Noninvasive Measurements of Glycogen in Perfused Mouse Livers Using Chemical Exchange Saturation Transfer NMR and Comparison to $^{13}$C NMR Spectroscopy

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ABSTRACT: Liver glycogen represents an important physiological form of energy storage. It plays a key role in the regulation of blood glucose concentrations, and dysregulations in hepatic glycogen metabolism are linked to many diseases including diabetes and insulin resistance. In this work, we develop, optimize, and validate a noninvasive protocol to measure glycogen levels in isolated perfused mouse livers using chemical exchange saturation transfer (CEST) NMR spectroscopy. Model glycogen solutions were used to determine optimal saturation pulse parameters which were then applied to intact perfused mouse livers of varying glycogen content. Glycogen measurements from serially acquired CEST Z-spectra of livers were compared with measurements from interleaved natural abundance $^{13}$C NMR spectra. Experimental data revealed that CEST-based glycogen measurements were highly correlated with $^{13}$C NMR glycogen spectra. Monte Carlo simulations were then used to investigate the inherent (i.e., signal-to-noise-based) errors in the quantification of glycogen with each technique. This revealed that CEST was intrinsically more precise than $^{13}$C NMR, although in practice may be prone to other errors induced by variations in experimental conditions. We also observed that the CEST signal from glycogen in liver was significantly less than that observed from identical amounts in solution. Our results demonstrate that CEST provides an accurate, precise, and readily accessible method to noninvasively measure liver glycogen levels and their changes. Furthermore, this technique can be used to map glycogen distributions via conventional proton magnetic resonance imaging, a capability universally available on clinical and preclinical magnetic resonance imaging (MRI) scanners vs $^{13}$C detection, which is limited to a small fraction of clinical-scale MRI scanners.
of both muscle and liver glycogen metabolism in the diabetic state (e.g., refs 9 and 10). Despite these advances, in vivo $^{13}$C NMR spectroscopy still remains handicapped by its inherently low signal-to-noise ratio (SNR) due to the low natural abundance and the low gyromagnetic ratio of the $^{13}$C nucleus, as well as the cost of the often required $^{13}$C labeled isotopes. Furthermore, the vast majority of clinical magnetic resonance imaging (MRI) scanners lack $^{13}$C detection capability, and clinical adaptation of this technology will likely remain within the research community only.

Recently, a novel MRI method for detection of tissue glycogen was reported$^{11}$ based on sensing the chemical exchange of glycogen hydroxyl protons with tissue water. This general method is relatively straightforward to implement on current MRI imaging systems, because only proton detection is required (e.g., refs 12 and 13). However, whether it can reliably measure physiological concentrations of glycogen and what other factors affect its accuracy have not been established. In this paper, we report experiments using perfused livers that evaluate the proposition that chemical exchange saturation transfer (CEST)-based measurements are able to reliably quantify glycogen levels with high sensitivity.

Proton-based CEST approaches benefit from increased SNR compared to $^{13}$C NMR spectroscopy and also from the wide availability of $^{1}$H MR hardware. The use of CEST-based approaches to detect other $\text{–OH}$ and $\text{–NH}$ containing metabolites has been recently reported for glycосaminoglycans$^{14}$, creatine$^{15}$, glutamate$^{16}$, glucose$^{17}$ and 2-deoxy-glucose$^{18,19}$. Despite the demonstration of proof of concept for detection of these metabolites, the optimization, calibration, and quantification (including glycogen) in the tissue of interest using CEST has, for the most part, not yet been reported. Furthermore, in the initial report of CEST-based detection of glycogen$^{14}$, there appeared to be a nonlinear and saturating relationship between the amount of glycogen and the CEST signal measured in phantoms over the expected physiological range, calling into question the utility of this approach for measurements of physiological levels of tissue glycogen. Therefore, the goal of this study was to measure the relationship between total liver glycogen (as measured with $^{13}$C NMR) and the CEST MR signal and to demonstrate that appropriate CEST methods can provide reliable and accurate measurement of liver glycogen. Specifically, our aims were (1) to optimize the acquisition parameters for CEST detection of glycogen over the expected physiological range in phantoms, (2) to use these parameters to perform measurements of perfused liver glycogen with CEST, and (3) to correlate these measurements with those obtained with $^{13}$C NMR and to investigate the inherent errors in each of these techniques.

## METHODS

### CEST Background.

The basis of CEST detection of glycogen hydroxyl protons is shown schematically in Figure 1 where the $^{1}$H NMR signal from a relatively small solute pool (glycogen in this case) with exchangeable protons (Figure 1a) can be indirectly detected via saturation with NMR pulses, transfer of these saturated protons to the hydroxyl functional groups to water, and subsequent measurement of the attenuation of the water $^{1}$H NMR signal (Figure 1b). The series of water peaks acquired at varied frequency offsets ($\omega$) of the saturation pulse is typically called a “Z-spectrum” (Figure 1c) and is used to calculate the magnetization transfer ratio asymmetry (MTR$_{\text{asym}}$) (Figure 1d) as

$$\text{MTR}_{\text{asym}}(\omega) = \frac{|S(-\omega) - S(\omega)|}{S_0}$$

Here, $S$ represents the water signal observed at a saturation offset $\omega$ from the water resonance, and $S_0$ is the water signal observed at a saturation offset far (>20 ppm) from the water resonance. (Note that we have adopted the standard convention in CEST studies of defining the water resonance to be 0 ppm.) Most reported measures of CEST are based on this MTR$_{\text{asym}}$ parameter; though for some choices of the saturation parameters, there may be factors other than exchange that influence measured values of MTR$_{\text{asym}}$.

### NMR Acquisitions.

All studies were performed on a Bruker 500 MHz (11.7 T) vertical bore NMR spectrometer using XWin-NMR 3.2 and a 20 mm TXO probe with $^{13}$C/$^3$P on the inner coil and $^1$H/$^2$H on the outer coil with a custom fabricated 20 mm NMR tube. Magnetic field lock was provided by a small (~0.5 mL) separate sealed tube of D$_2$O placed inside the 20 mm NMR tube. Natural abundance $^{13}$C NMR acquisitions
were performed using a $15^\circ$ square pulse, $^1$H broadband decoupling, an interpulse delay of 560 ms, and 1600 averages (22 min acquisition time). CEST NMR acquisitions were performed using 32 nonuniformly spaced frequency offsets as follows: $\pm 9$, $\pm 8.5$, $\pm 8$, $\pm 6$, $\pm 4$, $\pm 2.5$, $\pm 2$, $\pm 1.75$, $\pm 1.5$, $\pm 1.25$, $\pm 1$, $\pm 0.75$, $\pm 0.5$, $\pm 0.25$, $\pm 0.1$, 0, and 40 ppm from water. These were chosen to facilitate Lorentzian modeling of the Z-spectrum as described later in the Data Analysis protocol. Saturation pulse power and time for perfused liver studies were optimized using phantom studies (described below) and were 4 $\mu$T and 0.5 s. The total repetition time of the sequence was 30 s, which yielded a total scan time of 20 min.

**Phantom Studies.** Glycogen (oyster, Sigma-Aldrich P/N #G8751) was dissolved in a Krebs-Henseleit buffer supplemented with 0.25% bovine serum albumin, 0.1 mM palmitate, 0.5 mM glutamate, 0.5 mM glutamine, and 2 mM ATP (final pH = 7.4). As the maximum expected concentration of liver glycogen is approximately 400 $\mu$mol/g and the maximum expected mouse liver size for these studies was 2 g, phantoms were constructed with up to 800 $\mu$mole of glycogen, measured as glucose equivalents, in the sensitive volume of the NMR probe. CEST spectra were acquired with varying saturation pulse powers and times in order to investigate which combination of parameters yielded a linear relationship between total glycogen and the numerically integrated CEST MTR$_{asym}$ area under the curve (AUC), with maximal dynamic range. It should be noted that these phantom studies were performed only to determine the optimal saturation pulse parameters and were not used to calibrate the glycogen CEST signal. This is because tissue glycogen will likely have different structural (chain length, branching, protein binding) and relaxation characteristics compared to glycogen in solution.

**Perfused Liver Studies.** The animals were studied under the purview of an Institutional Animal Care and Use Committee, and all applicable regulations and laws pertaining to the use of laboratory animals were followed. The perfused liver procedure has been published in detail elsewhere and is summarized briefly here. C57/BL6 mice (3–6 months old) were fed either normal chow or a high fructose diet (described below) and were 4 moles. This protocol is shown in Figure 2. A total of 13 $^1$C NMR spectra of glycogen were analyzed with peak fitting programs written in Matlab. Briefly, the glycogen NMR resonances were each fit to a Lorentzian line shape model, the parameters of which were optimized using a least-squares minimization routine. Each modeled resonance was then integrated analytically and the sum of the areas was converted to an absolute amount of glycogen by comparison with a standard curve of glycogen phantoms acquired under identical conditions.

For the CEST acquisitions, all data analysis was performed using Matlab functions. Z-spectra were calculated by first numerically integrating each magnitude $^1$H NMR spectrum along the frequency dimension to yield the 2D NMR spectrum and then subtracting the water signal. The resulting Z-spectra were then normalized to the corresponding integral of the $^1$H NMR signal acquired with a 40 ppm frequency offset to form the Z-spectrum.

**Data Analysis.** $^1$C NMR spectra of glycogen were analyzed with peak fitting programs written in Matlab. Briefly, the glycogen NMR resonances were each fit to a Lorentzian line shape model, the parameters of which were optimized using a least-squares minimization routine. Each modeled resonance was then integrated analytically and the sum of the areas was converted to an absolute amount of glycogen by comparison with a standard curve of glycogen phantoms acquired under identical conditions.

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For the particular application, the Z-spectrum can be considered as the sum of the CEST contribution and the direct water saturation (DWS) contribution. Starting from the two-site model of chemical exchange and incorporating the weak saturation pulse (WSP) approximation, it can be shown that the total signal of the Z-spectrum is theoretically inverted Lorentzian in shape. Accordingly, experimental Z-spectra were fit over the nonglycogen signal containing regions with an inverted Lorentzian model given by

$$DWS(\omega) = L_0 - \frac{h}{4\left(\frac{\omega - \omega_0}{LW}\right)^2 + 1}$$

where $L_0$ is a small DC offset parameter, $h$ is the height, $\omega_0$ is the center frequency, and LW is the line width at 50% peak height. This fit was performed over the nonglycogen signal containing regions of the Z-spectrum using the following subset of saturation frequency offsets: $\pm 9$, $\pm 8.5$, $\pm 8$, $\pm 6$, $\pm 4$, $\pm 2.5$, $\pm 2$, $\pm 1.75$, $\pm 1.5$, $\pm 1.25$, $\pm 1$, $\pm 0.75$, $\pm 0.5$, $\pm 0.25$, $\pm 0.1$, 0, and 40 ppm from water.

The glycogen MTR$_{asym}$ function was then calculated as the difference between the modeled DWS spectrum and the experimental Z-spectrum, i.e.,

$$\text{glycogen MTR}_{asym}(\omega) = DWS(\omega) - S(\omega)$$

and this glycogen MTR$_{asym}$ curve was numerically integrated to yield the final measure of glycogen CEST signal. While this Lorentzian fitting procedure may be subject to small errors due to the potential inclusion of NOE effects in the region used to fit the DWS portion of the Z-spectrum, it should be noted that the direct calculation of the MTR$_{asym}$ (eq 1) would suffer from the same shortcoming. Furthermore, we observed that this procedure proved superior to the direct calculation of the MTR$_{asym}$ as it allowed for any slight deviations of the 0 ppm offset to be incorporated into the model and corrected for.
Monte Carlo Error Simulations. As each liver started with a different glycogen level, the calculation of error estimates with repeated measures was not feasible. As an alternative, we used Monte Carlo simulations based on the SNR of the $^{13}$C and $^1$H spectra to generate standard deviations for each $^{13}$C NMR and CEST measurement, respectively. For the $^{13}$C NMR errors, a normal distribution with a mean of zero and standard deviation equal to the root-mean-square (RMS) noise of the $^{13}$C NMR spectrum was created, and random values from this distribution were added to each resonance integral value. The total sum of the areas of the glycogen NMR peaks was then recalculated, and this procedure was repeated 50,000 times to generate a distribution of $^{13}$C NMR signal areas. The standard deviation of this distribution was used as the horizontal error bar in the plot of $^{13}$C NMR determined glycogen versus CEST MTR asym AUC.

For the CEST errors, a normal distribution with a mean of zero and a standard deviation equal to the RMS noise of the $^1$H NMR spectra in the Z-spectrum was created and random values from this distribution were added to each resonance in the Z-spectrum. The integral of each resonance in the new Z-spectrum was recalculated, and this new Z-spectrum was also separately studied the CEST MR signal from glucose in those reported in many other biomolecule CEST studies. We also investigated which region of the MTR asym curve would be optimal to use for correlation with the $^{13}$C NMR data by comparing the $R^2$ value for the correlation between $^{13}$C NMR determined glycogen and CEST MTR asym AUC as different regions of the MTR asym curve were incorporated into similarly prepared phantoms (data not shown) and found that the CEST signal was approximately 2-fold greater, consistent with the fact that free glucose has 5 exchangeable $-OH$ groups, while the glucosyl units in glycogen have 2–3 exchangeable $-OH$ groups depending on branching within the glycogen molecule.

Perfused Liver Studies. We then performed perfused liver studies using lean C57/B6 mice, which were fasted, fed normal chow, or fed a high fructose diet to generate a range of liver glycogen levels. Studies were performed using interleaved natural abundance $^{13}$C NMR and CEST acquisitions as shown in the protocol in Figure 2. After initial $^{13}$C NMR and CEST acquisitions, a 100 pM dose of glucagon was added to the perfusion system. Figure 5a,b shows raw $^{13}$C NMR and CEST data, respectively, acquired in a selected perfused liver experiment. Here, we can see that the 100 pM dose of glucagon stimulated glycogenolysis and served to generate a range of liver glycogen values in a single study. However, since glucagon will also cause glucose release from the liver and since glucose $-OH$ protons have also been shown to have a CEST signal, there was likely to be additional CEST signal following glucagon administration. To account for this, data from all the perfused liver studies were separated into two groups, those acquired before glucagon addition and those acquired after glucagon addition.

We also investigated which region of the MTR asym curve would be optimal to use for correlation with the $^{13}$C NMR data by comparing the $R^2$ value for the correlation between $^{13}$C NMR determined glycogen and CEST MTR asym AUC as different regions of the MTR asym curve were incorporated into
the AUC calculation. Figure 6 shows the dependence of this $R^2$ value on which region of the MTRasym curve was integrated (for fixed integration range of +2 ppm). Here, we see that the region that produced the maximum $R^2$ value is 0.5−2.5 ppm which is consistent with the glycogen −OHs being reported to resonate at approximately 1.2 ppm downfield from water.11

Figure 7 shows a plot of $^{13}$C NMR determined glycogen versus CEST MTRasym AUC$_{0.5-2.5}$ for data acquired before (blue) and after (red) glucagon. The $R^2$ values were 0.88 ± 0.054 and 0.87 ± 0.040; the slope values were 0.0091 ± 0.00078 and 0.0082 ± 0.00064, and the Y-intercept values were −0.50 ± 0.38 and 2.4 ± 0.21, respectively (mean ± SD). Error bars for the data points in Figure 7 as well as for the standard deviations for the correlation parameters were determined by Monte Carlo simulations (see Methods). We observed a strong linear relationship between $^{13}$C NMR determined glycogen and CEST MTRasym AUC$_{0.5-2.5}$ both before and after glucagon treatment. The slope of the relationship was similar in both groups as evidenced by the overlapping standard deviations. This slope value can be used in future studies as a calibration factor between CEST MTRasym AUC$_{0.5-2.5}$ and total perfused liver glycogen. The fact that the Y-intercept is within two standard deviations of zero (i.e., not statistically different from zero) in the data obtained before glucagon addition demonstrates that there are few competing endogenous CEST metabolites in this spectral region of the liver. The increased Y-intercept value observed after glucagon addition is attributed to glucose release from the liver adding an additional CEST signal during this period of the experiment.

Interestingly, this glucose CEST signal is much higher than would have been predicted on the basis of the expected perfusate glucose concentration of approximately 1 mM in the NMR tube (determined by the perfusion flow rate and observed glycogen breakdown rate) and differences in numbers...
of exchangeable −OH groups between glucose and glycogen. We not
ed that, while the linear relationship between total
glycogen and CEST MTR_{asy} that was observed in phantoms
was preserved in the perfused liver studies, the overall
magnitude of the MTR_{asy} signal was reduced approximately
by a factor of 4 in the perfused liver studies. Factors which
could account for this are the lower water relaxation (T_{1w})
values in liver compared to the phantom solutions, differences
in the molecular architecture of glycogen (e.g., branching
patterns, number of tiers) in liver compared to that in phantom
solutions (oyster glycogen), and also the protein-bound nature
of glycogen in tissue. The latter could reduce the CEST effect
either directly by removing exchangeable glycogen −OH
groups or by altering the relaxation properties of the glycogen
−OH groups. This observation of reduced glycogen CEST
signal in liver has two significant implications in the design of in
vivo studies using CEST-based measurements of tissue
glycogen. First, glycogen phantom solutions cannot be used to
calibrate in vivo tissue glycogen measurements as is often
done with the NMR detection of other metabolites/biomolecules. Second, the competing CEST signal from glucose
represents a much larger potential hurdle than
the amount of competing glucose CEST signal may be
different.

Relative Precision of CEST. The error bars for the plot
shown in Figure 7, along with the errors reported for the R²,
slope, and Y-intercept values, were determined by Monte Carlo
simulations (see Methods) based on the respective SNR of the
13C NMR glycogen spectra and 1H NMR Z-spectra. These
error values allow us to estimate and compare the relative
precision inherent to each approach. Defining the relative
precision as the ratio of the dynamic range to the average error
for each technique, we estimate values of 10:1 and 80:1 for 13C
NMR and CEST MTR_{asy}, respectively, implying that
CEST is inherently ~8-fold more precise than natural abundance
13C NMR. It is important to realize, however, that this
calculation is based solely on the SNR of the original 1H
and 13C spectra, and it is likely that other experimental factors
such as variations in shimming quality and variations in B1 pulse
power from experiment to experiment could reduce the
precision of the CEST measurement. As a preliminary
assessment of this, we studied a 40 mM glycogen phantom
(prepared as described in Methods) and measured the CEST
MTR_{asy} under the following three conditions: (1) optimal
shimming (1H line width = 40 Hz) with B1 = 4 μT, (2)
suboptimal shimming (1H line width = 50 Hz) with B1 = 4 μT,
and (3) optimal shimming with B1 = 3.6 μT (i.e., a 10% error in
the B1 pulse). We found that case (2) produced an error in the
CEST MTR_{asy} of ~5% while case (3) produced an error of
~10%. Both of these errors are larger than the SNR-based
errors estimated by the Monte Carlo simulations (~1–2%),
demonstrating that variations in experimental parameters are
likely to be the dominant source of error in CEST
measurements of liver glycogen. In contrast, we routinely
observe that errors in 13C NMR measurements of glycogen are
relatively insensitive to small variations in shimming and other
experimental parameters and are dominated mostly by the
inherently low SNR of the 13C nucleus.

CONCLUSION

In summary, we have implemented and optimized an
experimental protocol in which 1H CEST NMR was used to
accurately and noninvasively measure liver glycogen over the
normal physiological range in perfused livers. The CEST data
were calibrated by reference to 13C NMR spectroscopic data
and under appropriate experimental conditions varied in direct
proportion to the 13C NMR measurement. Using Monte Carlo
simulations, we found that inherent errors in glycogen CEST
measurements were small and likely to be less important than
ersors caused by variations in experimental conditions. We
found that the CEST signal from glycogen in liver was
significantly less than that observed from identical amounts in
solution. As a consequence of this, we assert that (1) phantom
solutions cannot be used to calibrate in vivo or whole tissue
glycogen measurements and (2) free glucose, despite its lower
physiological concentration, could still significantly interfere
with glycogen CEST measurements.

Our findings open the door for accurate and reliable
measurements of tissue glycogen in vivo, assuming that suitable
experimental protocols are chosen to keep circulating glucose
levels constant. The validated CEST method offers the
advantage of using conventional proton MRI scanners and
proton detection, which is widely available. Therefore, CEST-
based sensing has a strong potential for future clinical
translation, which can be used as a tool to noninvasively report
glycogen levels. Furthermore, unlike $^{13}$C detection inherently limited by low SNR, CEST-based imaging methods already demonstrated in vivo could additionally report on the distribution and heterogeneity of in vivo glycogen.

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Author Contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.

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