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Hexagonal phase with ordered acyl chains formed by a short chain asymmetric ceramide

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ABSTRACT

Ceramides constitute a group of lipids with usually high melting temperature that also favor negative curvature in membranes when mixed with other lipids. The short chain C10:0 ceramide is an asymmetric lipid which consists of an 18 carbon sphingosine base *N*-acylated with decanoic acid. According to high sensitivity differential scanning calorimetry, it shows a minor exothermic peak at 61 °C and a main endothermic transition at 75 °C. By small angle X-ray scattering and polarized light microscopy we found that, at temperatures below the main transition, the fully hydrated lipid dispersions are arranged in a tridimensional structure corresponding to an inverted hexagonal phase. Infrared spectroscopy and wide angle X-ray diffraction indicated that the acyl chains of ceramides exhibit a relatively high order in the hexagonal phase. As far as we know, this is the first report of a lipid hexagonal phase having highly ordered acyl chains. Molecular asymmetry due to the different length of the sphingosine and the *N*-acyl chains of C10:0 ceramide may explain why this novel phase is formed.

1. Introduction

Sphingolipids comprise a vast family of members composed by a long chain aminediol base *N*-linked to a fatty acid through an amide bond. The base most usually found is the 18 carbon sphingosine, with a *trans* double bond at position 4–5, (see Fig. 1), which is itself a bioactive lipid; however other variants with shorter or longer hydrocarbon chains, different hydroxylation level and saturation can be found [1–6]. In addition, the *N*-acyl chain can also vary in length (ten in the present case, see Fig. 1), hydroxylation and unsaturation level, although long saturated fatty acyl residues are more often encountered in sphingolipids than in glycerol-based lipids; such variations transduce into largely different molecular packing and phase state [7].

In addition, a great variety of polar groups attached to hydroxyl group at position C1 give rise to the different families of sphingolipids: ceramides, with a single hydroxyl group; sphingomyelins, with phosphorylcholine; cerebrosides, with different sugars; gangliosides, with sugars and sialic acid residues [8]; among others.

The chemical variety of the sphingolipids is responsible for the large structural polymorphism observed due to the intrinsic natural curvature that the polar head group, in relation to the hydrocarbon moiety, imposes to the different lipid families [9]. In this regard, while gangliosides with a complex and charged head group self-assemble as micellar structures of different shapes and size [10,11], sphingomyelins and cerebrosides form stable bilayers [3]. In the case of ceramides, it has been reported that natural extracts of bovine brain and *N*-palmitoylceramide can form lamellar stackings [12], a feature also induced by other complex glycosphingolipids and some of their mixtures with phosphatidylcholine [13]. However, when mixed with inverted phase-forming lipids such as phosphatidylethanolamine, it was shown that ceramides *N*-acylated with fatty acids equal or longer than 8 carbons decreased the temperature of lamellar Lα to hexagonal II transition [14], suggesting that this ceramide stabilized the negative curvature of membranes. On the other hand, when mixed with dipalmitoyl phosphatidylcholine, an increase of the main transition temperature and reduction of bilayer thickness due to chain interdigitation is induced by egg-Ceramide [15].

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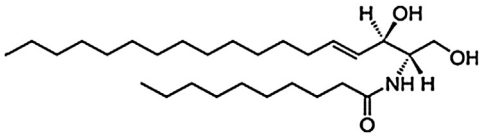

Fig. 1. Chemical structure of *N*-decanoyl-D-*erythro*-sphingosine (C10:0 ceramide).

Ceramides also display the highest melting transition temperatures described in lipid systems [16–18], owing to tight molecular packing, high Van der Waals interactions and subsequent hydrogen bonding [19]. In bilayer and monolayer membrane model systems, ceramides form highly ordered, solid phases [19–21] and, when mixed with either glycerophospholipids or sphingolipids, induce phase segregation of condensed domains and complex thermograms [18,22–24]. However it should be pointed out that even the condensed C16:0 ceramide can undergo liquid-expanded to condensed transitions depending on temperature, besides other structural transitions in the condensed state [21]. Also, when the *N*-acyl chain length of ceramides is shortened to less than 14 carbons, expanded phase and expanded to condensed monolayer phase transition at room temperature have also been found in Langmuir monolayers [21].

In the present work, we studied the phase behavior of an asymmetric ceramide *N*-acylated with the 10 carbons long decanoic acid, C10:0 ceramide, when dispersed in excess water in bulk. Previous studies [21] have shown C10:0 ceramide is able to form stable monolayers with an expanded to condensed transition at 25 mN m^{-1} at 24 °C. Combining differential scanning calorimetry (DSC), small angle X-ray scattering (SAXS), polarized light microscopy (PLM), wide angle X-ray scattering (WAXS) and Fourier transform infrared spectroscopy (FTIR) we could ascertain the presence of a novel lipid phase below the main transition that consists of an inverted hexagonal phase HII in which the acyl chains of C10:0 ceramide are highly ordered. As far as we know, this is the first report of a curved-tubular (hexagonal) phase with ordered acyl chains in pure lipid systems.

2. Materials and methods

The lipid *N*-decanoyl-D-*erythro*-sphingosine (C10:0 Ceramide, Fig. 1) was from Avanti Polar Lipids (Alabaster, Al, USA) and used without further purification. D_2O 99.9% was from Sigma-Aldrich.

C10:0 Ceramide was difficult to hydrate and generally three to four cycles of freeze and thawing should be carried out from −20 °C to 90 °C, followed by extensive bath-sonication. Samples at concentrations higher than 2 mg mL^{-1} looked opalescent. Unless otherwise indicated, measurements were done in triplicate, with freshly dispersed samples.

DSC was carried out in a MicroCal VP-DSC (MicroCal Llc) at a scanning rate of 0.5 °C.min^{-1} and overpressure of 26 psi. Lipid samples were resuspended in 145 mM NaCl at 0.5 mg mL^{-1} final concentration and degassed under vacuum. Some loss of material in the walls of the tubes and sampling syringe was unavoidable, which hampered quantitative treatment of data (enthalpy change determination).

Samples for SAXS measurements were prepared at 5 mg mL^{-1} in 145 mM NaCl and mounted between mica windows in a liquid cell. Temperature control was achieved by means of an external circulatory bath and a thermocouple, which sensed the block cell. SAXS measurements were carried out at the SAXS-2 beamline at the Brazilian Synchrotron Light Laboratory (LNLS) at Campinas, Brazil. Data was collected by means of a MarCCD detector and radially integrated by using FIT2D V 12.077 from Andy Hammersley at the European Synchrotron Radiation Facility.

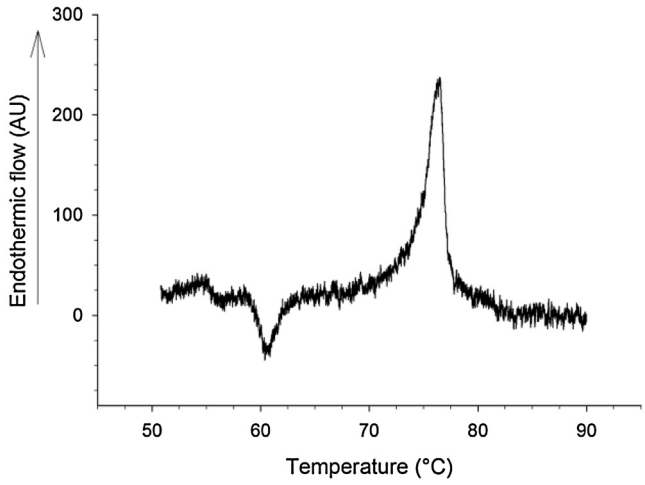

Fig. 2. DSC thermal trace of C10:0 ceramide at a nominal concentration of 0.5 mg mL^{-1}. A clear endothermic peak is observed at 75 °C, a less pronounced exothermic peak is observed at 60 °C. Scans were performed at 0.5 °C.min^{-1} in triplicate independent samples. A representative scan is shown.

The SAXS data were modelled by using the algorithm introduced by Schmidt-Rohr which allows to calculate the scattering intensity profile as a function of scattering vector [25]. Starting from a 2D electron density map made by modelling with SAXS data restrictions, the form factor function *F* was obtained by applying inverse Fourier transform.

Polarizing light microscopy was carried out in an Olympus BX51 system. Small amounts of C10:0 ceramide on a glass slide were covered with coverslips and heated above their main transition. Then, a drop of pre-heated 145 mM NaCl was placed at the edge of the coverslip, capillary adsorbed and the temperature was decreased slowly before examination under the microscope.

Samples for WAXS were prepared inside glass capillaries (Mark-Röhrchen, Germany). Measurements were carried out at the XRD-1 beamline at the Brazilian Synchrotron Light Laboratory (LNLS) in Campinas, Brazil at room temperature (25 °C). Data collection was performed with a system consisting of 24 detectors (MYTHEN 24K from Dectris) that covered 120°, and was carried out in two steps in order to overcome the gaps between the individual detectors.

FTIR was performed in a Nicolet 5700 spectrophotometer (Thermo Nicolet) equipped with a DTGS detector and dry-air purge (Parker-Balston). Lipid samples were prepared in 145 mM NaCl dissolved in D_2O at a final concentration of 5 mg mL^{-1}. A transmission liquid cell (Harrick Sci) equipped with CaF_2 windows and 56 μm PTFE spacers, was loaded with 23 μL of the lipid dispersions. Absorbance measurements were obtained by averaging 32 spectra at a resolution of 2 cm^{-1}.

3. Results

3.1. DSC

Ceramide *N*-acylated with decanoic acid (C10:0 ceramide) has not, to our knowledge, previously been studied by DSC, although thermograms of ceramides with shorter and longer *N*-acyl chains have been reported [12,14,18,22]. Due to difficulties in preparation of C10:0 ceramide dispersions a quantitative loading of the DSC cell was not reliable, thus values of ΔCp are not reported. Thermograms of C10:0 ceramide showed asymmetric and rather noisy peaks with a weak exotherm at 60 °C and a main endotherm at 75 °C (Fig. 2).