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Rapid report

Rectangular solid domains in ceramide–cholesterol monolayers – 2D crystals

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Abstract

Very small rectangular domains were observed by atomic force microscopy in binary monolayers of synthetic ceramides and cholesterol. When the cholesterol content is increased the domains are bigger although the rectangular shape is retained. The almost perfect shape of the domains indicates two-dimensional single ceramide crystals. Lipid domains in monolayers of this particular shape and size have to our knowledge not been reported in the literature previously.

Keywords: Two-dimensional crystal; Monolayer; Atomic force microscopy; Ceramide; Cholesterol; Domains

Among the diverse lipids occurring in biological membranes there is usually a fraction that in the pure state would form a gel phase with crystalline chains rather than the dominant lamellar liquid crystalline phase. There has for a long time existed a debate on to what extent crystalline domains play an important biological role [1]. Recently, the so called raft model has been proposed. It describes small size domains as membrane lipid rafts which can serve as platforms for lipid and protein transport or as relay stations in intracellular signalling [2]. Lipid monolayers as models for the membrane bilayers have been studied extensively and for example studies of mixed phospholipids and cholesterol [3–5]. The size, shape and lateral arrangement of the domains formed within a monolayer are dependent on the monolayer composition. It has been shown that phosphatidylcholines form chiral two-dimensional (2D) domains at the air–water interface. With a small amount of cholesterol (1%) the chiral domains become elongated and thin [6]. Fatty acids form irregularly shaped domains that are smaller than the phosphatidylcholines domains and the presence of cholesterol leads to an increased interfacial length [7,8].

The imaging technique limits the possible resolution. Most studies on lipid monolayer domain formation have been performed with fluorescence microscopy [3] and Brewster angle microscopy [9]. Both techniques image the air–water interface but the wave length of light limits the resolution. Atomic force microscopy (AFM) can resolve features on a nanometer scale. However, the sample has to be transferred onto a solid support. Yang et al. [10] have shown that the chiral domains of phosphatidylcholines with four-fold clockwise rotation symmetry

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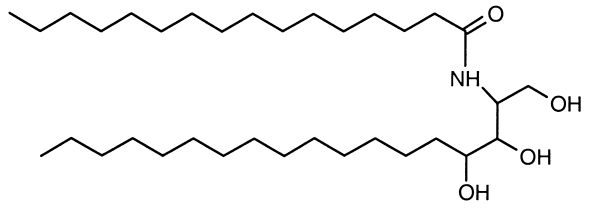

Fig. 1. The molecular structure of C16cerIII.

that have earlier been observed on the air–water interface [6] can be imaged at higher resolution with AFM. In the present study we have observed very small rectangular domains in mixed monolayers of ceramide and cholesterol with AFM. The domains resemble 2D crystals. Two-dimensional lipid domains of this rectangular shape and size have to our knowledge not been reported in the literature previously. The area of the domains are about 10 000 times smaller than the so-called stripe domains observed by epifluorescence microscopy in cholesterol, dimyristoylphosphatidylserine and dimyristoylphosphatidylcholine monolayers [11]. Lipid domains of the magnitude of 10 nm in binary lecithin bilayers have been visualised with AFM by Gliss et al. [12]. However, these domains are not as regular in shape as those reported here.

Binary monolayers of cholesterol and ceramides have been studied. Two synthetic ceramides were used, one with a palmitic (C16:0) (Fig. 1) and one with a lignoceric (C24:0) acid amide-linked to a phytosphingosine base. The ceramides were a gift from Joke Bouwstra prepared by Gist Brocades, Cosmoferm B.V. (Delft, the Netherlands) and are here called C16cerIII and C24cerIII, respectively. Cholesterol ($>99\%$ purity) was purchased from Sigma (St. Louis, MO, USA). Monolayers were prepared on a Langmuir–Blodgett trough type 611 from Nima Technology (Coventry, UK). A 0.1 M acetate buffer, adjusted to pH 4.0 was used as subphase. The water was deionized, distilled and filtered through a Millipore Q purification system (Millipore Corporation, Bedford, MA, USA). For deposition, sheets of freshly cleaved mica were immersed into the subphase. The lipids dissolved in a chloroform:methanol mixture (molar ratio 5:1, 1 mg lipid/ml solvent), were spread at the air–water interface. After the solvents had evaporated for 20 min, the monolayer film was compressed at a speed of 20 cm^2/min to 22 mN/m. The monolayer was kept at constant pressure for 20 min and during transfer to the substrate at a dipping speed of 2 mm/min. All samples were prepared in a cleanroom at a constant temperature of 19°C. The transfer ratios of the monolayers were close to unity. Constant force AFM measurements were performed on a commercial Nanoscope IIIa instrument (Digital Instruments, Santa Barbara, CA, USA) as previously described [7]. Dimensions of the domains were measured directly from the AFM height images. Area ratios, thickness variations and Fourier transformed images were calculated in the Nanoscope IIIa image analysis software.

In AFM images of monolayers of C24cerIII and cholesterol, molar ratio 1:0.4, small closely packed domains are observed (Fig. 2a). The domains are rectangular with edges of 25 and 175 nm, respec-

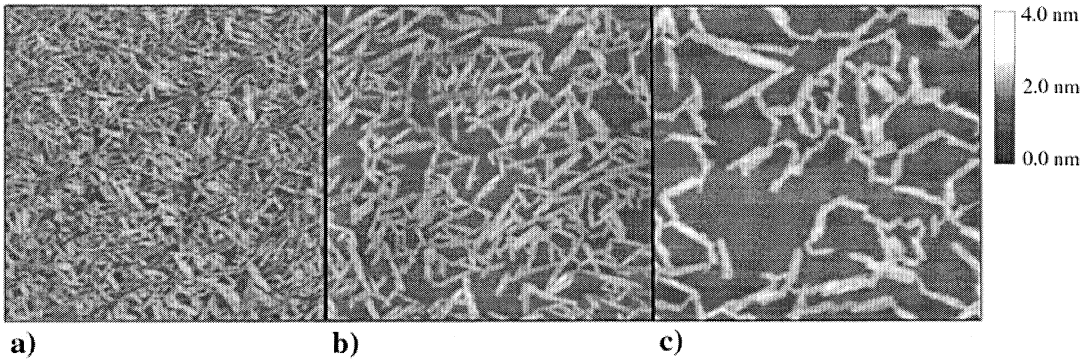

Fig. 2. Topographic AFM images (2×2 μm) of a transferred monolayer of C24cerIII:cholesterol. Two phases can be observed, one thick phase with small domains of stick-like shape is embedded in a thinner phase. (a) Molar ratio 1:0.4. (b) Molar ratio 1:1. (c) Molar ratio 1:2. The difference in thickness between the phases is measured to 0.8 ± 0.1 nm for all images. The films were deposited at a surface pressure of 22 mN/m. Z range, 4 nm.

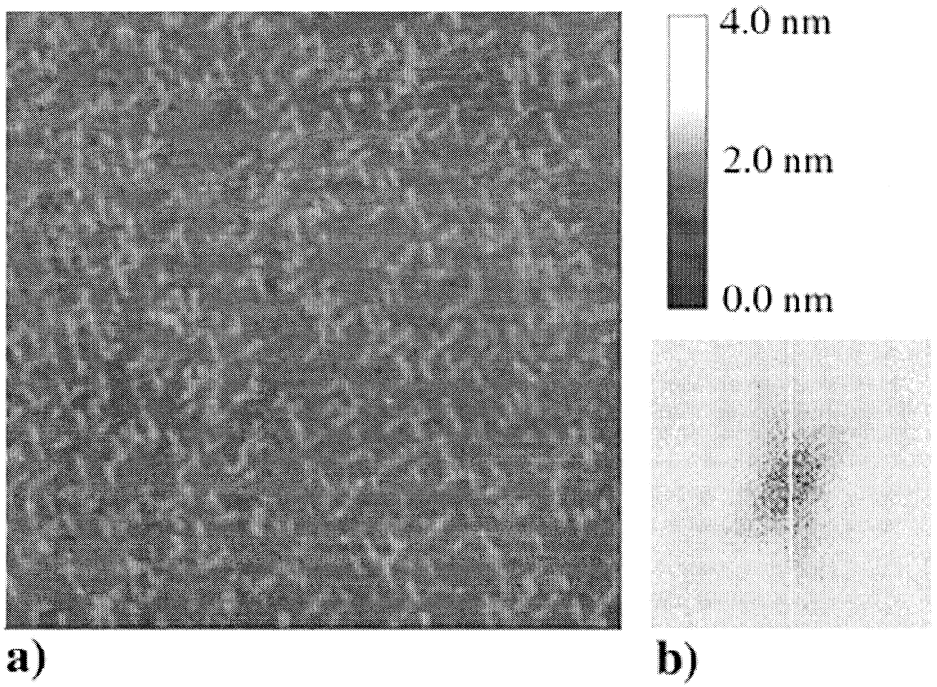

Fig. 3. (a) Topographic AFM image (2×2 μm) of C16cerIII:cholesterol molar ratio 1:2. Small stick-like domains that seem to be arranged with a preferred angle to each other. The sticks are 0.4 ± 0.1 nm thicker than the flat thin phase. The monolayer was deposited at a surface pressure of 22 mN/m. *Z* range, 4 nm. (b) A 2D Fourier transform of image (a).

tively, and the way they are arranged reminds of a network. When increasing the cholesterol content to a molar ratio C24cerIII:cholesterol of 1:1, the domains are bigger and seems more dispersed, although the shape and the network arrangement is retained (Fig. 2b). All domains are about the same size with edges of approximately 40 and 250 nm, respectively, and they are 8 ± 1 Å thicker than the thin flat phase. The domains seem to preferentially connect to each others short edges although a few domains are tightly arranged parallel to each other. At even higher cholesterol contents the size of the domains increase further. Fig. 2c shows the transferred monolayer of C24cerIII:cholesterol molar ratio 1:2 where the sizes of the domains are up to twice the size compared to the previous described sample. Domains of similar shape are observed in monolayers of the shorter ceramide, C16cerIII, and cholesterol although the domains are smaller. As in the C24cerIII–cholesterol system the domains are bigger at increased cholesterol content with typical sizes of 10×80 and 20×150 nm for C16cerIII:cholesterol molar ratio 1:1 and 1:2, respectively. A striking observation is that the lateral arrangement of the domains in the C16cerIII–cholesterol monolayers is of a higher order compared to the domains in the C24cerIII–cholesterol monolayers and the domains seem to intersect with a preferred angle (Fig. 3a). A 2D Fourier transformation of Fig. 3a shows a cross at an angle of 54° indicating two well defined directions in the plane (Fig. 3b). A corresponding lateral arrangement of rectangular ceramide domains was also observed in ternary monolayers of C16cerIII, cholesterol and lignoceric acid (Sparr et al., unpublished results).

To be able to further discuss the remarkable domains in the ceramide cholesterol monolayers, it is necessary to know the composition of the different phases. The total areas of the domains decrease with increasing amount of cholesterol indicating that the domains consist of ceramide. This is also supported by the differences in thickness within the monolayer. However, by comparing the molar composition of the monolayer and the area of the domains occupied it is clear that the thinner phase covers a greater fraction of the area than would be expected if the lipids were totally immiscible. Some ceramide must, therefore, be incorporated into the thinner cholester-

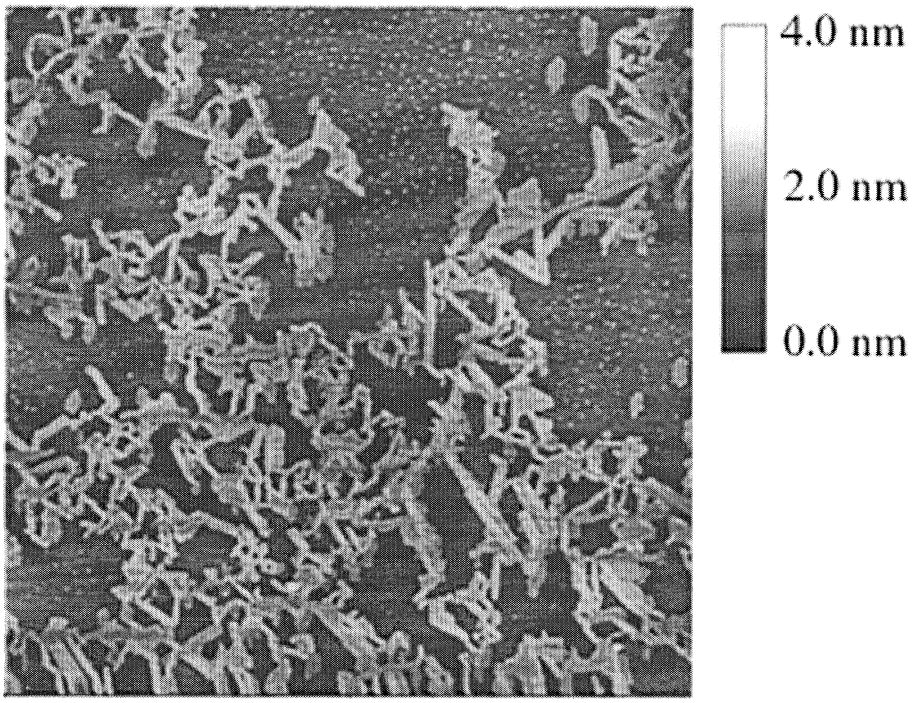

Fig. 4. Topographic AFM image (5×5 μm) of C24cerIII:cholesterol molar ratio 1:1. The lipids were left at 0 mN/m for 12 h before the film was compressed and deposited at a surface pressure of 22 mN/m. The difference in thickness between the two phases is 0.8 ± 0.1 nm. *Z* range, 4 nm.

ol rich phase. The perfect rectangular shape of the domains indicates crystalline packing of the ceramide molecules. An important observation is that in all the samples described the long edges of the rectangular domains are approximately 7 times longer than the short edges. This is true irrespective of the size of the domains and the composition of the monolayer. The constant ratio between the lengths of the edges indicates 2D crystals. Ideally, the equilibrium values of the length and the width of flat deformation-free solid domains are determined by the minimum in the boundary free energy [13]. As a consequence, the ratio between the lengths and widths of single rigid rectangular crystals has to be constant and equal to the ratio of line tensions at the crystal–liquid interfaces along the short and the long edges. According to this, the rectangular domains observed in this work can be seen as single ceramide crystals where the line tension along the short edge is around 7 times larger than the line tension along the long edge.

The process observed here most probably starts as a homogeneous nucleation of crystal growth in the monolayer. It is possible that cholesterol acts as an impurity providing surface to which the ceramide molecules can attach and thereby initiate nucleation. Also, the line active properties of cholesterol [3,8] may favour the nucleation process. When the cholesterol content is increased the number of nucleation centres decreases. This can explain the increased size of the domains at higher cholesterol contents. At very low cholesterol contents, the monolayer looks homogeneous. The film can be regarded as a polycrystalline monolayer of very many very small ceramide crystals. It is therefore not possible to detect segregation of cholesterol. Another interesting observation is that the rectangular domains are monodisperse in size. This implies that Ostwald ripening is not a dominant mechanism for the process demonstrated here.

The lateral arrangement of the rectangular domains differs significantly in the different systems. In the C24cerIII–cholesterol monolayers, the domain arrangement can be seen as a 2D analogy to association of clay particles, while the domains in the C16cerIII–cholesterol monolayers show a more well-ordered lattice arrangement. The lateral organisation of the domains is to a great part determined

by the diffusion in the monolayer. At high surface pressures one expects a slow diffusion in the film. Also the composition and packing of the continuous phase is crucial. The systems described here differ in respect to the amount of ceramide in the cholesterol-rich phase (Sparr et al., unpublished results). This can be an explanation for the differences between the two systems, although the exact mechanisms for the actual arrangements are not known.

To investigate the kinetics of the domain formation, monolayers of C24cerIII:cholesterol, molar ratio 1:1, were left at zero surface pressure for 12 h before compression and deposition onto mica. The domains in the transferred monolayer are rectangular although not as perfectly shaped as the domains in previously described samples (Fig. 4). One can observe that the domains are bigger and more aggregated than in the earlier described sample at corresponding composition (compare Figs. 4 and 2b). Under these conditions the lateral diffusion increases and the domains are allowed to cluster to a higher extent. The increased size can be due to a lower density of nucleation centres at a lower surface pressure.

The almost perfect shape of the domains as well as the constant ratio between the lengths of the edges indicates 2D crystals. As the thickness of the domains remains constant in relation to the thinner phase one can exclude the possibility of 'normal' 3D crystal growth. The surface pressure–area isotherms show that the ceramides spontaneously form a condensed monolayer already at low surface pressures. A condensed state in the monolayer is considered to correspond to a more crystalline state in the bulk phase. In 3D crystals of C24cerIII the double chained ceramide has a v-shaped arrangement [14]. Corresponding molecular arrangement in the monolayer can be excluded since the molecular area estimated from the surface pressure–area isotherms is too small to allow such a shape. However, it is interesting to note that when C24cerIII was crystallised from solvent at low temperatures a phase with needle-shaped crystals was observed, referred to as phase A in the work by Dahlén and Pascher [15]. This is the only reported crystalline phase of C24cerIII with parallel chain packing and would therefore best represent the bilayer structure in biological membranes. Unfortunately, no 2D crystal structure of the ceramides have been reported. It is also interesting to note that the 3D crystalline phases of C24cerIII grow with a preferred direction [14,15], thus giving elongated crystals.

This very simple system of synthetic ceramides and cholesterol might not be directly comparably to more complex biological systems. However, it should be noted that in AFM images of pigskin ceramide–cholesterol monolayers [16] domains of the same size have been observed although the shape of the domains were not as perfect as for those observed here. This is probably a consequence of the variation in chain lengths of the pigskin ceramides. Segregated crystalline ceramide have also been recognised in phospholipid–ceramide liposomes [17]. Small scale aggregation in lipid membranes has, as previously mentioned, been proposed to be relevant for different biological functions. The phenomenon illustrated in this work has also an interest from a physical point of view. Nucleation and 2D crystal single growth have been discussed. The rectangular shape of the monodisperse crystals follows from simple thermodynamic principles where the single crystal domain shape is determined by minimising the boundary free energy.

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