Functionalized Lipopeptide Micelles as Highly Efficient NMR Depolarization Seed Points for Targeted Cell Labelling in Xenon MRI

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Improving diagnostic imaging and therapy by targeted compound delivery to pathological areas and across biological barriers is of urgent need. A lipopeptide, P-CrA-A2, composed of a highly cationic peptide sequence (A2), an N-terminally attached palmitoyl chain (P) and cryptophane molecule (CrA) for preferred uptake into blood–brain barrier (BBB) capillary endothelial cells, was generated. CrA allows reversible binding of Xe for NMR detection with hyperpolarized nuclei. The lipopeptide forms size-optimized micelles with a diameter of about 11 nm at low micromolar concentration. Their high local CrA payload has a strong and switchable impact on the bulk magnetization through Hyper-CEST detection. Covalent fixation of CrA does not impede micelle formation and does not hamper its host functionality but simplifies Xe access to hosts for inducing saturation transfer. Xe Hyper-CEST magnetic resonance imaging (MRI) allows for distinguishing BBB endothelial cells from control aortic endothelial cells, and the small micelle volume with a seven-fold improved CrA-loading density compared to liposomal carriers allows preferred cell labelling with a minimally invasive volume (~16 000-fold more efficient than 19F cell labelling). Thus, these nanoscopic particles combine selectivity for human brain capillary endothelial cells with great sensitivity of Xe Hyper-CEST MRI and might be a potential MRI tool in brain diagnostics.

Nanoparticular carrier systems distinctly improve transport and delivery of drugs and diagnostic reporters into pathological areas.[1,2] The delivery of reporters that focus on the visualization of disease onset and spread is particularly important. Brain metastases are a highly relevant example as they affect the majority of breast cancer patients with poor survival outcome.[3] This diagnostic need extends to preclinical settings where therapy approaches for brain tumors are first tested in rodent models. Microvasculature proliferation is considered an early tumor marker and would highly benefit from nanoscopic reporters that visualize sprouting of new blood vessels. This mechanism occurs presumably even before increased energy uptake (monitored by positron emission tomography (PET)) as a subsequent step of malignant dedifferentiation.[4] Various techniques are used to visualize the brain (micro)-vasculature.[5] As PET, computed tomography (CT), and single photon emission computed tomography (SPECT) rely on ionizing radiation, they are often not the first choice for repetitive exams. Photo-acoustic imaging (PAI) is limited by the penetration depth of the optical excitation of the reporter and thus imposes challenges for the application in deep tissue. Angiographic applications of these four techniques rely on indirect assessment of changes in blood vessel formation by detecting reporters in the blood stream. Similarly, Doppler ultrasound uses the flowing blood itself as the detected medium. This also holds for magnetic resonance imaging (MRI) when applied as arterial spin labelling (ASL)[6] to visualize hot spots of cerebral blood flow (CBF). Cerebral blood volume (CBV) can be measured using dynamic contrast-enhanced MRI[7] (with Gd-based contrast agents, GBCAs). As such, MRI combines several advantages but lacks a sensitive direct access to microvascular tissue: Beside the ongoing discussion concerning the safety of GBCAs, both CBV and CBF measurements represent only an indirect access to microvasculature proliferation by detecting the contained blood and are prone to errors, particularly due to anesthesia impacts.[6,7] A direct detection of brain microvascular endothelial cells would thus be the preferred strategy.

We have previously designed a reporter for such purpose based on targeted liposomes with Xe hosts that enable switchable contrast in 129Xe MRI.[8] However, the ability of
influencing the surrounding detected Xe magnetization as efficacious as possible still left considerable room for improvement: only a small fraction of the nanocarrier was truly “NMR susceptible” and access of the detected nuclei to those sites was affected by restricted diffusion. We herein report on a type of targeted, modular NMR reporter that acts on hyperpolarized nuclei for addressing two critical challenges of NMR cell labeling protocols: Based on a compact micelle design, we achieve a) size-selective preferential uptake into endothelial cells of the blood–brain barrier (BBB) through surface-anchored peptides and b) provide a nanoscopic core with highly efficient spin depolarization sites that are readily accessible by the surrounding bulk magnetization of hyperpolarized Xe. This allows unprecedented minimally invasive cell labelling for $^{129}$Xe NMR detection. It combines the advantage of non-ionizing radiation with a unique reporter approach by incorporating emerging aspects of nanotechnology\(^9\) for improved contrast generation.

The group of cell penetrating peptides (CPPs) are most promising carrier-modifying compounds\(^10\) to transport cargos across cellular barriers\(^11–13\). One particular challenge is targeted transport across the BBB\(^10,14\) and specific labelling thereof for diagnostic imaging. Regarding selective recognition and uptake into human brain microvascular endothelial cells (HBMECs) of the BBB, we previously developed micelles composed of about 19 lipopeptides characterized by a high surface density of cationic amino acid residues\(^15,16\). These lipopeptides also rapidly incorporate into liposomes\(^15,17,18\) and comprise dipalmitoylated R-, K-rich apolipoprotein E-derived sequence (P2A2) with specific binding sites for the low-density lipoprotein receptor (LDLr), and also bind nonspecifically to cell-surface heparan sulfate proteoglycans (HSPGs).

While the micelle size of $\approx 10$ nm diameter is desirable to enter the BBB, it imposes the challenge of designing a highly efficient NMR reporter. In a previous conventional approach, micellar nanoparticles ($25–30$ nm $\varnothing$) have been loaded with Fe(III) ions to induce $T_1$ relaxation of surrounding water and thus cause hyperintense signal\(^19\). However, the achievable relaxivity was still only $<10$ mM$^{-1}$ s$^{-1}$ and thus requires high $\mu$M concentrations like commercially available GBCAs. Paramagnetic iron oxide nanoparticles\(^20\) for $T_1$ relaxation cause hypointense signals but do not allow a switchable image contrast to eliminate other causes for signal loss. Instead, we implemented a concept in which the detected spin labels themselves are only transiently associated with the reporter substance and the latter one simply serves as an intermediary that provides spin-receptive hosts to achieve manipulation of magnetization for many nuclei (see Figure 1a). Magnetic depolarization gates as introduced in the transplentor concept\(^21\) pursue this idea. Such molecular hosts act on spin-hyperpolarization from atomic $^{129}$Xe guests that are reversibly bound for Hyper-CEST\(^22\) detection. This allows to introduce nuclei with a limited hyperpolarization lifetime long after the reporter has been accumulated in the target cells. Hyper-CEST enables a switchable Xe MRI contrast through radiofrequency (RF)-activated saturation transfer, i.e., a controlled depolarization that propagates rapidly into the bulk magnetization pool. MRI of well perfused organs with dissolved Xe from inhalation has made important progress that demonstrates the potential for such a targeted vascular reporter\(^23–25\). The self-assembling micelle approach with a core of multiple hosts that are accessible for Xe but shielded from other interactions is very promising compared to the administration of individual, bare Xe hosts that have several shortcomings: examples such as cucurbit[6]uril (CB6) are problematic since they are accessible for competitive guests and are difficult to functionalize. Thus, they currently require unrealistic high (mM) concentrations for in vivo applications\(^26\).

We previously achieved self-embedding of Xe depolarization units into liposomal POPC (1-palmitoyl-2-oleoylphosphatidylcholine) nanocarriers to generate NMR contrast\(^9\). These liposomes were decorated with a dipalmitoylated oligo-arginine sequence (P2Rn) that shows favourable comparable targeting properties\(^8,27\) like P2A2\(^28\). However, this design has several shortcomings. First, the loading capacity of liposomes for the hydrophobic Xe host cryptocryptophane-A (CrA) is limited in order to avoid membrane destabilization. Second, the “NMR susceptible” CEST site-carrying POPC bilayer represents only $\approx$16% of the particle volume and leaves much room for improvement (Figure 1b). Third, the Xe atoms that enter the bilayer need to undergo restricted diffusion before and after they bind reversibly to a CrA cage. This process limits the release of saturated nuclei back into the bulk pool and depends highly on the membrane composition and fluidity\(^29–31\). Thus, condensing the liposome volume down to a minimal nanoscopic lipidic environment and achieving a high local Xe host load with immediate access of Xe to the hosts was the motivation behind developing a P2A2-derived micelle approach where the hosts are covalently linked to the lipopeptide units. P2A2 was fully characterized before\(^30\) and chosen over P2Rn because of the $\approx$fivefold lower critical micelle concentration (cmc) value\(^31\). CrA is considered an ideal host for this purpose because of two reasons: First, its cage-shaped cavity formed by the linkage of two macrocyclic cyclotriveratrylene units has a volume that yields a high binding affinity for Xe according to Rebek's rule\(^33\). This is more stable than binding of Xe between aliphatic chains as found in phospholipid membranes and thus efficiently confers a distinct chemical shift to transiently bound spins. Second, CrA is available with various handles for conjugation to other units\(^34\).

Essential aspects of this study include the investigation whether a) the CrA-lipopeptide compound assembles into micelles, b) such nanocarriers retain their preferential interaction and uptake into BBB cells, and c) Xe has sufficient and immediate access to the hosts for reversible binding and experiences a transient change in chemical shift. The latter aspect is crucial to enable Hyper-CEST signal amplification in which CrA binds xenon atoms for a few milliseconds. Each host then affects many Xe atoms with their $\approx 10^5$-fold enhanced spin polarization when applying a selective RF pulse. This driven depolarization yields another $\approx 10^3$-fold amplification. Importantly, recent studies have mentioned that the immediate molecular environment of CrA can significantly alter the exchange kinetics\(^35\) or Xe accessibility to the host as this might collapse in a hydrophobic environment\(^36\). Interaction with nearby tryptophan (W) has also been discussed\(^37\) and could impact accessibility for competitive guests and is difficult to functionalize in micelles since the P2A2 parent compound contains a W as one of the palmityloxy anchors (Figure 1c).

Successful implementation of CrA-bearing micelles would improve cell labelling compared to other attempts where individual Xe hosts were chemically coupled to a ligand for cell surface receptors\(^38,39\) or to an antibody via a biotin-avidin bridge\(^40\). Compared to simple self-embedding used in the
liposomal approach,[8] anchoring to the lipopeptide will also prevent CrA units from freely diffusing out of the carrier and as a consequence cause undesired side effects.

We chose a modular design in which the individual units are readily coupled through standard synthesis protocols. This allows flexible adaptation for other targeting units (known from nanoparticulate drug delivery systems[41]) and easy implementation as contract work. The designed lipopeptide was composed of the arginine- and lysine-rich A2 sequence modified with one palmitoyl chain at the N-terminal amino group and a CrA molecule covalently bound to the ε-amino group of lysine in position one (P-CrA-A2, Figure 1c). The fluorescent analogue (P-CrA-f-A2) contains carboxyfluorescein at the side chain of an additional lysine in position three. The peptides are C-terminally amidated. The determined masses of the synthesized peptides were 4215.3 (P-CrA-A2) and 4701.7 (P-CrA-f-A2) and thus corresponded to the calculated values. Peptide purity was higher than 95%.

Figure 2a demonstrates a cmc of 2.4 µM for P-CrA-A2 determined by an ANS (8-anilinonaphthalene-1-sulfonic acid) assay where fluorescence becomes detectable only at sufficiently high amount of micelle-incorporated dye molecules. This is identical with the cmc of the dipalmitoylated parent compound, P2A2, in phosphate buffer determined by the same method and additionally by fluorescence anisotropy and fluorescence correlation spectroscopy of carboxyfluorescein-labeled derivatives.[32] Critically, the replacement of one palmitoyl chain by the highly
hydrophobic and bulky CrA obviously does not influence the ability of forming micelles.

The hydrodynamic diameter of the lipopeptide micelles ranges between ≈10 and 12 nm without differences in the polydispersity index as a measure of the size distribution (Table 1). The stability of the P-CrA-A2 micelles is demonstrated by the conserved particle size during storage over weeks (Figure S1, Supporting Information). With 10.3 mV and 10.7 mV, the zeta potentials of P-CrA-A2 and P2A2 micelles, respectively, are virtually identical (Table 1). Knowing that 19 molecules of P2A2 form a micelle,\textsuperscript{[18]} we assume a comparable number of molecules in P-CrA-A2 micelles based on identical physical properties. This means that the average cage load per volume is ≈sevenfold higher in the micelles than for the liposomes (see Figure 1b).

No differences in the cytotoxicity pattern of P-CrA-A2 and P2A2 toward HBMEC or HAoEC (human aortic endothelial cells) were observed. Both peptides cause less than 80%
HBMEC viability above 6.25 µM and HAoEC viability decreases below 80% above 3.75 µM, i.e., at the one- to twofold cmc of the lipopeptides (Figure 2b). The used protocol with 1 h of incubation is even exceeding (twofold) the incubation used for previous liposomal formulations. This should carefully screen for toxicity effects presumably arising from the increased charge density of the micelles. Such an effect would match observations where the P2A2 monomer shows low toxicity but its micelles do affect cell viability. For P2-f-A2, a preferred and highly efficient uptake into endothelial cells of brain capillaries was found.

Uptake studies with P-CrA-f-A2 were performed at 2.5 µM lipopptide concentration. This concentration was chosen according to the cmc and the peptide toxicity. The fluorescence characteristics of the lipopeptide are described in the Supporting Information. Confocal laser scanning microscopy (CLSM) confirms the efficient internalization of P-CrA-f-A2 micelles into HBMEC (Figure 2c, d). The punctuated cytoplasmic fluorescence points to a clathrin-mediated endocytotic uptake route as reported for P2A2 micelles. While P-CrA-A2 micelles translocate the membrane of HBMEC, the micelles dissociate upon interaction with the HAoEC membrane and P-CrA-A2 accumulate as monomers in the cellular membrane as derived from the following observations: The fluorescence for HAoECs (Figure 2e) is significantly reduced after addition of trypan blue, which is localized in the plasma membrane (Figure 2f). This observation points to a pronounced peptide accumulation within the membrane and reduced uptake into the cytoplasm. An important aspect for quantification is the observation that the fluorescence for unquenched, membrane-bound P-CrA-f-A2 is ≈30-fold increased when compared to simple buffer conditions (Figure S2). FACS-derived data giving the geometric mean fluorescence for unquenched, membrane-bound P-CrA-f-A2 is 30-fold increased when compared to simple buffer conditions (Figure S2). FACS-derived data giving the geometric mean fluorescence for unquenched, membrane-bound P-CrA-f-A2 is 30-fold increased when compared to simple buffer conditions (Figure S2).

The Xe Hyper-CEST spectrum of P-CrA-A2 is characterized by direct saturation of free Xe in solution at 191 ppm and only one signal of P-CrA-A2-encapsulated Xe which is shifted by −121 ppm (Figure S4, Supporting Information). When acquiring spectra at different P-CrA-A2 concentrations, always one CEST signal at 70 ppm is observed. Hence, monomeric peptide (at 0.4 µM and 1 µM) and micelle structures (at 10 µM) yield the same chemical shift for encapsulated Xe inside the covalently fixed and micellar organized CrA. Thus, P-CrA-A2 preparations were considered appropriate to confirm the suitability of A2-lipopeptide-micelles for targeting and MRI tracking of endothelial cells of the BBB.

We incubated the target HBMECs and control HAoECs with P-CrA-A2 preparations for 1 h at 37 °C (as for the toxicity tests) and performed Xe Hyper-CEST measurements on a two-compartment setup with identical cell density in both volumes as described before. The used peptide concentration was 5 µM, of which we anticipated ≈2.5 nmol per 10^6 cells per 1 mL suspension to be internalized according to the aforementioned measured internalization efficiency of 50%. Hence, this concentration is a) beyond the cmc, b) should yield a comparable amount of internalized CrA as in the previous liposome study, and c) is within the range of acceptable toxicity. Figure 3 shows the MR images after applying an on-resonance saturation RF pulse at the Xe@CrA_\text{Hz} resonance at 70 ppm and an off-resonant saturation RF pulse at 312 ppm. For the derived Hyper-CEST effect image (for calculation, see Supporting Information, section S10), the inner compartment containing the HBMECs shows with (59 ± 5)% a significantly higher Hyper-CEST effect compared to the outer compartment containing the control HAoECs ((44 ± 5%); clearly displayed as two populations in the histogram evaluation, Figure 3d). The systematic stronger response from the target cells is also confirmed by localized z-spectra derived from a whole image series with different saturation offsets (Figure 3e).

### Table 1. Hydrodynamic diameter of P-CrA-A2 and P2A2 and their fluorescence labeled analogues determined at 100 µM lipopeptide concentration in DPBSG.

|               | P-CrA-A2 | P-CrA-f-A2 | P2A2 | P2fA2 |
|---------------|----------|------------|------|-------|
| Diameter [nm] | 11.2 ± 0.6 | 11.1 ± 1.1 | 9.6 ± 0.9 | 11.0 ± 0.7 |
| Polydispersity | 0.64 | 0.70 | 0.68 | 0.63 |
| Zeta potential [mV] | 10.3 ± 0.87 | nd | 10.7 ± 3.0 | nd |

*Mean and standard deviation of three independent samples with three measurements each. nd, not determined; Zeta potential, ζ determined at 25 µM peptide concentration in DPBSG.*
Xe itself can easily cross the plasma membrane\textsuperscript{[45]} and can thus readily reach CrA compounds that have been internalized\textsuperscript{[44]}. The higher uptake of P-CrA-f-A2 into HBMECs (deduced from FACS data with P-CrA-f-A2) should thus already warrant a stronger CEST effect. But this stronger Hyper-CEST response at identical cell density could also come from a larger cell volume that allows more uptake than in (hypothetically) size-limited HoAECs instead of truly preferred internalization mediated by the A2 units. However, such a scenario can be ruled out: In general, brain endothelial cells exhibit a low endocytic activity compared to endothelium from other tissue beds\textsuperscript{[46]}. This is in agreement with observations for untargeted LUVs carrying CrA where the saturation transfer was more pronounced for HAoECs as expected\textsuperscript{[8]}. Thus, the cell volume cannot be the limiting factor for the aortic cells. Both the larger Hyper-CEST response and the stronger uptake according to scaled FACS signal from HBMECs are therefore caused by the preferred uptake of P-CrA-A2 micelles into the endothelial cells of brain capillaries. A content of 20 mol\% of the carboxyfluorescein-labeled P-CrA-f-A2 in the preparation did not change the Hyper-CEST effect significantly (HBMEC compartment: (63 \pm 5)\% vs HAoEC compartment: (40 \pm 6)\%). Compared to our earlier study using CrA-loaded LUVs tagged with P2Rn\textsuperscript{[8]}, the difference in Hyper-CEST between HBMEC and HAoEC seems to be slightly reduced in this study. This might be due to the fact that the 19 CrA units per micelle are embedded into a relatively small surrounding lipid phase volume. While this phase typically provides favorable CEST conditions (as observed in bilayer systems\textsuperscript{[47]}), we purposely kept it small to enable immediate Xe access in and out of the bulk pool. The faster Xe exchange in and out of the hosts within the lipid environment also explains why the Hyper-CEST signal from HAoECs appears over proportionally large despite the 6.3-fold reduced peptide uptake.

Major advantages become obvious when putting the investigated micelle system in perspective. Covalent binding of CrA in the carrier allows controlling of the incubation concentration and the single resonance signal will facilitate future interpretation. Additionally, much lower CrA concentrations can be used. The micellar organization of about 19 molecules causes a locally very high concentration of CrA and enables very efficient depolarization seed points for Hyper-CEST with regard to the occupied volume. The internalized \( \approx 2.5 \) nmol lipopeptide for \( 10^6 \) cells corresponds to only \( 1.5 \times 10^9 \) CEST sites per cell and represents a total micelle volume of \( \approx 55 \) fL per cell (see Supporting Information, section S12). This is a rather small volume when compared to other methods: For comparison, \( ^{19} \)F-labelling of cells has become an established method for many NMR applications and benefits already (pre-)clinical use\textsuperscript{[48]}. The toxicity profile and safety of these probes is well studied and \( ^{19} \)F MRI detection can also often be done with the same RF hardware as conventional \(^1\)H detection. However, \( 10^{11}–10^{13} \) \(^{19} \)F nuclei are required for cell labeling\textsuperscript{[49]} and our estimation yields that this CEST approach is...
~ 16 000-fold more efficient than \(^{19}\)F labelling (see Supporting Information). Many studies have used PFCE nanodroplets with reported concentrations around 2 pmol per cell and the related uptake volume can reach a non-negligible volume fraction for many cell types (600–700 fL; protocols with surfactant agents even reach 40–50 pL; see Supporting Information). The volume of a cell measuring 12.4 µm in diameter, however, amounts only to \(\approx 1000\) fL (assuming spherical shape in suspension). The situation with SPIOS is somewhat better but provides no switchable contrast like CEST to validate the cause of hypointense signals: 15 fL particle volume per cell is but provides no switchable contrast compared to existing protocols. \(^{129}\)Xe is already quite established and has recently been expanded in non-exchanging thermally polarized nuclei. \(^{19}\)F agents, e.g., still require \(10^{-1}\) M concentrations of \(^{19}\)F atoms for moderate signal-to-noise ratios in MRT\(^{[50]}\) despite extensive signal averaging (\(\approx 300–500\) scans\(^{[50,51]}\)). Moreover, \(^{129}\)Xe MRI generates a switchable contrast compared to existing protocols. \(^{129}\)Xe is a nucleus with a relatively low gyromagnetic ratio. While this theoretically requires gradients stronger than those used for \(^{1}\)H MRI (factor \(\approx 3.6\)) to achieve a comparable image encoding, this is not really an issue in practice because the realistic spatial resolution is anyway lower than for \(^{1}\)H detection. Regarding the frequency selective saturation of \(^{129}\)Xe, this is also possible at lower (clinical) field strengths due to the large chemical shift range of \(^{129}\)Xe. However, CEST detection does come with certain limitations: the presaturation increases the imaging time, particularly when it is applied at multiple frequencies. It also requires a certain build-up time to release enough depolarized nuclei into the bulk pool. At first sight, this might seem problematic in a vascular environment where the blood stream “carries away” the saturation information. However, a pharmacokinetic model for brain tissue where \(^{129}\)Xe is delivered through the microvasculature has shown that this type of detection will be feasible with highly efficient depolarization seed points (see Supporting Information in ref.\(^{[52]}\)).

In conclusion, P-CrA-A2 conserves properties of P2A2 and combines the HBMEC selectivity and high uptake efficiency of A2 peptide-bearing micelles with the great sensitivity of \(^{129}\)Xe Hyper-CEST MRI. Covalent binding of CrA prevents diffusion out of the carrier, results in high local cage concentration and allows reliable quantification of the signal molecule. Additionally, integration into the micellar structure permanently reduces the toxicity of CrA and is not endangered by leakage. We consider P-CrA-A2 micelles highly promising as future potential MRI reporters for the monitoring of highly vascularized brain regions such as tumors. Clinical lung imaging with \(^{129}\)Xe is already quite established and has recently been expanded by novel applications for mapping the \(^{129}\)Xe distribution in other organs like the human brain\(^{[21,23,31]}\) as well as the kidneys.\(^{[45]}\) It has benefited from technical improvements found in modern polarizers,\(^{[49]}\) optimized RF antenna systems,\(^{[55,56]}\) and adapted readout protocols that will ultimately also enable (pre)clinical Hyper-CEST studies. Given such further advancements in MRI of \(^{129}\)Xe dissolved in tissue, this type of reporter would enable a first meaningful in vivo application of Hyper-CEST with the potential for novel insights beyond targets detectable with existing contrast agents.

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**Experimental Section**

See Supporting Information.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

blood–brain barrier, lipopeptides, magnetic resonance imaging, nanocarriers, targeting, \(^{129}\)Xe Hyper-CEST

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