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Communication

An ultrasensitive tool exploiting hydration dynamics to decipher weak lipid membrane–polymer interactions

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ABSTRACT

We introduce a newly developed tool, ^{1}H Overhauser Dynamic Nuclear Polarization (ODNP), to sensitively explore weak macromolecular interactions by site-specifically probing the modulation of the translational dynamics of hydration water at the interaction interface, in the full presence of bulk water. Here, ODNP is employed on an illustrative example of a membrane-active triblock copolymer, poloxamer 188 (P188), which is known to restore the integrity of structurally compromised cell membranes. We observe a distinct change in the translational dynamics of the hydration layer interacting with the lipid membrane surface and the bilayer-interior as P188 is added to a solution of lipid vesicles, but no measurable changes in the dynamics or structure of the lipid membranes. This study shows that hydration water is an integral constituent of a lipid membrane system, and demonstrates for the first time that the modulation of its translational diffusivity can sensitively report on weak polymer–membrane interactions, as well as mediate essential lipid membrane functions. ODNP holds much promise as a unique tool to unravel molecular interactions at interfaces even in the presence of bulk water under ambient conditions.

1. Introduction

The cell membrane is a dynamic interface where diverse biological functions occur. It comprises lipids, membrane proteins and other biomolecules held together with hydration water. This hydration water is more than just a solvent, but rather a responsive medium intimately coupled to biological functions [1]. Thus, interactions between lipid membranes and foreign biomolecules can be evaluated through the perturbation of hydration dynamics at the interacting interfaces. Although bound water with dynamics on the sub-nanosecond to nanosecond timescale has been characterized [2,3], it is the diffusion dynamics of loosely bound water with the translational correlation time, τ, on the picosecond to sub-nanosecond timescale that is critically modulated by these interactions [4,5]. So far, very few techniques have been employed to study the dynamics of this disordered water, and existing techniques are often not applicable to study lipid membranes under ambient conditions. For example, ^{2}H and ^{17}O magnetic resonance dispersion can probe the retardation of protein hydration dynamics, but only in supercooled water [6]. Femtosecond fluorescence spectroscopy of tryptophan probes was used to study protein hydration dynamics of solvent-exposed sites [4,5], but does not measure the unperturbed, native, diffusion dynamics, and is not applicable to study processes at the bilayer interface and interior due to extra quenching effects. Although a powerful tool has been recently reported by Wand and coworkers to study the hydration landscape of a protein confined in reverse micelles [7], it does not present a suitable platform to study lipid membrane systems, and their transient interactions with macromolecules. ^{2}H NMR spectroscopy [8,9], electron paramagnetic resonance (EPR) [10,11] and isothermal titration calorimetry (ITC) [12] have been successfully applied to lipid membrane studies. However, they are only suitable for identifying interactions strong enough to alter structural or dynamic features of the lipid membrane, but not for probing weak and transient interactions taking place at membrane interfaces. A fundamental challenge is to study these weak interactions under biologically relevant conditions in dilute solutions, where the molecular interface is a negligible fraction of the total sample. In fact, there are long standing efforts to use NMR techniques for studying weak molecular interactions, but their capabilities are limited. Among them, Nuclear Overhauser Effect (NOE) is only applicable when the distance between two interacting molecules is within a short range (*i.e.* ~5 Å), thus presenting still relatively strong interactions compared to the type of interactions of interest in this study [13,14]. Paramagnetic Relaxation

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Enhancement (PRE) can be very sensitive to detecting weak affinities up to 50 μM in some cases [15,16], but is often limited for the study of lipid membranes, unstructured macromolecular systems and their interactions, given insufficient NMR spectral resolution for these large complexes in solution [17,18].

Here, we introduce a new tool, ^{1}H Overhauser Dynamic Nuclear Polarization (ODNP), to selectively measure the dynamics of hydration water within ~10 Å (~3–4 hydration layers) of a nitroxide spin probe (SP) attached to a molecular site of interest [19–26]. As this hydration layer is intimately coupled to the lipid membrane interface, its dynamics is sensitively modulated by the interaction between the lipid membrane and foreign molecules. Our ODNP technique premises on the significant ^{1}H NMR signal enhancements of water induced by dipolar relaxation of the water protons through the saturation of the much higher polarized unpaired electrons of SPs. The efficiency of the dipolar coupling is directly connected to the water proton dynamics that must be on the same order of magnitude as the electron spin precession period of 100 ps (=1/10 GHz, inverse of electron Larmor frequency) at a magnetic field of 0.35 T, *i.e.* on the order of translational diffusion timescale of bulk and biological water. Because this dipolar coupling heavily weighs towards hydration water closest to the SPs (<10 Å) attached at specific sites of molecular interfaces, unperturbed bulk water is quasi *silent* in the NMR spectrum, rendering this technique highly sensitive only to interfacial water even in fully water-dispersed samples. It is, however, necessary to clarify that we can assume full exchange between hydration and bulk water within the timescale of ODNP build-up time that is on the order of the T_1 of water protons (*i.e.* seconds), so that there will be no pool of "unpolarized" bulk water skewing the DNP-measured local hydration dynamics. ODNP has already been applied to study several important biological processes through the site-specific monitoring of the interfacial hydration dynamics, *e.g.* the role of water in the initial collapse of protein folding [19], the early stages of protein aggregation [20], and the complex coacervation of polyelectolye polymers [21,22]. However, it must be emphasized here that this work is the first experimental demonstration that the modulation of the translational diffusion of the hydration layer can be employed to probe weak macromolecular interactions by ODNP, the effect that were not visible by, *e.g.,* EPR, ITC and PRE measurements.

We demonstrate the utility of the ODNP tool to sensitively detect weak molecular interactions on an illustrative example of a surface-active polymer, poloxamer 188 (P188) that interacts with lipid bilayer vesicles. P188 is an amphiphilic triblock copolymer with a nonpolar poly(propylene oxide) (PPO) midblock and two polar poly(ethylene oxide) (PEO) endblocks. It is known to act as a *membrane sealant*, restoring the integrity of structurally compromised cell membranes, and has been used for treatments of acute heart failure and necrosis of muscle cells [27,28]. However, the molecular and structural basis of this function is poorly understood. With ODNP detection of hydration water dynamics off the lipid headgroup as well as in the bilayer interior, we explored the nature of the lipid membrane/P188 interaction that may form the basis of the sealing function. We observe that the interfacial hydration dynamics is exquisitely modulated when P188 is introduced to lipid membranes in solutions, while there are no measurable changes in membrane dynamics/structure according to EPR, ITC and PRE measurements, in good agreement with earlier X-ray scattering and fluorescence leakage findings that the surface adsorption of P188 changes neither lipid packing nor lipid dynamics [29,30]. The combination of these results suggests that P188 exerts its resealing function by tightening the surface and bilayer-internal hydration dynamics, not by altering structural or dynamic features of the lipid membrane or through insertion.

2. Results and discussions

ODNP relies on the polarization transfer from electron spins to water protons via dipolar relaxation induced by the saturation of electron spin transitions through continuous-wave (cw) microwave irradiation. The maximal NMR signal enhancement E_{max} is expressed as [23,24]

$$E_{max} = E(p \to \infty) = 1 - \rho f s_{max} \frac{|\gamma_s|}{\gamma_I} \quad (1)$$

where ρ is the coupling factor, f is the leakage factor, s_{max} is the maximum electron spin saturation factor, γ_s and γ_I are the gyromagnetic ratios of the electron and proton spins, given by $|\gamma_s|/\gamma_I = 658$. ρ carries information on the translational correlation time τ, and it can be obtained by quantifying E_{max}, f, and s_{max}. E_{max} can be measured by acquiring enhanced ^{1}H NMR signal of water as a function of microwave power (p) and extrapolating the value to infinite p. f can be obtained by recording the longitudinal relaxation times of samples in the presence (T_1) and absence (T_{10}) of SPs, following $f = 1 - T_1/T_{10}$. To quantify s_{max}, full saturation of all EPR transitions, and thus complete exchange of the hyperfine lines of the nitroxide radical needs to be achieved. Indeed, for nitroxide radicals tethered to slow tumbling macromolecules, such as proteins or lipid vesicles, the condition of $s_{max} \approx 1$ is fulfilled, even at low SP concentrations (*i.e.* 1–2 mol%) [23–25]. Once ρ is determined, τ of hydration water with respect to the SPs can be extracted. Since the fluctuation of the dipolar interaction due to translational diffusion can be expressed with a single correlation time, it permits the use of a single spectral density function $J(\omega,\tau)$ to describe this interaction. Taken together, ρ is given by [31]

$$\rho = \frac{6J(\omega_s + \omega_I, \tau) - J(\omega_s - \omega_I, \tau)}{6J(\omega_s + \omega_I, \tau) + 3J(\omega_I, \tau) + J(\omega_s - \omega_I, \tau)} \quad (2)$$

where ω_s is the electron Larmor frequency, ω_I is the nuclear Larmor frequency, and τ is the translational correlation time between the electron and water proton, at the given distance of closest approach between the two spins. To extract τ from Eq. (2), an analytical expression for ρ is needed. Here we employ the force-free hard-sphere dynamic model that describes the surface water relaxation in the spin-labeled systems mediated by translational diffusion [32].

We used two types of phospholipids SPs, PC-TEMPO and 16-DSA [25,26], where in PC-TEMPO the SP is located *above* the lipid head group and extends into the hydration shell off the membrane surface, and in 16-DSA the SP is located *within* the core of the intrabilayer volume. In polymer-free dioleoylphosphatidylcholine (DOPC) vesicles, we found τ = 198 ps with PC-TEMPO and τ = 403 ps with 16-DSA largely impeded compared to bulk water dynamics that measures a translational correlation time τ = 42.5 ps, assuming ρ = 0.3 for bulk water [21], confirming significant retardation of the lipid membrane hydration dynamics [25,26]. All measured or calculated DNP parameters as a function of microwave power that led to these results are presented in Table S1 and S2 of the supplemental material. Upon addition of P188, both surface and intrabilayer water diffusivities are further and distinctly slowed. Such retardations are gradually enhanced with increasing P188 concentration, until a maximum τ is reached at the critical micelle concentration of P188 (CMC ~ 125 μM, see Fig. 1). As the amount of P188 available to interact with vesicles is roughly constant above the CMC, the retardation of hydration dynamics is found to reach a plateau. This supports the idea that P188 unimers are the species interacting with the membrane to cause retardation in both *surface* and bilayer *interior* water dynamics. The crucial information here is that earlier studies found P188 to *only weakly* adsorb onto the membrane surface, but not to insert into the bilayer over the time scale of the experiment [30]. Hence, direct collision between P188 and

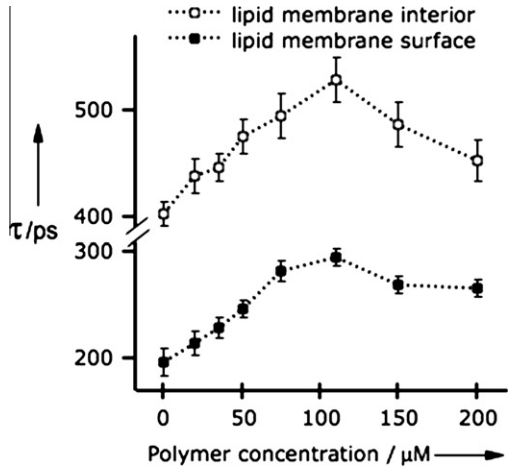

Fig. 1. Translational correlation time of hydration water on the surface and in the interior of DOPC lipid vesicles as a function of P188 concentration without incubation.

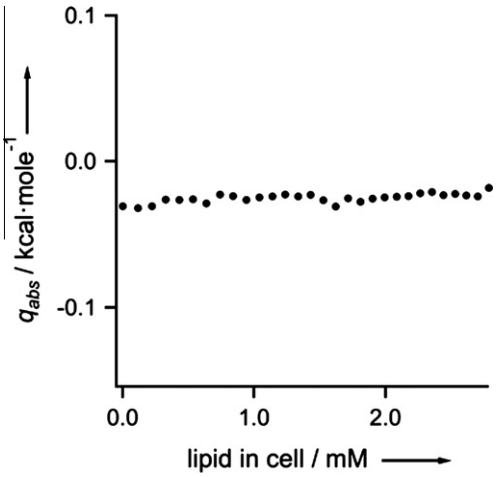

Fig. 3. Isothermal titration calorimetry of 50 μM P188 with POPC vesicles at 25 °C. The data are the integrated heat per injection (q_{abs}) normalized with respect to the number of moles of POPC injected.

the intrabilayer volume is excluded as the cause for the observed retardation of *intra*-bilayer hydration dynamics by up to 32%; we hypothesize that this is instead due to the slowed fluctuations of the lipid interface and surface hydration by P188 *surface*-adsorption. We have further experimental support for this mechanism: cw EPR spectra of both SPs in DOPC vesicles remain unaltered (Fig. 2) upon introducing P188 into vesicle samples, while it is known that EPR lineshape is extremely sensitive to changes in lipid dynamics and packing; ITC data reveal no measurable heat exchange (Fig. 3) upon titration of POPC vesicles with P188 at all relevant concentrations; and ^{1}H NMR signal from P188's methylene measured at 500 MHz shows no measurable PRE effects in the presence of SPs on the DOPC surface (see Supplementary materials, Fig. S3). Taken all together, the conventional wisdom would suggest the absence of any P188/lipid membrane interactions. However, our ODNP data clearly indicate a different reality: P188 does interact with the vesicle by weakly adsorbing to its surface (strong adsorption is ruled out as it is known to change EPR lineshape [33]) and affect the interfacial hydration layer, while P188 must not be inserting into the lipid bilayer [30].

In fact, similar retardation of intrabilayer hydration dynamics that is not accompanied by measurable changes in lipid dynamics has been observed, induced by the addition of low concentrations of salts (*e.g.* NaCl) or small amphiphilic molecules (*e.g.* ethanol) that interact with the lipid membrane interface [26,34]—it was concluded that slowed interfacial lipid fluctuations damp transient water pore formation and hydration dynamics [26].

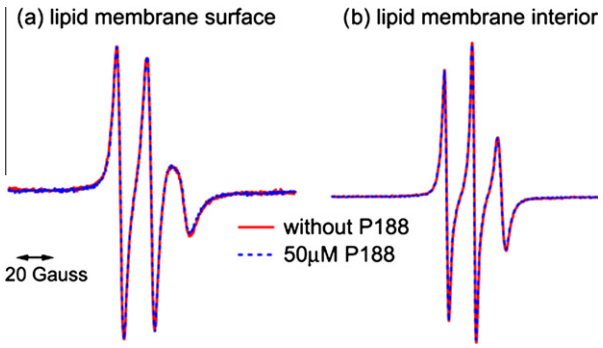

Fig. 2. X-band EPR spectra of spin-labeled DOPC vesicles (a) on the lipid membrane surface and (b) in the lipid interior in the presence or absence of 50 μM P188 at 25 °C.

To further verify our ODNP approach on a different system, we tested a widely employed membrane-fusion agent, polyethylene glycol (PEG), to characterize its interactions with DOPC membranes by probing the interfacial hydration dynamics. Previous studies have shown that there is a ~1 nm depletion layer between surface adsorbed PEG and the membrane surface, and that the lipid dynamics does not change significantly upon PEG approaching to the lipid membrane [35–37]. Our ODNP, however, reveals that the surface hydration dynamics of DOPC vesicles is measurably slowed by 17% (τ value changes from 198 ps to 232 ps) with the addition of 6.25 mM PEG, while the intrabilayer hydration dynamics is concurrently slowed by 12% (τ value changes from 403 ps to 453 ps). Taken together, this finding demonstrates that the modulation of diffusion dynamics of the hydration water at the membrane-polymer interface is an ultrasensitive means of probing these subtle, yet important, interactions that underlie membrane functions. In addition to its high sensitivity, another strength of ODNP is that it requires only a relatively dilute sample (*i.e.* ~5 μL and ~100 μM) to site-specifically probe the hydration dynamics of buried or solvent-exposed interfaces with ~1 s time resolution.

3. Conclusion

This is the first study to demonstrate that ODNP can be employed for the investigation of the weak interactions between foreign molecules and the lipid membrane, and the analytical tool developed herein serves as an important starting point for future studies. It is anticipated that ODNP is well-suited to unravel the nature of a much wider variety of interactions between foreign molecules and lipid membranes, and thus can guide us to a better understanding of their biological functions.

4. Experimental

4.1. Samples

Phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and a headgroup nitroxide spin-label, 1,2-dioleoyl-*sn*-glycero-3-phospho(tempo)choline (PC-TEMPO), were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). A lipid tail spin-label, 16-doxyl-stearic acid (16-DSA), contained a nitroxide spin label at the 16th carbon position was purchased from Sigma Aldrich. Poloxamer P188 (MW = 8400) was a generous gift from BASF (Wyandotte,

MI). PEG with 8000 g/mol molecular weight was purchased from Sigma Aldrich. DOPC and POPC are both in the fluid phase at room temperature. All chemicals were used without further purification, and their chemical structures are presented in Fig. S1 of the supplementary materials.

4.2. Lipid vesicle preparation

Large unilamellar vesicles (LUVs) were prepared according to the conventional thin-film hydration method. Basically, a stock lipid was dissolved in a chloroform:methanol (9:1, v/v) mixture and the desired amount of stock lipid solution was transferred into a glass vial. The solvent was then dried under a faint N_2 gas stream and the formed thin film was thoroughly dried in a desiccator connected to a mechanical vacuum pump. The dry lipid was hydrated using Millipore water at 37 °C for 1 h, followed by vortexing for 1 h. The extrusion was performed by passing the lipid dispersion through a polycarbonate membrane with a given diameter pore size (200 nm for DOPC; 100 nm for POPC) above the lipid transition temperature. The same batch of vesicle sample was used for each set of experiments. Fresh vesicle samples were used for all the measurements. For DNP, EPR, and PRE measurements, the final lipid concentration was 32 mM and the spin label concentration was 675 μM. For ITC measurement, the final lipid concentration was 15 mM.

4.3. Overhauser DNP measurement

^{1}H Overhauser DNP experiments were performed at a 0.35 T electromagnet, operating at 14.8 MHz ^{1}H Larmor frequency and at 9.8 GHz electron Larmor frequency. A ~5 μL sample was loaded in a 0.6 mm I.D. quartz capillary tube (Fiber Optic Center Inc., New Bedford. MA) and sealed at both ends with beewax. The capillary was mounting on a homebuilt NMR probe with a U-shaped radio-frequency coil. The EPR signal was then acquired by a Bruker X-band EMX EPR spectrometer with a rectangular TE_{102} cavity. All EPR spectra were acquired at room temperature with an incident microwave power of 20 mW. During DNP experiments, the center field of nitroxide hyperfine transition lines was pumping continuously by microwave irradiation at 9.8 GHz while ^{1}H NMR signal was recorded. The typical ^{1}H NMR spectra of samples with and without microwave irradiation can be found in Ref. [24,38]. Dry nitrogen gas was continuously purging into the EPR cavity throughout the experiment to avoid sample heating. T_1 relaxation measurements were carried out by an inversion-recovery pulse sequence operated by either Kea (Magritek Limited, Wellington, New Zealand) or Bruker Avance spectrometer in a 0.35 T superconductive magnet or an EPR electromagnet. A standard 90° pulse length was about 4 μs. The typical experimental time for T_1 experiments was about 20 min, and about 10 min for DNP experiments. All experiments were performed at 25 °C.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmr.2011.12.004.

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