Free-base porphyrins as CEST MRI contrast agents with highly upfield shifted labile protons

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Purpose: CEST has become a preeminent technology for the rapid detection and grading of tumors, securing its widespread use in both laboratory and clinical research. However, many existing CEST MRI agents exhibit a sensitivity limitation due to small chemical shifts between their exchangeable protons and water. We propose a new group of CEST MRI agents, free-base porphyrins and chlorin, with large exchangeable proton chemical shifts from water for enhanced detection.

Methods: To test these newly identified CEST agents, we acquired a series of Z-spectra at multiple pH values and saturation field strengths to determine their CEST properties. The data were analyzed using the quantifying exchange using saturation power method to quantify exchange rates. After identifying several promising candidates, a porphyrin solution was injected into tumor-bearing mice, and MR images were acquired to assess detection feasibility in vivo.

Results: Based on the Z-spectra, the inner nitrogen protons in free-base porphyrins and chlorin resonate from −8 to −13.5 ppm from water, far shifted from the majority of endogenous metabolites (0-4 ppm) and Nuclear Overhauser enhancements (−1 to −3.5 ppm) and far removed from the salicylates, imidazoles, and anthranillates (5-12 ppm). The exchange rates are sufficiently slow to intermediate (500-9000 s−1) to allow robust detection and were sensitive to substituents on the porphyrin ring.

Conclusion: These results highlight the capabilities of free-base porphyrins and chlorin as highly upfield CEST MRI agents and provide a new scaffold that can be integrated into a variety of diagnostic or theranostic agents for biomedical applications.

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1 | INTRODUCTION

MRI is a uniquely valuable tool for visualizing soft tissue with high spatial and temporal resolution. In the clinic, MRI contrast agents are in routine use for detecting lesions based on highlighting tissue through altering the T₁ or T₂ MR relaxation times of water.¹ ² CEST contrast agents represent an attractive alternative because these agents do not require inclusion of heavy metals and can instead provide contrast using exchangeable protons on metabolites or other organic diamagnetic molecules.³ ⁶ Molecules that produce CEST contrast include a number of natural biomolecules, which suggest that biochemical pathways should be detectable using this contrast mechanism. Indeed, a number of biomolecules have been reported over the past few years. Elegant examples of diamagnetic CEST (diaCEST) agents include glucose,⁷ ⁸ myo-inositol,⁹ ¹⁰ glutamate,¹¹ ¹² Cr,¹³ L-arginine,¹⁴ ¹⁵ glycosaminoglycans,¹⁶ protamine,¹⁷ glycogen,¹⁸ and glycoproteins.¹⁹ Critical to the success of CEST imaging is selective irradiation of the labile protons while avoiding perturbation of bulk water signal and protons found in tissue. However, exchangeable protons of the aforementioned metabolites fall within 4 ppm from bulk water, which limit the use of selective saturation pulses and increase background signal. Further shifted protons for enhanced CEST contrast on lower field scanners can be found on molecules such as barbituric acid,²⁰ iopamidol,²¹ ²³ and several thymidine analogues.²⁴ ²⁵ More recently, significant progress was made by employing the intramolecular bond–shifted hydrogens principle.²⁶ ³⁰ A number of anthranilates, imidazole-4,5 dicarboxamides, and salicylates show strong CEST contrast properties, with labile protons resonating up to 12 ppm from water. Despite these promising advances, the development of diaCEST agents with the highly upfield shifted protons, which presents sensitivity advantages that also are well tolerated after administration, remains elusive.

Herein, we show that several properly substituted free-base porphyrins and a chlorin possess two protons that resonate at a remarkable −9 to −13.5 ppm from water. These CEST peaks are shifted well outside of the range of other known labile protons, and exchange rates are sufficiently slow to intermediate on the NMR timescale, making these agents well suited for CEST imaging. As far as we are aware, no other diamagnetic CEST agent has been reported at such low frequencies. Based on this finding, we evaluate the feasibility of visualizing a water-soluble porphyrin in mice bearing A549 cell-derived xenografts.

2 | METHODS

Phantom preparation: δ-aminolevulinic acid, porphobilinogen, uroporphyrin I, coproporphyrin I, and protoporphyrin IX were purchased from Sigma Aldrich (St. Louis, MO). Tetraphenylporphine sulfonate (TPPS₄); 5, 10, 15, 20-tetrakis (4-carboxyphenyl) porphyrin (TCPP); and chlorin e₆ were purchased from J&K Scientific Ltd (Beijing, China). Hematoporphyrin were purchased from Binhai Hanhong Biochemical Co., Ltd (Shanghai, China). Metal salts were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). 5, 10, 15, 20-tetrakis(4-β-glucoylphenyl) porphyrin was synthesized according to a previous report.³¹ All samples were dissolved in 0.01 M phosphate-buffered saline at the desired concentrations and then titrated using high concentration HCl/NaOH to the desired pH values. The solutions were placed into 1 mm glass capillaries and assembled in a holder for CEST MR imaging. The samples were kept in 37 °C during imaging. Phantom CEST experiments were taken on a Bruker 9.4 Tesla vertical MR scanner (Bruker Avance 400, Ettlingen, Germany), using a 25 mm birdcage transmit/receive coil. CEST images were acquired using a rapid acquisition with relaxation enhancement factor (rapid acquisition with relaxation enhancement factor = 8) sequence with continuous wave saturation pulse length of 3 seconds and saturation field strength (ω₁) from 1.2 μT to 14.4 μT. The CEST Z-spectra were acquired by incrementing saturation frequency every 0.25 ppm from −16 to 16 ppm for phantoms; TR = 8 s, effective TE = 5.1 ms, matrix size = 128 * 96, and slice thickness = 3 mm. Water saturation shift referencing images were also acquired using 0.5 s continuous wave saturation pulse with field strength of 0.5 μT and saturation frequency from −1.6 ppm to 1.6 ppm with 23 scans.

Cell culture and cancer model: A549s, non-small-cell lung cancer cells, were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). The tumor cells were cultured in Iscove’s Modified Dulbecco’s Medium (Boster, Wuhan, China), supplemented with 10% fetal bovine serum (Boster, Wuhan, China), 100 U/mL penicillin (Boster, Wuhan, China), and 100 U/mL streptomycin (Boster, Wuhan, China) in a humidified air with 5% CO₂ at 37°C. BALB/c male nude mice (aged 5-6 weeks, approximately 20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were inoculated subcutaneously with A549 cells (1 * 10⁶ cells of each) on the legs and used for MRI after 3 weeks breeding.

Animal imaging: Animal experiments were carried out in accordance with the guidelines provided and approved by the
in situ and in vivo studies of porphyrins have been carried out using different techniques such as 1H-MRI and 1H-NMR. The results obtained from these studies have shown that porphyrins can act as effective contrast agents for MRI. The magnetic properties of porphyrins make them suitable for CEST imaging, which is a technique that relies on the magnetic properties of water to generate contrast. The present study aimed to characterize the CEST properties of a new porphyrin derivative, known as TPPS4, and to evaluate its potential as a contrast agent for CEST imaging.

### 3 | Results

CEST-MRI contrast is generated by applying a selective RF pulse (saturation pulse) on labile protons to annihilate their magnetization. Due to dynamic proton exchange of the “saturated” labile protons with water transferring the saturation, there is a progressive loss in water signal with continuous replacement of the saturated protons by unsaturated protons followed by renewed annihilation of their signal. As a result, the low concentration labile protons display an amplified influence on water signal, the source of MRI signal. As shown in Figure 1A, Cr, glutamine, and glucose display strongly overlapped CEST contrast between 1 to 4 ppm at a saturation field strength ($\omega_1 = 3.6 \mu T$). As described previously, on 3 Tesla scanners saturation transfer contrast is improved for compounds with chemical shifts more than 5 ppm away from water. Suitable labile protons are found in aromatic compounds with intramolecular hydrogen bonds with two representative contrast curves from the intramolecular bond shifted hydrogens scaffold shown in Figure 1B. In our search for high chemical shift labile protons, we became interested in investigating free-base porphyrins, which are aromatic macrocycles that possess a very large magnetic anisotropy. As shown in Figure 1C, the MTR$_{\text{asym}}$ spectra of free-base TPPS4, a known 2nd generation photosensitizer, is well suited for detection via CEST imaging due to the central nitrogen (NH) protons on this compound. In fact, these fall within a region of the chemical shift spectrum far removed from all other diaCEST agents to date. Unlike the labile protons on other compounds, which fit these criteria, the NHs in TPPS4 possess a large upfield chemical shift from water ($\Delta \omega = -9.75$ ppm).

### 3.1 | Characterization of porphyrin CEST contrast

We first measured the CEST properties of TPPS4 in vitro. Figure 2A shows a Z-spectrum and MTR$_{\text{asym}}$ spectrum for the compound. The proton exchange rate ($k_{sw}$) with water for TPPS4 was measured as a function of pH using the quantifying exchange using saturation power experiment. TPPS4 has a $k_{sw} = 0.21$ ks$^{-1}$ at 12.5 mM, pH = 7.0, with $k_{sw}$ strongly dependent on pH (Figure 2B). Above pH 6.0, $k_{sw}$ is below the chemical shift difference at 9.4 Tesla ($\Delta \omega = 3900$ Hz), placing the exchange rates in the slow-to-intermediate exchange NMR regime and making this agent well suited for CEST imaging (Supporting Information Figure S1) (Supporting Information Table S1). The concentration dependence of CEST contrast is linear, and 1.5% contrast was obtained at 2.5 mM using $\omega_1 = 5.4 \mu T$ (Supporting Information Figure S2).
3.2 | Porphyrins, chlorin and precursor CEST properties

To get a better handle on what the range of CEST properties are for free-base porphyrins and chlorin, we tested the series shown in Figure 3 including the porphyrin precursor porphobilinogen in vitro. Isolated porphobilinogen possesses a pyrrole NH and displays modest contrast at $\Delta \omega = 3$ ppm (Supporting Information Figure S3). In contrast, uroporphyrin I, which has 8 carboxyl groups conjugated to the $\beta$ positions on the porphyrin ring, has a pronounced CEST peak at $\Delta \omega = -9$ ppm, with $k_{sw} = 1.05$ ks$^{-1}$ at pH 7.4, placing the rates in the slow-to-intermediate NMR exchange regime and making this agent well suited for CEST imaging (Figure 3) (Supporting Information Figure S4) (Supporting Information Table S2). Coproporphyrin I is a downstream metabolite of uroporphyrin I, with methyl
substitutions for 4 of the carboxyls (Figure 3) (Supporting Information Figure S5). These substitutions decrease the Δω further to −13.5 ppm from water; however, the ksw also increases such that the CEST peaks are poorly detected until pH 9.0 (Supporting Information Figure S5) (Supporting Information Table S3), making this porphyrin unsuitable as a CEST agent. Similarly, hematoporphyrin with its hydroxyl and methyl substitutions at the meso-positions on the ring provides minimal CEST contrast until pH 8.0 (Supporting Information Figure S6) (Supporting Information Table S4). Protoporphyrin IX, the final free-base porphyrin in heme biosynthesis, could not be tested because the water solubility was too low. We also tested chlorin e6, which is not aromatic through the entire circumference of the ring and has carboxyls at the meso- and β-positions. Chlorin e6 can provide suitable CEST contrast at neutral pH values with 2 different Δω = −10.25 and −8.75 ppm, with ksw = 0.85 and 0.20 ks⁻¹, respectively (Supporting Information Figure S7) (Supporting Information Table S5). Because of these 2 frequencies, chlorin e6 has another interesting feature, a pH dependent contrast frequency, as can be seen in Supporting Information Figure S8. In addition to those natural porphyrins and TPPS₄, we tested 2 additional synthetic water-soluble tetraphenylporphyrins, as shown in Figure 3: TCPP and 5, 10, 15, 20-tetrakis(4-carboxyphenyl) porphyrin. The NH protons of TCPP show well-defined, sharp peaks at Δω = −10 ppm with sufficiently slow ksw values (TCPP, ksw = 0.56 ks⁻¹, pH = 7.4) (Supporting Information Figure S9) (Supporting Information Table S6), placing these in the slow-to-intermediate exchange regime (i.e., ksw < Δω). NH protons of tetrakis(4-β-g lucosylphenyl) porphyrin resonate at a similar chemical shift (Δω = −9.25 ppm) but have a fairly slow exchange rate at neutral pH (ksw = 0.1 ks⁻¹, pH = 7) (Supporting Information Figure S10) (Supporting Information Table S7). The magnitude of the CEST contrast increased when the pH dropped below 7 because of the particularly high exchange rates (TCPP, ksw = 3.3 ks⁻¹, pH = 6.6). The results of all the ksw measurements are listed in Supporting Information Tables S1 through 7. Based on all these data, uroporphyrin I, TPPS₄, and TCPP all display well-defined CEST peaks with excellent water solubilities. Unfortunately, the water solubility of TCPP is severely limited below pH 6.6, a problem for in vivo applications. Therefore, we settled on TPPS₄ for further studies.

As has been observed previously, porphyrins present several complications as contrast agents, including a tendency to ligate metals at the axial site, which would displace the labile protons used to create CEST contrast; a tendency to self-aggregate; and in general a limited water solubility. We tested this for TPPS₄ by acquiring Z-spectra after simply mixing 1 eq. metal salts with TPPS₄ in solution at 37 °C for 4 h under constant shaking and titrated to neutral pH using high-concentration HCI/NaOH. As shown in the Supporting Information Figure S11, this procedure did not result in the formation of metal complexes which eliminated the CEST signals. In addition, we have tested the influence of human serum on TPPS₄. MR data was acquired on 10% normalized human serum titrated to pH 7.3 with and without 12.5 mM TPPS₄. As seen in the
3.3 | In vivo imaging of tumor xenografts

To test how well these upfield CEST agents could be detected, we performed an in vivo study in live BALB/c mice bearing A549 cells-derived xenografts. We administered 50 µL of a 0.1 M TPPS₄ solution, a known photosensitizer for photodynamic therapy, through intratumoral injection. CEST images were acquired before and after administration, revealing the distribution of TPPS₄ within the tumor. The peak CEST contrast was 9.5% contrast at −9.75 ppm after injection of TPPS₄ and markedly decreased over 3 h (Figure 4C) (Supporting Information Figure S13) (Supporting Information Table S8-9). The advantage of the remarkable upfield signal is evident from Figure 4B, where the peak of TPPS₄ is far removed from background saturation transfer signals detected as negative MTR asym values from 0 to −6 ppm. In addition, using $\omega_1 = 3.6 \mu T$, the water signal at −9.75 ppm is 20% larger compared to at −5 ppm, which improves the contrast-to-noise ratio of the CEST images as well. The dynamics are similar to those found for intratumoral injection of CEST stealth liposomes previously, with $k_1 = 7.83 \times 10^{-1}$ hr⁻¹ and $k_2 = 9.98 \times 10^{-2}$ hr⁻¹ (rate constants with $k_1$ corresponding to the cellular uptake of the porphyrin from extracellular space through endocytosis; $k_2$ corresponding to release of the porphyrin into the intracellular vesicle space). For the larger lipoCEST agent, these values were $k_1 = 1.73 \times 10^{-2}$ hr⁻¹ and $k_2 = 3.82 \times 10^{-2}$ hr⁻¹ using Castelli et al.’s multicontrast kinetic analysis model. Overall, these CEST imaging results are encouraging and should enable use of more advanced saturation methods at clinical field strengths in future studies.

4 | DISCUSSION

We have shown that selected porphyrins and chlorin display excellent CEST MRI properties, with hematoporphyrin-possessing labile protons that are the furthest shifted diaCEST agent identified to date, further than the previously identified 3-nitrosalicylic acid. Normally, the protons on heteroaromatic rings such as pyrrole, aniline, and imidazole resonate downfield from water due to the strong deshielding effect from the aromatic ring. The deshielding effect can be further enhanced using intramolecular hydrogen bonding, which is a powerful strategy to increase the sensitivity of CEST imaging. As we show in this work, the inner NH protons in the center of aromatic porphyrins and chlorin instead are highly upfield-shifted, with inner labile NH resonating as much as 17 ppm higher field than NH resonances in pyrroles or on lysine or arginine-rich peptides. These unusual shifts are largely attributed to the effect of “ring currents” formed by the precession of 18 $\pi$-electrons in the porphyrin ring, as previously described by Jusélius and Sundholm using density-functional theory. The $k_{sws}$ for a number of these molecules are suitable to allow robust detection, with the substitution of

Supporting Information Figure S12, at this concentration human serum albumin does not interfere with the CEST signal of TPPS₄.

FIGURE 4  In vivo contrast for the TPPS₄. A, −9.75 ppm CEST contrast maps at pre- and post-20 min, post-60 min, post-100 min, and post-160 min injection of compound TPPS₄. B, MTR asym for a ROI enclosing the entire tumor with preinjection data (black), 20 min postinjection (red), 60 min postinjection (blue), 100 min postinjection (green), 160 min postinjection (pink). C, Temporal evolution of the MTR asym (−9.75 ppm) for ROIs enclosing the whole tumor after intrathecal injection of TPPS₄. $\omega_1 = 3.6 \mu T (n = 4)$. ROI, region of interest.
the ring playing an important role in determining both the labile proton shift and \( k_{\text{ex}} \), as shown by our data.

In a number of recent studies, the triiodobenzene analogues\(^{21,22} \) and thymidine analogues\(^{24,25} \) were extensively investigated and shown to have great potential for biomedical applications. These downfield shifted agents suffer a little still from overlap with background signals from endogenous exchangeable protons on clinical 3 Tesla scanners. There are less background signals occurring in upfield regions of the spectra, except for the relay nuclear Overhauser enhancements from \(-1\) to \(-3.5 \text{ ppm}\),\(^{46} \) which may be a major advantage for specific detection. While \( \text{Nd}^{3+} \) and \( \text{Pr}^{3+} \), \( \text{Tb}^{3+} \) and \( \text{Ho}^{3+} \) paramagnetic CEST agents can also contain labile protons or water molecules with strong upfield shifts,\(^{47,48} \) the \( k_{\text{ex}} \)s for these complexes are generally much faster than the 400 to 2000 \( s^{-1} \), which are well detected by the moderate strength saturation pulses generated using body coils on clinical 3 Tesla scanners.

A number of free-base porphyrins are metabolites from heme biosynthesis. Because of this, using CEST imaging to detect these compounds could provide information on metabolic disorders such as porphyria. Furthermore, photomedicine, which includes optical image guidance of surgeries\(^{49} \) and photodynamic therapies,\(^{50} \) employs these or similar compounds. Our findings potentially allow inserting MRI into photomedicine for enhanced visualization. In addition, because a wide variety of free-base porphyrins possess favorable properties for detection using CEST imaging, conjugation of these probes to water-soluble polymers,\(^{51} \) liposomes,\(^{52,53} \) or incorporation into micelles\(^{54,55} \) should preserve the CEST properties, allowing their use for a wide variety of multimodal diagnostic and theranostic studies.

5 | CONCLUSION

We have demonstrated that porphyrins and chlorin are a promising new set of diaCEST probes with chemical shifts far upfield from conventional organic CEST agents. The temporal evolution of the contrast detected is similar to observed before for paraCEST agents using a multicontrast kinetic analysis.\(^{32} \) This type of highly upfield shifted probe could improve the sensitivity of existing CEST methods. More MRI studies on the pharmacokinetics and tumor uptake of porphyrins are now under investigation in our labs.

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REFERENCES


**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**FIGURE S1** Influence of pH on the contrast of TPPS₄ (ω₁ = 5.4 μT, tₛₐₜ = 3 s, 12.5 mM)

**FIGURE S2** Influence of concentration on the contrast of TPPS₂₄. The concentration dependence of the contrast of TPPS₄ at pH 7.0–7.2 was measured at a saturation field strength (ω₁) = 5.4 μT. The Z-spectra and MTRₘₑₚₚₚ spectra at concentrations 0 mM, 1.25 mM, 2.5 mM, 5 mM, 7.5 mM, 10 mM, 15 mM and 20 mM were collected and are shown below. 1.5% contrast was obtained at 2.5 mM

**FIGURE S3** Influence of pH on the contrast of porphobilinogen (ω₁ = 5.4 μT, tₛₐₜ = 3 s, 12.5 mM)

**FIGURE S4** pH effect on the contrast of uroporphyrin I (ω₁ = 5.4 μT, tₛₐₜ = 3 s, 12.5 mM)

**FIGURE S5** Influence of pH on the contrast of coproporphyrin I (ω₁ = 5.4 μT, tₛₐₜ = 3 s, 12.5 mM)

**FIGURE S6** Influence of pH on the contrast of hematoporphyrin (ω₁ = 5.4 μT, tₛₐₜ = 3 s, 12.5 mM)

**FIGURE S7** Influence of pH on the contrast of Chlorin e6 (ω₁ = 5.4 μT, tₛₐₜ = 3 s, 12.5 mM)

**FIGURE S8** Z-spectra and MTRₘₑₚₚₚ of Chlorin e6 using different saturation field strengths. The saturation power dependence of the contrast of Chlorin e6 at pH 7.0 was measured using different saturation field strength. The Z-spectra and MTRₘₑₚₚₚ spectra with ω₁ = 1.2 μT, 2.4 μT, 3.6 μT, 5.4 μT, 7.2 μT, 10.8 μT and 14.4 μT were collected and are shown below. Two peaks were observed at −8.75 ppm and −10.25 ppm when weak saturation power (ω₁ = 1.2 μT, 2.4 μT, 3.6 μT or 5.4 μT) was employed.

**FIGURE S9** Influence of pH on the contrast of TCPP (ω₁ = 5.4 μT, tₛₐₜ = 3 s, 12.5 mM)

**FIGURE S10** Influence of pH on the contrast of 5, 10, 15, 20-tetrakis (4-β-glucosylphenyl) porphyrin (ω₁ = 5.4 μT, tₛₐₜ = 3 s, 12.5 mM)

**FIGURE S11** CEST contrast of TPPS₄ in the presence of various metal ions (1 eq.). The effect of metal ions on the contrast of TPPS₄ were tested at a concentration of 12.5 mM, pH = 7, ω₁ = 5.4 μT. After simply mixed metal ions with TPPS₄ solution at 37°C for 4 h under constant shaking and titrated using high-concentration HCl/NaOH to neutral pH, the Z-spectra in presence of 1 eq. metal salts include ZnCl₂, CaCl₂, MgCl₂, CdCl₂ and AlCl₃ were collected.

**FIGURE S12** In vitro test of TPPS₄ in the presence of 10% HSA (pH = 7.3, 37 °C, 5.4 μT, 3 s for saturation)

**FIGURE S13** In vivo Z-spectra and MTRₘₑₚₚₚ spectra for the tumor of mouse 2 with data collected pre-injection and post injection. a) Z-spectra of tumor at different time points (pre, 20 min, 40 min, 60 min, 80 min, 100 min, 120 min, 140 min and 160 min); b) MTRₘₑₚₚₚ of tumor at different time points. For these experiments, 50 μl of 0.1 M TPPS₄ solution was injected into mouse through Intratumoral (IT) injections. Before injection, the B₀ inhomogeneity was measured and corrected using the water saturation shift referencing approach. An 105-offset Z-spectrum (from 15 ppm to −15 ppm) was also acquired using saturation field strength of 3.6 μT. For the dynamic CEST contrast measurements, a series of whole Z-spectra and water saturation shift referencing experiments were acquired after injection. The CEST contrast map was calculated by MTRₘₑₚₚₚ = |S(+ω₁)–S(−ω₁)|/ S(+ω₁). The images at every three adjacent time points were averaged to increase the contrast-noise ratio. The maximum CEST contrast at -9.75 ppm was up to 14.5% after injection of TPPS₄ and markedly decreased over 3 hours. The contrast map before and after injection are shown in Supporting Information Table S8 and S9.

**TABLE S1** Measured proton exchange rates of TPPS₄ at different pH values

**TABLE S2** Measured proton exchange rates of uroporphyrin I at different pH

**TABLE S3** Measured proton exchange rates of coproporphyrin I at different pH

**TABLE S4** Measured proton exchange rates of hematoporphyrin at different pH

**TABLE S5** Measured proton exchange rates of Chlorin e6 at different pH

**TABLE S6** Measured proton exchange rates of TCPP at different pH

**TABLE S7** Measured proton exchange rates of 5, 10, 15, 20-tetrakis (4-β-g1ucosylphenyl) porphyrin at different pH

**TABLE S8** T₂w map and CEST contrast map of Mouse 1 and Mouse 2

**TABLE S9** T₂w map and CEST contrast map of Mouse 3 and Mouse 4