Effect of melittin on water diffusion and membrane structure in DMPC lipid bilayers

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Abstract – Quasielastic neutron scattering (QENS) is well suited for studying the dynamics of water in proximity to supported membranes whose structure can be characterized by atomic force microscopy (AFM). Here we use QENS to investigate the effect of an adsorbed peptide (melittin) on water diffusion near a single-supported zwitterionic membrane (DMPC). Measurements of the incoherent elastic neutron intensity as a function of temperature provide evidence of bulk-like water freezing onto the melittin, which AFM images indicate coalesces into peptide-lipid domains as the peptide concentration increases. Analysis of the QENS spectra indicates that, at sufficiently high melittin concentrations, a water component diffusing more slowly than bulk-like water first freezes onto the bound melittin.

Introduction. – Membrane proteins represent a significant frontier in structural biology – they are ubiquitous in nature and perform a variety of tasks that help govern cellular activity. Their structure, insertion mechanisms, and function largely depend on the interactions between peptide-lipid domains and the hydrating water. Therefore, the dynamics of the membrane-associated water and its interaction with embedded proteins remain some of the most fundamental issues in biological physics today.

Single-supported lipid bilayer (SSLB) membranes provide model systems of biological interest for experimental investigation by a variety of techniques, including atomic force microscopy (AFM) [1] and neutron scattering [2–4]. Our recent studies have shown that quasielastic neutron scattering (QENS) is well suited for studying water dynamics associated with SSLBs and have recently used QENS to compare water diffusion in proximity to bare membranes comprised of either zwitterionic or anionic single lipid species [4]. We have since enhanced the complexity and biological relevance of such systems by incorporating peptides into a single-supported membrane. Here we use QENS to investigate the effect on the diffusion of water in proximity to a model peptide (melittin) bound to a single-supported zwitterionic membrane (DMPC).

QENS has long been used to study water dynamics in the hydration layer surrounding proteins in solution [5]; however, to our knowledge, there have been no previous QENS investigations of the water dynamics near a protein bound to a bilayer phospholipid membrane. On the other hand, nuclear magnetic resonance (NMR), employing Overhauser dynamic nuclear polarization (ODNP)-enhanced NMR relaxometry, is capable of probing the local translational diffusion of hydration water around specific sites of biological samples under physiological conditions [6]. This technique was used to investigate the water diffusivity within 5–10 Å of spin labels tethered to lipid bilayers forming small unilamellar vesicles with proteins bound to them [6,7]. After presenting our results, we discuss how QENS measurements on single-supported membranes at various temperatures combined with AFM imaging can complement ODNP-enhanced NMR for investigating water dynamics near membrane-bound proteins.

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Melittin, the principal toxic component in bee venom, is a well-studied antimicrobial peptide (AMP) known to insert into DMPC membranes and has served as a prototype for understanding other pore-forming peptides [8]. Early measurements by Terwilliger et al. showed by x-ray diffraction [9] that each melittin chain consisting of 26 amino acids adopts a mostly α-helical conformation. Its shape can be described as a bent rod with two linear α helices joined near the middle at a “hinge”, making an angle of ~160° when bound to DMPC bilayers [10]. The conformation and amphiphilic properties of melittin and similar AMPs result in several possible mechanisms for insertion [11,12].

Terwilliger et al. proposed the so-called “wedge effect”, describing how melittin adsorbs at low concentrations with its principal helical axis parallel to the bilayer surface [9]. Hydrophobic residues oriented mainly toward the inside of the helical bend penetrate the lipid head group region, while hydrophilic residues on the outside of the bend tend to orient away from the membrane center [13]. As melittin concentration increases and aggregates form [14], the area of the outer leaflet of the membrane increases relative to that of the inner leaflet, creating tension in the membrane that can be released by the formation of transmembrane pores lined by the amphiphilic melittin peptide [9,13]. Based on their analysis of x-ray diffraction measurements on peptide-lipid multilayers, Lee et al. [13] have argued that the wedge model of Terwilliger et al. [9] applies to transient pores in vesicles through which melittin redistributes to both sides of the membrane prior to forming stable pores. It is such stable pores induced by AMPs which have been studied in measurements of lipid leakage kinetics [15].

The question that we address here is how the structure of the DMPC membrane and the diffusion of its hydration water are affected by the binding of melittin. Our AFM measurements provide evidence of melittin aggregation on the membrane surface with increasing melittin concentration. To establish the interaction of the hydration water with the adsorbed melittin, we demonstrate that the presence of melittin strongly affects the water’s freezing behavior compared to that in proximity to a bare DMPC membrane. Analysis of the QENS spectra provides evidence that, at sufficiently high melittin concentrations, a water component diffusing more slowly than bulk-like water first freezes onto the bound melittin.

**Methods and materials.** – Our sample fabrication began by using a vesicle fusion method identical to that described previously [4,16,17] to deposit uniform, single, bare DMPC membranes on two different substrates: single-crystal silicon wafers coated with their native oxide (SiO₂) and mica. Silicon substrates were chosen for neutron scattering samples because of their extremely low incoherent scattering cross-section whereas freshly cleaved mica was used for in situ AFM measurements due to its easy-to-clean and atomically flat surface. After lipid deposition, all samples were rinsed with deionized water to remove lipid material above the complete DMPC bilayer. Melittin (C₁₃H₂₂NO₃) powder purchased from Sigma Aldrich and Gen Script (purity > 96%) was used for the AFM samples and melittin from Sigma Aldrich (purity > 85%) was used for the QENS samples. The melittin was dissolved in an aqueous solution to form 600 µM aliquots, which were stored at 253 K. Upon thawing, these aliquots were diluted to obtain solutions with peptide concentrations ranging from 0.1 µM to 10 µM in a buffer containing 10 mM HEPES and 150 mM NaCl pH 7.2. These salt concentrations were found to be optimal in facilitating the deposition of homogeneous melittin-treated single-supported bilayers [18]. The previously prepared single-supported DMPC bilayers were submerged in the peptide solution and incubated at 328 K for 4 hours. The wafers were periodically flipped and the solutions gently stirred during deposition in order to ensure all the available membrane surface area was exposed to the peptide solution. We note that similarly prepared melittin-treated bilayers have been characterized in situ using a quartz-crystal microbalance where the time scale over which the peptide-membrane interactions occurred was measured to be on order of several hours for the same temperature used in our deposition [19]. After their exposure to the peptides, the wafers containing melittin-treated DMPC bilayers were carefully removed from the solution, rinsed with deionized water, and then placed in aluminum drying trays.

To control the hydration level, melittin-treated DMPC membranes were annealed in air at 328 K to evaporate excess water. A stack of 100 wafers was then sealed in an aluminum sample can under a helium atmosphere with a known amount of water (120 µl H₂O) [4] to ensure full hydration of the membranes. Before sealing the samples, we used AFM to confirm membrane coverage and sample homogeneity.

**Results and discussion.** – Topographical images of melittin-treated DMPC bilayers deposited on Si substrates for neutron scattering measurements were collected under ambient conditions using a Digital Instruments Nanoscope IIIa AFM at the University of Missouri. Imaging of the melittin-treated membranes was performed before and after they had been subjected to an anneal at 328 K. To visualize structural changes to the membrane induced by melittin during deposition, we also performed in situ imaging of similarly prepared bilayers supported on mica substrates using a Cypher ES Environmental AFM at the Center for Nanophase Material Sciences at Oak Ridge National Laboratory. In both cases, AFM images were recorded under tapping mode using silicon nitride tips (radius < 10 nm) possessing spring constants and resonant

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Fig. 1: (Colour online) AFM images and line scans of 1.0 µM melittin-treated DMPC supported bilayers. The upper panel displays in situ images of a DMPC membrane (a) collected under buffer at 303 K before the introduction of melittin and (b) after 60 minutes of exposure to melittin. AFM images in the bottom panel were collected in air at 295 K (c) before and (d) after annealing at 328 K. Line scans were performed over the 0.5 µm white scale bar shown in the images and reveal depths of roughly 1 nm for all dimple-like features. Upon annealing as shown in (d), the dimple diameters increase to as large as 500 nm without destroying the membrane. All images were collected in tapping mode and have a z-scale of 1 nm.

In situ AFM measurements were first conducted on a bare DMPC bilayer in order to confirm sample quality. Images of the bare membrane prior to its exposure to melittin show a featureless surface with an average roughness slightly greater than that of the mica substrate (< 0.1 nm). After imaging bare DMPC, the buffer was carefully removed and replaced with a new solution containing a melittin concentration of 1.0 µM. Figure 1 shows two in situ AFM images collected at 303 K from (a) a bare DMPC bilayer supported on mica under buffer solution and (b) the same sample 60 minutes after exposure to melittin.

After this exposure, dimple-like features can be seen on the DMPC surface (fig. 1(b)), which occupy roughly 6–10% of the total scan area. Line trace analysis over the dimples reveals diameters in the range of 60–80 nm and depths of roughly 1 nm. Upon further exposure, the dimple-like features remained stable for the rest of the measurement (∼80 min) before the buffer solution evaporated.

The lower two panels in fig. 1 show the effect of annealing on DMPC membranes exposed to a solution containing 1.0 µM melittin. Unlike those in the upper panels, these AFM images were taken from DMPC samples prepared for neutron scattering measurements. Images in fig. 1(c) and (d) were collected in the absence of buffer solution and at a slightly lower temperature of 295 K. Prior to annealing (fig. 1(c)), we again observe dimple-like features on the membrane surface as in fig. 1(b), although the longer exposure time of 4 hours and drier imaging conditions yield larger diameters (≤ 200 nm vs. ∼70 nm). Despite the drier state of the membrane, the diameters of the dimples increase with annealing up to 500 nm (fig. 1(d)), while maintaining the same depth of ∼1 nm as those observed initially and in the in situ case.

The annealing temperatures for our melittin-treated DMPC samples were determined previously from temperature-dependent AFM studies of a bare DMPC supported bilayer in air, revealing a shift in the gel-to-fluid phase transition to 328 K as indicated by a decrease in bilayer thickness [17]. Although during its anneal the membrane is in a dehydrated state, the molecular motions are faster at higher temperatures, which is believed to facilitate aggregation of surface-bound melittin, a hypothesis supported by measurements of the incoherent elastic neutron scattered intensity as a function of time [17]. The elastic intensity of DMPC membranes treated with melittin concentrations of 0.5 µM were found to increase a few percent during annealing at 328 K over a period of several hours. Annealing times exceeding 8 hours resulted in saturation of the elastic neutron intensity, indicating no further slowing of the hydrogen motion. This behavior is consistent with the diffusion and subsequent anchoring of melittin into domains, such as those observed in fig. 1(d).

Previous studies suggest that, prior to forming transmembrane pores, melittin and other AMPs can induce thinning of the membrane [20–22]. Mecke et al. [1] performed AFM measurements on supported DMPC bilayers treated with an AMP similar to melittin at concentrations of 1–10 µM. They also observed formation of distinct domains of a peptide-lipid phase of a well-defined thickness less than that of the surrounding bilayer and independent of the total amount of peptide bound to the membrane [1]. These authors found the average height difference of these domains to be 1.1 ± 0.2 nm comparable to the ∼1 nm depth of the dimple-like features in fig. 1. In addition, Rakowska et al. [23], have reported AFM images collected in air from a supported bilayer of DLPC:DLPG (3:1 mixture) treated with a synthetic AMP that reveal circular impressions in membrane as large as 10 µM in diameter and depths of less than < 1 nm as a result of its exposure to a 10 µM peptide solution.

We analyzed AFM images of samples similar to those used for our QENS measurements following the procedure that Mecke et al. [1] used to infer a peptide-to-lipid
ratio $P/L$. To estimate $P/L$ for our samples, each pixel of the AFM images in fig. 1 was binned according to whether it belonged to a dimple or not. Knowing the total area occupied by the dimples, we then calculated an approximate $P/L$ under the following assumptions: 1) the melittin peptides are bound to the DMPC membrane with their principal helical axis parallel to the bilayer plane [9,13]; 2) the peptide and the lipid occupy a cross-sectional area of 4 nm$^2$ [9] and 0.6 nm$^2$ [1], respectively; 3) within a dimple, the peptides completely displace lipids from the upper leaflet as in fig. 7(c) of ref. [1]; and 4) there are a negligible number of peptides outside the dimples. We note that the presence of peptides between dimples would increase our estimate of $P/L$; however, they are likely to exist as isolated monomers or small clusters too small to participate in pore formation.

Under these assumptions, we estimate a maximum $P/L$ of $\sim 1/120$ for the 1.0 µM melittin-treated sample in fig. 1(d). Despite the uncertainty in these estimates, we conclude that our SSLB QENS sample made with a 0.5 µM melittin concentration is substantially below the critical value of $P/L^* = 1/45$ determined for pore formation in multilayer membranes of DOPC/DOPG (7:3 mixture) exposed to melittin as determined by Lee et al. [13]. Therefore, in our analysis of the QENS spectra, we consider the possibility of water confined to pores for a 0.5 µM melittin-treated sample to be unlikely; nevertheless, we cannot completely exclude their presence. DMPC bilayers treated with melittin concentrations greater than 1.0 µM were also investigated by AFM. However, their surfaces were found to be highly disordered with insufficient homogeneity for neutron scattering measurements.

The large incoherent cross-section of hydrogen and the preponderance of H atoms in the water contained in our samples results in water molecules providing the dominant contribution to our QENS spectra. Before performing time-consuming QENS measurements, it is useful to measure the temperature dependence of the intensity of neutrons scattered elastically from a sample. These elastic scans aid in the identification of temperature ranges at which to collect QENS spectra for investigating the water dynamics. We conducted these elastic scans on the High Flux Back Scattering Spectrometer (HFBS) at NIST Center for Neutron Research (NCNR) in Gaithersburg, MD [24]. Its energy resolution of $\sim 1$ µeV allows only molecular motions occurring on a time scale longer than $\sim 4$ ns to contribute to the elastic intensity. Thus, an increase in the elastic intensity is proportional to the amount of frozen water in a sample.

Figure 2 shows the temperature dependence upon cooling of elastically scattered neutrons from water near melittin-treated DMPC membranes. Intensities were summed over all 16 detectors of the HFBS, spanning a wave vector transfer range $0.25 \text{ Å}^{-1} < Q < 1.75 \text{ Å}^{-1}$, and normalized to unity at a temperature of 274 K. The elastic scan from the sample made from the solution with the lowest melittin concentration, 0.1 µM, closely resembles that of the bare DMPC membrane (black squares in fig. 2) except that the bulk-water freezing transition at 265 K is now slightly broadened. We believe this broadening results from a dilute dispersion of melittin monomers on the membrane surface.

As the melittin concentration used to treat the DMPC membranes increases to 0.5 µM, a sharp freezing transition of the membrane-associated water appears at 270 K (green circles in fig. 2); and, concomitantly, there is a decrease in the height of the freezing step of bulk-like water observed at 265 K for the bare membrane. Furthermore, on progressing to the 1.0 µM-treated sample (blue triangles in fig. 2), the size of step in the elastic intensity at 270 K increases nearly in proportion to the melittin concentration. Upon further cooling, the bulk-like water freezing transition identified in the bare-membrane sample is recovered around 265 K, but with a smaller step upward in intensity. We propose that the newly observed freezing transition at 270 K results from water freezing onto melittin domains of the type observed in the AFM images of fig. 1 and as depicted in the inset of fig. 2.

Full quasielastic spectra were collected from melittin-treated DMPC bilayers on the Backscattering Silicon Spectrometer (BASIS) located at the Spallation Neutron Source (SNS) at Oak Ridge National Laboratory (ORNL) [25]. We chose the 0.5 µM melittin sample for further investigation based on the observation in the elastic scans of two distinct freezing transitions of the membrane-associated water at 270 K and 265 K,
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respective, that had comparable changes in elastic intensity (green circles in fig. 2).

On cooling the melittin-treated sample, we obtained a QENS spectrum with a counting time of 1 hour at intervals of 0.5 K over a temperature range that included the two freezing transitions. The spectra were fitted using the Data Analysis and Visualization Environment (DAVE) software [26] by folding the instrumental resolution function with a scattering law composed of three terms: a delta function corresponding to the elastic scattering and two Lorentzian terms representing the quasielastic scattering.

The $Q$ range accessible on BASIS ($0.3 \AA^{-1} < Q < 1.9 \AA^{-1}$) is comparable to that of the HFBS; however, BASIS possesses a much larger dynamic range ($\pm 120 \mu$eV), allowing us to resolve two distinct diffusive processes that occur on different time scales: a “fast” motion described by a broad Lorentzian and a “slow” motion represented by a narrow Lorentzian in addition to the elastic component. The temperature dependence of the intensity of the three spectral components is plotted in fig. 3. In panel (a), we see that the elastic component displays a two-step behavior on cooling in qualitative agreement with elastic measurements from a different sample on the HFBS (cf. fig. 2, green circles). There is again an abrupt upward step in the elastic intensity at $\sim 269$ K, which we have attributed to bulk-like water freezing onto the peptide. It is followed by a wider step near 267 K, which is somewhat weaker and broader than the one at 265 K in the HFBS elastic scan. We attribute these differences to a greater heterogeneity in the BASIS sample, its somewhat lower purity (85%), and possible differences in thermometry and method of temperature control.

Figure 3(b) shows that a step-like decrease in the intensity of the broad Lorentzian occurs at the same temperature as the step-like increase in the elastic component (fig. 3(a)) that was attributed to the freezing of bulk-like water onto the peptide. At lower temperatures, there is a narrow plateau in intensity followed by a more gradual decrease in the broad-Lorentzian intensity, matching the wider step upward in the elastic intensity near 267 K. Based on this correspondence between the intensities of the elastic and broad-Lorentzian components in the QENS spectra, we identify the broad Lorentzian as representing the diffusion of bulk-like water. Its temperature dependence below 269 K may result from the freezing of bulk-like water over regions of the DMPC membrane unoccupied by melittin.

The half-width at half-maximum ($\Gamma$) of both Lorentzian components in the BASIS spectra exhibits a $Q^2$-dependence at low $Q$, characteristic of translational diffusion. Performing a least squares linear fit to Fick’s Law ($\Gamma = DQ^2$) at low $Q$, we obtained a diffusion coefficient $D$ for both the broad- and the narrow-Lorentzian components in the spectra. The weaker intensity of the narrow Lorentzian (see fig. 3) made fitting the QENS spectra with two components difficult. Therefore, in order to resolve the narrow component and infer a diffusion coefficient from it, we began our fitting procedure by fixing the width of the broad Lorentzian at values corresponding to those of bulk supercooled water at temperatures above 269 K [27,28]. This constraint allowed a stable two-Lorentzian fit to the spectra initially, which subsequently could be relaxed. We were unable to fit spectra collected below 269 K reliably using two Lorentzians.

Figure 4 contains the diffusion coefficient as a function of temperature determined for bare DMPC (from ref. [3]) and for the two Lorentzian components in the spectra of the melittin-treated DMPC. Diffusion coefficients for bulk supercooled water obtained from QENS [27] and NMR [28] measurements have been included for comparison. At temperatures above 268 K, the diffusion coefficient obtained from the broad-Lorentzian component in the spectra of the melittin-treated sample (blue squares in fig. 4) follows the linear trend exhibited (dashed gray line in fig. 4) by values of bulk supercooled water in this
temperature range as determined by NMR (black triangles in fig. 4). Moreover, diffusion coefficients obtained from the broad-Lorentzian component in melittin-treated DMPC are comparable, within experimental uncertainty, to values of bulk-like water identified in the bare DMPC membranes [3]. Below 268 K, D begins to deviate from the bulk behavior, decreasing steadily before leveling off at 264 K, the same temperature at which the elastic intensity plateaus (see inset to fig. 4). We have previously identified the plateau in D of the bare DMPC membrane in the temperature range 262 K < T < 265 K with a water type termed “confined 1”, which remains mobile below the freezing transition of bulk-like water and which may be located in the region between bulk ice and the lipid head groups [4]. The final step downward in D of the bare sample between 260 K and 262 K was interpreted as the freezing of the confined 1 water. The absence of this final step in the diffusion coefficient inferred from the broad-Lorentzian of the melittin-treated sample suggests that the confined 1 water also freezes onto the peptide with ice growing upward from the water/membrane interface.

The diffusion coefficient inferred from the narrow-Lorentzian component (red triangles in fig. 4) has a magnitude roughly half that of the bulk-like component at 273 K and displays a qualitatively different temperature dependence, which tracks that of the narrow-Lorentzian intensity in fig. 3(c). Above 271 K, D is nearly temperature-independent to within measurement uncertainty, but at lower temperatures decreases to a value ~0.17 × 10⁻⁵ cm²/s at ~269 K where the intensity of the narrow component vanishes. We suggest that this more slowly diffusing water population is located closer to the melittin aggregates and interacts more strongly with them than the bulk-like water.

Conclusion. – We have investigated the diffusion of water in proximity to a DMPC membrane on which melittin peptides have been deposited. Our AFM measurements indicate that treating a DMPC membrane with melittin at concentrations ≥ 0.5 μM allows peptide domains to form whose thickness is ~1 nm less than that of the surrounding membrane. However, the melittin coverage is estimated to be below the threshold P/L ratio necessary for pore formation. At these concentrations, melittin strongly affects the freezing behavior of the membrane-associated water by introducing a newly observed freezing transition at 270 K, in addition to the freezing of bulk-like water at 265 K (cf. fig. 2). Upon analyzing QENS spectra from DMPC membranes treated with 0.5 μM melittin, we find evidence of two populations of water indicated by broad and narrow Lorentzians, representing fast and slow motion, respectively. Both Lorentzian components exhibit a Q dependence of their half-widths at half-maxima indicative of translational diffusion. The broad Lorentzian yielded diffusion coefficients similar to bulk water for 268 K < T < 274 K, whereas analysis of the narrow Lorentzian yielded D values that were roughly half those of bulk water at the same temperature. Below 269 K, the narrow Lorentzian vanishes from the spectra, indicating a stronger interaction with the peptide than the bulk-like water. We speculate that larger proteins may exhibit more than the two freezing transitions that we have found for water onto melittin and thereby reveal the relative strength of different binding sites for water. Also, a more slowly diffusing water component in proximity to the inserted peptide may be a feature common to other non-pore-forming antimicrobial peptides.

Our results suggest a role for QENS measurements on SSLBs in complementing the ODNP-enhanced NMR technique for investigation of water dynamics near membrane-bound proteins. In addition to the need to determine the effect of the spin-label on the system of interest, the NMR technique is limited to room temperature measurements. QENS is able to distinguish the dynamics of distinct water populations near room temperature provided that the time scale of their motion is sufficiently different as we have demonstrated near 274 K for the “fast” and “slow” water components in our melittin-treated sample. In addition, by performing QENS measurements at lower temperatures, it is possible to freeze out different types of motion such as that of bulk water. Also, in some cases, it may be desirable to probe the water dynamics beyond 5–10 Å from...
the spin label. Although a QENS measurement involves an average over a macroscopic sample, the wave vector and energy dependence of the QENS spectra allow one to separate molecular motions from distinct water populations characterized by their respective length and time scales. Therefore, by combining AFM with QENS measurements on single-supported membrane samples of sufficient homogeneity, one is able to probe both the structure of the bound peptides and the dynamics of the membrane-associated water on the nanoscale.

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REFERENCES


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