Chemical exchange saturation transfer (CEST) MRI is currently set to become part of clinical routine as it enables indirect detection of low concentrated molecules and proteins. Recently, intermediate to fast exchanging functional groups of glucose and its derivatives, glutamate and dextran, have gained attention as promising CEST contrast agents. To increase the specificity of CEST MRI for certain functional groups, the presaturation module is commonly optimized. At an early stage, this is performed in well-defined model solutions, in which, for instance, the relaxation times are adjusted to mimic in vivo conditions. This often involves agar, assuming the substance would not yield significant CEST effects by itself, which the current study proves to be an invalid assumption. Model solutions at different pH values and concentrations of agar were investigated at different temperatures at a 9.4 T human whole body MR scanner. High power presaturation of around 4 μT, optimal for investigating intermediate to fast exchanging groups, was applied. Postprocessing included spatiotemporal corrections for B0 and spatial corrections for B1+. CEST effects of up to 3 % of the bulk water signal were observed. From pH, concentration and temperature dependency, it was concluded that the observed behavior reflects a CEST effect of agar. It was also shown how to remove this undesirable contribution from CEST MRI data. It was concluded that if agar is involved in the CEST MRI parameter optimization process, its contribution to the observed effects has to be taken into account. CEST agent concentration must be sufficiently high to be able to neglect the contribution of agar, or a control sample at matched pH is necessary for correction. Experiments on pure agarose showed reduced CEST effects compared with agar but did not provide a neutral baseline either.

**KEYWORDS**

CEST effect agar, CEST MRI parameter optimization, gluCEST MRI optimization, glutamate CEST

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1 | INTRODUCTION

Chemical exchange saturation transfer (CEST) MRI enables noninvasive imaging of molecules and proteins at millimolar concentration in vivo. CEST effects occur after repeatedly applying off-resonant presaturation at the frequency offset of a proton pool that is in exchange with the bulk water protons.\(^1,2\) The resulting alteration of the bulk water signal through magnetization transfer can be detected by subsequent water proton MR image acquisition. In vivo, many different CEST active groups are apparent (e.g., see\(^3-8\)). To increase the specificity for a certain CEST active proton pool, the presaturation module can be optimized. This is due to the fact that the CEST labeling efficiency depends on both the exchange rate of specific CEST pools and presaturation power.\(^9,10\) Recently glucose,\(^11\) its derivatives,\(^12,13\) glucosamine,\(^14\) dextran\(^15\) and glutamate\(^5\), have gained attention as promising CEST MRI contrast agents. All of these five contrast agents have fairly high exchange rates of the order of a few kilohertz. This study will exemplarily investigate L-glutamic acid. Since the transfer of magnetization is governed by the exchange rates between the bulk water and the CEST pools, the CEST MRI signal is also sensitive to external parameters such as pH\(^16\) or temperature. Moreover, as CEST effects are measured indirectly via the bulk water signal, longitudinal and transversal relaxation time (T\(_1\), T\(_2\)) of the water pool also affect the CEST signal directly.\(^17-22\) Therefore, when in vivo optimization is not possible, it is necessary to perform the optimization of presaturation parameters in precisely defined model solutions that closely mimic in vivo conditions. At least T\(_1\), T\(_2\), pH, temperature and semisolid magnetization transfer have to be adjusted to ensure the optimized parameters transfer to in vivo application.

To control the temperature of the sample is a rather technical task. The pH value may be adjusted using suitable base and acid combinations along with a buffer such as phosphate buffered saline (PBS). T\(_1\) can be modified using paramagnetic MRI contrast agents. For modifications of T\(_2\), agar made its way into CEST MRI parameter optimization and is frequently used in model solutions. Besides altering T\(_2\), agar also introduces relaxation due to magnetization transfer effects.\(^21,22,24\) Adjustment of T\(_2\) is of major importance for the optimization process, since T\(_2\) directly influences spillover effects,\(^19\) which will finally be reflected in the presaturation module optimized for a specific CEST pool. All substances used to adjust the parameters of the model solutions share one prerequisite: they should not introduce significant, specific CEST effects themselves. For agar this was implicitly—in some cases even explicitly—assumed to be generally fulfilled. To date, only Li et al. have explicitly mentioned a significant proton exchange-related contribution of agar to the CEST MRI signal, but they did not provide any further discussion on this issue.\(^25\) Besides that study, an exchange rate of 4 kHz in agarose samples at an acid pH of 3.5 was reported by Chávez and Halle\(^26\) when characterizing the exchange-mediated orientational randomization (EMOR)\(^27\) model on MR spectrometers. In the current study, the contribution of agar to the observed CEST MRI signal was investigated in detail. This was carried out at a 9.4 T human whole body MR system at in vivo-like pH, T\(_1\) and T\(_2\) values using an established gradient echo-based CEST MRI sequence\(^28\) as it is also applied in vivo. With this setup it was shown that agar does not provide a neutral baseline for CEST MRI parameter optimization prior to translating the latter to in vivo. It was exemplarily demonstrated that optimizing the presaturation of CEST MRI for the CEST effect of L-glutamic acid was significantly biased by agar and provided misleading optimization results. So although agar was successfully used in model solutions for CEST MRI in the past, its CEST effect has to be taken into account again when faster exchanging agents are studied. Especially at a higher field, these have become increasingly important over the last few years. The detailed characterization of the observed CEST effects of agar help to interpret and correct the results of L-glutamic acid obtained in agar gels.

2 | EXPERIMENTAL

2.1 | Preparation of model solutions

Model solutions were prepared using agar (Agar-Agar, Kobe I, powdered, for microbiology, art. no. 5210.1, Carl Roth, Germany) that was dissolved in 1X PBS (prepared according to\(^29\) but containing only KCl, Na\(_2\)HPO\(_4\), KH\(_2\)PO\(_4\) and 140 mmol/L NaCl). After boiling, the pH value was adjusted while the mixture cooled down to \(\sim 44^\circ\)C under constant stirring. To minimize dilution, hydrochloric acid (HCl) and sodium hydroxide solution (NaOH) were both applied at 1 mol/L concentration (Sigma-Aldrich Laborchemikalien, Germany, and Fisher Scientific, UK). The pH value was monitored along with temperature using a pH electrode with an integrated temperature sensor (inoLab pH 720, WTW, Germany). To set T\(_1\) to in vivo-like values, 0.105 mmol/L gadoteric acid (gadoterate meglumine [dotarem 500 mmol/L], Guerbet, Germany) was added. If the model solution contained 10 mmol/L L-glutamic acid (Fluka Chemie, Switzerland), the latter was added at a temperature below 44°C to avoid damaging the molecule. Afterwards, the mixture was cooled further and solidified on an orbital shaker (Titramax 100, Heidolph Instruments, Germany) or was centrifuged for around 4 seconds at 3490 rpm (Multifuge 1 S-R, Heraeus, Germany) to remove residual air bubbles.

In addition, model solutions were made from pure agarose (Agarose NEEO ultra-quality, art. no. 2267.2, Carl Roth, Germany) in the same manner as described for agar. Agarose is a polysaccharide and together with agarosepectin forms agar. These samples also contained 0.105 mmol/L gadoteric acid to reduce T\(_1\) values.
2.2 CEST MRI parameters

Measurements were performed at a 9.4 T ultra-high field, human whole body MR scanner (Magnetom 9.4 T, Siemens Healthineers, Erlangen, Germany) using a custom-built 18/32 Tx/Rx head coil. To maintain stable temperatures ($\Delta T_{\text{max}} \sim 1$ K) during the measurements, a thermos jug was used, in which the model solutions were placed on a custom-built, circular-shaped rack surrounded by water that contained $\sim 50$ mmol/L sodium chloride.

MRI was performed using a 3D gradient echo-based sequence (centric reordered, rectangular-spiral readout) realized in a snapshot manner. The basic sequence parameters were: flip angle (FA) = $5^\circ$, TE/TR = 1.91/3.76 ms, nominal matrix size 96 x 78 x 8 (RO = AP x PE = LR x 3D = HF; GRAPPA = 2) with FOV = 147 x 119 x 40 mm$^3$. The presaturation module consisted of five Gaussian-shaped $t_p = 100$ ms pulses at a duty cycle (DC = $t_p/(t_p + t_d)$: $t_d$: delay after single pulse) of 50% or matched adiabatic spinlock pulses with the same duration and DC. The adiabatic tipping pulses swept over a bandwidth of 3 kHz and took 8 ms both for tip-up/-down and only the locking time is considered as $t_p$. The spinlock pulses were chosen because they provide reduced direct water saturation and are beneficial in terms of specific absorption rate (SAR) compared with Gaussian pulses. This is because the rectangular locking pulse requires a lower peak $B_1$ compared with a Gaussian-shaped pulse. The resulting Fourier width of both the spinlock and Gaussian-shaped pulses was $\sim 10$ Hz (Figure S1). For the widths of the resonances in the Z-spectrum, it is the continuous wave power equivalent which is important and not the pulse duration (e.g., see\textsuperscript{32}). The continuous wave power equivalent was found to be 20% smaller for the spinlock pulses (see the supporting information for more details). The transmit $B_1$ ($B_1^+$) values stated in this study are the average $B_1^+$ values of a single presaturation pulse, such that $\text{FA} = \frac{t_p B_1}{\gamma}$ independent of the pulse shape. The presaturation offset list can be found in the supporting information. For both pulse types the recovery times for un-/saturated images were 12/5 seconds. All measurements were performed for three different nominal $B_1^+$ values to enable interpolation-based $B_1^+$-correction.\textsuperscript{33} Interleaved WASABI\textsuperscript{34} scans (single rectangular pulse of duration $t_p = 3.7$ ms at $B_1^+ = 5$ $\mu$T; recovery times [un-/saturated]: 12/5 ms) before and after each CEST acquisition were performed to track spatiotemporal changes of the static magnetic field. Image readout parameters for WASABI were the same as for the CEST acquisitions and data were acquired with the same 3D gradient echo-based sequence.

2.3 Data evaluation

Unless stated otherwise, data were normalized, including multiple unsaturated images ($M_0$). These were acquired before the first, after half of all, and after the last saturated image. During postprocessing the intensities of the different $M_0$ images were interpolated (linear) to match the acquisition times of the saturated images. This yielded an individual $M_{0,i}$ for each presaturation offset $i$. So each saturated image was normalized to $Z_i = M_{\text{sat},i}/M_{0,i}$. Afterwards, data were corrected for spatiotemporal changes in $\Delta B_0(r,t)$\textsuperscript{35} combining the different $\Delta B_0(r)$ maps derived from the interleaved WASABI scans. Next, data were corrected for spatial inhomogeneity in transmit $B_1^+$($r$) using averaged relative $B_1^+$ maps derived from WASABI scans by a two-point Z-$B_1$ correction\textsuperscript{33} including data acquired at three different nominal $B_1^+$. Since the observed CEST effects were located close to the water resonance ($\delta \omega < 2$ ppm) and no discrete peaks could be resolved, a simple and therefore meaningful asymmetry analysis\textsuperscript{2} was performed on the corrected Z-spectra\textsuperscript{36}

$$MTR_{\text{asym}} = \frac{M_{\text{sat}}(-\Delta \omega) - M_{\text{sat}}(+\Delta \omega)}{M_0}$$

This is justified if (a) inhomogeneity of $B_0$ was completely corrected, (b) measurement was stable over time and (c) the Z-spectrum does not contain multiple pools both up- and downfield of the bulk water resonance.

2.3.1 Bloch-McConnell simulations

To determine the approximate CEST effect of agar at a clinically more popular field strength of 7 T, the Bloch-McConnell equations were simulated numerically.\textsuperscript{37} To determine suitable parameters, both measurements at 3 and 9.4 T were modeled with the same CEST and ssMT pool parameters but different longitudinal and transversal relaxation rates for the water pool. Afterwards, these values were interpolated to 7 T and the simulations were performed again. Additionally, the data measured at 3 and 9.4 T were directly interpolated to 7 T for comparison. Detailed model parameters can be found in the supporting information.
3 | RESULTS

3.1 | Agar

Figure 1 shows the effect of the suggested postprocessing on Z-spectra. It was found that normalization including multiple M₀ scans (yellow line) reduced MTR_{asym} for larger offsets compared with normalization including only a single M₀ image (orange line). The effect of both spatial \( \Delta B_0(r) \) and spatiotemporal \( \Delta B_0(r, t) \) correction (blue and purple lines) was most prominent directly around 0 ppm, where it shifted MTR_{asym} from negative to positive values. Comparing both methods, the spatiotemporal correction gave reduced MTR_{asym} values (Figure 1C). The applied \( \Delta B_1^+(r) \) correction (black line) did not substantially alter either the Z-spectra or MTR_{asym} in addition to the \( \Delta B_0(r, t) \) correction in this particular case.

To make sure the observed MTR_{asym} effects were not caused by contributions of PBS, NaOH, HCl or gadoteric acid, measurements of samples that contained solely these compounds were performed (Figure 2). For these samples pH was adjusted to comparable values between 6.90 and 7.00 and the measurement was performed at 25°C. Figure 2B shows that neither PBS, NaOH, HCl nor gadoteric acid yielded a maximum MTR_{asym} of more than 0.4%, but 2% (w/w) agar caused an almost 10-fold larger maximum MTR_{asym} of 3.3%. In Figure 2C,D the same samples are shown for Gaussian-shaped instead of matched adiabatic spinlock pulses but with identical nominal \( B_1^+ = 4 \mu T \), \( t_p \) and number of pulses. The average of absolute values of MTR_{asym} in the range of 0.5 to 10.0 ppm over samples that contained no agar was \((0.08 \pm 0.02)\%\) for Gaussian and \((0.18 \pm 0.04)\%\) for matched adiabatic spinlock pulses. For the sample that contained agar the same metric revealed 1.05% and 1.43% of average MTR_{asym}. In all cases, data were processed according to the suggested full postprocessing as described above and evaluated at a corrected \( B_1^+ \) of 4 \( \mu T \). From Figure 2A,C it can be observed that for some of the samples the Z-values do not approach Z = 1, even for offsets farther away from

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**FIGURE 1** A, effect of different postprocessing methods on Z-spectra of agar. B, the Z-spectra in an 0.72 ppm presaturation offset interval to emphasize differences between the postprocessing stages. C, the resulting MTR_{asym} for different postprocessing and the red dashed line highlights MTR_{asym} = 0. Key: “single M₀ scan”/orange – no \( \Delta B_0 \) or \( \Delta B_1^+ \) correction and single M₀ scan for normalization; “multiple M₀ scans”/yellow – no \( \Delta B_0 \) or \( \Delta B_1^+ \) correction but using three interleaved M₀ scans for normalization; “correction method 1”/blue – additionally including \( \Delta B_0(r) \) correction; “correction method 2”/purple – not only \( \Delta B_0(r) \) but spatiotemporal \( \Delta B_0(r, t) \) correction included; “full correction”/black – \( \Delta B_0(r, t) \) and \( \Delta B_1^+(r) \) correction included. Data acquired at \( B_0 = 9.4 \) T and \( T = 37°C \) using 5 x 100 ms matched adiabatic spinlock pulses with DC = 50%. All samples contained 2.0% (w/w) of agar, 1 X PBS and ~0.1 mmol/L gadoteric acid with adjusted pH = 7.00 at 43.5°C.
water of around ±10 ppm. This is a known relaxation effect, because some samples (the ones without gadoteric acid) had long $T_1$ of ~3.2 seconds but the recovery times were 5.5 seconds, which prevented full relaxation in these cases. More data on this observation can be found in Figure S4.

Figure 3 shows Z-spectra of agar samples acquired at 9.4 T including the described postprocessing along with the determined MTR$_{asym}$ for different temperatures (25°C in A and B and 37°C in C and D) and pH values. Twice as high maximum MTR$_{asym}$ was observed for lower temperature at nominal pH = 7.40 (Figure 3E). The maximum value of MTR$_{asym}$ constantly increased with decreasing pH at 37°C (Figure 3E). Compared with this, MTR$_{asym}$ was found to be rather stable across pH at 25°C with overall higher maximal values (Figure 3E). This temperature dependency of MTR$_{asym}$ at different pH values reveals more details of the underlying exchange reaction, as explained below.

First, MTR$_{asym}$ as a function of the exchange rate ($k_{ex}$), increases with $k_{ex}$ if $k_{ex} < \gamma B_1$ (correlation), but decreases with $k_{ex}$ if $k_{ex} > \gamma B_1$ (anticorrelation).32 Second, in the majority of cases, exchange rates correlate with temperature (Arrhenius equation), which is also assumed here. Additionally, in3 increasing $k_{ex}$ was confirmed experimentally for various exchangeable groups. As decreasing MTR$_{asym}$ was observed with higher temperature and therefore higher exchange rate (anticorrelation), it can be concluded that the exchange reaction was in the regime $k_{ex} > \gamma B_1$. The fact that MTR$_{asym}$ values at 25°C were almost unaffected by pH indicates that the exchange reaction was close to the regime of maximum possible MTR$_{asym}$, where $k_{ex} = \gamma B_1$ ($B_1 = 4 \mu T$ in this study).

At 37°C, MTR$_{asym}$ values showed highest signal for lowest pH. As MTR$_{asym}$ was still anticorrelated with $k_{ex}$, this means that the exchange rates correlate with pH. This makes the exchange reaction base-catalyzed.

The average observed shift in offset position of maximum MTR$_{asym}$ (Figure 3F) was 0.1 ppm, which was around four times smaller than the average shift introduced by altered concentrations (Figure 4F).
Z-spectra and MTR_{asyn} values for different concentrations and temperatures at constant pH are shown in Figure 4A,B, again for 25°C (A and B) and 37°C (C and D). All the samples were measured at the same time but over two different sessions for the different temperatures. The maximum MTR_{asyn} value, in general, increased with agar concentration (Figure 4E), on average by 18%, increasing from 1.0% to 2.5% (w/w) of agar. Whereas for low concentrations (increasing from 1.0% to 1.5% of agar) it did not change significantly (P > 0.9), it increased by ~13%, increasing from 1.5% to 2.0% (w/w) agar. The observed changes in maximum MTR_{asyn} were significant at a global \( \alpha = 0.003 \) (Holm-Bonferroni method) for pairwise comparison of concentrations except for the comparison of 1.0% and 1.5% (w/w). MTR_{asyn} was evaluated in single voxels at the presaturation frequency of maximum MTR_{asyn} of the ROI-averaged Z-spectrum. The frequency offset of maximum MTR_{asyn} shifted away from the bulk water resonance at 0 ppm for both temperatures as the agar concentration increased (Figure 4F). Shifts were 0.4 and 0.6 ppm for 25 and 37°C, respectively. More linear shift dependence on concentration was observed for higher temperatures.

The conclusions derived from maximum MTR_{asyn} could also be confirmed analyzing the area under the curve (AUC) of MTR_{asyn} within 0.5 to 4.0 ppm. This metric should be more stable against noise, though maximum MTR_{asyn} already represented a ROI average. The associated results are shown in Figure S10.

To determine the undesired contribution of agar to the observed CEST effect of L-glutamic acid, model solutions with matched pH values both with and without agar were investigated. As can be seen from Figure 5, the position of maximum MTR_{asyn} value shifted 0.7 ppm away from water when \( T_2 \) was reduced from \( (525 \pm 131) \) ms to more in vivo-like values of \( (60 \pm 15) \) ms using agar (\( T_2 \) values determined from repeated 2D spin echo sequences with different \( T_2 \)). Still, the absolute maximum value was reduced by less than 2% due to adjusted \( T_2 \). For the sample in which \( T_2 \) was not adjusted, two peaks can be seen in MTR_{asyn}. This is due to chemical shift averaging, as described by Cai et al.5 and can be understood by \( T_1^* \) theory (see equation 23 in Zaiss et al.19). To isolate the contribution of agar in these data, a control sample without L-glutamic
acid was created and Z-values were subtracted from the referring Z-spectrum of the sample that contained agar and L-glutamic acid. It was found that the maximum MTRasym decreased by 50% when the contribution of agar was removed (Figure 5B, green curve). This leads to two important conclusions. First, it was shown that (as expected from theory) T2 does significantly affect the observable CEST effect of L-glutamic acid. And second, without taking into account the CEST effect of agar, one could have falsely concluded that the applied presaturation would yield nearly identical MTRasym independent of T2.

From the Bloch-McConnell simulations (Figures S2 and S3) it was estimated that 2.0% (w/w) of agar should result in maximum MTRasym of the order of 2% at B0 = 7 T given a saturation with 5 x 100 ms matched adiabatic spinlock pulses (DC = 50%). It was found that both simulations and straightforward interpolation of 3 and 9.4 T data yielded comparable results even although the experimentally applied saturation differed in terms of number of pulses (n = 4 at 3 T vs. n = 5 at 9.4 T).

3.2 | Pure agarose

The agarose samples were measured with the same matched adiabatic spinlock pulses used for the agar samples: DC = 50%, tp = 100 ms, B1⁺ = 4 μT. Again, all samples were measured at both 25°C and 37°C. A detailed presentation of the Z-spectra and MTRasym for different concentrations and pH values of pure agarose is shown in Figures S5 and S6. It was found that the agarose samples yielded on average 61% /56% lower maximum MTRasym for the same concentrations measured at 25°C/37°C (Figure 6A,B) compared with agar. The maximum MTRasym observed in pure agarose was between 0.9% and 1.4%.
At different pH values, the maximum $\text{MTR}_{\text{asym}}$ was reduced on average by 60% for $T = 25^\circ\text{C}$ in pure agarose compared with agar (Figure 6C). For higher temperatures, $\text{MTR}_{\text{asym}}$ showed a stronger dependency on pH for agar compared with the dependency observed for agarose (Figure 6D). $\text{MTR}_{\text{asym}}$ showed a slight increase of $\Delta \text{MTR}_{\text{asym}} = 0.25\%$ when going towards more alkaline pH in agarose. For agar it decreased by $\Delta \text{MTR}_{\text{asym}} = 1.26\%$ over the same range of pH (Figure 6D).

As both agar and agarose may be used to adjust $T_2$ in model solutions, their influence on $T_2$ was compared (Figure S7A). It was found that at the same pH and concentration, both compounds yielded the same $T_2$ values. While the $T_2$ values did depend on the concentration, they were not significantly different within the investigated pH range (Figure S7B). The latter remark holds true for both pure agarose and agar, each at a global significance level of $\alpha = 0.05$.

4 | DISCUSSION

The observed $\text{MTR}_{\text{asym}}$ did not vanish even after application of multiple corrections during postprocessing (Figure 1). This proved that there is indeed a significant CEST contribution from agar that can be detected even with CEST MRI at a human whole body scanner. At the same time, control experiments did not yield significant $\text{MTR}_{\text{asym}}$ (Figure 2B,D) for samples that did not contain agar. This assured that no contribution from substances other than agar, which were used to adjust $T_1$ and pH, could be detected. The employed gadoterate meglumine had five potentially exchangeable hydroxyl groups per molecule, but still no significant $\text{MTR}_{\text{asym}}$ was observed. This could be attributed to the fact that the concentration necessary to decrease $T_1$ to in vivo-like values is very low ($\sim 0.1 \text{ mmol/L}$) and that exchange rates could potentially be very high. Therefore, a possible contribution could be expected to be not significant. This would be tolerable for optimization of CEST MRI parameters. Regarding the pulse shape, it was first shown that both Gaussian-shaped and matched adiabatic spinlock pulses yielded consistent results (Figure 2). Still, the matched adiabatic spinlock pulses provided 45% higher maximum $\text{MTR}_{\text{asym}}$, which is related to a reduced spillover contribution, which made it easier to investigate the CEST effects of agar.

The influence of pH, concentration and temperature further supported the hypothesis that agar shows a specific, significant CEST effect under in vivo-like conditions. The observed $\text{MTR}_{\text{asym}}$ increased for more acid pH values at $37^\circ\text{C}$ but was more stable at $25^\circ\text{C}$ (Figure 3). This is
because both temperature and pH are able to alter exchange rates and may balance with each other. For increasing agar/agarose concentrations $T_2$ was decreased (Figure S7A), which pronounced spillover effects. The latter is why adjusting $T_2$ properly in model solutions is of major importance for presaturation parameter optimization. As the concentration of agar increased, the contribution of semi-solid magnetization transfer also increased, to a factor of 2.8 (Figure S8).

It was observed that both $M_{TR\text{asym}}$ and the frequency offset of maximum $M_{TR\text{asym}}$ depended on the agar concentration (Figure 4). The shift of maximum observed $M_{TR\text{asym}}$ is expected as the spectrum becomes broader for shorter $T_2$. In particular, this interplay of both scaled CEST pool size, and because of this the modified $T_2$-related spillover effects for different agar concentrations, might cause problems if one is actually optimizing parameters for another CEST pool. For instance, for concentrations of 1.0% and 1.5% (w/w), the maximum $M_{TR\text{asym}}$ value was stable but increased by 13% for an additional 0.5% (w/w) of agar. This was most likely due to the fact that increased spillover effects at lower concentrations still counterbalanced the effects of increased pool size. The AUC (Figure S10) at 25°C already showed a significant ($P < 0.05$) increase for a concentration of 1.5% compared with one of 1.0% (w/w). This demonstrates that it is not trivial to predict the expected behavior of the agar CEST contribution in model solutions, especially if different presaturation schemes are to be compared. At least it could be concluded that for the pH (6.8 to 7.4) and concentration (1.0%-2.5% w/w) ranges studied, the underlying exchange rates are base-catalyzed, which might be of interest depending on the behavior of the other CEST pools for which parameters should actually be optimized.

The contribution of agar at lower powers ($B_{1+} < 1 \mu T$) may be negligible (Figure S9). On the other hand, recently published studies that deal with glucose or its chemical derivatives rely on high power presaturation for CEST MRI. Also, other compounds, such as glutamate$^{5,39}$ need a high presaturation power of $\sim3 \mu T$. In the current study, continuous wave power equivalents of 2.8 and 3.4 $\mu T$ for spinlock and Gaussian-shaped pulses were applied for a total saturation duration of 1 second. Given the SAR restrictions at 9.4 T, this was comparable with the continuous wave saturation ($B_{1,\text{rms}} = 3.6 \mu T$, optimal $t_{\text{sat}} = 1$ second) that Cai et al.$^5$ reported for in vivo GluCEST imaging at $B_0 = 7$ T.

As an alternative to agar one may use pure agarose, which has also been used in previous CEST MRI studies. We found that the use of pure agarose instead of agar can be beneficial as it showed less than half of the $M_{TR\text{asym}}$ observed for agar. Still, with maximum $M_{TR\text{asym}}$ of up to 1.4%
for certain concentrations and pH values, it does not yet provide a fully neutral baseline for CEST parameter optimization. In terms of adjusting $T_2$, it was found that both agar and agarose performed equally well. Therefore, pure agarose should be preferably chosen when performing parameter optimization for CEST MRI in model solutions.

Regarding the origin of the CEST effect, hydroxyl groups may be responsible for the observed saturation transfer. These are known for rather high exchange rates and resonate close to the bulk water (e.g., Jin et al.40), which is what was observed in the current study. Furthermore, agarose, which makes up the main part of agar, has five exchangeable hydroxyl groups (e.g., see Gamini et al.41) that could contribute. The observed effects in pure agarose were smaller in terms of both AUC and maximum $MTR_{asym}$ compared with agar. This shows that agarose by itself probably does not provide all the exchanging sites observed in agar. Agar is derived from a natural product and also contains agaropeptine. It is therefore likely that additional exchanging sites are available. Still, the exact composition of agaropeptine is unknown and the spectral resolution of CEST MRI does not enable any further insights. Therefore, it is not possible to exactly assign a particular exchanging group to the observed effects.

It should also be emphasized that the applied postprocessing is crucial, especially when $MTR_{asym}$ is evaluated. For instance, even spatial $\Delta B_0(r)$ or spatiotemporal $\Delta B_0(r,t)$ correction35 yielded a 10% difference in $MTR_{asym}$ (Figure 1C).

This study benefited from the availability of an ultra-high field 9.4 T whole body MR scanner that is not typically used in clinical CEST MRI studies. However, also at 3 T (Prisma$^\text{TM}$, Siemens Healthineers, Erlangen, Germany), $MTR_{asym}$ of up to 0.5%, most likely originating from agar, was observed (Figure S11). Due to reduced absolute frequency separation at lower field strength, CEST pools resonating close to the bulk water are strongly affected by spillover, making quantification challenging. From the 3 T data alone it would not have been possible to characterize the CEST contribution of agar and agarose. Still, it was at least shown that the AUC of $MTR_{asym}$ differed significantly from zero ($P < 0.001$) in the case of agar, but not for the control sample without agar ($P > 0.5$). Small effect sizes will occur for all CEST effects, but not only for agar or agarose at lower field strength. Although it will not be obvious to see, it is even more important to be aware that there is a possible contribution from agar and/or agarose, even at 3 T. As an estimate for the effect size at more commonly used 7 T scanners, numerical simulations of the Bloch-McConnell equations showed that the expected effect (maximum observed $MTR_{asym}$) size for an identical saturation scheme was reduced by $\sim 30\%$ compared with 9.4 T.

To show that neglecting the CEST effect of agar can lead to errors when optimizing CEST MRI parameters in model solutions, samples containing 10 mmol/L glutamic acid were prepared exemplarily. As shown, the effect of agar contributed 50% to the observed maximum $MTR_{asym}$ (Figure 5B). Considering $MTR_{asym}$ at 3 ppm, it was found that the observed $MTR_{asym}$ (3 ppm) value originated from agar by one-third and from glutamic acid by two-thirds. This means that even although larger contributions from agar were found closer to water, for offsets farther away, the contribution also remains significant. It is also shown (see the supporting information) that for the discussed setup the relaxation-compensated $MTR_{ex}$ and $MTR_{asym}$ provide the same results with regard to the relative contribution of agar. The contribution of agar to CEST MRI depended on $B_1^*$, pH, concentration and temperature in a nonlinear manner (Figures 3 and 4, Figure S9). To remove its contribution, a baseline sample which contained no glutamic acid was suggested for correction. Another way to bypass the bias introduced by agar would be to find another suitable way to modify $T_2$ in CEST model solutions. On the other hand, there are many practical reasons which encourage the continued use of agar for the creation of CEST MRI model solutions. Therefore, we suggest creating control samples with corresponding pH, $T_1$, and $T_2$ values to correct for the bias attributable to agar in CEST MRI parameter optimization by simply subtracting the corresponding Z-values. As agarose displayed $\sim 50\%$ smaller CEST effects, instead of using agar, pure agarose should be chosen as the first step in model solutions.

5 CONCLUSION

The presented findings proved that agar shows a significant CEST effect for in vivo-like pH and temperature at a human MR scanner. Therefore, it does not a priori provide a neutral baseline for CEST MRI parameter optimization in the case of strong presaturation. CEST effects of agar showed a complex dependency on the interplay of concentration, pH, temperature and $B_1^*$, which made the creation of control samples necessary. Otherwise, misleading results in presaturation parameter optimization may be derived, as well as incorrectly assigned CEST effect strengths. It was exemplarily shown—and corrected using the control sample—that for 10 mmol/L glutamic acid, 50% of the observed $MTR_{asym}$ was due to agar. It was also found that pure agarose yields 50% reduced CEST effects compared with agar and is therefore preferable when creating model solutions.

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SUPPORTING INFORMATION

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

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