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Original Paper

Evidence for the Formation of Symmetric and Asymmetric DLPC-DAPC Lipid Bilayer Domains

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Key Words
DLPC • DAPC • Lipid bilayer • Lipid domain • Lipid raft • Atomic force microscopy

Abstract
Background/Aims: We investigated if mixtures of the phosphatidylcholine (PC) lipids 1,2-dilauroyl-sn-glycero-3-phosphocholine (C12:0 PC; DLPC) and 1,2-diarachidoyl-sn-glycero-3-phosphocholine (C20:0 PC; DAPC), which differ by eight methylene groups in acyl chain length, lead to the spontaneous formation of distinct lipid rafts and asymmetric bilayers. ***Methods:*** The experiments were performed using Atomic Force Microscopy (AFM). ***Results:*** We show that DLPC and DAPC mixed at a molar ratio of 1:1 lead to the formation of single, double and triple bilayers with peaks at 6.14 ± 0.11, 13.27 ± 0.17 and 20.54 ± 0.46 nm, respectively (n=750). Within these formations discrete height steps of 0.92 nm can be resolved (n=422). ***Conclusion:*** The most frequently observed height steps value of 0.92 nm matches best with the calculated mean lipid hydrophobic thickness difference for asymmetric C12:0 PC and C20:0 PC lipid bilayers of 0.88 nm. This indicates the ability of DLPC and DAPC to form asymmetric lipid bilayers.

Introduction

Despite its canonical basic structure a cell membrane constitutes a sophisticated landscape of highly diverse and dynamic areas which differ in architecture, composition of constituents, size and lifetime, called domains and rafts [1, 2]. Transmembrane thickness, as well as lipid bilayer asymmetry are characteristics and essential biological features of such

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domains, important for a variety of biological processes such as membrane fission, vesicle fusion and transport, signal transduction, membrane trafficking, cell sorting and function of transmembrane proteins as well as processes like e.g. secretion, cell migration, cell volume regulation, cell proliferation, differentiation and apoptosis (reviewed in [3-6]. Moreover reconstitution of ion channels in lipid bilayers is frequently used to assess their biophysical properties and it has been shown that - among other factors - the composition and thickness of the bilayer are important factors influencing ion transporters as well as the gating and selectivity of the reconstituted channels [5, 7-11]. Accordingly, deranged cell membrane asymmetry may lead to a variety of pathophysiological conditions and diseases [3, 12-14].

Several techniques allow to visualize and quantify the dynamics of nano-domain formation and to track the lateral movement of even single molecules with a spatio-temporal resolution as low as ~10-20 nm and ~1ms, respectively, like e.g. fluorescence correlation spectroscopy (FCS), stimulated emission depletion (STED) far-field fluorescence nanoscopy, reversible saturable optical (fluorescence) transitions (RESOLFT), reversible saturable optical (fluorescence) transitions (RESOLFT), ground state depletion microscopy followed by individual molecule return (GSDIM), stochastic optical reconstruction microscopy (STORM), or photoactivated localization microscopy (PALM) [2, 15-20]. Accordingly the generally very high rate for in-plane lateral diffusion of membrane/nano-domain constituents can be accurately tracked by these methods. However, the observation of intrinsic transversal movements between the membrane leaflets asks for spatial resolution at the sub-nanometer scale. Atomic force microscopy (AFM) is a powerful method to address this issue [21-23]. As the rate of transbilayer movement of lipids meets thermodynamically unfavorable conditions it is fairly low [24] and therefore usually achieved by the aid of specific enzymes like ABC transporters, P4-ATPases, flippases, floppases and scramblases, as well as by interactions of lipids with the cytoskeleton [3, 24-32]. However, it is less clear whether formation of lipid asymmetry can also occur spontaneously and this issue is a matter of intense investigation [24, 28].

In the present study we investigated by AFM if mixtures of phosphatidylcholine lipids of different acyl chain length using DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine; C12:0 PC) and DAPC (1,2-diarachidoyl-sn-glycero-3-phosphocholine; C20:0 PC) leads to a spontaneous formation of distinct lipid domains, rafts and/or asymmetric bilayers.

Materials and Methods

Chemicals

DAPC and DLPC were purchased from Avanti Polar Lipids. All other chemicals were from Sigma-Aldrich.

Preparation of supported lipid bilayers

Vesicles were prepared by mixing DAPC and DLPC 1:1 from 2 % (w/v) chloroform stock solutions. Chloroform was evaporated under a stream of nitrogen gas. The lipid mixtures were rehydrated overnight in Milli-Q™ Ultrapure water (Millipore) to yield a final concentration of 2 mg/ml. The lipid mixture was vortexed for 5 min to produce multilamellar vesicles and subsequently sonicated for 30 min at 50°C (Decon Laboratories, Hove, UK) to produce small unilamellar vesicles. 7 µl of the vesicle solution were placed on freshly cleaved mica (Goodfellow, UK) and allowed to adhere for 1 min at room temperature (22°C). Subsequently, 50 µl of Hepes-buffered solution (HBS; 150 mM KCl, 5 mM Hepes, pH 8.0, adjusted with KOH) were added for 3 min. Thereafter HBS was removed by incubation for 3 min in Milli-Q™ water followed by 3 times gently rinsing. Samples were then dried under a stream of nitrogen gas and transferred to the AFM.

Atomic Force Microscopy (AFM)

AFM imaging was performed as described previously [7]. Briefly, a Multi-Mode Atomic Force Microscope (Digital Instruments, Santa Barbara, CA) equipped with a J-scanner and with an in-line electronics extender module, controlled by a Nanoscope IIIa controller was used. The microscope was placed on a silicone-gel

vibration isolation pad and on a pneumatic table and placed under an acoustic hood to reduce ambient room sound interferences. All experiments were performed at room temperature (22°C) in the air tapping mode with commercially available n+-silicon sensors on cantilevers with a specified spring constant of 42 N/m (NCH-50 Pointprobes; Nanosensors™, Wetzlar-Blankenfeld, Germany) tuned at a drive frequency of ~300 kHz. The drive amplitude was adjusted to produce a root mean square amplitude of ~1.7 V. Force was minimized by adjusting the set point just below the jump-off point of the tip. The images were captured at a 512 × 512 pixel resolution and the scan rate was 1 Hz. Images were flattened and analyzed using the algorithm provided by the Nanoscope software.

Analysis and Statistics

The preparation led to the formation of clearly detectable multiple patches of lipid formations representing single bilayers and bilayer-stacks of different heights (Fig. 2). 750 section line analyses of randomly chosen patches were performed to determine absolute heights with respect to the supporting surface and 422 individual line section analyses were made to analyze height differences within those lipid formations displaying one or more discrete height steps, using the integrated software of the AFM. Subsequent analysis was performed by assessment of frequency distributions using IgorPro version 6.31 (WaveMetrics, OR, USA). The bin width was set to 45 for the absolute heights histogram (Fig. 2B) and 21 for the height difference histogram (Fig. 4). Where appropriate, data are given as mean ± SEM.

Results

Using different experimental approaches DLPC (C12:0 PC) and DAPC (C20:0 PC) bilayers have been reported to have a thickness ranging from ~3.0 to ~4.7 nm (DLPC) and ~6 nm (DAPC), respectively [4, 7, 33-39]. According to Fig. 1 it can be assumed that Gaussian distributions of different combinations of single symmetric and asymmetric DLPC-DAPC bilayers yield peaks around these values, whereas stacks of bilayers should yield heights of multiples thereof. As shown in Fig. 2, mixtures of DLPC-DAPC at a molar ratio of 1:1 led to the formation of single bilayers and bilayer-stacks of different heights. Analysis of these heights revealed a frequency distribution with three prominent peaks at 6.14 ± 0.11, 13.27 ± 0.17 and 20.54 ± 0.46 nm, respectively. These values correspond well to the expected ones and therefore most likely represent single bilayers and stacks of double and triple bilayers.

Within the first peak (~3-9 nm) accumulations of observations occur at ~4.6 nm, 5.6 nm and 6.5 nm, respectively. This suggests the existence of discrete height differences of approximately 1 nm, which might arise from the formation of asymmetric bilayers. As shown in Fig. 3, bilayer formations contained sharp edged islets and/or rims of a discrete higher level of ~1 and 2 nm. 422 line section analyses of the height steps occurring in lipid preparations showing such islets and/or rims revealed a most frequently observed value of 0.92 nm as shown in Fig. 4.

Discussion

In giant unilamellar vesicles prepared from DLPC and DAPC a linear relationship between the phase state of the lipid domain and the difference in acyl chain length has been demonstrated. Accordingly it was concluded that the fraction of DLPC that cocrystallizes with a second phospholipid component as well as the amount of the second phospholipid component present in the DLPC fluid matrix decreases with increasing acyl chain length. It has been shown that DLPC-DAPC mixtures at a molar ratio of 1:1 have a fluid to fluid-solid phase transition temperature of 63°C and display domain coexistence between pure fluid and pure gel lipid domains below this temperature. It was concluded, that the fraction of the low melting temperature phospholipid (DLPC, Tm -1°C) is negligible in the solid domain (DAPC; Tm 66°C) [40]. Accordingly it might be assumed that the formation of asymmetric DLPC-DAPC bilayers at the experimental conditions given is rather unlikely to occur. In a recent

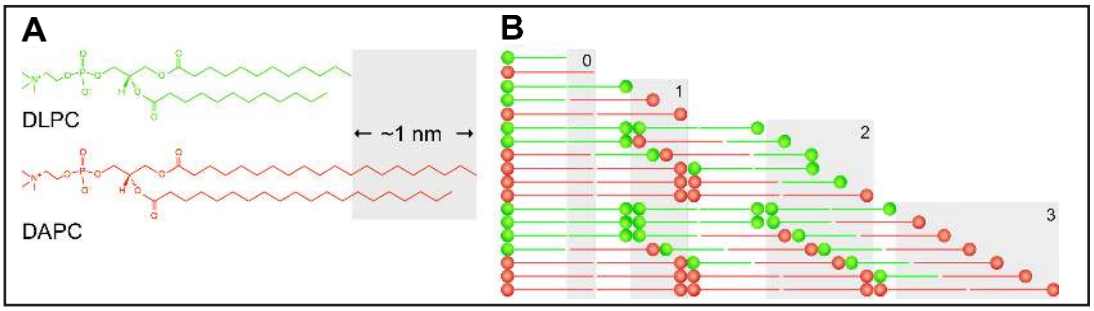

Fig. 1. A: Space fill-model of DLPC (C12:0; green) and DAPC (C20:0; red). The theoretical height difference caused by a mismatch of four methylene group yields a value of ~1 nm (gray area). B: Simplified theoretical model of combinations of monolayers, single, double and triple bilayers of DLPC (green) and DAPC (red). Gray areas mark the range of the minimum (left area margin) and maximum (right area margin) height of monolayers (area 0), symmetric and asymmetric single (area 1), double (area 2) and triple (area 3) bilayers.

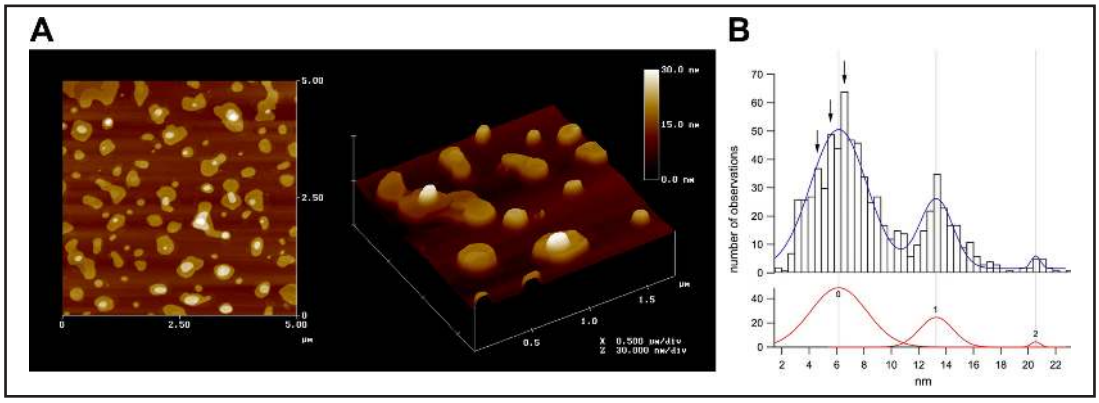

Fig. 2. A: 2D- (left) and 3D- (right) overview of a 1:1 DAPC-DLPC mixture, showing lipid domains of different heights, reflecting monolayers, bilayers and bilayer stacks, respectively. B: Height section profile of DAPC-DLPC mixture formations (n=750; bin width 45). Peak 0, 1 and 2 represent the mean heights of single-, double and triple bilayers, respectively. Note the slight discrete accumulation of observations within peak 0 at ~4.6 nm, ~5.6 nm and ~6.5 nm (arrows), possibly indicating the presence of discrete height steps of ~1nm and ~2nm, respectively.

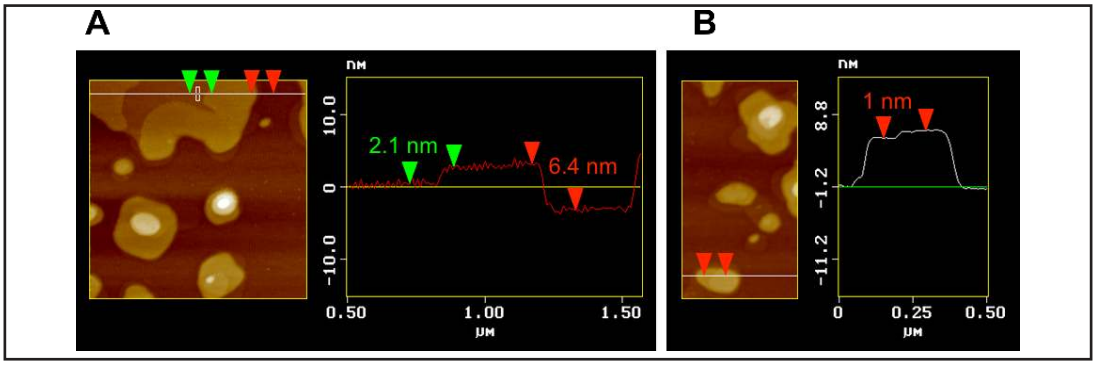

Fig. 3. A: Height section profile (right) of a lipid raft in a DAPC-DLPC mixture (left), most likely reflecting a symmetric DAPC bilayer of 6.4 nm height (red arrows) and a symmetric DLPC bilayer of 4.3 nm height (difference of lower green and lower red arrow). Green arrows (2.1 nm) reflect the height difference between the two bilayers. B: Height section profile (right) of a lipid raft in a DAPC-DLPC mixture (left) with a height difference of ~1 nm, most likely representing the height difference occurring in asymmetric DLPC-DAPC bilayers.

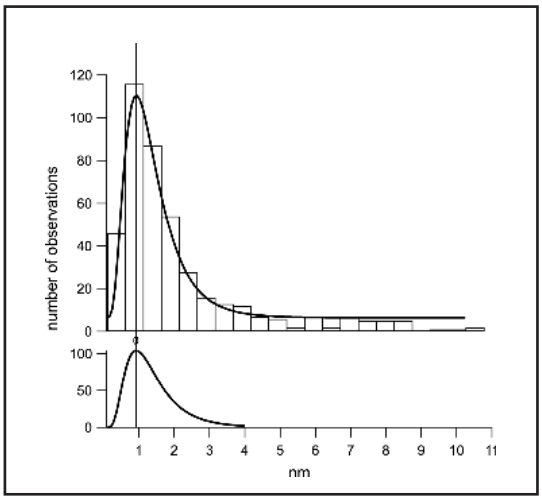

Fig. 4. Frequency histogram of measured height steps in DAPC-DLPC lipid rafts. Number of bins = $422^{1/2} \approx 21$. Log Normal fitting reveals a peak at 0.92 nm, indicating the ability of the phosphatidylcholine lipids to form asymmetric bilayers.

study the spontaneous formation of completely symmetric and asymmetric supported lipid bilayers formed of mixtures of DLPC (C12:0 PC) and DSPC (C18:0 PC; Tm 55°C) has been demonstrated [27]. Similarly, the formation of asymmetric bilayers has been demonstrated for phospholipid mixtures of DLPC-DPPC (C16:0 PC, Tm 41°C), DMPC (C14:0 PC, Tm 23°C)-DPPC and DOPC (C18:1 PC, Tm -21°C)-DPPC using neutron reflectivity and quartz crystal microbalance measurements [39]. Given the theoretical all-trans-length difference between C12:0 and C20:0 lipids of ~1 nm [4, 5, 34, 38, 41-43], symmetric and asymmetric bilayers with a chain mismatch of eight methylene groups as used in this study should yield height differences of ~2 nm for symmetric and ~1 nm for asymmetric bilayers, respectively (Fig. 1). The most frequently observed height step of 0.92 nm in the present study fits best to the calculated mean lipid hydrophobic thickness difference for asymmetric C20:0 PC and C12:0 PC lipid bilayers of 0.88 nm [43].

As mentioned above, the composition, area and lifetime of membrane nano-domains in living cells are highly dynamic. Clearly the results obtained in this study are confined to an artificial membrane model which is kept as simple as possible to allow for capturing snapshots of events which demonstrate the principle thermodynamically possibility of lipids with highly different biophysical properties to spontaneously form asymmetric membranes without the aid of specific enzymes. Further studies are necessary to substantiate this finding and to test, whether it might be extended from *in vitro* observations to *in vivo* conditions in living cells. Of note, recent studies have demonstrated that driven by membrane deformations during vesicle fusion and fission membrane lipids can flip their hydrophobic tails from the outer leaflet of one bilayer to the adhering outer leaflet of the opposing bilayer, i.e. to favor trans-monolayer movement and thus creating a possibility for asymmetric bilayer formation for cellular events *in vivo* [44].

In conclusion the absolute heights and hight step differences of bilayers prepared from of 1,2-dilauroyl-sn-glycero-3-phosphocholine (C12:0 PC; DLPC) and 1,2-diarachidoyl-sn-glycero-3-phosphocholine (C20:0 PC; DAPC) with an acyl chain length difference of eight methylene groups, as measured in this study indicate the spontaneous formation of symmetric bilayers composed of DLPC-DLPC and DAPC-DAPC and asymmetric bilayers built by DLPC-DAPC as well as different bilayer-stack combinations thereof.

Abbreviations

AFM (Atomic Force Microscope/Microscopy); DAPC (1,2-diarachidoyl-sn-glycero-3-phosphocholine); DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine); DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine); DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine);

DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine); DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine); Tm (main gel- to fluid-phase transition temperature).

Conflict of Interests

The authors declare no conflict of interests.

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