Research Article

SURFACE and INTERFACE ANALYSIS

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Probing peptide–membrane interactions using AFM

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Atomic force microscopy (AFM) has become a powerful addition to the range of instruments available to probe the organization of lipid monolayers and bilayers. Currently, AFM is the only tool that can provide nanoscale topographic images of supported lipid membranes under physiological conditions, enabling researchers to resolve their detailed structure and to monitor their interaction with drugs, peptides and proteins. Here, we survey recent data obtained by our research groups that demonstrate the power of the technique for exploring peptide–membrane interactions, with an emphasis on microbial lipopeptides and on tilted peptides.

Keywords: AFM; membranes; nanoscale resolution; peptides; real-time imaging; supported lipid films

Introduction

Lipid monolayers and bilayers supported on solid supports are valuable model systems to mimic biological surfaces. They are widely used in biophysical research to investigate the properties of biological membranes and processes such as molecular recognition, enzymatic catalysis, cell adhesion and membrane fusion.[1,2] Supported lipid films are made either by the Langmuir-Blodgett (LB) technique or by fusion of lipid vesicles.[3] In the first method, a Langmuir trough consisting of a rectangular Teflon bath equipped with moveable barriers is used to compress the lipid molecules at the air–water interface.[4] Lipids are usually spread at the air–water interface in hexane/ethanol or chloroform/methanol mixtures, and then compressed after solvent evaporation for 15 min. A sensor records the surface pressure at the interface, which can be expressed as a function of the interfacial area. The surface pressure *versus* area isotherms provide useful information on the packing and organization of the lipid molecules. In the LB technique, the monolayer of amphiphilic molecules is transferred at constant surface pressure and constant speed onto a solid support, usually mica. Careful control of surface pressure and lifting speed is essential to avoid artefacts such as defect formation or feature alignment of deposited structures. Lipid monolayers interact with mica through the polar heads, thus exposing the hydrophobic tails to the environment. These systems are stable in air, but not in water. Transferring a second lipid layer onto a mica-supported lipid monolayer yields a supported bilayer that better mimics cellular membranes. These supported bilayers should always be analyzed in aqueous solution since they are not stable in air.

Fusion of lipid vesicles on solid supports is another approach to obtain supported lipid bilayers.[5,6] Typically, lipids are first dissolved in an organic solvent. After solvent evaporation under nitrogen and subsequent desiccation under vacuum, the dried lipid film is resuspended in aqueous buffer solution (usually Tris or PBS) yielding a multilamellar vesicles (MLVs) suspension. From this suspension, small unilamellar vesicles (SUVs) can be obtained using various approaches, sonication being the most popular one. The suspension is sonicated to clarity (e.g. five cycles of 2 min) using a titanium probe sonicator while keeping the suspension in an ice bath, and then filtered on 200 nm mesh nylon filters to eliminate titanium particles. Fusion is achieved by heating the SUV suspension in contact with freshly cleaved mica for 1 h at a temperature between 45 and 60 °C. The supported bilayers are finally cooled gently to room temperature and rinsed abundantly with the appropriate image buffer. Compared to LB deposition, the drawbacks of the fusion method are the impossibility to prepare asymmetric bilayers composed of two layers of different nature as can be easily obtained with the LB technique, and the lack of control of the lateral pressure in the lipid layers. However, because the fusion approach is much simpler and permits lipid diffusion as in free-standing bilayers, it is the most widely used method in supported lipid bilayer research.

A variety of approaches are available to probe the structure, composition and properties of lipid films, including fluorescence[7] and Brewster angle microscopy[8]; X-ray reflection[9] and diffraction[10] methods; neutron reflectivity[11] and fluorescence recovery after photobleaching techniques.[12] Transferring lipid films onto solid substrata offers the possibility to apply a range of surface analytical techniques that could not be used to study

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real biological membranes, such as ellipsometry,[13] X-ray photoelectron spectrometry,[14] and time-of-flight secondary ion mass spectrometry.[15] However, little was known until recently about the structure and properties of lipid films at the nanometer level, due to a lack of high-resolution surface imaging techniques. In this respect, the advent of atomic force microscopy (AFM) has opened up exciting new possibilities, enabling researchers to reveal the molecular structure and nanoscale organization of supported lipid films, as well as their interaction with solvents, peptides, proteins, and antibiotics (for reviews see Refs [3,16–19]). In lipid film research, the commonly used AFM imaging mode is the so-called contact mode, in which the AFM tip is raster scanned over the sample while the cantilever deflection, thus the force applied to the tip, is kept constant using feedback control. In this mode, lateral forces which measure variations of probe-sample friction can also be detected to reveal chemical or mechanical heterogeneities of the sample. Besides contact mode, dynamic modes in which the tip is oscillated while being scanned over the surface may also be used. Because lateral forces during imaging are greatly reduced, these modes are advantageous for imaging fragile lipid films. Hereafter, we discuss recent AFM studies focusing on two classes of membrane-interacting peptides, i.e. microbial lipopeptides and tilted peptides.

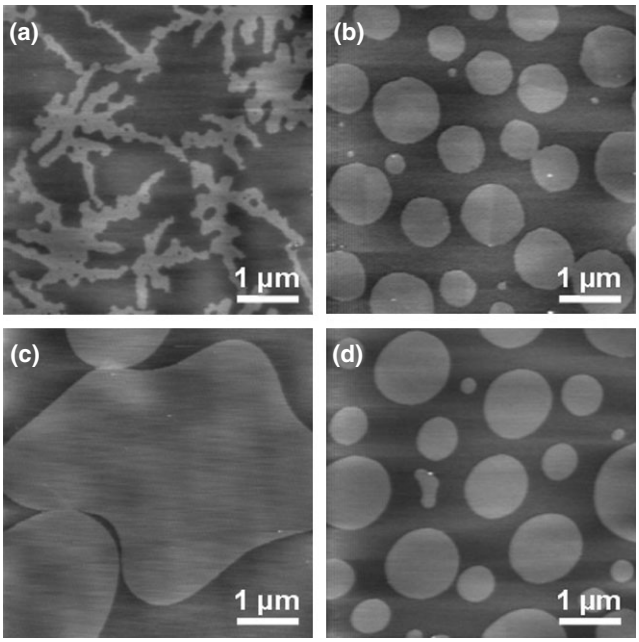

Figure 1. AFM height images (z-scale: 3 nm) in air of mixed surfactin/phospholipids (0.25 : 0.75) LB monolayers supported on mica, while varying the phospholipid chain length (a: DPPC, b: DSPC) and polar head (c: DPPE, d: DPPS). Reprinted with permission from Bouffioux *et al*.[25]

Lipopeptide–Membrane Interactions

Surfactins are surface-active lipopeptides produced by *Bacillus subtilis* strains, which are attracting more and more attention due to their high surface activity and remarkable biological properties, including antiviral and antibacterial activities.[20–24] As the biological activity of surfactin directly relies on its interaction with membranes, understanding the molecular interactions, mixing behavior and domain formation of this molecule within lipid monolayers and bilayers is an important challenge.

AFM was used in combination with molecular modeling and surface pressure-area isotherms to probe the molecular organization of surfactin-phospholipid monolayers (Fig. 1),[25] while varying the phospholipid chain length (dimyristoylphosphatidylcholine – DMPC, dipalmitoylphosphatidylcholine – DPPC, distearoylphosphatidylcholine – DSPC) and polar head group (dipalmitoylphosphatidylethanolamine – DPPE, dipalmitoylphosphatidylserine – DPPS). Topographic images showed phase-separation for all surfactin-phospholipid systems except for surfactin–DMPC, which was in good agreement with compression isotherms. On the basis of domain shape and line tension theory, it was concluded that the miscibility between surfactin and phospholipids is higher for shorter chain lengths (DMPC > DPPC > DSPC) and that polar headgroups influence the miscibility of surfactin in the order DPPC > DPPE > DPPS. Molecular modeling data showed that mixing surfactin with DPPC has a destabilizing effect on DPPC monolayers while it has a stabilizing effect toward DPPE and DPPS molecular interactions. In summary, these data emphasize the important role that phospholipid chain length (14, 16, and 18 carbon atoms) and polar head groups (large and zwitterionic PC, small and zwitterionic PE, negatively charged PS) play in modulating surfactin–phospholipid interactions.

Fengycin is another bioactive lipopeptide produced by *B. subtilis* that has received much less attention because of the difficulty to produce and to purify this molecule.[26,27] It has been demonstrated that fengycin has a strong surface activity and interesting antifungal property with low haemolytic activity, indicating that this molecule has a real potential in the pharmaceutical field, particularly in dermatology. In this context, mixed monolayers composed of fengycin and of the skin lipid ceramide were investigated using combined AFM (monolayers supported on mica) and surface pressure-area isotherms (monolayers at the air–water interface).[28] AFM topographic and friction images revealed phase-separation in mixed monolayers prepared at 20 °C/pH 2, in the form of two-dimensional (2-D) hexagonal crystalline domains of ceramide surrounded by a fengycin-enriched fluid phase (Fig. 2(a)). Surface pressure-area isotherms as well as friction and adhesion AFM images confirmed that the two phases had different molecular orientations. While ceramide formed a highly ordered phase with crystalline chain packing, fengycin exhibited a disordered fluid phase with the peptide ring lying horizontally on the support. Increasing the temperature

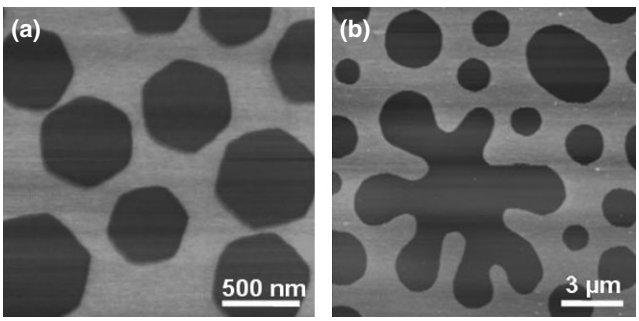

Figure 2. AFM friction images in air of mixed fengycin/ceramide LB monolayers supported on mica prepared at 20 °C/pH 2 and 37 °C/pH 5. The images reveal phase-separation in the form of two-dimensional hexagonal or circular domains of ceramide surrounded by a fengycin-enriched fluid phase and demonstrate the dramatic influence of the environmental conditions (i.e. temperature, pH) on the molecular organization of the films. Reprinted with permission from Eeman *et al*.[28]

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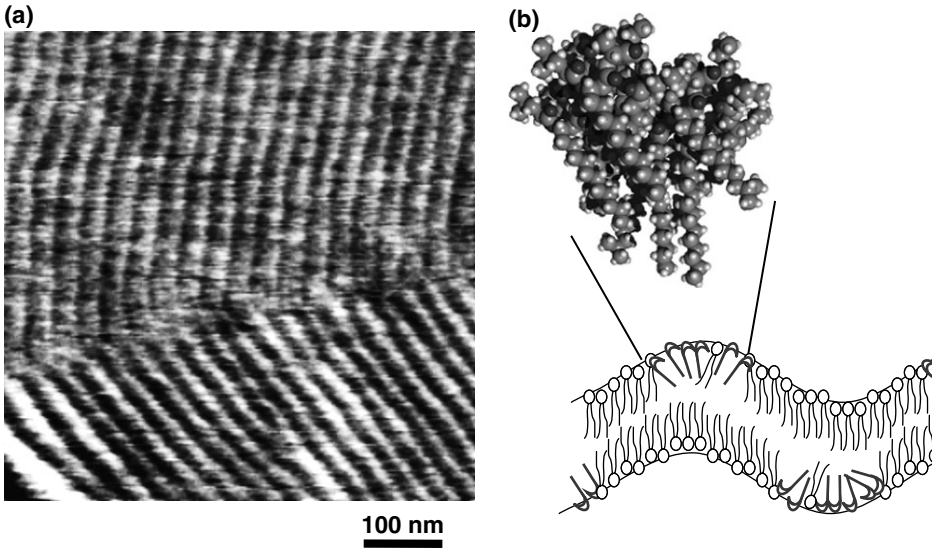

Figure 3. Imaging surfactin-induced ripple phases in lipid bilayers. (a) High-resolution height image (z-scale: 2 nm) of a mixed surfactin/DPPC (0.15 : 0.85) bilayer in Tris buffer, showing ripples structures with straight orientations that change in direction with a 120° angle. (b) Mixed assembly of interacting DPPC and surfactin molecules, indicating the formation of stable cone-shaped structures that may promote positive curvature in the bilayer by accumulating in the concave regions of the undulations, thereby favoring the ripple phase. Reprinted with permission from Brasseur *et al.*[29]

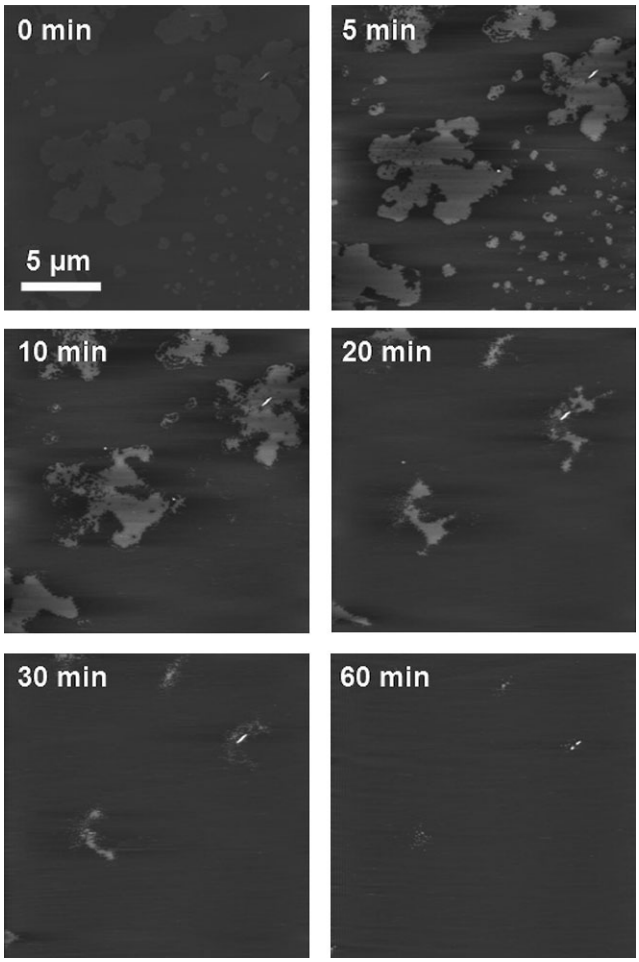

Figure 4. Real-time monitoring of the interaction of surfactin with supported lipid bilayers. AFM topographic images (z-range: 20 nm) of a DOPC/DPPC (1 : 1) bilayer recorded in Tris buffer prior (0 min) and after (5, 10, 20, 30, and 60 min) addition of a 1 mM surfactin solution.

and the pH to values corresponding to the skin parameters, i.e. 37 °C/pH 5, was found to dramatically affect the film organization, the hexagonal ceramide domains transforming into round-shaped domains (Fig. 2(b)). At higher fengycin concentration these domains were shown to melt into a continuous fengycin/ceramide fluid phase. Consistent with this, the monolayer properties at the air–water interface supported the formation of complexes between individual fengycin and ceramide molecules. These results indicate that fengycin is a natural surface-active agent with strong membrane activity, thereby confirming its potential in pharmacology.

Notably, real-time AFM imaging allowed us to visualize the interaction of lipopeptides with supported lipid bilayers, thereby providing novel insight into the structuring/destructuring effects of these molecules. For instance, surfactin was shown to induce nanoripples of 30 nm periodicity in DPPC bilayers at 25 °C, i.e. well below the pretransition temperature of DPPC (Fig. 3).[29] While most undulations formed straight orientation of ripple phases with characteristic angle changes of 120°, as previously observed by AFM,[30–33] some of them also displayed unusual circular orientations. Ripple structures were formed at 15% surfactin, but were rarely or never observed at 5 and 30% surfactin, emphasizing the important role of surfactin concentration. Theoretical simulations corroborated the AFM data by revealing the formation of stable surfactin/lipid assemblies with positive curvature. To our knowledge, these are the first data revealing the formation of a ripple phase induced by a lipopeptide.

We also showed that injection of surfactin on preformed DOPC/DPPC bilayers dramatically alters their nanoscale organization in a concentration-dependent manner. Below the critical micelle concentration (CMC), surfactin caused a time-dependent erosion of DPPC gel domains, leaving the DOPC phase unaltered, while above the CMC the lipopeptide led to an immediate solubilization of the DOPC phase and progressive erosion of the remaining DPPC domains (Fig. 4). These findings may be of great biological relevance since the bioactive properties of surfactin

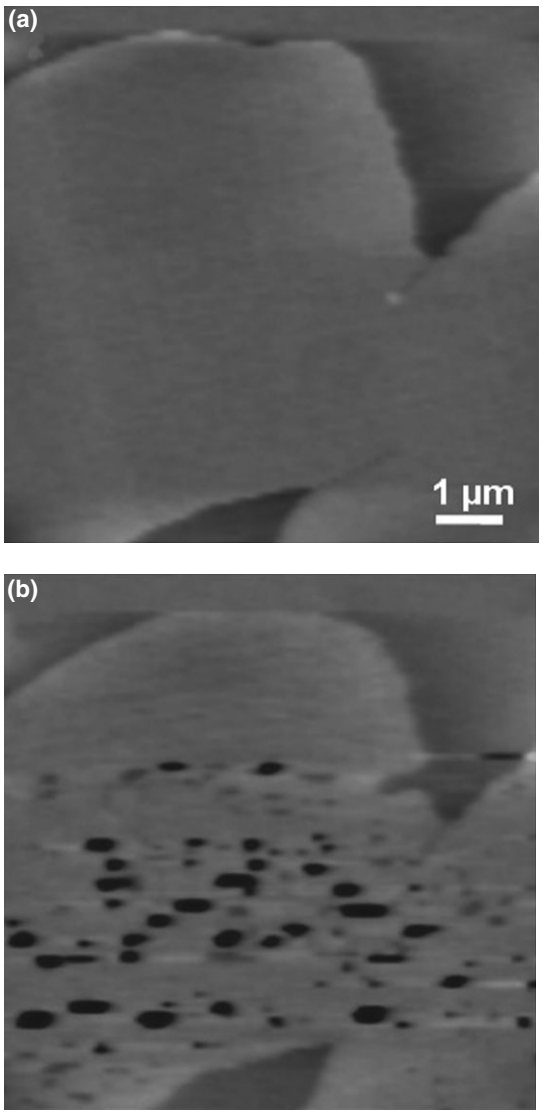

Figure 5. Interaction of the SIV tilted peptide with supported DOPC/DPPC bilayers. AFM height images (z-scale : 10 nm) of a mixed DOPC/DPPC (1 : 1) bilayer recorded in Tris/EDTA buffer (a) and in Tris/EDTA buffer containing the SIV peptide at 10 µM (b). Incubation with SIV lead to the rapid appearance of nanometer scale holes within DPPC gel domains. Reprinted with permission from El Kirat *et al.*[38]

are thought to involve membrane permeabilization by so-called detergent-like action.

Tilted Peptide–Membrane Interactions

Elucidation of the molecular mechanism leading to biomembrane fusion is a challenging issue in current biomedical research in view of its involvement in controlling cellular functions and in mediating various important diseases.[34] Many fusion events are known to involve the active participation of hydrophobic peptides, which help destabilizing the membrane lipid bilayer. Tilted peptides[35–37] represent a special class of fusogenic peptides in many membrane-interacting proteins such as viral fusion proteins, neurotoxic proteins and proteins involved in lipoprotein metabolism. These short peptides (10–20 residues) have a hydrophobicity gradient that runs along the axis of their helical structure. Hence, not only are they amphipathic but their hydrophobicity increases from one end of the helix to the other, a property that causes them to insert at an angle of 30°–60° at hydrophobic/hydrophilic interfaces. Despite the vast body of literature that has accumulated on tilted peptides, little is known about the molecular mechanisms underlying their interactions with lipid membranes.

Using *in situ* AFM, we demonstrated that the simian immunodeficiency virus (SIV) peptide induces stable nanoholes in lipid bilayer domains (Fig. 5).[38] Incubation of preformed DOPC/DPPC bilayers with SIV lead to the rapid appearance of nanometer scale holes within DPPC gel domains, while keeping the domain shape unaltered. This behavior was attributed to a local weakening and destabilization of the DPPC domains due to the oblique insertion of peptides and was directly correlated with the fusion activity of the peptide as determined using fluorescent-labeled DOPC/DPPC liposomes. By contrast, nontilted control peptides did not promote liposome fusion and did not induce holes, emphasizing the important role played by the tilted character of the peptides. The local destabilization and desorption of bilayer nanopatches may be of great biological relevance in that they may represent a key event leading to membrane fusion.

According to the generally admitted stalk mechanism described for membrane fusion, negatively curved lipids may play a central role during the early steps of the process. In this context, AFM enabled us to elucidate the influence of the negatively curved lipid dioleoylphosphatidic acid (DOPA) on the ability of SIV fusion peptides to perturb the organization of lipid bilayers.[39] To this end, mixed DOPC/DPPC bilayers containing 0.5% DOPA were imaged in real-time in the presence of the peptide (Fig. 6). At short incubation time, we observed a 1.9 nm thickness reduction of the DPPC domains, reflecting either interdigitation or fluidization of lipids. At longer incubation times, these depressed DPPC domains evolved into elevated domains, composed of nanorod structures protruding several nanometers above the bilayer surface and attributed to cylindrical reverse micelles. Such bilayer modifications were never observed with nontilted peptides. These *in situ* AFM images clearly demonstrate that negatively curved lipids play a key role in promoting modifications of membrane phases and structures by the SIV peptide.

In summary, the data reviewed here point to the power of AFM for imaging peptide–membrane interactions at high resolution. AFM images demonstrate that lipopeptides and tilted peptides are able to perturb the membrane organization in very different ways: formation of monolayer domains, promotion of ripple phase, solubilisation by a detergent-like action, induction of stable nanoholes and creation of reverse micelles. These nanoscale investigations provide novel insight into the molecular basis of events such as miscibility, domain formation, permeabilization and fusion, and offer exciting prospects in biomedicine for testing the membrane activity of peptides and drugs. Since most AFM studies have focused on pure lipid membranes, a crucial challenge for future research will be to extend these investigations to more biologically relevant membranes that incorporate proteins and carbohydrates.

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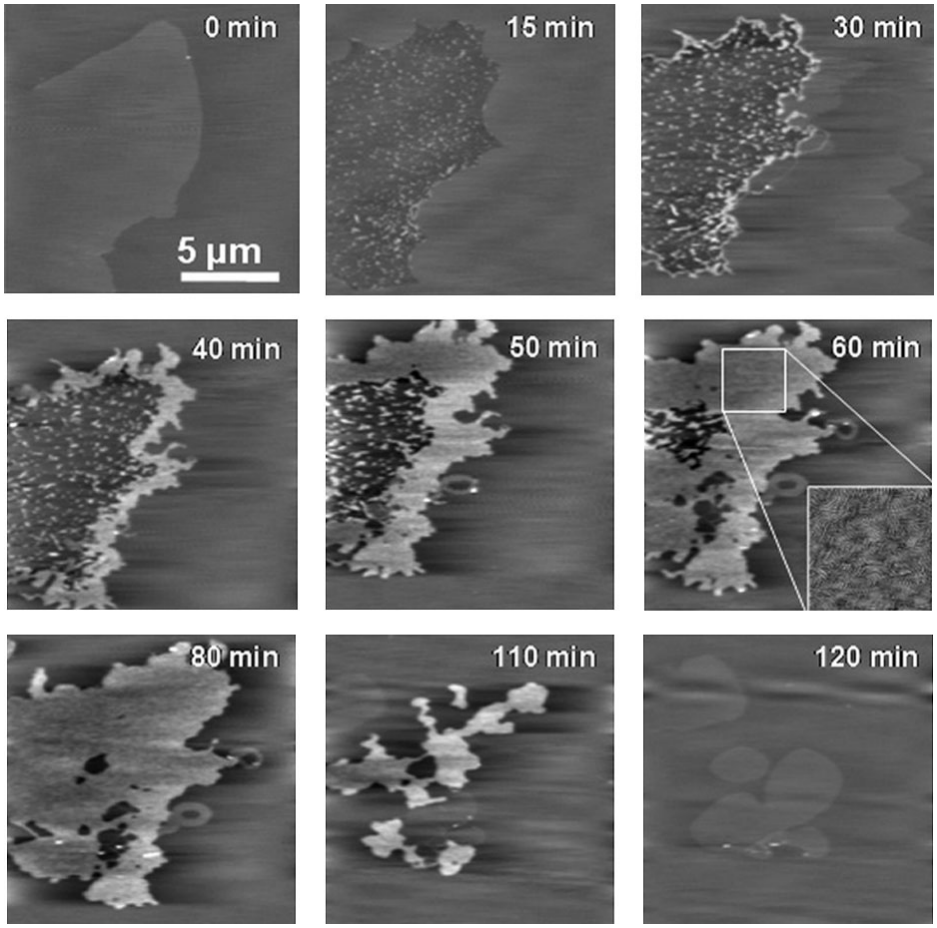

Figure 6. Interaction of the SIV tilted peptide with supported DOPC/DPPC/DOPA bilayers. AFM height images (z-range: 10 nm) of a DOPC/DPPC/DOPA (495 : 500 : 5) bilayer recorded in Tris/EDTA prior (0 min) and after (15, 30, 40, 50, 60, 80, 110, and 120 min) addition of a 10 µM SIV peptide solution. The inset (60 min) is a higher magnification (3 µm × 3 µm; z-range: 2 nm) of the white box region that reveals nanostructures attributed to cylindrical reverse micelles. Reprinted with permission from El Kirat *et al*.[39]

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