

Visualizing detergent resistant domains in model membranes with atomic force microscopy

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Abstract Evidence is accumulating that in cell membranes microdomains exist, also referred to as rafts or detergent resistant membranes. In this study, atomic force microscopy is used to study supported lipid bilayers, consisting of a fluid phosphatidylcholine, sphingomyelin and cholesterol. Domains were visualized of which the morphology and size depended on the cholesterol concentration. The presence of cholesterol was found to induce bilayer coupling. At 30 mol% cholesterol, a change in percolation phase was observed, and at 50 mol%, when both fluid lipids and solid lipids are saturated with cholesterol, phase separation was still observed. In addition, we were able to directly visualize the resistance of domains against non-ionic detergent. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Raft; Sphingolipid; Cholesterol; Phase separation; Planar lipid bilayer; Percolating

1. Introduction

Since biological membranes consist of many different lipids and proteins, and thus form heterogeneous mixtures, it has been speculated that lateral domains can form in biomembranes [1,2]. Especially domains that consist of sphingolipids and cholesterol have received much attention lately [3–5]. Such domains are usually referred to as rafts [6] and they are believed to correspond to parts of biological membranes that are resistant to non-ionic detergent in the cold [7]. Detergent resistant membranes (DRMs) [8,9] have indeed been found to be enriched in sphingolipids and cholesterol.

Eukaryotic plasma membranes consist of glycerophospholipids, sphingolipids and sterols. The glycerophospholipids usually have a low melting temperature (T_m) while sphingolipids in biomembranes usually have a high T_m . Cholesterol, the sterol present in mammalian cells, is able to induce the liquid ordered phase [10,11]. In this phase, lipid acyl chains are extended and tightly packed, as in the solid phase, but the lateral diffusion is almost as high as in the fluid phase [12,13]. The basis of the idea that rafts exist in cell membranes is that,

because sphingolipids and cholesterol preferentially interact [14,15], they phase separate from glycerophospholipids in the fluid phase, and form domains in the liquid ordered phase, which are insoluble in non-ionic detergents, such as Triton X-100, in the cold.

Liquid ordered phases have been studied in model systems with different techniques such as fluorescence quenching [16,17], fluorescence polarization [17,18], electron spin resonance [11] and fluorescence microscopy [13]. Atomic force microscopy (AFM) can image biological samples under aqueous conditions with high resolution in three dimensions without the use of any probes. AFM has been successfully used to image isolated DRMs [19], phase separated bilayers [20,21] and peptide-lipid domains in supported bilayers [22,23]. Also monolayers containing glycosphingolipids and cholesterol have been imaged [24], but so far membrane mimicking bilayers consisting of phospholipids, sphingolipids and cholesterol have not been investigated by AFM. We have, for the first time, used AFM to image bilayers consisting of a fluid glycerophospholipid, sphingomyelin (SpM) and varying amounts of cholesterol and directly visualized domains and their resistance against cold non-ionic detergent extraction. In addition we report on some novel findings concerning these systems.

2. Materials and methods

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and egg SpM were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Egg SpM is enriched in sphingosines with C_{16:0} acyl chains. All lipids were >99% pure. Cholesterol was from Merck (Darmstadt, Germany). For all experiments MilliQ water was used.

2.1. Vesicle preparation

All lipids were dissolved in chloroform/methanol (3:1, v/v) and subsequently mixed at predetermined ratios. The ratio of SpM and DOPC was always 1:1 (mol/mol) and cholesterol concentrations varied between 0 and 50 mol% of the total amount of lipid. The lipid mixtures were dried in a rotary evaporator followed by overnight storage under high vacuum. To the dried mixed films, 1 ml of 20 mM NaCl solution was added, which resulted in a lipid concentration of 1 mM. The hydrated film was freeze-thawed and sonicated in a bath sonicator (Branson, Danbury, CT, USA) at maximum power for at least 30 min, to obtain small unilamellar vesicles (SUVs). Possible remaining large vesicles were spun down at 20 800 × g for 1 h, at 4°C. The supernatant containing SUVs was used for the preparation of supported bilayers.

2.2. Preparation of supported bilayers

The supported bilayers were prepared using the vesicle fusion method. 75 µl of SUV suspension was applied onto freshly cleaved mica (diameter 10 mm). The vesicles were allowed to adsorb and fuse on

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Abbreviations: DRM, detergent resistant membrane; AFM, atomic force microscopy; SpM, sphingomyelin; T_m , melting temperature; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; SUV, small unilamellar vesicle; DSC, differential scanning calorimetry; TLC, thin layer chromatography

the mica while the sample was heated to 60°C for 60 min. Afterwards, the sample was left to cool down to room temperature at ambient conditions and subsequently rinsed. In this way bilayers were obtained suitable for scanning with AFM.

Bilayers made of vesicles that were not heated, but left to adsorb for 5 h at room temperature, had the same morphology as the heated bilayers. The same holds true for bilayers made of unsonicated, multilamellar vesicles (MLVs), but in this case the quality of the AFM images was bad, most likely due to the fact that multiple bilayers were present underneath the scanned bilayer.

2.3. AFM

The sample was mounted on an E-scanner, which was calibrated on a standard grid, of a Nanoscope III (Digital Instruments, Santa Barbara, CA, USA). A quartz flow cell was used without the O-ring. All samples were scanned with oxide sharpened tips with a spring constant of 0.06 N/m, as estimated by the manufacturer (Digital Instruments). Scans were recorded with a scan speed of 6 lines/s and at a minimal force where the image was stable and clear, which was usually smaller than 500 pN. All images shown are flattened raw data. All bilayers were imaged at room temperature. With the Nanoscope software it is possible to study cross-sections and height distributions of AFM images. We used the cross-sections to determine the height of the observed domains and the height distributions to quantify the amount of area occupied by domains.

2.4. Detergent extraction

Samples of DOPC/SpM (1:1) with 0, 10 or 25 mol% cholesterol were, after scanning, left to cool down in the refrigerator to 4°C for 1 h. Subsequently 50 µl of the solution on the bilayer was replaced by cold 10% Triton X-100, and the sample was left to incubate for 1 min. After rinsing with 10×75 µl cold 20 mM NaCl solution, the sample was left to warm up to room temperature and scanned again with AFM. The exact same area that was scanned before Triton extraction could be found again by using an electron microscopy grid, glued under the mica.

2.5. Differential scanning calorimetry (DSC) and thin layer chromatography (TLC)

DSC measurements were done on MLVs of pure SpM and of SpM/DOPC (1:1), with a SpM concentration of 5 mM, in a MCS (Microcal Inc., Northampton, MA, USA) with a scan rate of 60°C/h. TLC was performed on silica plates (Merck) with an eluent of chloroform/methanol/water (65:25:4). Lipids were stained with iodine and with short heating after spraying with 10% H₂SO₄.

3. Results and discussion

We have prepared supported bilayers consisting of DOPC

and SpM (1:1), with increasing concentrations of cholesterol, 0–50 mol% of the total amount of lipid. Of each system, several samples were made and scanned, and we show here the most representative AFM images. Occasionally, in all bilayers, small pinholes were seen, indicating that a bilayer was indeed present, but they were always too narrow to measure the bilayer thickness [25]. In the gray-scale used to visualize height differences, black is low, and white is high.

Fig. 1A depicts an AFM image of a DOPC/SpM (1:1) bilayer, without cholesterol. This bilayer is expected to show phase separation because egg SpM has a T_m of 38°C, as measured with DSC (data not shown) and thus is in the solid phase at room temperature. DOPC has a T_m of −15°C [14] and thus is in the fluid phase at room temperature. Since bilayers in the solid phase are thicker than bilayers in the fluid phase, higher domains consisting of SpM can be seen in Fig. 1A. The size of the solid domains was in the order of 10–100 nm. In Table 1, the height difference between the domains and the surrounding bilayer, and the areas of the domains, as percentages of the total amount of area, are listed for all bilayers.

The average amount of area occupied by domains was 21% of the total area, which is relatively small as compared to the amount of SpM present. However, in a DOPC/SpM mixture, the T_m of SpM decreases [14]. The thermogram of mixed DOPC and SpM showed a broad phase transition peak of SpM, starting at 10°C and finishing at 30°C (data not shown), indicating that at room temperature only a fraction of SpM is in the solid phase. It is this fraction that is seen as higher domains in AFM images of such bilayers (Fig. 1A), while the remaining part of the SpM is in the fluid phase, present in the surrounding bilayer, probably dissolved in DOPC.

The average height difference between these solid domains and the fluid bilayer is 1 nm, which is in agreement with other results obtained with AFM on phase separated fluid–solid bilayers [20]. Occasionally some intermediate levels, appearing 0.6 nm above the level of the fluid bilayer, were seen. This is illustrated in Fig. 1B, which shows an image of a DOPC/SpM (1:1) bilayer at higher magnification. Fig. 1C depicts the cross-section of the line drawn in Fig. 1B. This indicates that some parts of the domains are asymmetric in their mono-

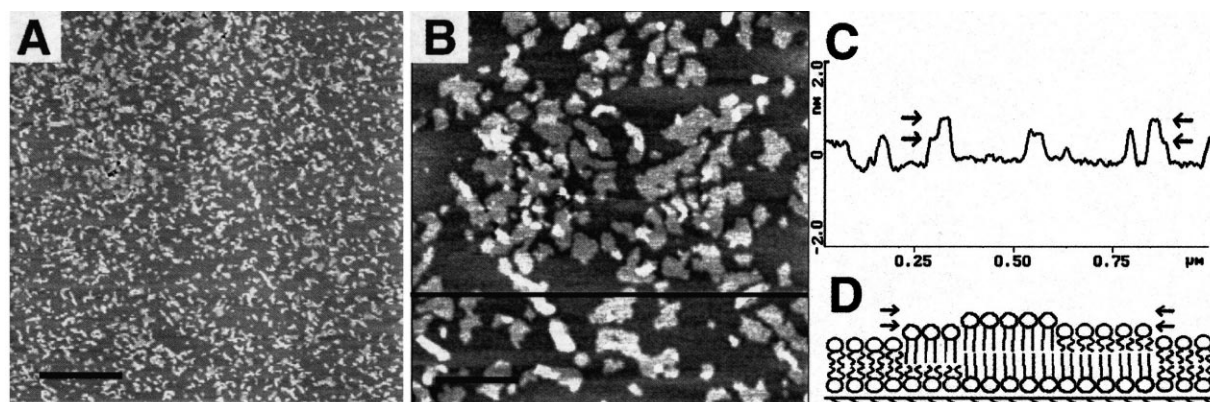


Fig. 1. Bilayer of DOPC/SpM (1:1). A: AFM image showing phase separation, image size 5×5 µm, scale bar is 1 µm and z-scale is 10 nm, visible as a gray scale in which low is black and high is white. B: DOPC/SpM (1:1) bilayer at larger magnification. The domains appear in two different heights, visible as white and light gray. The dark gray represents the level of the fluid bilayer. Image is 1×1 µm, scale bar is 200 nm and z-scale is 5 nm. C: Cross-section of the line drawn in B. The two different height levels of the domains are denoted with arrows. D: Molecular model of the domains showing the two height levels. It is not known whether in the domains of intermediate thickness, the ordered molecules face the support or not.

Table 1

Amount of area occupied by domains and height differences between the domains and surrounding bilayer in DOPC/SpM bilayers containing different amounts of cholesterol

Cholesterol concentration (mol%)	Area domains (% of total area)	Height difference domains and bilayer (nm)
0	21 ± 11 (<i>n</i> = 7)	1.0 ± 0.1 (<i>n</i> = 8)
2	25 ± 6 (<i>n</i> = 3)	0.9 ± 0.3 (<i>n</i> = 3)
5	24 ± 9 (<i>n</i> = 6)	0.8 ± 0.2 (<i>n</i> = 6)
10	28 ± 5 (<i>n</i> = 4)	0.8 ± 0.1 (<i>n</i> = 3)
15	21 ± 10 (<i>n</i> = 5)	0.9 ± 0.2 (<i>n</i> = 5)
25	41 ± 7 (<i>n</i> = 21)	0.8 ± 0.2 (<i>n</i> = 20)
30	58 ± 2 (<i>n</i> = 3)	0.6 ± 0.2 (<i>n</i> = 3)
50		0.4 ± 0.1 (<i>n</i> = 4)

layer composition. Apparently not in all domains, both monolayers are made up of solid lipid, but some also of fluid lipid. This is clarified in Fig. 1D.

The chain length asymmetry in SpM has been found to cause monolayer coupling, for SpM with a difference in chain length of 11 carbons [26]. Chain length differences of five carbons were not sufficient to induce this effect. In our system, where the SpM chain length difference is even smaller, namely three carbons, monolayer coupling should not occur, which is supported by our data (Fig. 1B–D).

Bilayers of DOPC/SpM (1:1) with 2, 5, 10 or 15 mol% cholesterol had an appearance comparable to the bilayers without cholesterol, except that the domains were slightly larger. Also, some large domains (500 nm) were present that seemed to consist of coalesced smaller domains. In Fig. 2A, a

bilayer containing 10 mol% cholesterol is shown with arrows denoting such ‘clusters’ of domains. The average height difference between the domains and the surrounding fluid bilayer is slightly lower than for domains without cholesterol, while the average amount of area occupied by domains is slightly higher (Table 1). For all bilayers containing 5 mol% cholesterol or more, the intermediate levels described for the system without cholesterol (Fig. 1) were never observed. This implies that cholesterol has the ability to couple monolayers in membranes, at least in the presence of SpM. Previous observations have suggested that in cell membranes, the rafts in the outer leaflet are coupled to the cytoplasmic leaflet, although the latter is sphingolipid poor [27]. Our findings indicate that cholesterol may play a role in this leaflet coupling.

Fig. 2B shows a bilayer of DOPC/SpM (1:1) with 25 mol%

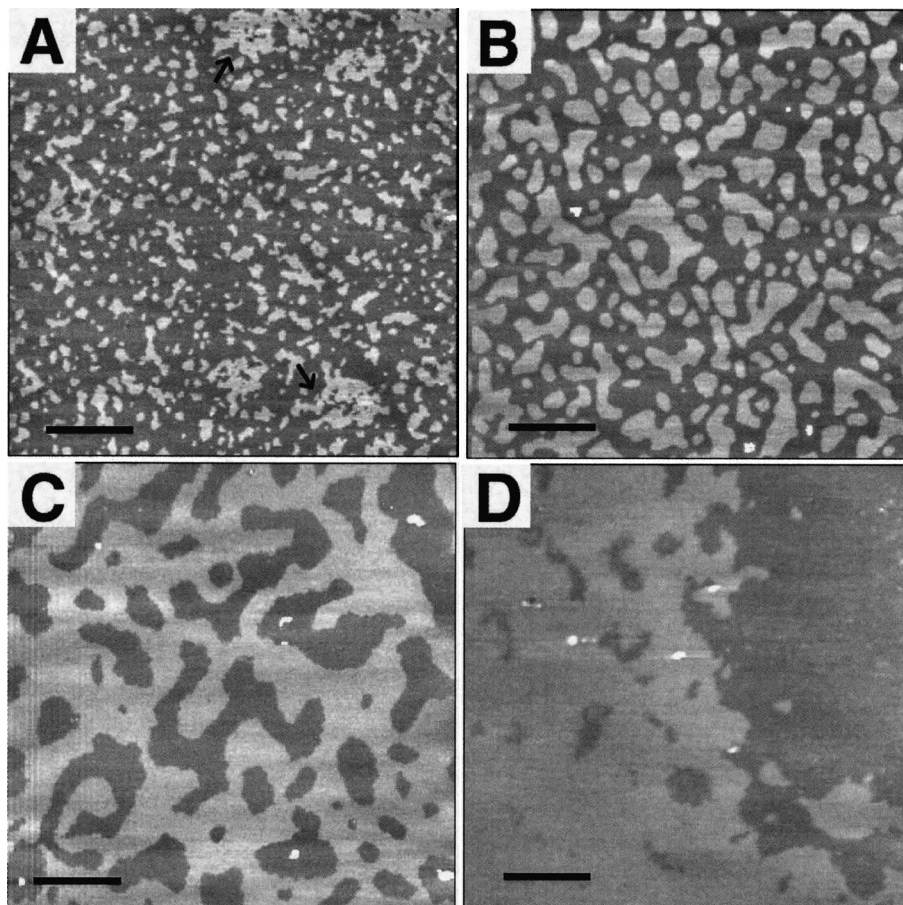


Fig. 2. AFM images showing domains in DOPC/SpM (1:1) bilayers. A: 10 mol% cholesterol. B: 25 mol% cholesterol. C: 30 mol% cholesterol. D: 50 mol% cholesterol. All images are 5 × 5 μm, scale bars are 1 μm and z-scales are 10 nm.

cholesterol. Domains formed in such bilayers are larger in size (up to 1 μm) and less irregular in shape than the domains formed at lower cholesterol concentrations. The height of the domains is comparable to the height of the other domains containing cholesterol (Table 1). The average amount of area occupied by these domains is significantly higher, namely 41% of the total area. Possibly all SpM molecules in the solid phase are saturated with cholesterol, and now also SpM, originally dissolved in DOPC in the fluid phase, is incorporated in the higher domains.

At 30 mol% cholesterol (Fig. 2C), the amount of higher area has increased further (Table 1). The average height difference between high and low areas decreased slightly, to 0.6 nm. At this point, the higher phase is in the majority and apparently the percolation threshold has been passed, since the higher phase is now the percolating phase. A change in percolating phase has been proposed to be biologically functional [28], and this is the first time such a change is visualized directly in bilayers of phospholipids, sphingolipids and cholesterol.

The maximum concentration of cholesterol in lipid bilayers is 50 mol%. At this concentration bilayers showed large phase separated areas with sizes of more than 10 μm . The amount of high area could not be quantified reliably, but by eye it seemed to be about 50% of the total area. The average height difference between the two phases was found to be merely 0.4 nm. In these systems SpM as well as DOPC are saturated with cholesterol. Yet, still two phases can be distinguished in these systems (Fig. 2D), one consisting of SpM and cholesterol, and the other of DOPC with cholesterol which appears

as the lower areas. This implies that in biomembranes rich in cholesterol, such as myelin, phase separation, and thus domain formation, can occur.

Cholesterol is known to thicken fluid bilayers [29] which explains the decrease in height difference between the two phases to 0.4 nm. Since the height difference at low cholesterol concentrations was 0.8 nm, this means that cholesterol thickened the fluid bilayer with 0.4 nm. This is in agreement with other studies, which found cholesterol to cause an increase in bilayer thickness of 0.4–0.6 nm [30].

To test whether the domains shown in this study are resistant to non-ionic detergent extraction in the cold, we treated several samples with Triton X-100 at 4°C. An example is presented in Fig. 3.

A DOPC/SpM (1:1) bilayer with 25 mol% cholesterol is depicted in Fig. 3A (compare to Fig. 2B), before detergent extraction. Fig. 3B shows the cross-section of the line drawn in Fig. 3A, showing the height difference between the domains and the surrounding bilayer. Fig. 3C depicts an image of the same area after detergent extraction, and Fig. 3D shows the cross-section of the line drawn in Fig. 3C. It is clear that after detergent extraction, the fluid phase has disappeared, revealing the underlying mica substrate, and that the domains are still present. The height difference between the domains and the mica was found to be 5–6 nm. This corresponds to the thickness of a solid bilayer and a layer of water between the mica and the lipid layer [25].

When the detergent extraction was performed at room temperature, nearly the whole bilayer had disappeared (data not shown). Domains formed in bilayers with a concentration of

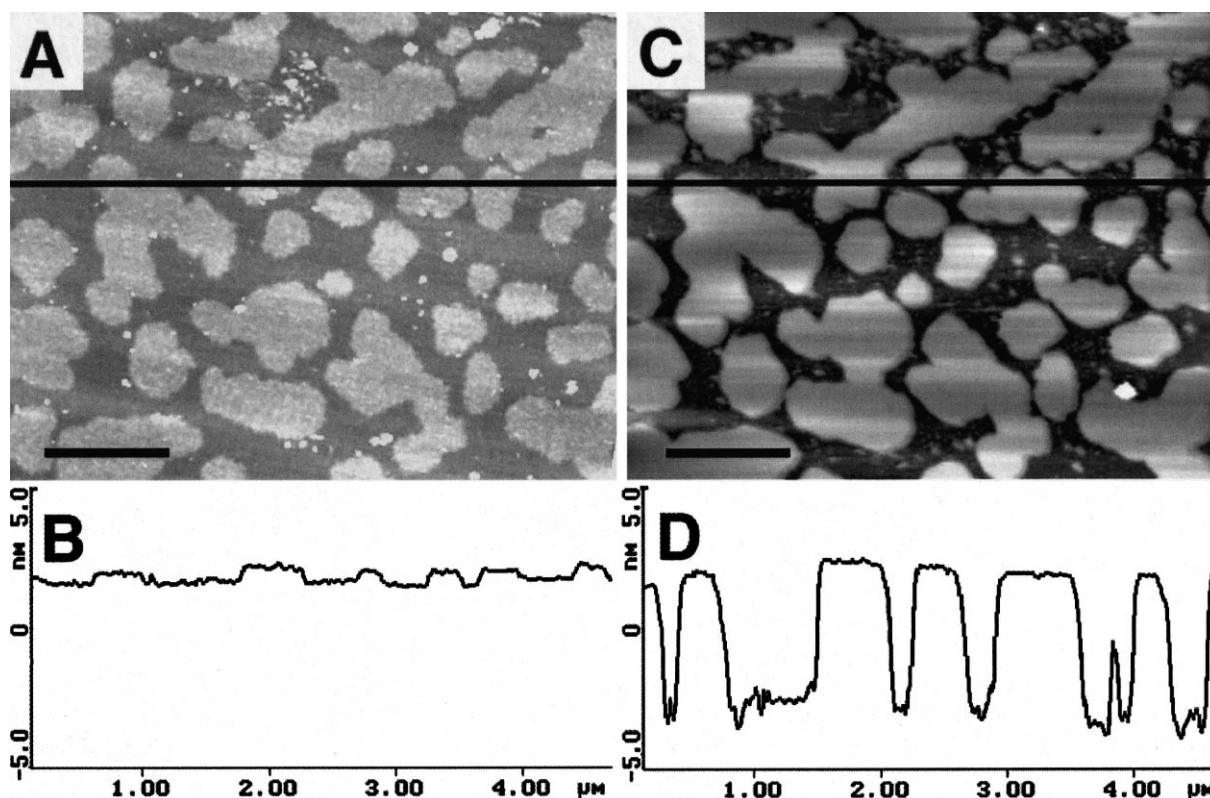


Fig. 3. Visualization of the resistance against Triton X-100. A: DOPC/SpM (1:1) bilayer containing 25 mol% cholesterol. B: Cross-section of line drawn in A showing the height difference between liquid ordered domains and surrounding bilayer is less than 1 nm. C: The same bilayer as in A, after treatment with Triton X-100 at 4°C. The domains are present, but the surrounding bilayer is dissolved. D: Cross-section of line drawn in C. The height difference between the domains and the underlying mica is 5.5 nm. Both scale bars are 1 μm , z-scale is 10 nm.

10 mol% cholesterol were also found to be resistant to detergent extraction in the cold, while the surrounding fluid bilayer was not (data not shown). The same was found for bilayers without cholesterol, illustrating that indeed also domains in the solid phase are detergent resistant [31,18]. Detergent treated bilayers with varying amounts of cholesterol were collected and their lipid composition was checked with TLC. This showed that indeed the domains were depleted of DOPC.

It has been postulated that DRMs isolated from cell membranes by detergent extraction in the cold may have been formed during cooling or by the detergent extraction itself [3]. The domains in our system after the detergent extraction (Fig. 3C) are merely slightly larger than before the detergent extraction, possibly either due to the cooling, or due to the fact that the lateral support of the surrounding fluid bilayer has disappeared, causing the domains to sag. This increase in size has caused some domains to coalesce. However, altogether the domains still have roughly the same size and shape. This means that detergent treatment in the cold hardly influences the cholesterol/SpM domains present in our model membranes and that any domain found after the treatment was already present before the treatment. Extrapolating this to biological membranes, domains found after detergent treatment of mammalian cell membranes were present as such within the membranes before the treatment.

This study shows that it is possible to directly visualize SpM/cholesterol domains and their resistance to detergent using AFM, illustrating the usefulness of this technique in raft studies. Also our results promise the possibility to directly visualize rafts in cell membranes in the near future.

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