biosensors for MR diagnostics that pair the high sensitivity of hyperpolarized xenon with the high specificity of a functionalized targeting unit.

Xenon biosensors were only part of proof-of-principle studies at the beginning of this project. The only targeted version that had been used to acquire MR imaging (MRI) data relied on the idealized biotin-avidin interaction. Hence, this worked aimed to advance the status of xenon biosensors into applications of biomedical relevance. Significant contributions, both on the sensor design and the detection and characterization level, could be achieved. Two different strategies were pursued in terms synthesis of novel, ultra-sensitive Xe biosensors: type 1 as an antibody-based modular setup with individual Xe hosts as detectable MR reporters and type 2 as a nanodroplet-based design with a compartmental approach where a multitude of Xe is bound and can be used for cell labeling. These efforts were accompanied by some hardware development to implement an MR-compatible bioreactor that allows live cell studies within the magnet under continuous perfusion with Xe-enriched medium.

The combination of hardware and sensor improvement allowed to demonstrate the first ever MR live cell studies with Xe biosensors. Moreover, impressive sensitivity could be achieved with a nanomolar detection threshold, i.e. approx. 1000-fold improvement compared to achievements with the first sensor generation used before the start of this project. Another integral component was the demonstration of multi-channel imaging, i.e. the different “staining” of cells with different sensor types 1 and 2 that allowed channel-selective MRI detection of two different cell lines side by side.

Co-validation of cellular labeling with these new sensor types has been achieved through fluorescence techniques. To do so, we also implemented novel protocols with fluorescence-labeled Xe hosts as building blocks for correlating sensor uptake in the MR-investigated cells with fluorescence cytometry. Such dual-mode (optical and NMR) detection enabled sensitive cell profiling in cell lines for different disease models.

Overall, the achieved results represent a significant advancement of the Xe biosensor concept and established the Berlin-based labs as leading groups for future biomedical applications of this technique. The published studies are impressive illustrations for the future potential of Xe MR imaging with targeted sensors to close the sensitivity gap between modalities of nuclear medicine like PET/SPECT and MRI without using ionizing radiation or making compromises in penetration depth like in optical methods.

Background and Aims

Biomedical imaging has made great progress in developing methods that allow for visualization of tissue contrast beyond pure morphological information. In magnetic resonance imaging (MRI) the design of new contrast agents for disease-specific molecular targets or physiological parameters[1–6] has been an active field of research. Most of them rely on the detection of the ubiquitous water proton (^{1}H) signal rather than a direct detection of a specific disease-related biomolecule and therefore face limitations like a strong (unspecific) background signal and low sensitivity. Many markers that would be potential imaging reporters are simply too dilute to be detectable with MRI. Positron emission tomography (PET) as the seemingly better method (with respect to sensitivity) also suffers from strong unspecific background signals.[7]

Recent focus in MRI development has been on so-called hyperpolarized agents, i.e. specially prepared tracers with up to 10'000-fold increased magnetization to allow for detection of dilute concentrations. Another class of novel reporters was based on switchable relaxivity agents or frequency-selective saturation transfer contrast agents that alter the abundant water signal. However, many of these methods have only been demonstrated as in vitro studies with no further tests under physiological conditions like living tissue or even cell cultures. Although the community is aware of this problem,[8] few attempts have been made to bring the chemistry of novel agents into biomedical applications.

Part of the problem is the inherent low sensitivity of conventional MRI that requires relative high concentrations of both the contrast agent (ca. 50-500 µM[8]) and the target molecule to induce detectable changes of the water signal. Even the hyperpolarized agents were still facing challenges in terms of sensitivity and were not detectable at sub-micromolar concentrations in cellular environments. Contrary to MR, fluorescence microscopy and imaging is largely applied at the molecular level, ranging from genetically encoded sources (e.g. green fluorescent protein) to fluorescently-labelled antibodies. Developments such as two-photon detection or infrared fluorescence have enabled better tissue penetration, however, there are still severe limitations for the detection of these probes in deep tissues and whole organs.

This project was therefore designed to combine several amplification strategies to achieve unprecedented sensitivity and to demonstrate novel MRI biosensors with biological relevance. Ultimately, the key questions asked in this project are related to the utilization of novel probes for magnetic resonance imaging in vitro and in vivo. Starting from the inherently low sensitivity of conventional MR probes, signal amplification was partially achieved through hyperpolarized xenon as a means to interrogate molecular binding events or enzymatic reactions by MR. This was combined with chemical exchange saturation transfer (CEST) in which the noble gas constantly switches between two different chemical environments (free in solution and sensor-associated) and therefore jumps between two resonance frequency. These can be selectively addressed with radio frequency pulses to "label" sensor-related Xe and accumulate its signal in the solution environment. The method is called Hyper-CEST[9] because of the participation of the hyperpolarized noble gas and needed further improvement in sensor design. This need was actually considered as an opportunity to improve the amount of exchanging Xe per sensor unit significantly to incorporate a third measure for signal enhancement. Overall, we originally formulated the following aims based on this:

- Design of peptide- and antibody based novel sensors
- Magnetic resonance characterization (in particular Hyper-CEST studies) of targeted biosensors
- Cellular and perfused organ studies with targeted biosensors

Development oft he Project

Part of the aims were pursued in parallel, in particular the sensor design, synthesis and initial NMR characterization. Development of the bioreactor setup for the planned cell culture studies also started early on and was initially just tested for optimum perfusion with hyperpolarized Xe and sufficient cell viability. Once the optimized sensors had been obtained, cell labeling studies started with fluorescence tests to quantify cellular uptake and estimate NMR outcome, followed by verification of achievable sensitivity in simplified experiments with diluted sensor solutions.

Eventually the efforts resulted in multiple live cell studies that currently define the state of the art in Xe biosensor research and that will represent important reference studies for future translation into small animal studies. Considering the three defined aims, the project developed as follows:

Design of peptide- and antibody based novel sensors

We have achieved the production of an antibody-based sensor by expressing an avidin-conjugated antibody against CD14, a macrophage marker for inflammation studies. The modular design of our constructs allowed for either (i) detection by an optical probe when using the antibody in combination with a biotinylated fluorophore (ii) detection by magnetic resonance when utilizing a biotinylated cryptophane-A or (iii) the dual mode detection by using a cryptophane-A-fluorophore-biotin construct. The strength of this approach becomes especially clear in the context of alternative peptide-based designs. This approach was also pursued initially but it turned out that coupling of the cryptophane cages directly onto a peptidic backbone comes with rather limited yield and requires optimization for each type of sensor. We therefore decided to switch to a modular setup in which the cryptophane as an established Xe host is coupled to a biotin unit which itself enables ligation to many other types of modules that carry avidin as the coupling partner. This design was motivated by commercially available coupling kits that provide avidin conjugation to accessible lysine residues, e.g. on antibodies. CD14 was chosen as a target because stimulating macrophage cells with bacterial lipopolysaccharide (LPS) in vitro and pairing them with unstimulated fibroblasts provides an ideal model system for comparing a target with a control cell group.

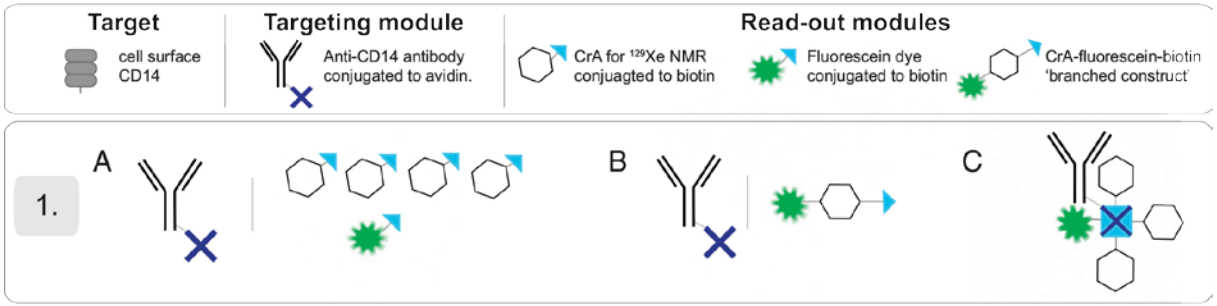

Fig. 1: The CD14 biosensor is comprised of an avidin-conjugated antibody targeting module and a set of biotin-conjugated readout modules for dual functionality (NMR/fluorescence) to selectively label high CD14-expressing cells. Multiple avidin molecules are conjugated to each targeting module, an anti-CD14 specific antibody, but for schematic simplicity, only one avidin is depicted per antibody. The biosensor can be applied in two different ways: sequential incubation (A and B), in which cells are incubated with the targeting module, followed by the readout modules, or via incubation with the complete construct (C), in which the targeting modules and readout modules are preconnected.

Upon the successful antibody-based designs, peptide-based approaches were not further followed, since they do not achieve the high specificity and affinity that can be obtained by antibodies. Furthermore, synthesis costs for peptides are relatively high compared to the relatively low costs for commercially available antibodies. However, part of the insights gained from the peptide-based approach was used to initiate a collaboration with the groups of Profes. Pines, Wemmer, and Francis at the University of California at Berkeley. One team member was successfully trained at UC Berkeley in grafting cryptophane cages onto bacterial phages that can be targeted to breast cancer cell lines. We acquired Hyper-CEST z-spectra with quality far superior to the initially published data.[10] This was accompanied by MRI data sets with significant image contrast that could not be achieved by the Berkeley team alone. A joint publication with these results is currently in preparation. This collaboration will be continued beyond the duration of the SAW grant.

The modular sensor design already allowed to have multiple Xe exchange sites (ca. 20) per antibody and therefore incorporated our third above-mentioned signal amplification component. However, we decided to develop another biosensor class that would allow even more efficient Xe exchange, hence better Hyper-CEST capabilities. This part of the project focused on perfluorooctyl bromide (PFOB) nanodroplets that are known for their gas binding properties and used as ultrasound contrast agents. The resonance frequency for droplet-bound Xe is distinct from free Xe and therefore enables selective saturation and Hyper-CEST quantification (see Fig. 2). Furthermore, the spectral selectivity for these nanodroplets had been reported to be different from Xe@cryptophane, so we decided to try them out for a multiplexing experiment in which to cell lines are labeled either with a PFOB based reporter or

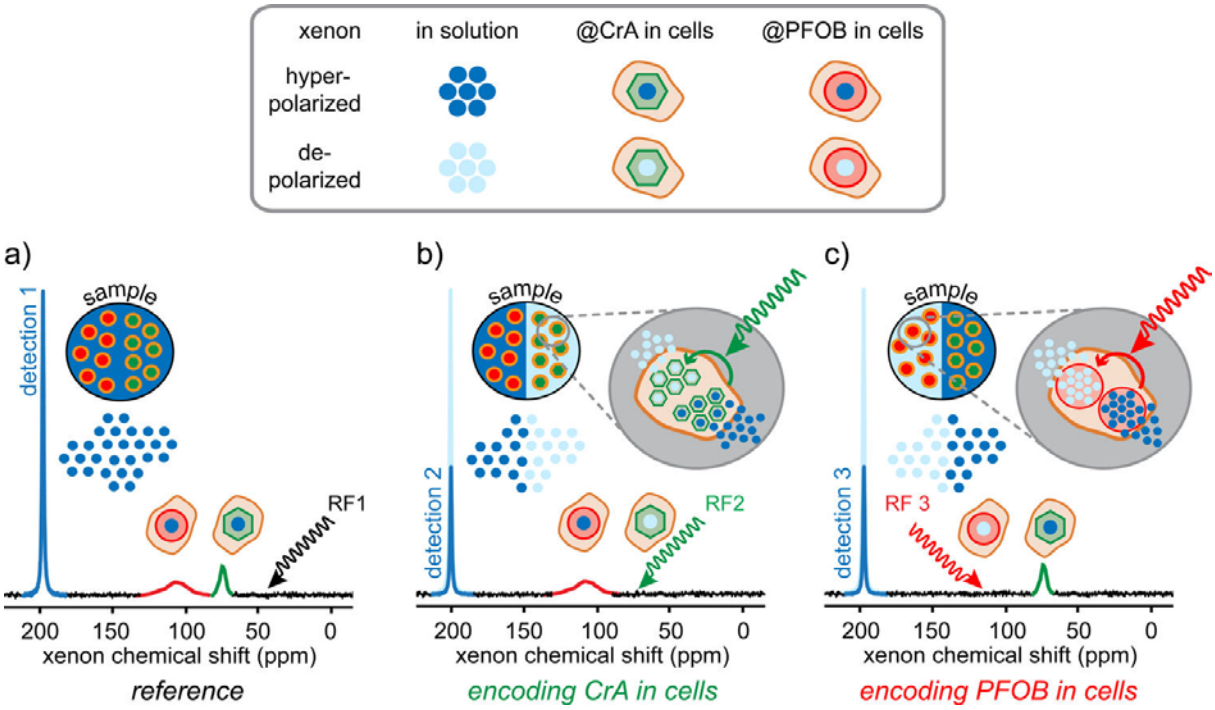

Fig. 2: Principle of multichannel Hyper-CEST detection for cell labeling. The MRI signal of hyperpolarized xenon in bulk solution acts as a sensing medium. It reports on the spatial distribution of two distinct cell-internalized xenon inclusion complexes (CrA and PFOB) both characterized by a unique xenon chemical shift. A reference scan visualizes the unaffected distribution of xenon in solution ((a) off-resonant saturation). The localization of cells either labeled with CrA ((b) on-resonant saturation for xenon@CrA in cells) or PFOB ((c) on-resonant saturation for xenon@ PFOB in cells) is encoded within the sensing medium by a frequency-specific saturation transfer acting on xenon atoms temporarily entrapped within the respective cell-internalized nanocarrier. Schematic spectra are shown for clarification as are participating xenon pools on top. Peak widths differ due to different exchange conditions.

with a cryptophane-A based one.

Magnetic resonance characterization of targeted biosensors

Novel biosensors were characterized in multiple ways. First, they were characterized spectroscopically with regard to the specific xenon NMR frequency. Tests verified that the CEST performance of conjugated cryptophane cages did not significantly differ from unconjugated cages. Next, optimized saturation transfer conditions were identified to achieve strong but still somewhat selective saturation responses under different conditions. Dilution series have been recorded to identify the detection limit for subsequent live cell experiments. As a general component, different MRI encoding schemes were tested to obtain reliable image contrast before application of the saturation transfer pulses at feasible measurement time. For all simple test solutions, it turned out that turbo spin echo sequences with large turbo factors (up to the max. number of k-space lines) and centric encoding were the method of choice because of sufficient long T_2 relaxation. The addition of cell material (see also next section) shortened T_2 noticeably and made accelerated acquisition with echo planar encoding (EPI) necessary, albeit this comes with some of the typical image distortions.

Biosensor building blocks have been tested with different dyes to compare the overall impact of the fluorescence label on the CEST performance and later the cellular uptake.[11] Small differences in the resonance frequency were identified between FITC- and TAMRA-containing compounds. The cell experiments later revealed that the TAMRA version facilitated cellular internalization but also came with higher toxicity. The achievable CEST effect was comparable for free sensor in solution and contrary to some preliminary results it turned out that the dye did not influence the NMR characteristics enough to address TAMRA and FITC compounds selectively from each other. Because of the similar NMR performance and the easy availability of many FITC building blocks, this became the fluorescent label of choice for most of the studies.

Cellular and perfused organ studies with targeted biosensors

The developed sensor compounds were tested for different uptake scenarios. In general, all compounds were tested at different incubation conditions with respect to concentration and cell viability.[11] A typical protocol is shown schematically in Fig. 3 for the CD14-targeted sensor. Initial tests were done with cell suspensions and direct bubbling of the Xe into solution. This turned out to reduce cell viability significantly, presumably due to strong shear forces caused by bursting gas bubbles. We therefore initiated substantial work on the bioreactor setup early on.

The two major aspects of the bioreactor were a) sufficient Xe delivery and b) retention of the cells within the detection volume of the NMR coil. With regard to the Xe delivery, gas dispersion chamber was implemented directly upstream from the imaging volume (see Fig. 4) that enabled dissolution of the freshly hyperpolarized gas immediately before entering the detection area. To achieve improved signal intensity, tests with an increased Xe fraction (5% instead of 2% of the total gas mixture) were performed and considered to be useful as the signal practically doubled. Initial test with gas exchange membranes were not followed up further since the observed signal intensity was far inferior to the bubbling setup.

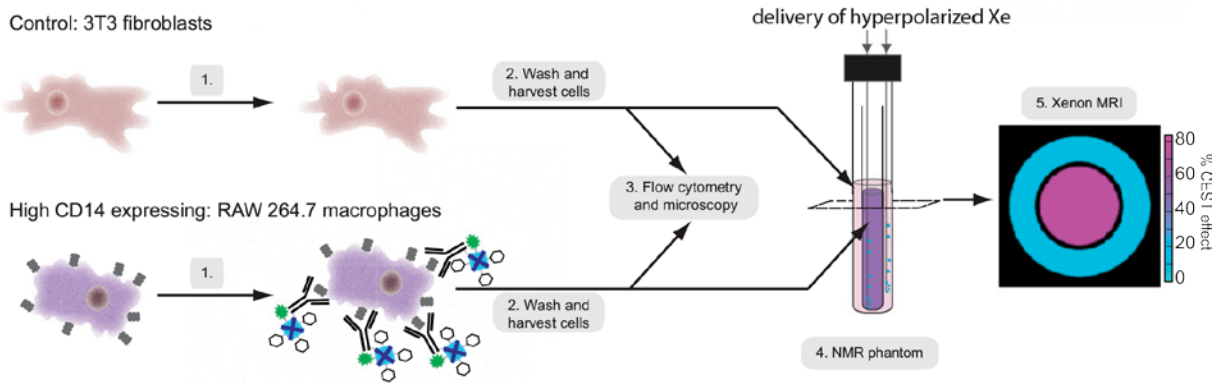

Fig. 3: Experimental design for live cell studies. Step 1: Cell incubation with biosensor compound. Step 2: Cells are washed to remove any unbound biosensor and harvested. Step 3: Cellular uptake, biosensor specificity and cellular localization can be evaluated via the fluorescence readout module. Step 4: The cell suspensions are placed into separate compartments within an NMR double phantom. The hp-Xe is bubbled through the samples for xenon MRI measurements. Step 5: Illustrative xenon MRI shows a cross-section of the NMR double phantom. The CEST effect encodes the localization of the CrA readout module in the compartment containing the high-CD14-expressing cells.

Next, a suitable cell immobilization protocol had to be developed. Various porous media such as glass beads and sponge-like structures were tested first. However, they turned out as unsuitable because they caused either problems with the magnetic field inhomogeneity or the cells were washed off during perfusion because of shear forces from the medium flowing at a few mL/min. Encapsulation of cells into alginate beads was finally identified as the method of choice. A remaining potential problem was the presence of Ca ions required for forming of the beads because Ca also binds to cryptophane cages to some extent. This problem could be solved by incubating the cells after fixation into beads and washing off the excess Ca that is not incorporated into the alginate. The beads showed high enough porosity to allow sensor uptake into the cells after bead formation.

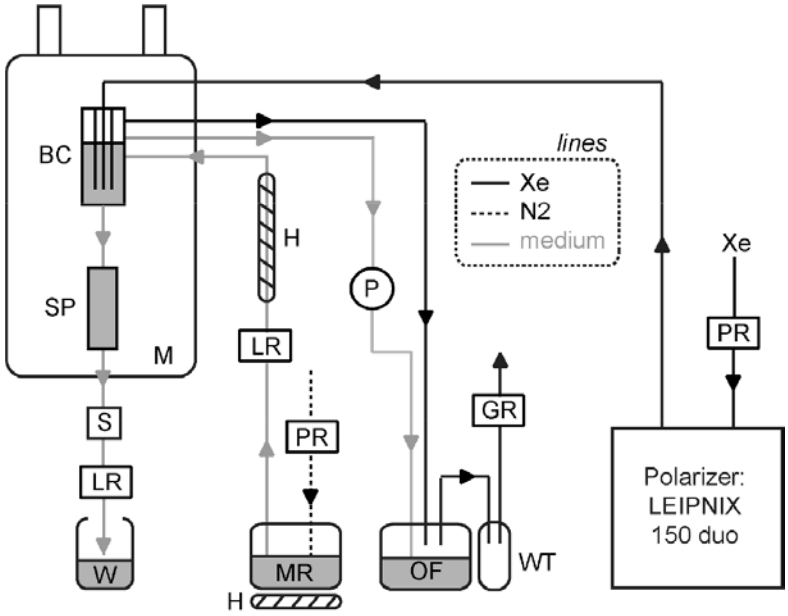

Fig. 4: Continuous-flow bioreactor setup for indirect delivery of hp-xenon. Alginate encapsulated cells are perfused with hp-xenon enriched medium. The dissolution of xenon takes place within a bubbling chamber sitting upstream the sample phantom. BC: bubbling chamber; SP: sample phantom; M: magnet; S: in-line sensors for temperature, pH and dissolved oxygen; LR: liquid flow regulator; W: waste tank; H: heating device; MR: medium reservoir; PR: pressure regulator; P: high pressure pump; GR: gas flow regulator; WT: water trap; OF: overflow tank.

The bioreactor setup was then successfully used for various designed sensor compounds. Initial tests for untargeted cell tracking were successful at micromolar concentrations.[12] The reliability of our protocol initiated collaborations, both internal and external ones, to test for novel sensor constructs. Results from the CD14-targeted sensor made clear that further improvement in sensitivity would still be beneficial and the PFOB-based sensor indeed allowed cellular labelling for MRI detection with further improved sensitivity, i.e. incubating the cells at 250 pM concentration.[13] Moreover, this study clearly demonstrated the aspect of multiplexing, i.e. the selective detection of differently labeled cell lines with two sensors in the same MRI study (see Fig. 5).

The originally intended projects on perfused organs were eventually omitted after a few tests on small organisms that demonstrated that Xe delivery might be too challenging within the time frame of this project. Since there was an extremely successful tool on hand in terms of the biosensor that also produced some demand from collaboration partners, aspects from live cell studies were substantially expanded to gain better insights in what is needed for carefully designing the first in vivo studies. This also included using the biosensor setup developed in this project for another collaboration that could successfully demonstrate MRI of cell surface glycans.[14] Thanks to the performance stability of the bioreactor, these previously inaccessible MRI targets could be visualized for the first time at nanomolar concentrations, hence with 1000-fold improved sensitivity compared to conventional contrast agents. This clearly demonstrated the potential of the xenon biosensor approach. Perfused organs might be useful as an intermediate step towards animal studies but we do actually think that the cell culture studies give enough insights for approaching the direct transition to in vivo experiments. The delivery of Xe, which was hard to mimic in a setup for perfused organs, will actually be enabled by the lungs and cardiovascular system in a much more efficient way.

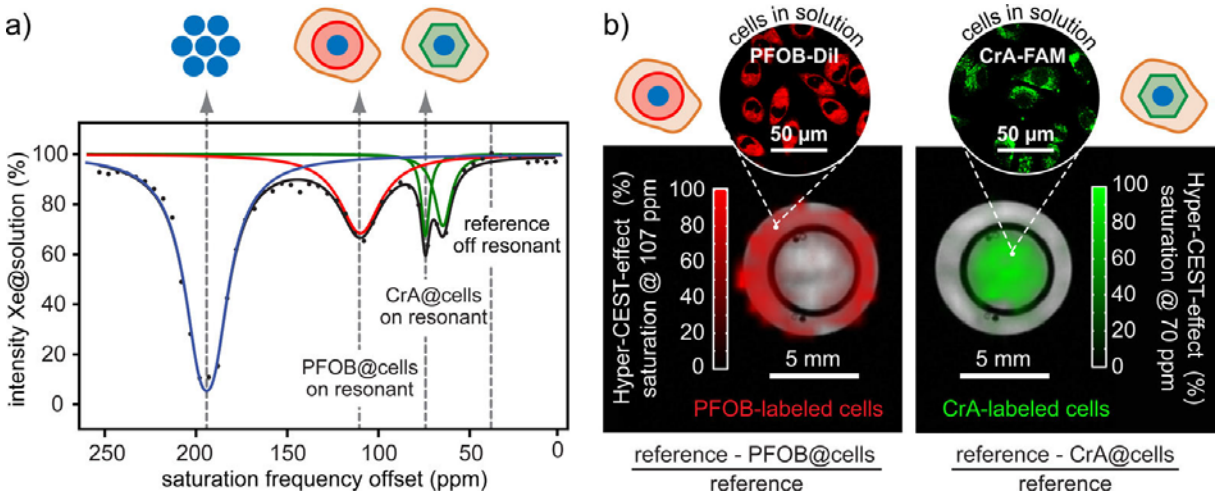

Fig. 5: PFOB- and CrA-labeled cells can be selectively localized by multichannel xenon-MRI as demonstrated for a two compartment phantom (outer compartment, PFOB-labeled cells; inner compartment, CrA-labeled cells). (a) The saturation frequency offsets used for Hyper-CEST imaging are indicated within the associated CEST-spectrum (8 s, 10 µT cw saturation) by gray dotted lines. Exponential Lorentzian fits are shown as solid lines. Schematics are used according to Figure 2. (b) ^{1}H-MR-Images are shown in overlay with pseudocolored Hyper-CEST-effects derived for both contrast agent-specific saturation conditions. Cell labeling was confirmed by laser scanning microscopy with fluorescence labeled versions of both Hyper-CEST agents as depicted within the circular insets.

Discussion oft he Results

The results obtained from the SAW project have pushed the field of Xenon-based magnetic resonance imaging forward in several ways:

- Novel designs could be validated that will be useful for a number of other laboratories around the world. For example, the modular design of our antibody-based constructs allow the coupling of virtually any cancer- or inflammation-relevant specificity to be equipped with the caged xenon.
- The construction of a new bioreactor now allows for the sensitive and reproducible MR imaging of cells. The dual chamber design of the reactor thereby allows the direct comparison of two types of cells, may this be tumor vs non-tumor, receptor-expressing vs non-expressing, stressed vs non-stressed cells etc. Possible applications range from testing mutant cell lines to small molecule or drug-based assays. Especially in combination with other MR-sensitive probes, such as perfluoroctyl bromide used by Klippel et al.[13], the multiplexed detection of cellular parameters can be investigated.

Further studies on sensors with even improved sensitivity, like the phage-grafted and anti-EGF directed sensors in a joint project with UC Berkeley, will be carried out in an ongoing collaboration between the applicants beyond the duration of this grant. Overall, the obtained results provided important insights to further advance the biosensor concept into in vivo applications and the applicants currently prepare the necessary setup and change in hardware components to be one of the first groups worldwide for performing this exciting transition.

The achieved results were published with much attention from the scientific community, both with respect to the paper and on international conferences like the 2014 World Molecular Imaging Congress in Korea. This work was honored with travel stipends for this conference to team members H. M. Rose and S. Klippel as well as with a Suraj Manrao Arward for Stefan Klippel at the ENC 2013 in Asilomar, USA

Follow-up projects were already initiated before the end of this SAW project. As mentioned above, interest by other groups was remarkable and triggered a collaboration with the California Institute of Technology on genetically expressible MRI contrast agents and some in-house collaborations on future in vivo studies. The results were used in preparing grant applications to the DFG and HFSP.

Economic Merit

The methodology developed here harbors a great potential to become a tool in diagnostic clinical application. However, we refrained from filing a patent for any particular aspect of the results from this project. This is mainly motivated because General Electric/Polareon currently still holds patents in the production of hyperpolarized Xe (which will expire within the next 3 years) and due to the fact that Lawrence Berkeley National Laboratory hold a patent on the use of caged Xe for biosensing applications (with priority date in July 2000).

Cooperation Partners

As mentioned above, the reliable performance of the bioreactor setup initiated a collaboration with the groups of Prof. Pines, Wemmer, and Francis at the University of California at Berkeley for which initial results have been obtained during the duration of this project.

Academic Qualifications

The biotechnology master student Stefan Klippel obtained his PhD as part of the work defined in the SAW proposal.

Related Publications

The following papers were published in the context of this project, either directly as part of Stefan Klippel'S PhD project or indirectly by using the tools developed therein in close timely relation (detailed information in the attached list of references):

- Klippel et al. in *Angew. Chem. Int. Ed.*[12] highlighted as cover article
- Klippel et al. in *Nano Lett.*[13] highlighted as cover article
- Rose et al. in *Proc. Natl. Acad. Sci. USA*[15]
- Rossella et al. in *ChemPlusChem*[11]
- Witte et al. in *Angew. Chem. Int. Ed.*[14] highlighted as cover article, very important paper and recommended by F1000 prime

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12. Klippel, S. *et al.* Cell Tracking with Caged Xenon: Using Cryptophanes as MRI Reporters upon Cellular Internalization. *Angew. Chem. Int. Ed.* **53,** 493–496 (2014).
13. Klippel, S., Freund, C. & Schröder, L. Multichannel MRI Labeling of Mammalian Cells by Switchable Nanocarriers for Hyperpolarized Xenon. *Nano Lett.* **14,** 5721–5726 (2014).
14. Witte, C. *et al.* Live-cell MRI with Xenon Hyper-CEST Biosensors Targeted to Metabolically Labeled Cell-Surface Glycans. *Angew. Chem. Int. Ed.* **54,** 2806–2810 (2015).
15. Rose, H. M. *et al.* Development of an antibody-based, modular biosensor for 129Xe NMR molecular imaging of cells at nanomolar concentrations. *Proc. Natl. Acad. Sci.* **111,** 11697–11702 (2014).

Data Management

Al data has been stored on local hard drives and in addition on servers either located at FMP or FU Berlin. The dissertation by Stefan Klippel with extensive documentation beyond the publication is available through

http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000099677

Media Coverage

For the publication by Klippel et al. in *Angew. Chem. Int Ed.*[12]
http://www.fmp-berlin.info/press-media/press-releases/press-releases-single-view1/article/das-mrt-der-zukunft-bildgebende-diagnostik-mit-xenon.html
reported in

- http://www.archiv.medizin-aspekte.de/Das-MRT-der-Zukunft-Bildgebende-Diagnostik-mit-Xenon_45586.html
- https://www.facebook.com/optikphotonik/posts/567842043294542
- http://www.invitrojobs.com/index.php/de/neuigkeiten/news-archiv/item/475-bildgebendes-mrt-mit-xenon-detektiert-winzige-details
- http://www.radiologiejournal.de/ARCHIV/Inhalt_3_2014.pdf
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For the publication by Klippel et al. in *Nano Lett*.[13]
http://www.fmp-berlin.info/press-media/press-releases/press-releases-single-view1/article/kernspin-in-farbe.html
reported in:

- http://www.healthcare-in-europe.com/de/artikel/14119-welches-potential-hat-die-xenon-mrt.html
- http://www.healthcare-in-europe.com/en/article/14038-xe-mri-advances-body-exploration.html
- http://www.gesundheitsstadt-berlin.de/mrt-in-farbe-fuer-individuellere-therapien-4860/

For the publication by Witte et al. in *Angew. Chem. Int. Ed.*[14]
http://www.fmp-berlin.info/press-media/press-releases/press-releases-single-view1/article/krebs-zuckermolekuele-weisen-den-weg.html
reported in:

- https://www.google.de/url?sa=t&rct=j&q=&esrc=s&source=web&cd=4&ved=0ahUKEwjcmbmj2eLKAhXEXQ8KHQtfDFUQFgg4MAM&url=http%3A%2F%2Fwww.bbb-berlin.de%2Fde%2Fdct%2F216%2Fdownload&usg=AFQjCNGS__cfiZnbL_-RibN9GIOGraNkCw&sig2=EVptKlsbf2rcvuOc3o-TSA

Report at RSNA Meeting 2013:
http://schroeder.fmp-berlin.info/RSNA.html
http://www.auntminnie.com/index.aspx?sec=rca&sub=rsna_2013&pag=dis&itemId=105910

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