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# Phase behavior of palmitoyl and egg sphingomyelin

Zoran Arsov[a,1], Emilio J. González-Ramírez[b,1], Felix M. Goñi[b], Stephanie Tristram-Nagle[c], John F. Nagle[c,*]

[a] Department of Condensed Matter Physics, Laboratory of Biophysics, Jozef Stefan Institute, 1000 Ljubljana, Slovenia
[b] Instituto Biofísika (CSIC, UPV/EHU) and Departamento de Bioquímica, Universidad del País Vasco, 48080 Bilbao, Spain
[c] Physics Department, Carnegie Mellon University, Pittsburgh, PA 15213, United States

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ABSTRACT

Despite the biological significance of sphingomyelins (SMs), there is far less structural information available for SMs compared to glycerophospholipids. Considerable confusion exists in the literature regarding even the phase behavior of SM bilayers. This work studies both palmitoyl (PSM) and egg sphingomyelin (ESM) in the temperature regime from 3 °C to 55 °C using X-ray diffraction and X-ray diffuse scattering on hydrated, oriented thick bilayer stacks. We observe clear evidence for a ripple phase for ESM in a large temperature range from 3 °C to the main phase transition temperature ($T_M$) of ~38 °C. This unusual stability of the ripple phase was not observed for PSM, which was in a gel phase at 3 °C, with a gel-to-ripple transition at ~24 °C and a ripple-to-fluid transition at ~41 °C. We also report structural results for all phases. In the gel phase at 3 °C, PSM has chains tilted by ~30° with an area/lipid ~45 Å$^2$ as determined by wide angle X-ray scattering. The ripple phases for both PSM and ESM have temperature dependent ripple wavelengths that are ~145 Å near 30 °C. In the fluid phase, our electron density profiles combined with volume measurements allow calculation of area/lipid to be ~64 Å$^2$ for both PSM and ESM, which is larger than that from most of the previous molecular dynamics simulations and experimental studies. Our study demonstrates that oriented lipid films are particularly well-suited to characterize ripple phases since the scattering pattern is much better resolved than in unoriented samples.

## 1. Introduction

The major lipids in biological membranes are glycerophospholipids, sphingolipids and sterols. While sphingolipids include glycosphingolipids and sphingomyelins (SMs), the latter are the most abundant sphingolipids in mammalian cells (van Meer et al., 2008) reaching concentrations of 15% of the total phospholipid content in the outer leaflet of the plasma membranes (Shaw et al., 2012). Structurally, SMs are similar to phosphatidylcholines (PCs) in that both lipids have phosphatidylcholine as the polar headgroup and both have long hydrocarbon tails, but there are considerable differences in the linkages between these parts of the lipids (Ramstedt and Slotte, 2002).

Recent interest in SMs arises from their interaction with cholesterol in generating cholesterol-rich lateral membrane domains, their specific binding to and regulation of particular membrane proteins, and their involvement as precursors to simpler sphingolipids in cell signaling events (Goni and Alonso, 2006; Slotte, 2013). The preferential mixing of sterols with SMs over PCs is mainly attributed to two properties: Firstly, SMs usually have saturated or *trans*-unsaturated tails and they apparently pack more tightly (van Meer et al., 2008); secondly, the amide and hydroxyl groups in SMs can act as hydrogen bond donors and acceptors, so they can form both intra- and intermolecular hydrogen bonding (Slotte, 2016; Venable et al., 2014).

Since pure SMs are more expensive than pure PCs, many experimental studies of systems involving them are performed using natural SM extracts e.g., egg, brain and milk SMs, which have mixtures of hydrocarbon chains. On the other hand, simulation studies use pure SM lipids with homogeneous chains. Egg SM (ESM) stands out as the most homogeneous of the natural SMs; it has predominantly 86% N-palmitoyl (16:0) acyl chain (Filippov et al., 2006) with 93% of the sphingosine (18:1) long-chain base (Ramstedt et al., 1999). Therefore from the viewpoint of composition, ESM is the closest natural extract to the pure palmitoyl sphingomyelin (PSM), so experimental properties using ESM are frequently compared to simulation studies using PSM (Niemela et al., 2004); such comparisons should be made carefully, especially as the main transition temperature of PSM ($T_M$ = 41 °C) (Barenholz et al., 1976) is somewhat higher than for ESM ($T_M$ = 38 °C) (Jimenez-Rojo et al., 2014). This paper will further address differences between ESM

* Corresponding author.
*E-mail address:* nagle@cmu.edu (J.F. Nagle).
[1] Contributed equally as first co-authors.

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and PSM.

Even the nature of the generic SM main transition has been unclear. By analogy with saturated PC lipids, the transition is often inferred to be from ripple phase to fluid (liquid-crystalline or liquid-disordered) phase if a pretransition is observed and gel-to-fluid phase if it is not. Many thermotropic studies did not observe a pretransition, either in PSM (de Almeida et al., 2003; Maulik and Shipley, 1996), or in ESM (Anderle and Mendelsohn, 1986; Arsov and Quaroni, 2008; Chien et al., 1991; Epand and Epand, 1980; Garcia-Arribas et al., 2016; Jimenez-Rojo et al., 2014; Mannock et al., 2003; Mckeone et al., 1986; Rujanavech et al., 1986; Steinbauer et al., 2003; Veiga et al., 2001), and have described the transition as from a gel-to-fluid (liquid-crystalline or liquid-disordered) phase.

Other PSM studies (Barenholz et al., 1976; Calhoun and Shipley, 1979) observed a pretransition using DSC. Turning to structural studies one of the first low- and wide-angle X-ray scattering (LAXS and WAXS) studies on PSM reported a gel phase structure below $T_M$ despite observing a pretransition using DSC (Calhoun and Shipley, 1979). (Maulik and Shipley, 1996) also reported no X-ray evidence for a ripple phase in PSM. However, other X-ray studies have reported that ESM is in a ripple phase below $T_M$ (Chemin et al., 2008; Quinn and Wolf, 2009; Shaw et al., 2012). All of these X-ray studies employed unoriented MLV samples where ripple reflections are either small or weak. By contrast, X-ray diffraction from oriented lipid samples is particularly well suited to differentiate between flat gel or interdigitated phases and the ripple phase (Akabori and Nagle, 2015; Guler et al., 2009; Katsaras et al., 2000; Sun et al., 1996b). To clarify the above mentioned differences in the literature for both ESM and PSM individually, as well as what the actual differences are between ESM and PSM, the aim of this work is to characterize the structure of the phases, particularly for $T < T_M$, using oriented hydrated samples.

A broad temperature interval was investigated and our results show that ESM persists in a ripple phase at all studied temperatures below $T_M$ (3–35 °C), i.e., no gel phase or gel-to-ripple phase pretransition was observed. This persistence enabled us to study the temperature dependence of the ESM ripple phase structural parameters. In contrast, we find that PSM is in a gel phase below 24 °C and in a ripple phase between 24 °C and the main transition. Our study confirms that it is particularly valuable to study oriented lipid films, especially for ripple phases, since their scattering patterns are much better resolved than in unoriented MLV samples. It will also serve to alleviate a relative dearth in structural studies of PSM and ESM.

## 2. Materials & methods

### 2.1. Reagents & lipids

Egg sphingomyelin (ESM) (Lot Egg-SM 860061-01-115) and palmitoyl sphingomyelin (PSM) (Lot 860584-01-017) were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. Avanti estimates 1–1.5% L-erythro impurity, no D- or L-threo impurity, and greater than 99% purity with respect to total sphingomyelin content in PSM, whereas as much as 10% L-threo may occur when synthesizing by hydrolysis of natural SM. HPLC organic solvents were purchased from Sigma/Aldrich (St. Louis, MO).

### 2.2. Sample preparation

#### 2.2.1. Oriented samples

4 mg of lyophilized lipid was dissolved in 200 mL chloroform:TFE (1:1) (v/v) and plated onto a silicon wafer (15 × 30 × 1 mm) via the rock and roll method (Tristram-Nagle, 2007) to produce stacks of ~1800 aligned bilayers (Tristram-Nagle et al., 2002). Solvents were removed by evaporation in a fume hood, followed by 2 h under vacuum at room temperature. The oriented samples were prehydrated at close to 100% RH in a polypropylene hydration chamber at 60 °C for 2 h and allowed to slowly cool to room temperature. Thin layer chromatography indicated no degradation due to this annealing procedure. Approximately 10 μm thick samples were trimmed to leave 5 mm (in the direction of the X-ray beam) by 30 mm (to provide many locations for the ~1 mm wide beam to minimize radiation damage).

#### 2.2.2. Unoriented samples

2–10 mg of lyophilized lipid was mixed with 100 μL of MilliQ water. Samples were hydrated by vortexing and temperature cycling 3 times between 60 °C and 0 °C to form multilamellar vesicles (MLVs). Hydrated lipids were mildly centrifuged using a desk-top centrifuge in a glass test tube at 1000 rpm for 10 min and the concentrated lipid was loaded into 1 mm diameter thin-walled X-ray capillaries (Charles Supper Company, Natick, MA) and flame-sealed. The X-ray capillaries were centrifuged in a capillary holder at 1000 rpm for an additional 2 min to further concentrate the lipid in the beam. The final ratio of water to lipid was at least 2:1 so the MLVs were fully hydrated as evidenced by their contact with excess water in the capillary.

#### 2.2.3. Volumetric samples

48.9 mg lyophilized ESM was added to 1.204 g MilliQ water and hydrated by vortexing and cycling three times between 60 and 0 °C. ~10 mg lyophilized PSM was added to ~1.1 g MilliQ water and hydrated by vortexing and cycling three times between 60 and 0 °C.

### 2.3. Methods

#### 2.3.1. X-ray scattering

Oriented stacks of membrane mimics were hydrated through the vapor phase in a temperature-controlled hydration chamber described in (Kučerka et al., 2005). Low angle X-ray scattering (LAXS) and wide angle X-ray scattering (WAXS) were obtained from a Rigaku RUH3R rotating anode as described in (Tristram-Nagle et al., 2002; Tristram-Nagle et al., 1993). Wavelength was 1.5418 Å, and sample-to-detector (*S*)-distance was 280.6 mm (LAXS) and 133.7 mm (WAXS). Data were collected using a Rigaku (The Woodlands, TX) Mercury CCD array detector (1024 × 1024, 68 μm pixels) during 5- or 10 min dezingered scans. Temperature was controlled using a Julabo Model F25 circulating water bath. Data analysis for the ripple phase was performed as in (Sun et al., 1996b; Akabori and Nagle, 2015) using our proprietary TiffView software.

#### 2.3.2. Volume measurements

The 4.1% aqueous ESM or ~1% aqueous PSM suspension was loaded into the Anton-Paar 5000 M DMA scanning density meter and heated at 12°/h.

## 3. Results

Fig. 1A shows the typical X-ray pattern obtained for ESM. Oriented samples clearly show off-specular peaks below $T_M$, indicated by white arrows; such peaks are required for in-plane periodicity that occurs for the ripple phase. The peaks are arranged symmetrically around the meridian because the sample is a powder average, in-plane only. This rippled pattern was present at every temperature investigated in this work from 3 to 35 °C. Additional oriented ESM data are shown in the Supplementary material (Figs. S1–S8). Following the literature (Akabori and Nagle, 2015; Sun et al., 1996b; Wack and Webb, 1989) these peaks can be indexed as (h,k) where the (h,0) peaks are the usual lamellar peaks that occur on the meridian with $q_r = 0$. The ripple side peaks are labeled with the k index. In Fig. 1A, the visible side peaks are, from the top, $(4,-2)$, $(3,-1)$, $(3,-2)$, $(2,-1)$, $(1,-1)$ and a weak (1,1). Fig. 1B shows that ESM is also in the ripple phase at 35 °C.

Fig. 2 shows LAXS data for PSM. At 37 °C (Fig. 2B) PSM is clearly in the ripple phase, however, at 3 °C (Fig. 2A) the absence of side peaks is consistent with a gel phase. In Fig. 2A, arcs, instead of spots, are due to