

## Research Letter

## Imaging domains in model membranes with atomic force microscopy

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**Abstract** Lateral segregation in biomembranes can lead to the formation of biologically functional domains. This paper reviews atomic force microscopy studies on domain formation in model membranes, with special emphasis on transbilayer asymmetry, and on lateral domains induced by lipid–lipid interactions or by peptide–lipid interactions. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Model peptides; Supported lipid bilayers; Phase separation; Rafts

## 1. Introduction

Biological membranes consist of membrane proteins and lipids. The lipids are organized as a bilayer, providing a barrier between the inside and outside of a cell or organelle, and an optimal environment for membrane proteins to function in. Since in biomembranes many different protein and lipid species are present, membranes are heterogeneous mixtures in which lateral segregation could occur, leading to the formation of domains [1,2]. Indeed domains have been found to occur in membranes, the most well-known one being the separation of apical and basolateral sides of plasma membranes of epithelial cells. Another example would be the transmembrane asymmetry of most cell membranes [3]. A vexed question lately is whether specific cholesterol-containing lipid domains, which are also referred to as rafts, exist. Domain formation can arise from lipid–lipid interactions or protein–lipid interactions. Since all domains discussed so far are biologically functional, it is important to gain more insight in the mechanisms behind the formation of domains.

In this paper we discuss heterogeneities in supported model membranes, observed by atomic force microscopy (AFM). AFM can image biological samples under aqueous conditions with a high resolution (less than 1 nm, see also Müller et al, this issue, [41]), and hence is an excellent tool to study domains in bilayers [4]. We will focus on the results we obtained from transbilayer asymmetry on the formation of raft-mimicking domains and on the formation of peptide–lipid domains in supported lipid bilayers.

## 2. Supported model membranes

In order to image model membranes with AFM, they have to be deposited on a solid support or a substrate, usually mica or a hydrophilized silicon wafer. Both of these substrates are hydrophilic and negatively charged. There are two established methods to prepare a supported phospholipid bilayer [5], each with their own advantages. One method to prepare supported bilayers is the Langmuir–Blodgett (LB) method, developed by Katharine Blodgett [6]. After spreading a monolayer on an aqueous phase in a Langmuir trough, the monolayer can be deposited on the solid support by pulling this substrate up through the air–water interface, from the aqueous phase into the air. A second layer can be deposited by dipping the substrate, coated with a monolayer, through the air–water interface again, from the air into the aqueous phase [7]. With the Langmuir–Schaeffer (LS) method, the second leaflet is deposited by pushing the substrate, coated with the first leaflet, horizontally through the air–water interface. With both methods, asymmetric bilayers can be prepared.

Another method is vesicle fusion [8,9]. With this method, a droplet of vesicle suspension of lipids, possibly with incorporated peptides, is deposited on the solid support. After adsorption of the vesicles to the substrate, they spontaneously form a bilayer [10]. Due to the negative charges on the substrate, it is recommended to prepare bilayers of anionic phospholipids with this method in the presence of divalent cations [5].

Single component, symmetric phospholipid bilayers consisting of phosphatidylcholine (PC) [9,11–13] and phosphatidylethanolamine (PE) [14,15] have been imaged. Such bilayers look flat and, when they are in the solid phase, they usually contain holes. Such defects are used to measure the bilayer thickness, which is usually 5–6 nm, corresponding to the thickness of a solid phase bilayer with a water layer between the bilayer and the substrate [16]. Bilayers in the fluid phase do not contain defects due to the high in-plane mobility of the lipids [12]. An example of a solid phase dipalmitoylphosphatidylcholine (DPPC) bilayer prepared with the LB method is shown in Fig. 1A. In all the AFM images depicted in this paper, a gray-scale is used to denote the height, with black corresponding to low and white corresponding to high.

The described bilayers expose zwitterionic lipids to the aqueous phase, potentially available for interaction with membrane proteins. However, many membrane proteins interact preferentially with anionic phospholipids [17]. Annexin A5, which participates in cell processes involving membrane fu-

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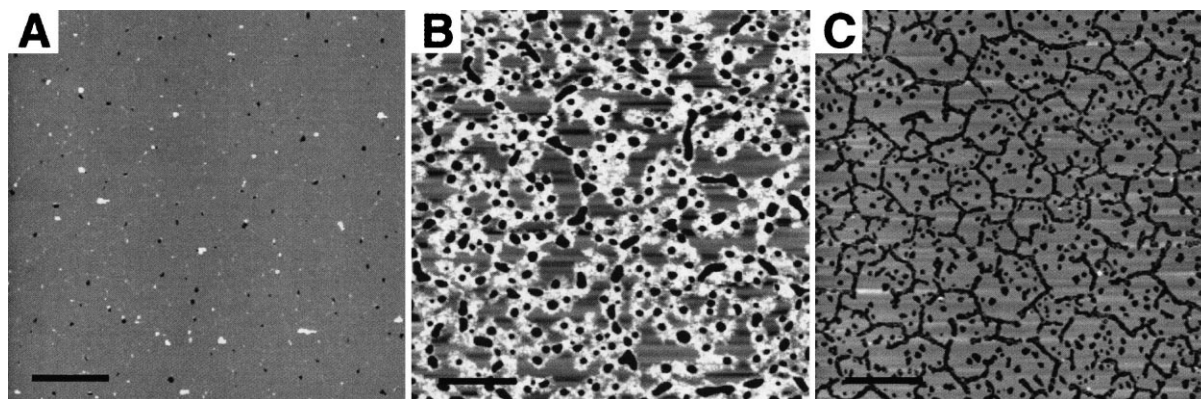


Fig. 1. Bilayers with a first leaflet of DPPC. A: Image of a symmetric bilayer with a second leaflet of DPPC, in which some contaminations on top of the bilayer (white spots) and defects (black spots) can be seen. B: In asymmetric bilayers with a second leaflet of anionic lipids (dimyristoylphosphatidylglycerol (DMPG)) in the solid phase, the defects are surrounded by elevations. C: These elevations were not present in asymmetric bilayers with a second leaflet of anionic lipids (DMPG) in the fluid phase. Scale bars are 2  $\mu\text{m}$ ; z-scale 10 nm.

sion and trafficking, is an example of such a protein. The association of annexin A5 with bilayers was imaged successfully using symmetric bilayers containing anionic lipids, made with the vesicle fusion method [18]. In this case, the required presence of divalent cations in order to prepare the bilayers was not a problem, because binding of annexin A5 is dependent on the presence of a divalent cation,  $\text{Ca}^{2+}$ . This is not the case for all membrane-associating proteins. Since divalent cations can severely effect bilayer morphology [19], it is desirable to have bilayers containing anionic lipids in the absence of divalent cations at ones disposal. The LB and LS methods offer the possibility to prepare bilayers with a first leaflet of zwitterionic, uncharged phospholipids, and a second leaflet of anionic, negatively charged phospholipids in the absence of divalent cations.

### 3. Asymmetric bilayers

Rinia et al. [20] describe the preparation and imaging of such asymmetric phospholipid bilayers, consisting of a first leaflet of zwitterionic phospholipids in the solid phase. The second leaflet consisted of anionic phospholipids in the solid phase. For all these systems, it was found that the defects, present in the bilayers, were surrounded by elevations of about 2 nm high. Fig. 1B shows an example of a bilayer with a second leaflet of an anionic phospholipid, dimyristoylphosphatidylglycerol, in the solid phase. The elevations were found to reversibly disappear in the presence of divalent cations. It was proposed that around the defects in supported phospholipid bilayers, the two leaflets are in contact. On the border of the lipid layer and the aqueous phase in the defect, lipids form a convex curvature, such that their headgroups shield the acyl chains from the aqueous surrounding in the defect [20,21]. This contact enables lipid exchange between the two leaflets. In the case of a second leaflet of anionic phospholipids, this means that negatively charged lipids end up in the first leaflet, facing the negatively charged surface. Due to electrostatic interactions, the bilayer edges around the defects are repelled and appear as elevations. As in the case of vesicle fusion using anionic lipids, divalent cations 'stick' the lipids to the substrate. The elevations were found to expand in time, and their formation was called 'bilayer blistering'. Bilayers with a first leaflet of PC and a second leaflet of anionic

lipids in the fluid phase did not show these elevations (Fig. 1C).

Other asymmetric bilayers were prepared by Czajkowsky et al. [22] to study the formation of ripple phases in these bilayers induced by components of PBS buffer. In other studies of the same authors, asymmetric bilayers were prepared to study the insertion and structure of proteins [23,24]. These bilayers consisted of a first leaflet of egg-PC, and a second leaflet of bovine heart lipids, which contain anionic lipids and are in the fluid phase, deposited by a LS-like procedure. Also, these bilayers with a second leaflet containing anionic phospholipids in the fluid phase looked smooth without elevations [23]. Furthermore, they found that the vacuolating toxin VacA, from *Helicobacter pylori*, only associated with bilayers with a second leaflet containing anionic phospholipids, at low pH.

### 4. Lateral segregation: lipid domains in supported bilayers

Apart from transbilayer heterogeneities, also lateral heterogeneities can occur in bilayers. A well-known cause of this lateral segregation is phase separation, for example fluid–solid phase separation. Bilayers in the solid phase are thicker than bilayers in the fluid phase, which implies that it is possible to visualize fluid–solid phase separation with AFM. Indeed, bilayers consisting of a DPPC mixed with a fluid phase PC or phosphatidylserine, showed higher domains, consisting of DPPC in the solid phase surrounded by lower areas, consisting of fluid phase lipid [12,19]). The height difference between DPPC and POPC was found to be 1 nm. Fig. 2 shows an example of a DPPC/POPC phase-separated bilayer, which we prepared using the vesicle fusion method. Bilayers of distearoylphosphatidylethanolamine (DSPE) and dioleoylphosphatidylethanolamine (DOPE) also showed phase separation. In this case the domains consisted of DSPE and were surrounded by fluid DOPE [25]. Giocondi et al. [26] imaged bilayers consisting of DMPC and DSPC, which at room temperature are both in the solid phase. Upon increasing the temperature, they were able to visualize the melting of DMPC, leading to phase-separated bilayers.

Also, fluid–liquid ordered phase separation can be visualized [27]. This phase separation is of particular interest since it is suspected to give rise to biologically functional domains in

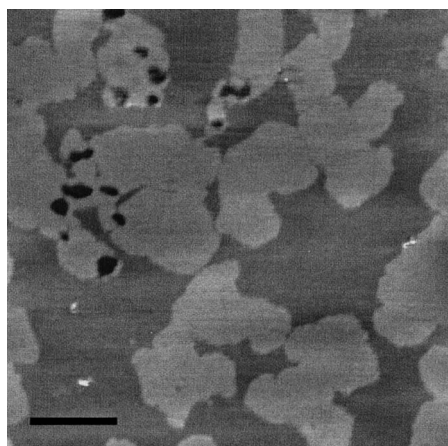


Fig. 2. Phase separation in a DPPC/POPC bilayer. The light (high) domains consist of DPPC in the solid phase, surrounded by fluid POPC. Only in the solid domains were defects present. Scale bar is 300 nm;  $z$ -scale 10 nm.

biological membranes [28]. Such domains are also referred to as rafts [29] or as detergent-resistant membranes (DRMs)[30], because they are insoluble in non-ionic detergent in the cold. Isolated DRMs have been imaged by Le Grimellec and co-workers [31]. In the liquid ordered phase, the lipids are ordered as in the solid phase, but they have a high mobility, as in the fluid phase. The liquid ordered phase can be induced by

the presence of cholesterol, which preferentially interacts with lipids with saturated acyl-chains, like naturally occurring sphingomyelins (SpM). Fig. 3A shows a bilayer consisting of a mixture of a fluid phase lipid, dioleoylphosphatidylcholine (DOPC) and a solid phase lipid, egg-SpM in a 1:1 ratio, with 25 mol percent cholesterol. In this bilayer, the higher domains consist of SpM and cholesterol in the liquid ordered phase, which are surrounded by lipids in the fluid phase. Fig. 3B depicts the cross-section of the line drawn in Fig. 3A, showing that the height difference between the domains and the bilayer is 0.8 nm, and Fig. 3C depicts the molecular organization of the lipids in such bilayers. In DOPC/SpM (1:1) bilayers, in the absence of cholesterol, pure SpM domains appeared 1 nm above the level of the fluid monolayer. However, intermediate heights were also observed, indicating that the two leaflets in the bilayer were decoupled. In the presence of cholesterol, such intermediate heights were not observed, demonstrating that the domains are coupled through both leaflets. Hence, it was suggested that cholesterol can induce bilayer coupling [27].

Also, the resistance against detergents of these domains could be visualized. This is illustrated in Fig. 3D, which depicts the same area as in Fig. 3A, after treatment with Triton X-100 at 4°C. Fig. 3E shows the cross-section of the line drawn in Fig. 3D, showing that the height of the domains is now 5–6 nm. Thus, the fluid bilayer has been dissolved, revealing the underlying substrate, but the liquid ordered domains are still present, as depicted in Fig. 3F.

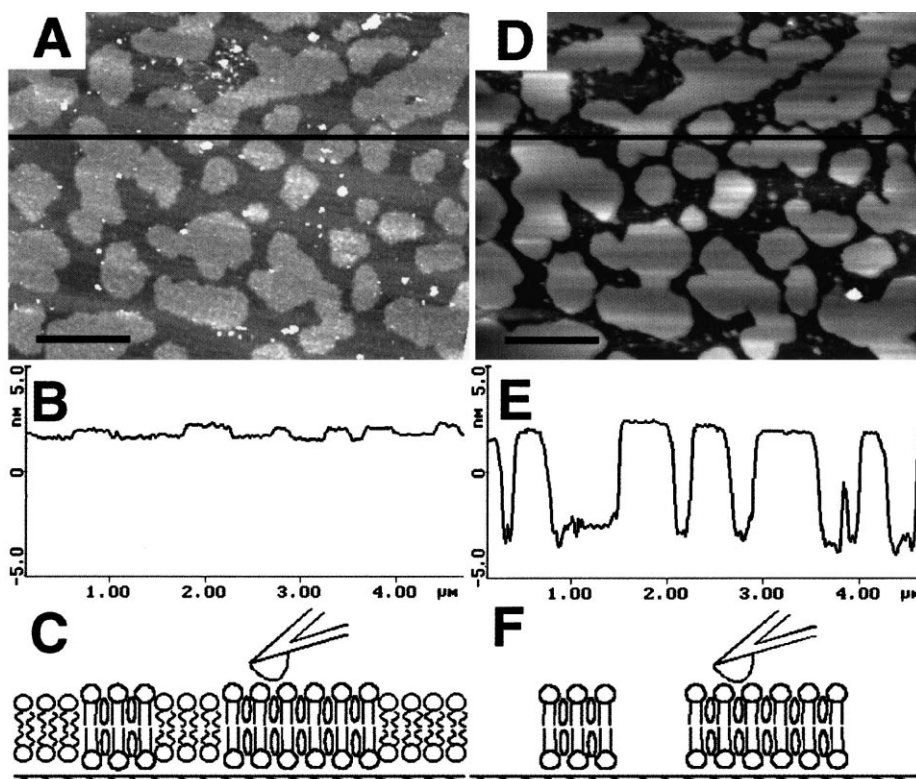


Fig. 3. Visualization of detergent resistant domains. A: Bilayer of SpM and DOPC (1:1) with 25 mol percent cholesterol. The high domains consist of SpM and cholesterol, surrounded by fluid lipids. B: Cross-section of the line in (A), showing that the height difference between the domains and the surrounding bilayer is 1 nm. C: Molecular organization of the bilayer depicted in (A). D: The same bilayer as in (A) after treatment with Triton X-100 at 4°C. The fluid bilayers have disappeared, but the SpM-cholesterol domains are still present and have nearly the same shape and size as before the detergent treatment. E: Cross-section of the line drawn in (D), showing that the height of the domains is now 6 nm. F: Molecular organization of the bilayer after treatment with detergent, shown in (D). Scale bars are 1  $\mu$ m;  $z$ -scale 10 nm.

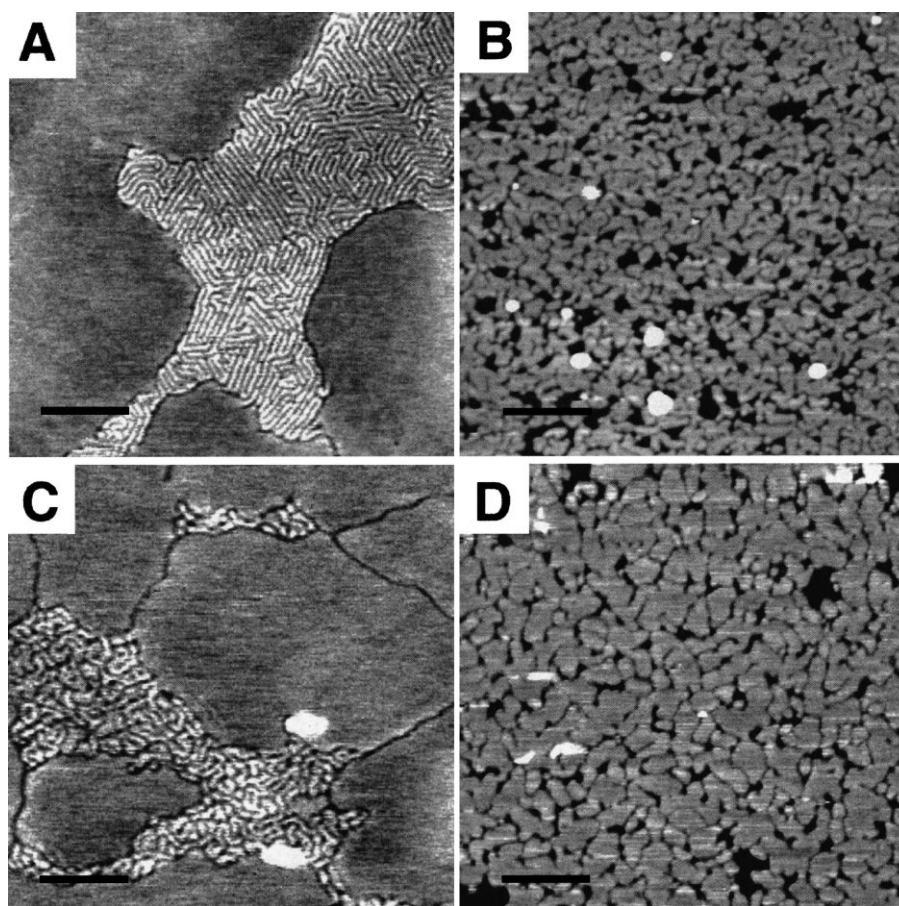


Fig. 4. DPPC bilayers with 2 mol percent model peptides incorporated. A: WALP23, with tryptophans as flanking residues, induces line-type depressions and striated domains exhibiting an ordered pattern. B: KALP23 has positively charged lysines as flanking residues and induces irregularly shaped depressions. C: HALP23 at pH 8 has uncharged histidines as flanking residues and also induces line-type depressions and striated domains. D: At pH 5, histidine is positively charged, and at this pH HALP23 induces irregularly shaped depressions. Scale bars for (A) and (C) are 100 nm; (B) and (D) 200 nm; z-scale 3 nm.

## 5. Peptide–lipid domains in supported lipid layers

Not only lipid–lipid interactions, but also protein–lipid interactions, can lead to the formation of lateral domains. A well-known example of naturally occurring domains, highly enriched in proteins is the purple-membrane patch. This two-dimensional crystal of bacteriorhodopsin has been extensively studied with AFM [32]. There are also several AFM studies on domain formation in model systems, induced by proteins or peptides. They comprise monolayers or bilayers with surfactant proteins, amphipathic peptides, gramicidin A or transmembrane model peptides.

Pulmonary surfactant is a protein–lipid mixture present in mammalian lungs, where it maintains the desired surface pressure in the alveoli during breathing. Monolayers consisting of DPPC, DPPG and surfactant protein C (SPC) have been studied by fluorescence microscopy and AFM [33]. At low surface pressure they showed three distinct phases: a smooth one consisting of lipid, a protein rich phase, consisting of filaments, and an intermediate phase. Also, monolayers consisting of DPPC, DPPG and SPB have been studied [34]. SPB-containing monolayers also show a smooth lipid phase and an intermediate phase, but no protein-rich phase. SPB was found to fluidize lipid monolayers and to reduce the solid lipid domain size. Also, SPC was found to induce a reduction in lipid

domain size in monolayers [33] and in bilayers [35]. A reduction in solid domain size is believed to render the monolayer collapse in the lungs more reversible and flexible, which is necessary to facilitate breathing [34].

In another monolayer study, DOPC films were imaged containing various amounts of a synthetic amphipathic peptide, which is used to study the cellular uptake of drugs [36]. At low molar fractions, this peptide was found to induce the presence of small (12 nm), round particles, while at high molar ratios long, thin (10 nm) filaments covered the monolayer. Interestingly, intermediate concentrations were found to induce phase separation, yielding domains, which appeared about 1 nm above the level of the bilayer. From this study, it is not clear what phase these higher domains are in.

Janshoff et al. [37] found that an  $\alpha$ -helical, amphipathic, virus-derived peptide induced irregularly shaped lower areas in DPPC bilayers. The amount of lower area increased with increasing peptide concentration, until at 0.5 mol percent, most of the bilayer appeared low, surrounding higher, solid domains. With support from other techniques, it was concluded that the lower area consisted of interdigitated lipids.

In the latter system, the peptides are suspected to be oriented parallel to the plane of the lipid layer. However, many membrane proteins span the membrane and are oriented nearly perpendicular to the plane of the bilayer. Gramicidin

A (GA) is a small channel forming protein, which spans the bilayer as a dimer, one monomer in each leaflet, oriented perpendicular to the plane of the membrane. This protein has been incorporated in PC bilayers with saturated acyl chains of varying length [38]. In DPPC bilayers with 1 mol percent GA, line-type depressions were present, and at 2 mol percent, higher domains were formed, in which point-like and line-type depressions were present, forming a distinct polygonal pattern. At 5 mol percent GA, the whole bilayer was covered by point-like depressions and interconnected line-type depressions, with a pattern similar to the one of the domains described for 2 mol percent. The average width of the depressions was found to be 3.7 nm, while the average shortest distance between two depressions was 4.6 nm.

GA forms a  $\beta$ -helix. In a similar study, aggregation of synthetic,  $\alpha$ -helical, transmembrane model peptides in PC bilayers was imaged [39]. These so-called WALP peptides consisted of an alternating alanine–leucine (AL) stretch, forming a hydrophobic  $\alpha$ -helix, flanked by tryptophans (W). A WALP peptide consisting of 23 amino acids in total (WALP23) was incorporated in DPPC bilayers at different peptide concentrations. At 1 mol percent it was found to induce line-type depressions, as described for the GA-containing bilayers, but in this case, already at this low concentration, small higher domains were present, containing line-type depressions. Upon increasing the peptide concentration, these domains increased in amount and size, from which it was deduced that the peptides were present in the striated domains. The larger domains showed that they consisted of high (light) and low (dark) lines, which formed an extremely ordered pattern exhibiting a three-fold symmetry (Fig. 4A). These domains were referred to as striated domains. At 10 mol percent, the whole bilayer seemed to consist of lines forming this pattern. The repeat distance of these lines, i.e. the width of a low and a high line, was found to be 8 nm. This is nearly the same value as Mou and co-workers [38] found for the width of the depressions and the shortest distance between two depressions (together 8.3 nm).

It is striking that both peptides yield such similar results, and that GA also contains tryptophans, which are, as in the case for WALP peptides, located at the hydrophobic–hydrophilic interface of bilayers. Hence, it was investigated if the tryptophans were involved in the domain formation [35]. This

was not the case since peptides with tyrosines (YALP) or phenylalanines (FALP) as flanking residues instead of tryptophans, were also found to be able to induce striated domains in DPPC bilayers. However, peptides flanked by positively charged residues like lysine (KALP) and arginine (RALP), gave rise to a completely different morphology (Fig. 4B). They induced line-type depressions and irregularly shaped lower areas, both increasing with increasing peptide concentration, hence, it was concluded that these peptides are present in the depressions and the lower areas. Peptides with histidines (HALP) as flanking residues offer the possibility to see if the change in morphology is due to the presence of positive charges. Histidines are uncharged at high pH and positively charged at low pH. Indeed, at high pH HALP peptides induced striated domains, and at low pH they induced a morphology comparable to the one induced by KALP peptides (Fig. 4C,D). This suggested that uncharged peptides are able to induce striated domains in DPPC bilayers, while positive charges are not.

The morphology of these bilayers was explained by assuming that the solid-state DPPC lipids form rigidly packed domains, in which the lipids are known to be tilted, and from which the peptides would be excluded. All the peptides have a length that is shorter than the thickness of a DPPC bilayer. The excluded peptide would be, possibly together with some fluidized lipid, present in the line-type depressions, bordering these lipid domains. In the case of uncharged peptides, excess peptide is accommodated in the striated domains. In these domains the peptides are present as one-dimensional aggregates, probably flanked by fluidized lipids, visible as the low lines. The high lines are formed by untilted DPPC lipids, which therefore appear higher than the surrounding DPPC bilayer. A model of the molecular organization of bilayers with incorporated uncharged peptides is shown in Fig. 5A as a cross-section, and in Fig. 5B as a top-view. In the case of positively charged peptides, the packing in one-dimensional aggregates is prevented due to the electrostatic repulsion between the peptides. Hence, they form, together with fluidized lipids, irregular-shaped depressions distributed over the bilayer, breaking up the bilayer in small, solid DPPC domains. Fig. 5C depicts the proposed molecular model of the organization of DPPC bilayers containing positively charged peptides as a cross-section, and Fig. 5D depicts a top-view.

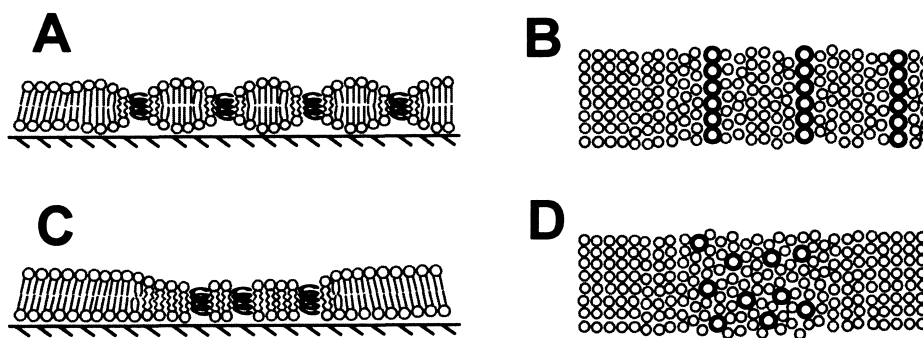


Fig. 5. Proposed molecular models of DPPC bilayers with model peptides incorporated. The lipids are depicted gray, and the peptides are depicted black. A: side-view of a striated domain, formed in DPPC bilayers containing uncharged peptides. The tilted packing of saturated PC in the solid phase (left) is disturbed by the presence of the peptides. This results in disorder and in a decrease in tilt of the lipids between the peptide arrays, hence they appear higher than the bulk gel-state bilayer. B: Top-view of a striated domain, showing that the peptides form arrays. C: Side-view of a depression in a DPPC bilayer containing positively charged peptides, which repel each other, and thus cannot be forced in arrays. They segregate with fluid-like lipids between the small gel-state patches. D: Top-view of the depression between two gel-state lipid patches.

The proposed fluidizing effect of the peptides on the lipids was supported by DSC data, and the  $\alpha$ -helical transmembrane conformation of the peptides was confirmed by circular dichroism measurements on oriented samples. The fact why the striated domains have such striking ordered patterns remains unclear. It was proposed that the geometry of the involved molecules play a role [39,40].

## 6. Concluding remarks

In this paper, we summarized the studies done on domains in model membranes using AFM. This technique has proven to be not only useful to directly visualize domains, but also to image processes related to domain formation, such as loss of asymmetry in supported bilayers, the detergent resistance of raft-mimicking domains, and the effect of peptides on the organization of lipid bilayers.

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