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# Effect of ceramide structure on membrane biophysical properties: The role of acyl chain length and unsaturation

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ABSTRACT

Ceramide is an important bioactive sphingolipid involved in a variety of biological processes. The mechanisms by which ceramide regulates biological events are not fully understood, but may involve alterations in the biophysical properties of membranes. We now examine the properties of ceramide with different acyl chains including long chain (C16- and C18-), very long chain (C24-) and unsaturated (C18:1- and C24:1-) ceramides, in phosphatidylcholine model membranes. Our results show that i) saturated ceramides have a stronger impact on the fluid membrane, increasing its order and promoting gel/fluid phase separation, while their unsaturated counterparts have a lower (C24:1-) or no (C18:1-) ability to form gel domains at 37 °C; ii) differences between saturated species are smaller and are mainly related to the morphology and size of the gel domains, and iii) very long chain ceramides form tubular structures likely due to their ability to form interdigitated phases. These results suggest that generation of different ceramide species in cell membranes has a distinct biophysical impact with acyl chain saturation dictating membrane lateral organization, and chain asymmetry governing interdigitation and membrane morphology.



## 1. Introduction

Sphingolipids (SLs) are important components of biological membranes and are involved in a variety of biological functions ranging from cell proliferation to apoptosis [1–3]. Ceramide is one of most important bioactive SLs, and comprises a fatty acid (which can be either saturated or monounsaturated) of variable chain length (C14–C26) linked by an amide bond to C2- of the long chain base, sphingosine (Fig. 1) [4]. In mammals, de *novo* ceramide synthesis is regulated by a family of six ceramide synthases (CerS) that have different specificities towards fatty acyl CoAs with various acyl chain lengths. Thus, CerS1 and CerS5 use long acyl chain CoAs (C18- and C16-, respectively), while CerS2 and CerS3 are involved in the synthesis of very long acyl chain ceramides (>C22) [5]. Ceramide can also be generated in the plasma membrane through the hydrolysis of sphingomyelin (SM). Both pathways can be activated in response to stress stimuli [6], driving an increase in cellular ceramide and consequent activation of cellular processes, including apoptotic signaling pathways [2].

It has been postulated that the distinct biophysical properties of ceramide play a major role in the regulation of ceramide-dependent signaling pathways [2]. Moreover, ceramides with different acyl-chains appear to have distinct physiological actions, which may be due to their different impact on the biophysical properties of membranes. As an example, mixing ceramide with fluid lipids [7–9], or generating ceramide through SM hydrolysis [10–13] in model membranes, drives an increase in the order of the membrane [14], induces gel-fluid phase separation [9,14,15], promotes the formation of non-lamellar structures [9] and/or induces morphological alterations of the vesicles [16]. Although some studies have explored the effect of different ceramides on the biophysical properties of model membranes [7,9,17], information is still lacking regarding the differential impact of long chain versus very long chain ceramides and the role of unsaturated chains compared to saturated chains. To date, most biophysical studies have focused on the chain length [7,18], the role of chain asymmetry [19,20] or on the properties of the non-physiological short chain (i.e. C2-6) ceramides [9]. In addition, some of these studies were performed using ceramide from natural sources which contain a mixture of different

*Abbreviations:* C16-ceramide, N-palmitoyl-D-*erythro*-sphingosine; C18:1-ceramide, N-oleoyl-D-*erythro*-sphingosine; C18-ceramide, N-stearoyl-D-*erythro*-sphingosine; C24:1-ceramide, N-nervonoyl-D-*erythro*-sphingosine; C24-ceramide, N-lignoceroyl-D-*erythro*-sphingosine; CerS, ceramide synthases; DPH, 1,6-diphenyl-1,3,5-hexatriene; FCS, Fluorescence correlation spectroscopy; GUV, giant unilamellar vesicles; MLV, multilamellar vesicles; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Rho 110, rhodamine 110; Rho-DOPE, N-rhodamine-dipalmitoylphosphatidylethanolamine; SLs, Sphingolipids; SM, sphingomyelin; t-PnA, trans-parinaric acid

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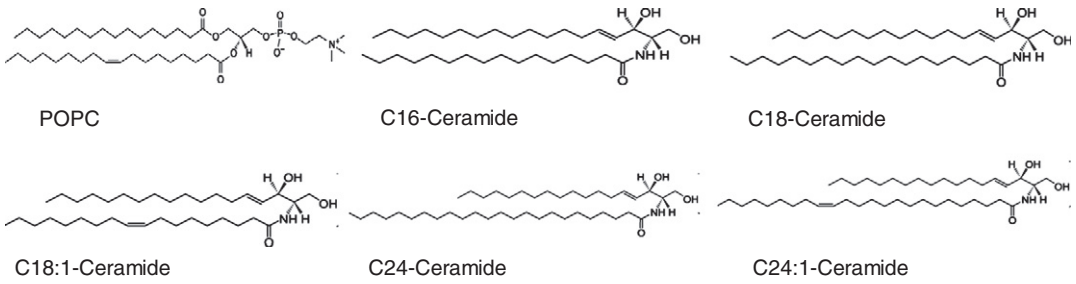


**Fig. 1.** Structures of the lipids used in this study.

acyl chain lengths. These are possible reasons for some of the discrepancies between different studies [20–22].

To address the impact of ceramide acyl chain structure, it is important to systematically analyze the effects of varying the acyl chain length and the degree of unsaturation. We now show that i) saturated ceramides have a stronger tendency to increase the order of the fluid membranes and to segregate into highly ordered gel domains compared to their unsaturated counterparts; ii) the impact of saturated ceramides in the properties of the fluid membrane is similar, changing only to a small extent with acyl chain length. However, the size and morphology of the domains formed by each saturated ceramide is significantly different; iii) very long chain ceramides are able to promote the formation of interdigitated phases and induce strong morphological alterations, such as tubules, independently of the degree of unsaturation; iv) the long chain unsaturated ceramide is unable to increase the order of the membrane or promote gel-fluid phase separation at physiological temperatures. Therefore, it is expected that generation of distinct ceramides in cell membranes would have a different biophysical impact with acyl chain saturation dictating membrane lateral organization and chain asymmetry governing interdigitation and membrane morphology. The global biophysical effect of increasing ceramide levels might therefore depend on the balance between long chain versus very long chain, and saturated versus unsaturated ceramide species.

## 2. Materials and methods

### 2.1. Materials

POPC, C16-, C18-, C18:1-, C24-, C24:1-ceramide (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, N-palmitoyl-D-*erythro*-sphingosine, N-stearoyl-D-*erythro*-sphingosine, N-oleoyl-D-*erythro*-sphingosine, N-lignoceroyl-D-*erythro*-sphingosine; N-nervonoyl-D-*erythro*-sphingosine) and Rho-DOPE (N-rhodamine-dipalmitoylphosphatidylethanolamine) (Fig. 1) were obtained from Avanti Polar Lipids (Alabaster, AL). DPH (1,6-diphenyl-1,3,5-hexatriene), t-PnA (trans-parinaric acid), and Rho 110 (rhodamine 110) were from Molecular Probes (Leiden, The Netherlands). All organic solvents were UVASOL grade from Merck (Darmstadt, Germany).

### 2.2. Fluorescence spectroscopy

To evaluate the effect of ceramide structure on membrane properties, multilamellar vesicles (MLV) (total lipid concentration of 0.1 mM) were prepared as described [8]. The suspension medium was 10 mM sodium phosphate, 150 mM NaCl, 0.1 mM EDTA buffer (pH 7.4). Fluorescence anisotropy of t-PnA or DPH (at a probe/lipid ratio of 1/500 and 1/250, respectively) was measured in a SLM Aminco 8100 series 2 spectrofluorimeter with double excitation and emission monochromators, MC400 (Rochester, NY). All measurements were performed in 0.5 cm × 0.5 cm quartz cuvettes under magnetic stirring. The excitation ($\lambda_{exc}$)/emission ($\lambda_{em}$) wavelengths were 305/405 nm for t-PnA and 358/430 nm for DPH. Constant temperature was maintained using a Julabo F25 circulating water bath controlled with 0.1 °C precision directly inside the cuvette with a type-K thermocouple (Electrical Electronic Corp., Taipei, Taiwan). For measurements performed at different temperatures, the heating rate was always below 0.2 °C/min.

Time-resolved fluorescence measurements with t-PnA were performed using $\lambda_{exc} = 295$ nm (using a secondary laser of Rhodamine 6 G) and $\lambda_{em} = 405$ nm. The experimental decays were analyzed using TRFA software (Scientific Software Technologies Center, Minsk, Belarus). For a decay described by a sum of exponentials, where $\alpha_i$ is the normalized pre-exponential and $\tau_i$ is the lifetime of the decay component i, the mean fluorescence lifetime is given by $<\tau> = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i$.

### 2.3. Confocal fluorescence microscopy and fluorescence correlation spectroscopy (FCS)

POPC/ceramide giant unilamelar vesicles (GUV) were prepared by electroformation [16]. Confocal fluorescence microscopy was performed using a Leica TCS SP5 (Leica Mycrosystems CMS GmbH, Mannheim, Germany) inverted microscope (DMI6000) with a 63× water (1.2 numerical aperture) apochromatic objective. Rho-DOPE (probe/lipid ratio 1/500) excitation was achieved using the 514 nm line from the $Ar^+$ laser. The emission was collected from 550 to 680 nm.

FCS measurements were performed using the same optical path described for the fluorescence imaging, with the exception that the fluorescence signal from the sample was detected by the avalanche photodiodes (APD) in the FCS unit, rather than the PMT (photomultiplier). A filter-cube containing a dichroic filter was used to separate the emitted light into two different detection channels and band pass filters 470–500 and 535–585 were used for spectral selection. GUVs labeled with Rho-DOPE (probe/lipid ratio 1/50,000–1/100,000) were used in different experiments and only the top side of the GUV was evaluated. Before starting the measurements, the Z-plane with higher intensity was selected. Six to ten successive autocorrelation curves were obtained for a single GUV and recorded during 20 s per FCS measurement. The average correlation curves were normalized for a better comparison between vesicles with different lipid composition. Autocorrelation curves were fitted using a two-dimensional Gaussian model in the absence of a triplet state [23]. To estimate the radius of the detection area $\omega_0$ on the focal plane (calibration of the apparatus), the three-dimensional diffusion coefficient of Rho 110 freely diffusing in water was measured. A new calibration was performed on each day of the experiments.

## 3. Results

### 3.1. Impact of ceramide acyl-chain structure on gel domain formation

We previously demonstrated that t-PnA fluorescence lifetime and anisotropy are suitable parameters to characterize ceramide-induced alterations in simple [8,16] and complex [13,24] model membranes.