RESEARCH ARTICLE

Determination of an optimally sensitive and specific chemical exchange saturation transfer MRI quantification metric in relevant biological phantoms

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The purpose of this study was to develop realistic phantom models of the intracellular environment of metastatic breast tumour and naïve brain, and using these models determine an analysis metric for quantification of CEST MRI data that is sensitive to only labile proton exchange rate and concentration. The ability of the optimal metric to quantify pH differences in the phantoms was also evaluated.

Novel phantom models were produced, by adding perchloric acid extracts of either metastatic mouse breast cancer cells or healthy mouse brain to bovine serum albumin. The phantom model was validated using 1H NMR spectroscopy, then utilized to determine the sensitivity of CEST MRI to changes in pH, labile proton concentration, T1 time and T2 time; six different CEST MRI analysis metrics (MTRasym, APT*, MTRRex, AREX and CESTR* with and without T1/T2 compensation) were compared.

The new phantom models were highly representative of the in vivo intracellular environment of both tumour and brain tissue. Of the analysis methods compared, CESTR* with T1 and T2 time compensation was optimally specific to changes in the CEST effect (i.e. minimal contamination from T1 or T2 variation). In phantoms with identical protein concentrations, pH differences between phantoms could be quantified with a mean accuracy of 0.6 pH units.

We propose that CESTR* with T1 and T2 time compensation is the optimal analysis method for these phantoms. Analysis of CEST MRI data with T1/T2 time compensated CESTR* is reproducible between phantoms, and its application in vivo may resolve the intracellular alkalosis associated with breast cancer brain metastases without the need for exogenous contrast agents.

KEYWORDS
brain, CEST, metastases, MRI, pH

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Abbreviations used: APT*, three-offset amide proton transfer metric; AREX, apparent relaxation due to exchange metric; BayCEST, Bayesian chemical exchange saturation fitting algorithm; BSA, bovine serum albumin; CEST, chemical exchange saturation transfer; CESTR*, chemical exchange saturation transfer ratio metric; EPI, echo planar imaging; MTRasym, magnetisation transfer asymmetry metric; MTRRex, magnetisation transfer ratio relaxation due to exchange metric; NOE, nuclear Overhauser enhancement; PCA, perchloric acid; ppm, parts per million; qCEST, quantitative CEST

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1 | INTRODUCTION

Chemical exchange saturation transfer (CEST) is an MRI contrast mechanism that measures changes in signal from water protons owing to their association with other biomolecules and metabolites, particularly via exchange of protons with hydrolysable functional groups such as amides and amines.\(^1,2\) CEST MRI has the potential to make non-invasive measurements of pH,\(^3\) inform on areas of infiltrating tumour,\(^4\) differentiate tumour from radiation necrosis,\(^5\) and provide information concerning the conformation of proteins.\(^6\) Since the pH of tissues is highly regulated, and numerous pathologies interfere with this regulation, the application of CEST MRI to generate pH maps \textit{in vivo} has received significant research interest recently.\(^7\)

There is a clear need to develop a reliable, non-invasive method of measuring tumour pH \textit{in vivo}. One hallmark of cancerous tumours is their dysfunctional regulation of pH,\(^8\) leading to an acidic extracellular space and slight alkalosis in the intracellular space.\(^9\) This change in pH has consequences for the efficacy of various treatments for tumours, and measurement of tumour pH may be useful in stratification of patients based on their expected responsiveness to particular therapies. In particular, brain metastasis, or secondary tumour spread to the brain, represents a major clinical problem, with poor prognosis and few therapeutic options. The development of better methods for interrogating the tumour microenvironment and targeting therapy may greatly enhance our ability to treat these tumours.

It is widely recognized that CEST is sensitive to more than just pH, which has led to various studies offering different explanations for the source of CEST contrast seen in tumours. Some studies claim that an increased protein concentration in tumour cells generates contrast between tumour and surrounding tissue.\(^10,11\) However, others have measured insignificantly different protein concentration between rodent brain and implanted tumour, and suggest that the contrast is a result of T\(_1\) contamination of the signal.\(^12\) In addition, evidence suggests that the pH and labile proton concentration are difficult to separate from CEST measurements.\(^13\) Despite this difficulty, a metric that is not contaminated by relaxation time changes and only depends on the relevant physiological parameters is needed before the potential of using CEST MRI for pH measurement in tumours can be assessed.

To develop such a metric, previous studies have used simple phantoms with a single exchanging pool of protons.\(^13,14\) These phantoms allow useful insights into the CEST MRI signal source, but do not adequately represent the complex \textit{in vivo} intracellular biomolecular environment. Methods such as quantitative CEST (qCEST)\(^14\) and the Omega plot\(^15\) have been used in these phantoms to successfully quantify labile proton exchange rate and concentration independently. However, these methods require many Z spectra to be acquired with varying saturation parameters, which is impractical in a clinical environment. The apparent relaxation due to exchange (AREX) metric has also been proposed to correct for contamination of CEST effects by T\(_1\) relaxation of water,\(^12,16–18\) but this method has not been validated in physiologically relevant phantoms.

The aims of this study, therefore, were the following: (i) to develop novel phantom models from cellular extracts that are representative of the \textit{in vivo} intracellular environment of both normal brain and brain metastases; (ii) to use these phantoms to determine the sensitivity of the CEST MRI signal as measured by a number of different analysis metrics to changes in pH, labile proton concentration, T\(_1\) and T\(_2\); (iii) to identify an optimally specific analysis metric for quantification of CEST MRI data and (iv) to evaluate the ability of the optimal metric to quantify pH differences in these phantoms.

2 | METHODS

2.1 | Phantom preparation

Phantoms were prepared to represent the intracellular environment of naïve mouse brain and \textit{in vitro} cultured 4T1-GFP mouse metastatic breast carcinoma cells. The 4T1-GFP cell line is commonly used as a mouse model of metastatic breast cancer, including studies of metastatic spread to the brain.\(^19\) It is an appropriate tumour model here as previous studies have suggested that intracellular pH is difficult to measure in tumours. Some studies claim that an increased protein concentration in tumour cells generates contrast \textit{in vivo},\(^16\) while others have used in-vitro phantom models to study the effect of pH on contrast.\(^16\) Here, we aimed to investigate the effect of pH and protein concentration on contrast \textit{in vivo}.

Phantom preparation

Phantoms were prepared to represent the intracellular environment of naïve mouse brain and \textit{in vitro} cultured 4T1-GFP mouse metastatic breast carcinoma cells. The 4T1-GFP cell line is commonly used as a mouse model of metastatic breast cancer, including studies of metastatic spread to the brain.\(^19\) It is an appropriate tumour model here as previous measurements of human breast cancers have displayed intracellular alkalosis.\(^20\) For the mouse brain phantoms, female BALB/c mice aged 6–8 weeks (n = 6) were terminally anaesthetized with sodium pentobarbital and transcardially perfused with 20 mL heparinized saline. Subsequently, the brains were removed, frozen in liquid nitrogen and stored at −80°C until further use. For the 4T1-GFP cell phantoms, cells were cultured in Dulbecco’s modified Eagle medium and passaged every second day to grow a sufficient number of cells.

Perchloric acid (PCA) extracts of either naïve brain or 4T1-GFP cells were prepared. Briefly, either naïve mouse brains (n = 6) or a pellet of 4T1-GFP cells (8 g) were homogenized and washed with 0.1 M HCl in methanol in a dry ice bath. Subsequently, the mixture was warmed to wet ice bath temperature, 0.02 M HCl and 3 M PCA added to the homogenizer and the precipitated protein sedimented by centrifugation (4800 g, 20 min, 4°C). The supernatant was neutralized to pH ~ 7 with KOH and the precipitated potassium perchlorate sedimented by centrifugation (4800 g, 20 min, 4°C). The supernatant was lyophilized. All volumes of solutions added were scaled to the weight of starting material to match metabolite concentration to their \textit{in vivo}/intracellular values.

The lyophilized samples were divided into 26 equal fractions and added to bovine serum albumin (BSA) to produce phantoms representative of the intracellular environment of naïve mouse brain and 4T1-GFP tumour cells. The pH (6.0–7.6, n = 6), BSA content (4–16% w/v, n = 7), T\(_1\) time (0.3–1.7 s, n = 7) and T\(_2\) time (29–140 ms, n = 6) of each phantom was serially varied for a total of 52 phantoms (n = 26 for each cell type). T\(_1\) and T\(_2\) relaxation times were varied by addition of gadolinium-DTPA (Omniscan, GE Healthcare) and iron nanoparticles (25–30 nm diameter), respectively. Where pH and BSA content were not varied, the phantoms were pH 7.4 with a BSA concentration of 4% w/v. BSA has been used in previous studies as a protein representative of the intracellular environment of brain protein pool, and 8% w/v is a reasonable assumption of the protein content of the rodent brain.\(^21,22\) The BSA was not cross-linked to avoid macromolecular magnetization transfer effects being introduced, and the phantom pH was titrated after addition of BSA.

2.2 | Validation of tissue/cell extract phantoms

The validity of the PCA extract supplemented with BSA model as a reasonable representation of the intracellular environment of brain/
tumour cells in vivo was confirmed using high-resolution NMR spectroscopy. One-dimensional $^1$H spectra with WATERGATE solvent suppression were acquired (see Section 2.3) from samples of PCA-extracted naïve mouse brain, in vitro cultured 4T1-GFP cells, and subcutaneous 4T1-GFP tumours. The subcutaneous tumours were grown by injecting $5 \times 10^5$ 4T1-GFP cells in 100 μL PBS subcutaneously into female BALB/c mice aged 6–8 weeks ($n = 6$). Tumours were allowed to grow until 10 mm geometric mean diameter, at which point the mice were terminally anaesthetized with sodium pentobarbital and transcendally perfused with 20 mL heparinized saline. The tumours were then isolated from the surrounding skin and fat, frozen in liquid nitrogen and stored at −80°C until further use.

Additional spectra were obtained from the lysate of in vitro cultured 4T1-GFP cells and the respective phantom model (PCA-extracted in vitro cultured 4T1-GFP cells supplemented with 8% w/v BSA). For the PCA extracts, the lyophilized samples were dissolved in pH 7.4 potassium phosphate buffer (1 M) to provide suitable buffering capability over the range required for this study. The cell lysate sample was produced by suspending 4T1-GFP cells in NP-40 lysis buffer (2.74 mL 1 M NaCl, 2 mL 200 mM pH 7 Tris HCl, 80 μL 0.5 M EDTA, 200 μL NP-40, 4.98 mL dH2O) and sedimenting the precipitated cellular membranes by centrifugation (21 000 g, 20 min, 4°C). All samples were prepared to a final volume of 600 μL with 5% D2O.

In addition, samples of 8% w/v BSA in potassium phosphate buffer (1 M, pH 7.4) were supplemented with varying concentrations of PCA-extracted in vitro cultured 4T1-GFP cells (1×, 1.5× and 2× metabolite concentration) to confirm that the presence of metabolites from the PCA-extracted cells in the sample influences the measured CEST spectrum.

2.3 Solution NMR experiments

Proton spectra were acquired using a vertical bore 600 MHz (14.1 T) spectrometer (Agilent Technologies, Santa Clara, CA, USA) using a WATERGATE sequence with a relaxation delay of 2 s, an acquisition time of 2 s and 128 transients per free induction decay recorded. The carrier was centred on water (4.7 ppm) with a sweep width of 9551 Hz. Spectra were processed using NMRPipe.23

2.4 MRI experiments

All MRI experiments were performed using a horizontal bore 400 MHz (9.4 T) spectrometer (Agilent Technologies) with a volume transmit-receive coil (internal diameter 40 mm, RAPID Biomedical, Rimpar, Germany). Shimming was performed prior to each experiment to minimize the B0 field inhomogeneity. CEST images were acquired of 26 phantoms simultaneously using a saturation scheme of 300 Gaussian pulses of 26 ms duration and 180° flip angle each (50% duty cycle, equivalent continuous wave saturation power 0.8 μT) at 85 saturation frequencies spaced equally between ±10 ppm, followed by an eight-shot spin-echo echo planar imaging (EPI) readout. Additional images were acquired following saturation at ±100 ppm for normalization; field of view 38 mm × 38 mm, matrix size 32 × 32, slice thickness 2 mm, echo time ($T_E$) 8.22 ms and repetition time ($T_R$) 7.85 s. Total scan time for each set of phantoms was 3 h 6 min.

In addition to CEST imaging, the $T_1$ and $T_2$ relaxation times of each phantom were measured using inversion recovery ($T_R = 10$ s, $T_E = 8.22$ ms, inversion time ($T_I$) varied in nine steps from 13.14 ms to 8 s, signals fitted to $M_z = M_0 (1 - 2 \exp(-T/T_I))$ and spin echo ($T_R = 10$ s, $T_E$ varied in 10 steps from 30 ms to 160 ms, signals fitted to $M_z = M_0 \exp(-T/E/T_2)$ experiments, respectively. In both cases eight-shot spin-echo EPI readout was used to acquire images.

2.5 MRI data processing

All MRI data were processed in MATLAB (MathWorks, Natick, MA, USA). The relaxation maps of the water pool were obtained by least square fitting of the measured intensity against the inversion time ($T_1$ map) and echo time ($T_2$ map). Six metrics were used to analyse the Z spectra: conventional asymmetry analysis ($\text{MTR}_{\text{asym}}$),1 multiple-offset analysis ($\text{APT}^*$),24 inverse Z-spectrum multiple-offset analysis ($\text{MTR}_{\text{Res}}$),12 AREX,12 and two variants of a Bayesian model-based analysis (CESTR*),13 as defined in Equations 1–5, respectively. $B_0$ inhomogeneity was corrected prior to $\text{MTR}_{\text{asym}}, \text{APT}^*$ and $\text{MTR}_{\text{Res}}$ analysis by shifting the minimum point of the Z spectrum to 0 ppm on a voxel-wise basis; CEST* corrects for $B_0$ inhomogeneity during the analysis. All analyses were performed on a voxel-wise basis, and the data presented for each phantom are the mean ± standard deviation for a fixed-area region of interest (ROI) over each phantom.

\[
\text{MTR}_{\text{asym}}(\omega) = \frac{Z(\omega) - Z_{\text{ref}}(\omega)}{Z_0} \quad (1)
\]

\[
\text{APT}^*(\omega) = Z_{\text{ref}}(\omega) - Z(\omega) \quad (2)
\]

\[
\text{MTR}_{\text{Res}}(\omega) = \frac{1}{Z(\omega)} - \frac{1}{Z_{\text{ref}}(\omega)} \quad (3)
\]

\[
\text{AREX}(\omega) = \frac{\text{MTR}_{\text{Res}}(\omega)}{T_1} \quad (4)
\]

\[
\text{CESTR}^*(\omega) = \frac{S_{1\text{-pool}}(\omega) - S_{2\text{-pool}}(\omega)}{Z_0} \quad (5)
\]

In Equations 1–5, $\omega$ is the offset frequency of interest, $Z(\omega)$ is the signal measured following saturation at the frequency $\omega$, $Z_0$ is the signal measured following saturation at ±100 ppm, $Z_{\text{ref}}(\omega) = \frac{Z(\omega + \delta\omega) - Z(\omega - \delta\omega)}{2\delta\omega}$, and $S_{n\text{-pool}}$ is the signal from a simulated Z spectrum with $n$ pools, and labile pool properties defined by those measured by a Bayesian model-based algorithm13 implemented in BayCEST as part of the FMRIB Software Library (www.fmrib.ox.ac.uk/fsl/baycest). CEST effects were measured for all metrics at $\omega = 2.8$ ppm with $\delta\omega = 1.4$ ppm.

Three pools were fitted using BayCEST: the water pool at 0 ppm (W), the labile amine proton pool at 2.8 ppm (CEST) and an exchange pool centred at −3.5 ppm. BayCEST fits the Bloch–McConnell equations to the measured Z spectra with the exchange rate and relaxation times for each pool defined by Bayesian prior distributions. BayCEST measures fitted values of the exchange rate and relative concentration of the protons in each pool, and $T_1$ and $T_2$ relaxation times for all proton pools (Supplementary Figure S1). BayCEST was run for
each phantom with ‘default’ values for the mean of the prior distributions for water $T_1$ (1.8 s) and $T_2$ (100 ms). Subsequently, the water $T_1$ and $T_2$ times measured from each phantom in the $T_1$ and $T_2$ maps were included as the means of the associated prior distributions in the model fitting (Supplementary Table S1). In both cases the $T_1$ and $T_2$ values remained as parameters within the fitting procedure to be estimated from the data.

CESTR* was calculated from the 2.8 ppm pool by simulating one-pool (W only) and two-pool (W + CEST) systems using only the fitted estimates of exchange rate and relative concentration, and measuring the difference in signal at 2.8 ppm (Supplementary Figure S2). This produced two values for CESTR*—one calculated with ‘default’ priors for water $T_1$ (1.8 s) and $T_2$ (100 ms), and a second with phantom specific measured $T_1$ and $T_2$ prior means—hereafter called ‘CESTR* with measured $T_1/T_2$ priors’. The fitted exchange rate was not used directly, as a degree of correlation exists between the fitted exchange rate and concentration. Further details of the fitting and analysis procedure for CESTR* can be found in the Supplementary Methods.

### 2.6 Determination of optimal metric

The relationship between the calculated CEST effect using each metric and the serially varied parameters was determined by linear regression, and its absolute value (in [% M₀/parameter unit change]) compared between phantom models using a $t$ test corrected for multiple comparisons using the Holm–Sidak method. Statistical significance was defined as $P < 0.05$, which after correction gave an effective significance level of $P < 0.003$. These comparisons were used to determine whether pooling the data from the two phantom models was appropriate.

Subsequently, a further multi-parameter linear regression model was used to determine the optimal analysis metric in terms of specificity. Data from both phantom models (tumour and naïve brain) were combined and fitted to a model of the form of Equation 6, which describes the response of an ideal CEST quantification metric (i.e. only dependent on pH and [BSA]).

$$\text{CEST effect (}\%\text{M}_0\text{)} = \alpha \text{pH} + \beta \text{[BSA]} + \varepsilon. \quad (6)$$

The coefficients $\alpha, \beta, \varepsilon$ of Equation 6 are constants (units)$\alpha = \frac{(\%\text{M}_0)}{\text{pH}}, \beta = \frac{(\%\text{M}_0)}{\text{[BSA]}}, \varepsilon = \%\text{M}_0$ that describe the level of sensitivity of the experimentally measured data to the varied parameters in the phantoms. Using these coefficient values and the known pH and [BSA] for each phantom, theoretical CEST effects were calculated and correlated to the experimentally measured CEST effects for each metric. The $R^2$ values for these correlations were used as indicators of the specificity of the metric to changes in only pH and [BSA]. The optimal metric was defined as that with the highest $R^2$ value for the correlation between calculated and experimentally measured CEST effects, which represents a metric with minimal contamination by $T_1$ and $T_2$ time.

### 2.7 Measurement of pH differences using optimal metric

For the optimal metric, differences in experimentally measured CEST effects were calculated between each pair of phantoms ($\Delta\text{CEST} = \text{CEST}_1 - \text{CEST}_2$). These differences were tested for statistical significance, defined as a difference larger than the standard deviations on each CEST effect measurement added in quadrature ($\Delta\text{CEST} > \sigma_{\text{CEST}_1} + \sigma_{\text{CEST}_2}$).

For statistically significant differences in CEST effect, pH differences as measured by pH probe (Experimental $\Delta$ pH) and back calculated from the linear regression coefficients from Equation 6 (‘Calculated $\Delta$ pH’) were correlated to verify the suitability of the metric for measuring pH differences.

### RESULTS

#### 3.1 Phantom validation

NMR spectra of the tissue and cellular models were compared. The metabolite peaks in spectra obtained from PCA-extracted in vitro cultured 4T1-GFP cells and the same cells grown in vivo as a subcutaneous tumour were very similar in magnitude (Figure 1A). Thus, in vitro cultured 4T1-GFP cells are metabolically similar to an in vivo tumour. However, clear differences were seen in the distribution and magnitude of metabolite peaks from PCA-extracted naive mouse brain and

![Figure 1](https://example.com/figure1.png)
in vivo tumour, revealing their distinctly different metabolite compositions (Figure 1B). Upon comparing the spectra from the lysate of in vitro cultured 4T1-GFP cells and the representative phantom model (Figure 1C), the marked similarities in the magnitude of the broad protein lineshapes indicate that the phantom (extract) model is a reasonable approximation of the intracellular environment of 4T1-GFP cells.

Clear differences between the Z spectra from phantoms of BSA only and those containing 4T1-GFP cell PCA extracts were evident in the -1.0 to -5.0 ppm region, and indicates nuclear Overhauser enhancement (NOE)-mediated saturation of the water signal owing to the presence of the intracellular metabolites (Figure 2). The CEST effect centred at 2.8 ppm is also altered with increasing concentration of PCA-extracted tissue.

### 3.2 | Quantification of CEST effects by multiple metrics

The relationships between the measured CEST effect at 2.8 ppm and the pH, BSA concentration, T1 time and T2 time were determined using multiple analysis metrics for phantoms containing BSA supplemented with extracts from either 4T1-GFP cells or naïve mouse brain (Figure 3). The T2 time was considered constant for increasing concentrations of gadolinium-DTPA, and the T1 time constant for increasing concentrations of iron nanoparticles, as these varied minimally compared with the objective relaxation time (Supplementary Figure S3).

Significance levels for all linear regression relationships are shown in Table 1. Significant decreases in MTR\textsubscript{sym}, APT\textsuperscript{*}, and MTR\textsubscript{relax} were observed with increasing pH, which is expected for the fast-exchanging amine protons at this frequency\textsuperscript{21} (Figure 3A.i–C.i). Conversely, however, since CEST\textsuperscript{*} estimates the exchange rate and concentration directly from the Z spectrum, an increase in CEST\textsuperscript{*} (calculated with default T1/T2 priors) was seen as pH increased (Figure 3D.i). The AREX metric also showed a significant decrease as pH increased (Figure 3E.i), and the increase in CEST\textsuperscript{*} became significant when measured T1/T2 priors were included (Figure 3F.i).

Significant increases in all metrics apart from MTR\textsubscript{sym} were evident with increasing protein concentration (Figure 3B.ii–F.ii). In contrast, MTR\textsubscript{sym} decreased as protein concentration increased (Figure 3A.ii). APT\textsuperscript{*}, MTR\textsubscript{relax} and CEST\textsuperscript{*} (default T1/T2 priors) were found to be sensitive to changes in both proton relaxation times in both phantom models (Figure 3B.iii,iv–D.iii,iv). whilst MTR\textsubscript{sym} was sensitive only to changes in T1 in the 4T1-GFP phantom model (Figure 3A.iii,iv). Some measurements of MTR\textsubscript{relax} and APT\textsuperscript{*} were negative because in these instances the relaxation time change resulted in no discernible CEST peak at 2.8 ppm (see Supplementary Figure S4). Since a negative CEST effect measurement is unphysical, these measurements were set to zero. As expected, AREX was insensitive to T1 relaxation time variations in both phantom models, but sensitive to T2 time changes (Figure 3E.iii,iv). CEST\textsuperscript{*} (measured T1/T2 priors) was the only metric insensitive to both relaxation times in both models (Figure 3F.iii,iv).

Significance levels for the comparisons of linear regression gradients between the two phantom models are shown in Table 2. Significant differences were found between phantom models for MTR\textsubscript{sym}, APT\textsuperscript{*} and MTR\textsubscript{relax} as pH was varied, and for CEST\textsuperscript{*} with default T1/T2 priors as T1 time was varied (Figure 3A.i–C.i,D.iii, P values in Table 2).

### 3.3 | Determination of optimal metric

MTR\textsubscript{sym}, APT\textsuperscript{*}, MTR\textsubscript{relax} and CEST\textsuperscript{*} with default T1/T2 priors all showed a significant sensitivity to serial variation in T1 and T2 time, indicating the contaminant effect that the water relaxation times have on these metrics when quantifying CEST effects. Of the two methods that incorporate compensation for relaxation times (AREX and CEST\textsuperscript{*} with measured T1/T2 priors), the CEST\textsuperscript{*} measurements showed minimal sensitivity to serial variation in either T1 or T2 time (Figure 3F.iii,iv). As expected, AREX was not significantly sensitive to variations in T1 time, but did vary with T2 time. Notably, CEST\textsuperscript{*} with measured T1/T2 priors was the only metric to show no significant dependence on T1 or T2 time in either phantom model, indicating the specificity of CEST\textsuperscript{*} to measuring changes in only the CEST pool properties (Table 1).

Correlation analysis between the experimentally measured CEST effects and the theoretically calculated CEST effects from Equation 6 yielded the highest R\textsuperscript{2} value (0.88) for CEST\textsuperscript{*} with measured T1/T2 priors (Figure 4A–F). This high R\textsuperscript{2} value means that CEST\textsuperscript{*} with measured T1/T2 priors is dependent only on pH and protein concentration. MTR\textsubscript{sym}, APT\textsuperscript{*}, MTR\textsubscript{relax} and CEST\textsuperscript{*} with default T1/T2 priors (Figure 4A–D) display constant calculated CEST effect in many cases, because these metrics do not directly account for the effects of T1 or T2 variations, making it difficult to use these metrics for pH estimation. One contributing factor to the low R\textsuperscript{2} values observed for MTR\textsubscript{sym} (0.53), MTR\textsubscript{relax} (0.14) and APT\textsuperscript{*} (0.45) may be the significant difference in linear regression gradients between tumour and naïve brain phantom models as pH is varied. However, it is unlikely that this is the only factor, since AREX and CEST\textsuperscript{*} with default T1/T2 priors both display similarly low R\textsuperscript{2} values (0.48 and 0.07 respectively) with non-significant differences between phantom models. On this basis, the optimal metric was found to be CEST\textsuperscript{*} with measured T1/T2 priors.

### 3.4 | Measuring pH differences using CEST\textsuperscript{*}

Differences in the experimentally measured CEST\textsuperscript{*} values (with measured T1/T2 priors) from each phantom were measured and

![Figure 2](image-url)
tested for statistical significance. A total of 28 $\Delta$CESTR* values were found to be significant. For these differences, the experimentally measured $\Delta pH$ and the calculated $\Delta pH$ using a rearrangement of Equation 6 were correlated (Figure 5). These findings validate CESTR* as a reliable measure of $\Delta pH$ by CEST MRI, with a root mean square deviation for the correlation in Figure 5—and expected quantitative pH accuracy—of 0.6 pH units for the case of no variation in protein concentration.

FIGURE 3  Linear regression relationships of MTR$_{ asym}$ A, APT* B, MTR$_{ Rex}$ C, CESTR* calculated with default $T_1/T_2$ priors D, AREX E, and CESTR* calculated with measured $T_1/T_2$ priors F, as a function of pH (i), BSA content (ii), $T_1$ time (iii) and $T_2$ time (iv) for the CEST effect at 2.8 ppm in phantoms containing 8% w/v BSA and PCA extract from 4T1-GFP cells (solid circles) and naive mouse brains (open squares). Solid and dashed lines are the fitted linear regression relationships to tumour and naive brain phantoms, respectively.
TABLE 1  

P values for the linear regression fits of CEST effect as measured by the four analysis metrics compared, for each of the varied parameters (pH, [BSA], $T_1$ and $T_2$), in both phantom models. P values marked * are statistically significant results, defined as $P < 0.05$.

|                     | pH       | [BSA]    | $T_1$ Time | $T_2$ Time |
|---------------------|----------|----------|------------|------------|
| **4T1-GFP cell phantoms** |          |          |            |            |
| $MTR_{asym}$        | <0.0001* | 0.06*    | 0.04*      | 0.25       |
| $MTR_{Rex}$         | <0.0001* | <0.0001* | <0.0001*   | 0.001*     |
| $APT^*$             | <0.0001* | 0.0004*  | <0.0001*   | 0.001*     |
| $CESTR^*$ (default $T_1/T_2$ priors) | 0.12     | <0.0001* | <0.0001*   | 0.56       |
| $AREX$              | <0.0001* | <0.0001* | 0.12       | 0.003*     |
| $CESTR^*$ (measured $T_1/T_2$ priors) | 0.02* | <0.0001* | 0.32       | 0.11       |
| **Naïve brain phantoms** |          |          |            |            |
| $MTR_{asym}$        | 0.02*    | 0.02*    | 0.33       | 0.46       |
| $MTR_{Rex}$         | 0.05     | <0.0001* | <0.0001*   | 0.006*     |
| $APT^*$             | 0.04*    | <0.0001* | <0.0001*   | 0.006*     |
| $CESTR^*$ (default $T_1/T_2$ priors) | 0.243 | <0.0001* | <0.0001*   | 0.72       |
| $AREX$              | 0.03*    | <0.0001* | 0.06       | 0.006*     |
| $CESTR^*$ (measured $T_1/T_2$ priors) | 0.03* | <0.0001* | 0.06       | 0.96       |

TABLE 2  

P values for the results of t tests comparing the linear regression gradient values for CEST effect measurements from 4T1-GFP and naïve brain phantom. $P < 0.05$ was defined as statistically significant, with correction for multiple t tests using the Holm–Sidak method. P values marked * are statistically significant differences.

|                     | $MTR_{asym}$ | $MTR_{Rex}$ | $APT^*$ | $CESTR^*$ (default $T_1/T_2$ priors) | $AREX$ | $CESTR^*$ (measured $T_1/T_2$ priors) |
|---------------------|--------------|-------------|---------|-------------------------------------|--------|-------------------------------------|
| pH                  | 0.001*       | 0.001*      | 0.002*  | 0.02                                | 0.02   | 0.45                                |
| [BSA]               | 0.04         | 0.01        | 0.02    | 0.10                                | 0.003  | 0.04                                |
| $T_1$ time          | 0.05         | 0.10        | 0.06    | 0.001*                              | 0.54   | 0.03                                |
| $T_2$ time          | 0.49         | 0.36        | 0.36    | 0.99                                | 0.09   | 0.16                                |

FIGURE 4  
Correlations between calculated CEST effects from Equation 6 and the experimentally measured CEST effects in each phantom as measured by each analysis metric: $MTR_{asym}$ A, $APT^*$ B, $MTR_{Rex}$ C, $CESTR^*$ calculated with default $T_1/T_2$ priors D, $AREX$ E, and $CESTR^*$ calculated with measured $T_1/T_2$ priors F. $R^2$ values are $MTR_{asym} = 0.53$, $MTR_{Rex} = 0.14$, $APT^* = 0.45$, $CESTR^*$ with default priors =0.07, $AREX =0.48$ and $CESTR^*$ with measured priors =0.88.
Within ±0.6 pH units of zero, CESTR* was successful in 75% of the magnitude of the experimental

dicted the sign of pH in 94% of cases (16/17) where experimental

ΔpH < −0.6 pH units and 100% of cases where experimental

ΔpH > 0.6 pH units (3/3). For the group where experimental ΔpH was within ±0.6 pH units of zero, CESTR* was successful in 75% of cases (6/8).

Further, considering the data points in distinct groups based on the magnitude of the experimental ΔpH value, CESTR* correctly predicted the sign of ΔpH in 94% of cases (16/17) where experimental ΔpH < −0.6 pH units and 100% of cases where experimental ΔpH > 0.6 pH units (3/3). For the group where experimental ΔpH was within ±0.6 pH units of zero, CESTR* was successful in 75% of cases (6/8).

4 | DISCUSSION

The current study examines the sensitivity and specificity of various CEST MRI quantification methods, in novel phantom models that closely reflect the intracellular environment of brain metastases and naïve mouse brain. In contrast to all other metrics, CESTR* with measured $T_1/T_2$ priors was found to be sensitive only to variations in pH and [BSA] in both tumour and naïve brain phantom models. CESTR* can remove the effects of $T_1$ and $T_2$, which are known to vary with pathological changes in vivo,\textsuperscript{25} from the CEST measurement, allowing a more accurate quantification of the CEST effect. We propose, therefore, that CESTR* with measured $T_1$ and $T_2$ priors is the most specific metric for quantification of CEST MRI data. CESTR* with measured $T_1/T_2$ priors can be applied robustly both between samples, and between voxels within a single experiment, to measure pH differences with an accuracy of 0.6 pH units in these phantoms.

Other studies have suggested that the $T_1$ contamination of the CEST signal is counteracted by changes in water content in vivo, and that the CEST contrast in tumours originates from a higher mobile protein concentration.\textsuperscript{27} If this is the case, it may be difficult to quantify pH in tumours as both the pH and protein concentration may be changing simultaneously. However, as shown by this study and others,\textsuperscript{12,28} $T_1$ correction is absolutely necessary for reliable, specific quantification of the CEST signal, regardless of the countereacting effect of water content. Assuming that the effects of $T_1$ and water content perfectly cancel may lead to inaccurate quantification of CEST effects in vivo in pathologies where only one of these parameters changes.

MTR\textsubscript{asym} measured from our phantoms decreased as the protein concentration increased, in contrast to previous studies.\textsuperscript{5,10,21} This discrepancy can be explained by the difference in saturation parameters used in the studies. In this study, a 7.8 s pulse train with CW equivalent power 0.8 μT was employed, which is preferentially sensitive to slowly exchanging protons. The effect of these saturation parameters is to enhance the NOE effects seen from the BSA and extracted metabolites in the phantoms. As the protein concentration increases, these NOE effects increase more than the CEST effects at 2.8 ppm, leading to a decreasing MTR\textsubscript{asym}. Other studies employing BSA phantoms\textsuperscript{21} or in vivo\textsuperscript{5,10} all used much shorter (4 s) and higher powers (1.3–1.5 μT), which are less sensitive to the slowly exchanging NOE effects.

Other methods not examined in this study can also be used to quantify the various contributions to a measured CEST effect, such as qCEST.\textsuperscript{14} However, qCEST separates the contributions of labile proton exchange rate and concentration to the Z spectrum by measuring the CEST effect as a function of the saturation power. When applied to a clinical setting this approach is impractical owing to acquisition duration and specific absorption rate (SAR) concerns. In addition, the phantom model used in this study does not include a contribution from macromolecular magnetization transfer, which may remain as a confounding issue in interpreting changes in CEST metrics in vivo. However, macromolecular magnetization transfer effects can be accounted for by CESTR* with measured $T_1/T_2$ priors by including another pool in the BayCEST fitting algorithm.

4.1 | Sensitivity of CESTR* to pH changes in tumours

One major result of this phantom study is that CESTR* measurement is dependent on a good choice of prior values of $T_1$ and $T_2$ time used by the BayCEST fitting algorithm. This implies that there is insufficient information in a single Z spectrum for the BayCEST algorithm to accurately estimate water $T_1$ and $T_2$ times when provided with generic, rather than individually measured, values for the prior distributions. The CESTR* metric with default $T_1/T_2$ priors has been used to identify the ischemic penumbra in acute stroke patients,\textsuperscript{7} and to generate quantitative pH maps in healthy volunteers and acute stroke patients.\textsuperscript{3} The apparent success of these pH maps (i.e. that the anticipated drop in pH was observed in known stroke regions) may be due to the lack of variation in $T_2$ across the healthy brain and in the acute stage of stroke. In addition, the simulated CESTR*–pH calibration in that study was generated using a constant amide concentration of 100 mM.

However, assumptions of constant $T_1$ and amide concentration may not be valid when assessing tumours. Consequently, accurate estimations of both $T_1$ and labile proton concentration are necessary for reliable pH quantification in tumours using CESTR*. While $T_1$ is easy to measure in vivo, the labile proton concentration is much harder to quantify reliably. We have shown in this phantom study that CESTR*, with knowledge of the $T_1$ time and labile proton concentration, provides a means for immediate, quantitative and non-invasive pH measurement, with an accuracy of 0.6 pH units based on the root mean square deviation of Figure 5. However, as the ΔpH measurements in Figure 5 were made on phantoms with known BSA concentration, application of this method in vivo is limited. Importantly, the non-significant difference in sensitivities of CESTR* to pH in both
tumour and normal brain phantoms implies that no manual tumour segmentation should be necessary when evaluating the pH of tumours using CEST\(^*\) in vivo.

Extracellular pH acidifications of the order of 0.6 pH units are not uncommon in tumours in vivo.\(^9\) Though intracellular pH changes of this order are rare, there are numerous cellular environments (mitochondria and other organelles) where the pH is very alkaline, and which may contribute to the CEST signal by virtue of the majority of their protein content being largely mobile.\(^{29,30}\) The exact contribution of each of these compartments to the CEST signal measured in vivo remains to be elucidated.

5 | CONCLUSION

Novel, realistic phantom models of the in vivo intracellular environment of brain metastases and naive mouse brain have been developed to determine an analysis metric for quantification of CEST MRI data that is sensitive to only labile proton exchange rate and concentration. We demonstrate that the CEST\(^*\) metric with \(T_1\) and \(T_2\) time compensation overcomes many challenges facing interpretation of CEST MRI data. When combined with prior knowledge of protein concentration, CEST\(^*\) with \(T_1/T_2\) compensation allows quantification of pH differences with a mean accuracy of 0.6 pH units. These results suggest that CEST MRI may enable pH differences between tumour and normal tissue to be quantified in vivo without the need for exogenous contrast agents.

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