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Quantitative Analysis of Molecular Transport across Liposomal Bilayer by J-mediated ^{13}C Overhauser Dynamic Nuclear Polarization

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Abstract

We introduce a new NMR technique to dramatically enhance the solution-state ^{13}C NMR sensitivity and contrast at 0.35 T and at room temperature by actively transferring the spin polarization from Overhauser Dynamic Nuclear Polarization (ODNP)-enhanced ^1H to ^{13}C nuclei through scalar (J) coupling, a method that we term J-mediated ^{13}C ODNP. We demonstrate the capability of this technique by quantifying the permeability of glycine across negatively charged liposomal bilayers composed of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG). The permeability coefficient of glycine across this DPPC/DPPG bilayer is measured to be $(1.8 \pm 0.1) \times 10^{-11} \text{ m/s}$, in agreement with the literature value. We further observed that the presence of 20 mol% cholesterol within the DPPC/DPPG lipid membrane significantly retards the permeability of glycine by a factor of 4. These findings demonstrate that the high sensitivity and contrast of J-mediated ^{13}C ODNP affords to measure the permeation kinetics of small hydrophilic molecules across lipid bilayers, a quantity that is difficult to accurately measure with existing techniques.

Molecular transport across cell membranes is responsible for critical biochemical processes and functions. Similarly, the permeability of lipid membranes plays an important role in drug delivery and controlled release applications. Especially because the major transport mechanism of marketed drugs across cell membranes is mediated by passive diffusion¹, a better understanding of how drugs permeate across various types of lipid bilayers can optimize the drug's bioavailability. The existing techniques, such as Parallel Artificial Membrane Permeation Assay (PAMPA)² and fluorescence spectroscopy³, are primarily applied for measuring the molecular permeability across synthetic liposomal membranes. PAMPA is originally developed to evaluate the drug's passive transmembrane permeability by measuring the UV absorption of drugs. Even though PAMPA provides high-throughput

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Supporting Information. Sample preparations. Chemical structures. DNP experiment. NMR pulse sequences. T₁ Relaxation data. Experimental procedures for DNP, EPR and ascorbic acid assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

permeability screening at low cost, hydrophilic molecules are excluded due to their inherently insufficient UV absorption amplitudes.² Fluorescence spectroscopy has also been used to study the transmembrane permeability of molecules.³ However, many natural biomolecules do not have intrinsic fluorophores, and thus require the chemical tethering of highly lipophilic fluorescent label, which is typically larger or equal in size (~ 20 Å) compared to the small molecule itself.⁴ Thus, the measured permeability rate of this labeled molecule would be skewed. Furthermore, various magnetic resonance approaches have been used to measure the mobilities of solutes in or through the lipid membranes.⁵ For example, Gawrisch et al. have integrated ^1H NOESY NMR spectroscopy and magic angle spinning (MAS) to determine the partitioning of ethanol^{5a, b} and tryptophan^{5c} in the lipid bilayers. The rotational correlation time of the solute protons located in different positions of the lipid molecule on the nanosecond timescale can be measured from the cross-relaxation rates of the NOESY spectra.^{5a} Still, this method more reliably provides the solute distribution in lipid bilayers, while the dynamics information of solute within the lipid membrane systems is limited. Additionally, magic-angle spinning pulse-field gradient (MAS-PFG) NMR has been applied to unoriented liposome samples to determine the diffusion rates of solute and lipid molecules with chemical shift resolution.^{5d} However, PFG-MAS has been shown to be rather insensitive to the diffusion of solute across the bilayer^{5d}, because the diffusion rate of solute perpendicular to the bilayer surface is found to be about two orders of magnitude slower than the diffusion rate of solute parallel to the bilayer surface.⁶ Besides, the standard PFG NMR measurement cannot easily measure the directional permeability of a solute across the liposomal bilayer, while MAS-based measurement requires packed solid samples, and thus does not afford to study the dilute samples dispersed in bulk water.

Despite the notoriously poor sensitivity of NMR, its sensitivity continues to be dramatically boosted by a number of novel methods by creating “hyperpolarized” nuclear spin states.⁷ Among them, ^1H ODNP is a viable method to directly amplify solution-state NMR signatures under ambient conditions by relying on polarization transfer from highly polarized unpaired electrons of localized nitroxide radicals to surrounding water protons.^{7e,8} Although it has been demonstrated that ODNP can be conducted at high magnetic fields due to recent methodological and instrumental advances,⁹ ^1H NMR signal of water can still be more routinely and efficiently amplified via the ODNP mechanism by up to 300-fold at 0.35T, that is the most common magnetic field used for X-band EPR studies.¹⁰ Besides sensitivity enhancement, ^1H ODNP at 0.35T has a unique feature to probe the translational dynamics of water at biological interfaces with high sensitivity and site-specificity.^{7d, e} However, the direct ODNP polarization of other nuclei of biological interest, such as ^{13}C and ^{15}N , in aqueous samples does not appear to be particularly promising at the low magnetic field, given low sensitivity and moderate DNP efficiency observed.

We have previously studied the direct ^{13}C ODNP effect of small aqueous molecules via $e \rightarrow ^{13}\text{C}$.¹¹ There, we discussed the complexity of the three-spin effect, where the interactions between $e \rightarrow ^{13}\text{C}$ and $^1\text{H} \rightarrow ^{13}\text{C}$ contribute with opposite signs to the overall DNP signal.¹¹ Thus, the enhanced ^{13}C ODNP signal can be strongly suppressed in a three-spin system, depending on the magnitude of these contributions.¹¹ Here, we present an approach that can circumvent the low or inconsistent ^{13}C ODNP sensitivity problems by an *active* polarization

transfer from DNP-enhanced protons to the directly bonded ^{13}C nuclei of small aqueous molecules via $e \rightarrow ^1\text{H} \rightarrow ^{13}\text{C}$, using J-based polarization transfer NMR pulse sequences, including INEPT (Insensitive Nuclei Enhanced by Polarization Transfer)¹² and DEPT (Distortionless Enhancement by Polarization Transfer)¹³, sequence elements which are routinely implemented in conventional multidimensional NMR spectroscopies of thermally polarized nuclei in solution. Notably, the J-based polarization transfer combined with hyperpolarization has been previously demonstrated by using ^1H singlet order of enriched parahydrogen, in which $\sim 10^4$ -fold ^{13}C signal enhancement was achieved when employing a modified INEPT pulse sequence.¹⁴ Although it has been successfully implemented to follow the metabolism of tricarboxylic acid cycle in vivo¹⁵, its mechanism and utility are very different than our approach in that the polarized molecule needs to contain unsaturated substrates for transferring the spin order of parahydrogen via hydrogenation^{7c}, and subsequent J-based polarization transfer is through two or three bonds.¹⁴ Thus, the sample system for which parahydrogen induced ^1H v.s. ODNP polarized ^1H NMR signal can be employed is nearly mutually exclusive.

Here, we present the first experimental demonstration of combining the J-based polarization transfer with the generally applicable ^1H ODNP mechanism at 0.35T and at room temperature, whose utility is demonstrated by determining the permeability of a hydrophilic amino acid, glycine, across a negatively charged liposomal bilayer, as well as comparing permeability coefficients of the glycine in the presence and absence of 20% cholesterol in the bilayer composition. In principle, this method can be anticipated not only to efficiently overcome the low or unpredictable enhancement caused by the above-mentioned three-spin effect, but also to amplify the ^{13}C signal by as high as 2632-fold, compared to the thermally-polarized ^{13}C signal. Moreover, the INEPT-based method combined with spectral editing differentiates the multiplicities for the different carbons in a molecule, thereby providing a handle for monitoring biological processes, such as carbon metabolism¹⁶, of molecules with high sensitivity and contrast, even at the low field of 0.35 T where the chemical shift resolution is largely lost.

Figure-1 compares the J-mediated ^{13}C ODNP and direct ^{13}C ODNP amplified NMR spectra of two ^{13}C isotope labeled molecules, $[2, ^{13}\text{C}]$ -glycine (Gly) and ^{13}C -dimethylamine (DMA), acquired at 25 °C and in a 0.35 T magnetic field by an electromagnet. The ^{13}C -DEPT-ODNP spectrum exhibits triplet and quartet signals in Gly and DMA, respectively, whereas an antiphase ^{13}C signals were observed in ^{13}C -INEPT-ODNP spectra for both molecules. Remarkably, about 30-fold higher ^{13}C signal enhancement is achieved when using J-mediated ^{13}C ODNP, compared to the signal obtained by the direct ^{13}C ODNP. This comparison is made for ^{13}C signal that is accumulated over a 10 min experimental time. Given the leakage factor, a parameter which typically takes the contribution of proton T_1 relaxation mechanism driven by the interaction with electron spin into account, was found to be close to 1 in both Gly and DMA samples (Table-S1), we can assume that the paramagnetic spin label at the concentration used in this study can efficiently relax and polarize the entire proton spin bath of the sample. Although the J-coupled protons represent a minor population of the entire proton bath of the sample, we have verified that the DNP-hyperpolarization of the protons can be efficiently transferred to ^{13}C through J-coupling (Figure-S3). Because protons with much shorter T_1 relaxation time than ^{13}C T_1 are rapidly

pre-polarized via the ^1H ODNP mechanism, it leads to 10 to 30-fold faster DNP build-up rates¹¹ compared to directly polarizing ^{13}C spins, yielding much higher sensitivity upon signal averaging over the same measurement time. Thermally-polarized ^{13}C signals are not shown in Fig. 1, as they cannot be detected under our experimental condition.¹¹ If the nitroxide radicals can be localized to exclusively polarize the nuclear populations of the volume in which the radicals are residing, this provides contrast between the different nanometer scale compartments, *e.g.* the liposomal interior and exterior.

The J-mediated ^{13}C ODNP is well suited for the study of molecular permeation kinetics across liposomal bilayers of complex biological systems. This is especially true because the applications of ODNP and nitroxide radicals are not limited by the size or complexity of the biological system studied.¹⁷ Here, we demonstrate the capability of this powerful technique to quantify the transmembrane permeability of a hydrophilic ^{13}C labeled amino acid, Gly. Two negatively charged DPPC/DPPG liposome samples (400 nm in diameter), with and without 20 mol% cholesterol embedded in the bilayer, were employed as model membrane systems. A membrane-impermeable nitroxide radical, CAT-1 (4-trimethylammonio-2,2,6,6-tetramethylpiperidine-1-yloxy iodide)¹⁸, was introduced to polarize the protons in the external volume of liposomes via ^1H ODNP. CAT-1 is a stable nitroxide radical and has been reported to be strongly unfavorable for penetrating the membrane, given its charged state.^{18a, b} We have verified that CAT-1 is indeed impermeable to membrane bilayers under our experimental conditions by using an ascorbic acid assay (see Fig.-S4).¹⁹ In the permeability measurement conducted by J-mediated ^{13}C ODNP, a solution containing CAT-1 and Gly was first mixed with the liposomal solution immediately prior to the measurement. Gly is initially located outside the liposomes upon mixing, where the Gly concentration in liposomal exterior is registered via J-mediated ^{13}C ODNP signal amplitude. After Gly gradually diffuses across the liposomal membrane and enters the liposomal interior, the ^{13}C spin of entrapped Gly is depolarized and provides no ^{13}C ODNP signal, given the absence of CAT-1 in the liposomal interior. By recording the J-mediated ^{13}C ODNP signal as a function of time, the signal decay was observed (Fig.2). Least-squares fits to a monoexponential decay function were applied, yielding an influx rate constant k of $275 \pm 13 \mu\text{s}^{-1}$ for 1.5M Gly across the DPPC/DPPG bilayer. The permeability coefficient of glycine was determined as follows. For a spherical liposome with internal volume V and surface area A , the permeability coefficient of the solute P can be expressed as $P = (V/A)k = (r/3)k$, where r is the radius of the vesicle and k is the influx rate constant.²⁰ According to the above expression, given $k = 275 \pm 13 \mu\text{s}^{-1}$ and $r = 200 \text{ nm}$, the permeability coefficient of Gly is $P = (1.8 \pm 0.1) \times 10^{-11} \text{ m/s}$ through the DPPC/DPPG bilayer, which is comparable to the literature value of $2 \times 10^{-11} \text{ m/s}$ found in DMPC liposomes under similar conditions.^{3b} Next, we sought to modulate the membrane permeability through a biologically relevant modification with expected alterations, and test whether our measurement picks out the change. It is widely accepted that the addition of cholesterol into liposomal bilayers promotes the formation of a lipid-ordered phase (L_o) with specific lipid composition.²¹ Thus, the permeability of ions or small solutes (*e.g.* water and glucose) is significantly reduced due to a tighter packed arrangement of the acyl chains in the L_o phase.²² It has been shown that the presence of cholesterol in lipid bilayer can reduce the water permeability by 5–10 fold, depending on the unsaturation of lipid acyl chain and cholesterol

concentration.^{22b, c} However, the effect of cholesterol content in lipid bilayer on the permeability of small molecules, other than water, has been rarely measured.²³ Our findings reveal that, indeed, the permeability coefficient is significantly lower with $(4.6 \pm 0.4) \times 10^{-12} \text{ m/s}$ for Gly in the presence of 20 mol% cholesterol embedded in the DPPC/DPPG bilayer, which is about 4-fold impeded compared to the permeability coefficient of Gly across the same bilayer membrane without cholesterol. To provide a reference value, the cholesterol content is typically around 20–30 mol % of plasma membrane²⁴, making our modification an alteration of biological relevance. A control experiment was further performed using a mixture of Gly and CAT-1 in the absence of liposomes (Fig.2). The controlled ^{13}C ODNP signal slowly decreases up to ~3% within the experimental time scale of 6 hrs. This slow reduction of ODNP signal is likely due to a slow evaporation of the sample during the long period of measurement time, and can be regarded negligible compared to the 30–50% signal decay attributed to the transmembrane permeation of Gly in this study.

In conclusion, we present a new method, J-mediated ^{13}C ODNP, that measures the permeability of small hydrophilic molecules across phospholipid bilayers, and enables to provide more reliable information on transmembrane permeability of small hydrophilic molecules than what has been available with existing techniques, such as PAMPA and fluorescence measurements. We found that added 20% cholesterol significantly impedes the permeability of glycine by a factor of 4, compared to that without cholesterol. It should be pointed out that in order to obtain the maximum ^{13}C signal, the permeability measurements in this work were carried out with 1.5 M ^{13}C labeled glycine. However, it is encouraging that high lipid concentration of 150 mg/mL and the minute sample volume of 4 μL were used in this work. Thus, a moderate change, such as a simple optimization of the experiments and/or a small increase in sample volume, will make the measurable solute concentration down to ~20–100 mM readily feasible even with our current setup, while, in the future, the sample concentration can be further significantly reduced by optimization of the hardware (e.g. by increasing the absolute detection sensitivity of the NMR probe), as well as improved experimental methods (e.g. by optimizing of pulse sequences for polarization transfer²⁵). The permeability measurement conducted by this technique is limited by the experimental condition in which the molecular influx rate needs to be slower than the ^1H DNP build-up rate, that is on the order of ^1H T_1 (i.e. ~100 ms). This condition is generally fulfilled for the permeation of hydrophilic small molecules across lipid bilayers.^{3b, 26} Although our method is currently not sufficiently sensitive to detect physiological protein or spin-label concentration of a few μM at low magnetic fields, it is still applicable to the measurement of solute mobility in other biological systems, in which the radical probe at concentration of tens mM is confined to a specific site or compartment of the sample system. This is a rather generally applicable method to enhance the contrast, as routinely carried out for tagging biological systems with radical, fluorescence, or other functional probes. Finally, this technique is also feasible to sensitively detect other nuclei than ^{13}C . For instance, the detection of hyperpolarized ^{15}N NMR signal, mediated by chemical exchange between amide protons of proteins or polypeptides and DNP-enhanced water protons, is anticipated to provide site-specific contrast for biomacromolecules that have different solvent exposures in biological relevant environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Proton-coupled ^{13}C NMR spectra of 2.5 M $[2,^{13}\text{C}]$ -glycine (Gly, left column), and 10 M ^{13}C -dimethylamine (DMA, right column) with 25 mM stable nitroxide radical, 4-amino-TEMPO, accumulated in the same experimental time (10 mins) at 25 °C and 0.35 T.

Figure-2.

(a) Time course of the influx of glycine across DPPC/DPPG (80:20 molar ratio) and DPPC/DPPG/Cholesterol (64:16:20 molar ratio) lipid bilayers, monitored by the decay of relative magnitude of J-mediated ^{13}C ODNP signals acquired at 25 °C and 0.35 T. Dash lines are the least-squares fits of a monoexponential function to the data points. The data points were extrapolated to time zero in order to compare the curves. The control experiment was conducted on the sample of glycine and CAT-1 without liposomes. (Final concentration: 150mg/mL Lipid, 30mM CAT-1, 1.5M glycine) A schematic presentation of glycine (Gly) permeation across the liposomal bilayers (b) in the absence and (c) presence of 20 mol% cholesterol. The orange arrows represent the capability of glycine permeation in two liposomal systems. The permeability coefficients P are indicated.