Trehalose as an alternative to glycerol as a glassing agent for in vivo DNP MRI

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Purpose: In dynamic nuclear polarization (DNP), the solution needs to form a glass to attain significant levels of polarization in reasonable time periods. Molecules that do not form glasses by themselves are often mixed with glass forming excipients. Although glassing agents are often essential in DNP studies, they have the potential to perturb the metabolic measurements that are being studied. Glycerol, the glassing agent of choice for in vivo DNP studies, is effective in reducing ice crystal formation during freezing, but is rapidly metabolized, potentially altering the redox and adenosine triphosphate balance of the system.

Methods: DNP buildup curves of 13C urea and alanine with OX063 in the presence of trehalose, glycerol, and other polyol excipients were measured as a function of concentration. T1 and Tm relaxation times for OX063 in the presence of trehalose were measured by EPR.

Results: Approximately 15–20 wt% trehalose gives a glass that polarizes samples more rapidly than the commonly used 60%-wt formulation of glycerol and yields similar polarization levels within clinically relevant timeframes.

Conclusions: Trehalose may be an attractive biologically inert alternative to glycerol for situations where there may be concerns about glycerol’s glucogenic potential and possible alteration of the adenosine triphosphate/adenosine diphosphate and redox balance.

KEYWORDS
dynamic nuclear polarization, glassing, OX063, EPR, relaxation

1 INTRODUCTION

Dissolution dynamic nuclear polarization (DNP) has opened a unique window into cellular metabolism by allowing the noninvasive tracking of exogenous metabolic tracers.1,2 By probing the activity of specific targeted enzymes rather than measuring absolute concentrations of metabolites, dissolution DNP has an advantage in isolating specific metabolic activities over static techniques such as magnetic resonance spectroscopic imaging (MRSI), which measure an equilibrium state that may stem from many processes. However, the widespread use of dissolution DNP is limited by the availability of suitable metabolic tracers. Most dissolution DNP studies to date have used pyruvic acid as a tracer. Pyruvate’s dominance in DNP research is partly because of the central position pyruvate occupies in glucose metabolism,3 but also because of its physical properties. In contrast to most other metabolites, pyruvic acid is a liquid
at room temperature. The liquid state of pyruvic acid ensures the radical is evenly mixed throughout the sample. In a solid sample dissolved in water, phase separation can occur during freezing. This is problematic as transfer efficiency from the radical to the nuclei of interest in DNP is steeply nonlinear with respect to the radical-nuclear spin distance. Clustering of the radical species caused by phase separation increases the median radical-nuclear spin distance and may result in sharply reduced transfer efficiencies and poor signal in DNP.

To reduce phase separation during freezing, glassing agents are commonly employed in studies of non-self–glassing tracers. Glycerol has emerged as the glassing agent of choice for in vivo studies based on its favorable safety profile and relatively high glassing efficiency. Although glycerol is safe at the concentrations used in DNP studies, it is not metabolically inert. Glycerol is rapidly transported into the cell by aquaporin and is readily metabolized to glyceraldehyde-3-phosphate, consuming adenosine triphosphate (ATP) and flavin adenine dinucleotide (FAD) in the process. It may, therefore, potentially perturb measurements of the citric acid cycle by the consumption of ATP during the phosphorylation to glycerol-3-phosphate and by alteration of the FAD/FADH2 and NAD (nicotinamide adenine dinucleotide)/NADH cytosolic redox balance from the glycerol-3-phosphate shuttle.

An improved glassing agent with a favorable safety profile that does not affect metabolism would help expand the clinical translation of DNP beyond pyruvate to other metabolic tracers. Trehalose is a nonreducing disaccharide commonly used as a pharmaceutical excipient to stabilize proteins in an immobile glass matrix. Trehalose has an unusually high glass transition temperature on account of its anisotropic hydrogen bonding pattern, which inhibits ice nucleation and favors the formation of amorphous glasses over ordered crystalline solids or liquids. Clinical tests of trehalose intravenous (IV) injections have not shown side effects with concentrations as high as 9% wt%, far exceeding the 0.5–1% range expected in dissolution DNP after the filtration step. In contrast to glycerol, trehalose is only metabolized in the small intestine where trehalase is present and is, therefore, metabolically inert in IV injections (<0.5% is absorbed by passive diffusion in humans). We show here that glasses containing ~15% trehalose give equilibrium polarizations similar to the commonly used glycerol formulation, but at much faster polarization rates.

2 METHODS

2.1 Dynamic nuclear polarization

We hyperpolarized 4.5M [1-13C]urea or 3M [1-13C]glycine (30 μL), containing 15mM OX063, and the indicated amounts of glassing agents, at 3.35 T and 1.4 K using the Hypersense DNP polarizer (Oxford Instruments, Abingdon, UK) according to the manufacturer’s instructions using a microwave frequency of 94.116 GHz. Polarization buildup rates...
were calculated assuming single exponential growth. For the mouse imaging experiment, 100 μL of hyperpolarized urea was rapidly dissolved in 4.5 mL of HEPES-based buffer before being intravenously injected through a catheter placed in the tail vein of the mouse (12 μL/g body weight). Spectra were recorded on a 3T MR Solutions (Guildford, UK) scanner.

2.2 | EPR studies

Electron spin relaxation times of 15mM OX063 trityl samples in water:trehalose solutions were measured at 5K using a Bruker E580 pulsed EPR spectrometer (Bruker, Kontich, Belgium) with the sample cooled in a He gas atmosphere using an Oxford CF935 cryostat and a Bruker/ColdEdge “Stinger” closed-cycle He cooling system. With trehalose present, Tm and T₁ were measured by spin-echo methods. T₁ values were calculated from UPEN analysis of inversion recovery experiments using 100 exponentials without variable smoothing and an additional nonnegativity constraint with most measurements made near the peak of the field-dependent spectrum. The signal detection gate encompassed about half of the width of the echo and free induction decay (FID) measurements integrated most of the FID intensity. Without trehalose, the EPR signal exhibited an FID that could not be suppressed with attempts to decrease the magnetic field homogeneity, so an echo could not be observed for this sample, and Tm was not measured. The absorption spectrum was obtained by field-swept FID detection, followed by calculation of the power spectrum. Samples of OX063 (sodium salt) and trehalose in water were prepared gravimetrically with the OX063 concentration kept at 15mM. Samples containing trehalose were frozen by cooling the 4-mm outer-diameter quartz tube in liquid nitrogen before inserting it into the cooled cryostat. The sample without trehalose was placed in a 1.1-mm inner-diameter Teflon tube inside the 4-mm outer-diameter quartz tube to prevent breaking the tube and inserted into the cold cryostat in an He atmosphere without prefreezing. Air was not removed from the samples before freezing, as previous studies found no effect of air on spin lattice relaxation of trityls in frozen solution.

3 | RESULTS

To test the potential of trehalose as a biocompatible glassing agent, we recorded the polarization buildup curves of 4.7M 13C urea, a potential marker for perfusion in patients with compromised renal function who cannot tolerate gadolinium or iodinated contrast agents, in the presence of increasing amounts of trehalose. In the absence of trehalose, polarization of urea was slow and the buildup of polarization was negligible in clinically relevant timeframes (Figure 1A, orange}
Imaging of a mouse leg xenograft with hyperpolarized urea without trehalose yielded only noise with no discernable signal whatsoever (Figure 2A,C).

The addition of trehalose as a glassing agent markedly increased both the equilibrium polarization and buildup kinetics. Starting at 6.7%, the final signal increased rapidly with concentration before leveling off at approximately 15% trehalose (Figure 1B). A similar concentration dependence was also seen with the buildup rate (Figure 1C). In comparison with the standard glycerol preparation, trehalose solutions built up polarization much more quickly, but had a lower equilibrium polarization. The increase in polarization led to greatly enhanced imaging in vivo. Although the urea image without a glassing agent was completely noise, the addition of 20% trehalose in the polarization mixture resulted in a clear image with urea localized within the tumor (Figure 2B,D).

To confirm that the results were not confined to urea, we repeated the polarization experiments with another difficult-to-polarize, non-self–glassing substrate, 13C-labeled glycine, with similar results (Figure 1D).

Although trehalose is often cited as having unique molecular properties that give it anomalous glass-forming and ice-breaking capabilities, the molecular properties of trehalose have been the subject of considerable contention. Other studies have suggested trehalose is not unique among carbohydrates in this regard. To evaluate other potential carbohydrate glassing agents besides trehalose, we measured the polarization buildup of urea in the presence of equivalent amounts of analogous mono- and disaccharides (Figure 3). Trehalose was not unique in this context; fructose, glucose, and PEG400 all had slightly higher equilibrium polarization at an equivalent wt%. Sucrose had significantly lower equilibrium polarization, but similar buildup kinetics. In comparison, 20% trehalose was an effective compromise between fast buildup and equilibrium polarization, allowing efficient polarization within a clinically relevant timeframe.

The complex dependence of the polarization efficiency on the trehalose concentration in Figure 1 suggests a possible change in the microenvironment of the radical through a phase separation during freezing or other process. To gain a greater understanding of how trehalose may affect changes in the microenvironment of the OX063 radical during the DNP process, we recorded the EPR spectra and spin-lattice (T1) and phase-memory dephasing (Tm) relaxation times of OX063 at 5K as a function of trehalose concentration (Figure 4). With roughly 10–60 times more trehalose than OX063 and the overall high concentrations of spins, it is likely that there are multiple environments for the radicals, some of which may be rather close neighbors of other radicals. Although the complexity of the system makes defining a quantitative physical model difficult, the differences in the microenvironment will likely be reflected in the T1 relaxation times, as the most isolated of the radicals will have very long relaxation times based on the distance dependence of the dipolar interaction, and clusters of several radicals will have very short relaxation times from cross relaxation within the clusters.

Changes in trehalose concentration resulted in dramatic changes in both the magnitude and distribution of relaxation times (Figure 4) of OX063. Without trehalose, the sample yielded an FID that could not be suppressed with attempts to decrease the magnetic field homogeneity and a short T1 (~6–7 μs). This was also the narrowest spectrum, and discrete structure was evident in the form of dipolar sidebands. A narrow linewidth and short T1 relaxation time are both consistent with the water crystallizing upon freezing, causing high local concentrations of OX063 as the radical is preferentially excluded from the newly formed ice crystal. The echo-detected line shapes of the samples containing trehalose were considerably broader, likely reflecting a more inhomogeneous environment as the OX063 was no longer strictly confined to fast relaxing clusters, but was instead dispersed, albeit unevenly, throughout the sample. Inhomogeneity was evident in the relaxation as well—all of the trehalose samples exhibited wide ranges (orders of magnitude).
magnitude) in $T_1$ relaxation times. Multiple choices of time windows, therefore, had to be measured to include both the short relaxation times and the long relaxation times. Values of $T_m$ were short, consistent with the high OX063 concentrations, and echo decays fit well with single exponentials.

At 5% trehalose, where DNP efficiency was similar to samples without trehalose (Figure 1), the $T_1$ relaxation was dominated by a fast relaxing population with a very short relaxation time (~0.8 μs)\(^3(\text{Figure 4B})\), similar to that found in the trehalose-free sample (data not shown). Two other minor populations, a distribution centered on 450 μs (10%) and another at 90 μs (~2%), were also detected in addition to the fast relaxing species. Increasing concentrations of trehalose caused both a population shift towards the slower relaxing species (Figure 4C) and within the slower relaxing population, a shift towards slower relaxation times (Figure 4E). At 10% and 15% trehalose, where DNP efficiency was close to optimal (Figure 1), the long relaxing species was dominant (95% and 70%, respectively). The highest concentration, 30%, partially reversed this trend as there was a large shift in the distribution back to short relaxation times (55%). This concentration was near the eutectic point of trehalose/water mixtures,\(^1(\text{Figure 4F})\), and the reversal in trend for $T_1$ and linewidth may reflect the preferential freezing of water during the freezing process, although the exact interpretation is uncertain as the relative partitioning of OX063 between trehalose rich and poor phases is unknown. In contrast to the complex concentration dependence of the $T_1$ relaxation times, the linewidth decreased and $T_m$ increased modestly and monotonically with the trehalose concentration (Figure 4F). Overall, as expected, there was a rough correspondence between the $T_1$ relaxation times and DNP buildup kinetics. In this case, longer relaxation times were associated with faster buildup kinetics, as expected for narrow linewidth radicals like OX063, where rapid spin diffusion exists and the solid effect is an important component in DNP transfer. Although not tested here, annealing may prove an efficient method for reducing inhomogeneities during the freezing process.\(^7(\text{Figure 4F})\)

4 | DISCUSSION

Solutions of trehalose have several potential advantages over the standard concentrated glycerol protocol. Although the equilibrium polarization is lower in the urea test case, the buildup rate is higher and the actual polarization is similar on the
The authors declare that they have no conflicts of interest.

CONFLICT OF INTEREST

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