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SURFACE TOPOGRAPHY OF MEMBRANE DOMAINS

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ABSTRACT

Elucidating origin, composition, size, and lifetime of microdomains in biological membranes remains a major issue for the understanding of cell biology. For lipid domains, the lack of a direct access to the behaviour of samples at the mesoscopic scale has constituted for long a major obstacle to their characterization, even in simple model systems made of immiscible binary mixtures. By its capacity to image soft surfaces with a resolution that extends from the molecular to the microscopic level, in air as well as under liquid, atomic force microscopy (AFM) has filled this gap and has become an inescapable tool in the study of the surface topography of model membrane domains, the first essential step for the understanding of biomembranes organization. In this review we mainly focus on the type of information on lipid microdomains in model systems that only AFM can provide. We will also examine how AFM can contribute to understand data acquired by a variety of other techniques and present recent developments which might open new avenues in model and biomembrane AFM applications.

I. Introduction

Elucidation of biomembranes molecular structure remains a major challenge for cell biology. As for simpler biological objects, like soluble proteins, understanding of structure-function relationships in cell membranes represents an essential step in the development of drugs directed not only against pathologies resulting from membrane dysfunction but also against all the intracellular processes which require drugs internalization. In plasma membrane, the slow transmembrane movement of most polar lipids, in particular in absence of energy source (flip-flop), has early allowed to establish, via chemical labelling or enzymatic treatment, their distribution between the cytoplasmic (inner) and external (exoplasmic, outer) membrane leaflets [1]. In contrast, the fast flip-flop of cholesterol (Chol), in the second time scale [2], has so far led to contradictory results about its transmembrane distribution, a situation expected for other neutral lipids. For the same kinetic reasons, the in plane dynamics of membrane constituents is responsible for our limited knowledge of the membrane lateral molecular organization. Even in a gel phase, the slowest rate for a freely diffusing single phospholipid is still > 100 nm^2sec^{-1} (Fig.1) [3, 4]. For the last three decades, existence of membrane microdomains resulting from either lipid-lipid, lipid-protein or protein-protein interactions [5-11] was the object of animated debates. Thermotropic transitions starting around room temperature and ending between ~39 and 42°C were early characterized either in purified apical brush border membranes (BBM) from renal and intestinal epithelial cells [12-14], or in situ [15], by differential scanning calorimetry (DSC), fluorescence polarization and electron spin resonance (ESR). These data strongly suggested that a sphingolipids-dependent [16] lipid phase separation could account for the existence of a category of membrane domains. The biochemical and biophysical characterization of

detergent resistant plasma membrane fractions (DRMs) isolated from MDCK cells [17, 18] and the concept of rafts, a category of microdomains enriched in sphingolipids (SL) and cholesterol (Chol) [8], as functional membrane platforms controlling a large variety of cell functions [19], have generated a huge amount of work on membrane heterogeneity in a great number of cell types. Determination of the lipid composition of the first DRMs led to a SL/Chol/glycerophospholipids (GPL) molar ratio close to 1/1/1 (Table 1) [17]. With this Chol concentration, DRMs membrane lipids are expected to be in a liquid ordered phase (L_o) (Fig.1). The L_o phase is formed by the interaction of phospholipids with Chol [20-22]. It is characterized by a high degree of acyl chains order associated with lateral diffusion properties close to those determined for lipids in the liquid-crystalline or fluid phase (L_α or L_d for lipid-disordered) where the acyl chains are kinked and loosely packed. For lipids in the gel phase ($L_{\beta'}$ or s for solid), acyl chains are even more ordered than in the L_o phase but lateral diffusion (D) is much slower (Fig.1). The formation of Chol-enriched domains would be driven by a L_o-L_α phase separation process in which L_o SL/Chol-enriched lipid domains are surrounded by a fluid L_α matrix enriched in more unsaturated GPL species. Despite all this information on various membrane domains, many questions about microdomains like the existence of families of domains sharing the enrichment in SL and Chol but with large individual variations in acyl chains and polar headgroup composition, the coupling between the exoplamic and cytoplasmic leaflets, their kinetics of formation, lifetime, and their size range(s), remain open. Thus, domains in the micrometer range were reported using fluorescence photobleaching recovery (FRAP), fluorescence digital imaging microscopy and single molecule fluorescence microscopy [23-25], while sizes ~ 20 nm [26, 27] or < 5nm [28] for domains associated with glycosylphosphatidylinositol (GPI) anchored proteins were estimated using the photonic force microscopy (PFM), stimulated emission depletion (STED) far field fluorescence microscopy and fluorescence resonance energy transfer (FRET)

methods, respectively. Difficulty in characterizing lipid-dependent domains of cell membranes is not surprising considering that the phase behaviour of lipid mixtures is still poorly understood [29]. Elucidating the properties of these mixtures with, or without, inserted peptides or proteins, is therefore of primary importance, even considering that in model membranes lipid domains are at, or close to, equilibrium which contrasts with the transient non equilibrium structures found in biomembranes [30]. The obvious first step is the study of Langmuir/Langmuir Blodgett (LB) monolayers properties [31] since a biomembrane can be considered as two "weakly" coupled monolayers (see however [32]) and because in plasma membranes SL, a key constituent of SL/Chol-enriched microdomains, is practically exclusively found in the exoplasmic leaflet. The next step is the characterization of lipid mixtures in bilayers which, since Gorter and Grendel [33], has followed the development of new tools as well as of new models, from supported bilayers to small (SUV), large (LUV) and, more recently, micrometer size giant (GUV) unilamellar vesicles [34]. Among these new tools, atomic force microscopy (AFM) [35] has become very popular in surface science by giving access, in air or in liquid, to topography at a molecular scale. This holds true for soft surfaces where the AFM high resolution of ~1nm in lateral and ~0.1 nm in the vertical direction was initially applied in P.Hansma laboratory to image phospholipids in LB and supported lipid bilayer (SLB) model membranes [36, 37]. Lipid domains in various binary and ternary mixtures under phase separation, containing or not inserted peptides/proteins, were thus later imaged in LBs and SLBs [38-42]. Recent reviews have exposed numerous AFM data obtained these last years both on the formation of solid-supported bilayers and on the imaging of domains in various lipid mixtures [43-47].

In this review we will essentially focus on the type of information on model systems that only AFM can provide. We will then examine how AFM can contribute to our understanding of data obtained on similar samples by lower resolution techniques. Some of

the intriguing properties of SLBs microdomains revealed by AFM and the new questions on membrane-substrate relationships they raise will be discussed in a next section. Finally, we will present AFM recent developments which might open new avenues in model and biological membrane applications of AFM imaging.

II AFM characterization of lipid films and membrane hemi-leaflets.

IIa) Single lipid and binary mixtures of lipids.

Langmuir and LB films have been extensively used to study the properties of monolayers as models for membrane biophysics [48-50]. Following fluorescence microscopy and FRAP methods [49, 51-53], total internal reflection fluorescence (TIRF) [54], Brewster-angle microscopy (BAM), x-ray and neutron scattering techniques, Fourier transform infrared (FTIR) spectroscopy, and polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) [55, 56] were applied to monolayers studies. Together, these techniques have provided invaluable data of liquid expanded (LE) and liquid condensed (LC) phases in monolayers and their dynamic properties down to the microscopic scale (for a detailed description of structure and phase transitions in Langmuir monolayers see [50]). In many experiments, planar supported lipid monolayers prepared from vertical Langmuir-Blodgett (LB) or horizontal Langmuir-Schäfer (LS) transfers from the air-water interface to a hydrophobic silanized glass slide [48, 57] have been examined under aqueous buffer. The transfer was performed at a chosen surface pressure which, when modelling biological membranes, was generally comprised between 30 and 40 mN/m [58, 59]. *Because the dynamics properties of samples are essentially maintained under these transfer conditions, FRAP, total internal reflection fluorescence microscopy (TIRF)[60, 61] and fluorescence*

correlation spectroscopy (FCS) [62] have been applied to study lipid-lipid, lipid-protein interactions and properties of model rafts [63, 64]

In contrast with the experiments mentioned above, upward transfer of lipid films onto hydrophilic substrates like mica, quartz and glass resulting in the exposure of acyl hydrophobic chains to air has been so far practically exclusively used for AFM analysis (Fig. 2). *}. It is worth noting that the use of fluorescently labelled phospholipid films presenting large phase separated domains showed that the microscopic monolayer topology was preserved for transfer pressures greater than 10 mN/m [49, 65]. Thus, although details of molecular arrangement might be affected, the difference in packing density before and after transfer is generally negligible for molecules closely packed before the transfer from water interface is accomplished [66]* [67]. *For lower surface pressures, transfer of monolayers from the LE phase or in the LE/LC coexistence region often results in the formation of a substrate (and pH)-dependent close-packed domains in the corresponding LB films [66, 68-70](however see [71, 72]).* AFM, and more generally scanning probes techniques, have given access to the missing essential information on monolayers organization at the mesoscopic and eventually molecular scales [36, 73, 74]. For these highest resolutions imaging, care has to be taken to use scanning forces as low as possible to prevent orientational ordering of structures by the AFM tip [75]. Sub-micrometer organization of phase-separated fatty acids in the LC-LE coexistence region and corresponding determination of local mechanical and tribological properties early illustrated the usefulness of AFM in the characterization of heterogeneous soft surfaces [76, 77]. As illustrated by Fig. 2A, AFM images of films made of a single phospholipid species in the LE phase, transferred at 32 mN/m, show a homogeneous surface of low roughness (< 0.4 Å for 1-palmitoyl-2oleoyl-sn-glycero-3-phosphocholine, POPC). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) films transferred in the LC phase at the same surface pressure also exhibit a low roughness (~0.6 Å) but with the presence of line

defects (0.1-0.5 Å depth, 15-20 nm in width) at their surface (Fig. 2B, arrows), which might correspond to a border between different domains, that only the incredible resolution of the AFM can reveal. Transferring DPPC films labelled by a fluorescent phospholipid probe, at a surface pressure where LC-LE coexist, demonstrated the existence of both large and mesoscopic LC domains and indicated that the presence of the dye reduced significantly the total amount of the LC phase [78]. Analysis of natural sphingomyelin (SM) films, the major SL found in eukaryotic cells, under identical transfer pressure conditions (Fig. 2C), clearly demonstrates the existence of a marked heterogeneity of the surface, with darker zones generally less than 200 nm in size and ~ 1 nm lower than the lighter surface. The presence of such domains in the film which, according to DSC thermograms of SM [79], correspond to LE regions surrounded by LC zones, would not be detected by optical techniques including fluorescence microscopy and their topographical details would escape all other analysing techniques. For SM, the height difference between domains can be explained by the fact that the shortest or unsaturated disordered acyl chains are in the LE phase. In the most common used contact mode for AFM imaging, the tip remains continuously in contact with the sample during the raster scan of the surface [80, 81]. Consequently, the relative height of surface structures can also be affected by the scanning force applied during scanning, which has to be minimized, and the local mechanical properties of the sample [82]. It is worth noting that when imaging in air under ambient conditions strong adhesion forces between the tip and the film, linked to water condensation at the air/sample interface, impose the use of scanning forces significantly higher than those required for imaging under liquid. Before imaging, samples are often kept in a dry cabinet or maintained in the laboratory atmosphere where relative humidity (RH) is generally less than 50%, a value sufficient to block the lateral diffusion of lipids in films transferred onto polymer cushions. For films transferred onto glass, the lateral diffusion is at the lower resolution limit of FRAP technique even at 90% RH [83].

Accordingly, in contrast with films exposing their polar head group to water, AFM in air gives topographical information on films where lateral diffusion is blocked. Immersion of the monolayer in water increases the diffusion coefficient by two orders of magnitude and the film remains stable even though the hydrophobic tails of the lipid molecule are exposed directly to water [83, 84], allowing AFM imaging [85]. Frictions forces between the tip and the sample [86], as well as energy dissipation which reports on local viscoelastic properties when using AFM oscillating modes, can also probe the existence of phase separation in monolayers in an original way [82, 87]. So far, however, the friction and viscoelastic signals are affected by the topography signal and, for lipid films, give only qualitative information. Moreover, improvement of the imaging quality by reducing the scan force is associated with minimizing friction in contact and phase shift in oscillating modes. AFM characterization of LC-LE coexistence regions in LB films made of single phospholipids species has been applied to various binary and ternary lipid mixtures and has established the usefulness of this technique for the detection of membrane domains ranging from the nanometer to the micrometer scale. Figure 3 illustrates an example of contact mode AFM imaging of a LB film of made of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC,LE)/DPPC (LC) now commonly used in studies on phase-separated binary mixtures, because their respective transition temperature of – 20 and 41 °C insures a large zone of order-disorder phase coexistence [88-92]. A large scan, at the AFM scale, shows the presence of two categories of light domains protruding by ~0.6 nm from a darker matrix (Fig. 3A). The larger ones, ~1 to 2.2 µm in lateral size, could have been detected by optical microscopy. This is not the case of the abundant smaller domains ~100-400 nm in size. The taller larger domains exhibit irregular, often linear and angular boundaries (white arrows), indicating they correspond to LC phase domains. Imaging of two other samples confirms the general characteristics of the mixture, showing the coexistence of large and small domains, but also indicates that their

form and relative size can vary (Fig. 3B&C). Decreasing the scan size demonstrates that angular boundaries are also found in smaller domains (Fig. 3D, white arrows). Local variations in friction force images (black arrows) obtained on LC domains in the forward (Fig. 3E) and the backward scanning direction (Fig. 3F), not associated with significant height modification, further suggest the existence of heterogeneity in their physical state as a function of the distance from the boundary. LB films made of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)/1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) [87], DOPE/1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) [82], 1-palmitoyl-2oleoyl-sn-glycero-3- phosphoethanolamine (POPE)/1-palmitoyl-2oleoyl-sn-glycero-3-phospho-L-serine (POPS) and POPE/POPS/SM [93], DPPC/1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-*rac*-glycerol) (DPPG) [94], DPPC/ POPC/cardiolipin (CL), POPE/CL [95] and POPC/POPE [96] are among the various binary mixtures examined by AFM. In many occasions, the detailed surface structure could not be predicted from the other approaches. *For example, this is the case of the phase topography of ceramides, Chol and free fatty acids mixtures that mimic the lipid composition of stratum corneum [97, 98]*.

IIb) Lipid mixtures related to microdomains enriched in sphingolipids and cholesterol

AFM has allowed to establish the distribution of ganglioside GM1, a glycosphingolipid (GSL) which is the natural receptor for cholera toxin, between ordered/disordered two-phase monolayers [88, 99]. GSL acts as a receptor for numerous biologically active agents and its distribution in phase-separated lipid mixtures had been previously extensively investigated by various indirect methods leading to contrasting conclusions (see references in [88]). AFM examination of LE/LC DOPC/DPPC monolayers doped by physiological, low concentrations (<5%) of GM1, shows that it forms round shaped (15-30 nm) and filamentous nanodomains, preferentially localized in the DPPC-enriched LC

phase (Fig.4) which eventually fuse to form fence-like structures at the interface (black arrows). This indicates the existence of a DPPC/GM1 LC/LC immiscibility, most likely driven by hydrophobic mismatch and strongly suggests that GM1 may also form nanodomains within larger ordered microdomains. In addition, a few GM1 nanodomains also localize in small LC microdomains (white arrows). Although in some cases GM1 nanodomains seem to be located in the LE phase, it cannot be excluded they are standing on LC nanodomains (white arrows). This complex distribution at the nanoscale probably explains at least partly the difficulties encountered before in defining GM1 distribution. These studies, in agreement with preferential localization of GM1 in the ordered "rafts" domains in plasma membranes, have been extended to higher GM1 concentrations [100] and to other gangliosides [101]. Alone, AFM cannot answer the questions concerning the monomeric/aggregates state of GM1 in domains. The possibility to analyse phospholipid monolayers labelled with fluorescent probes by near-field scanning optical microscopy (NSOM) was early recognized [102]. Association of AFM with confocal and NSOM was applied to DPPC LB films and SLB [70]. Combination of AFM and NSOM in one single equipment has further allowed to demonstrate, using Bodipy-labeled GM1, that the addition of ganglioside produced significant changes in the phase-separation behaviour of the binary DPPC/DOPC and the ternary DPPC/DOPC/Chol monolayers [103]. Moreover, with the Bodipy fluorophore, monomeric and aggregated gangliosides could be distinguished, which provided new insight into the complexity of GM1 partitioning. This work also demonstrated that, independently of a lipid oxidation process which affects the size of domains [104, 105], the fluorescent label affected the partition of GM1 between LC and LE phases.

Lipid composition of detergent resistant membrane fractions (DRMs), whose relationships with native "rafts" remain a matter of debates [106-109], is close to that of the apical membrane of intestinal and renal epithelial cells (Table 1). The asymmetry of SL distribution

in plasma membranes indicates that exoplasmic leaflet of intestinal and renal cells BBM is essentially made by mixtures of GSL/Chol and SM/GPL/Chol, with a predominant fraction of SM, respectively (Table I). A SL/GPL/Chol ratio close to unity, often chosen to model cells exoplasmic leaflet, rather corresponds to the composition of the outer leaflet of non polarized human erythrocytes and platelets [32]. Accordingly, LB films made of SM/GPL ratio modelling the exoplasmic leaflet of kidney BBM were examined by AFM. Because the saturated/unsaturated fatty acid ratio of phosphatidylcholine (PC) in these membranes is close to 1 [16], POPC was chosen rather than DOPC only present as a minor PC species in biomembranes [110]. For these BBM models AFM could establish the presence of concentration-dependent LC SM-enriched nanodomains [111] which would have escaped optical detection. Neither the size nor the spatial distribution of these domains could have been obtained by other approaches. The same series of experiments strongly suggested that LC/LE lipid phase separation may occur in the renal BBM exoplasmic leaflet in the absence or upon depletion of Chol. Furthermore, Chol-induced connection of nanodomains resulting in the formation of a lipid-ordered network was also demonstrated (Fig.5).

IIc) Lipid interaction with peptides and proteins can create domains in monolayers

Numerous studies on the interactions of amphipathic peptides and water-soluble proteins with membranes start with monolayers to investigate the lipid-water interface step. To surface pressure and electric potential measurements, they associate various techniques like fluorescence and Brewster angle microscopy, vibrational spectroscopy, x-ray and neutron scattering techniques and PM-IRRAS. Here again, AFM brings unique invaluable information. For example, investigation of the interaction with monolayers of some amphipatic peptides, that act as very efficient drug carriers, has shown concentration and lipid headgroup-dependent α to β conformational transitions. AFM analysis of samples further

revealed that these transitions were associated with formation of nanofilaments and nanodomains supramolecular arrangements, providing a new interpretation of data [112] [113]. Similarly, the Human calcitonin fragment 9-32, another efficient carrier spontaneously forms supramolecular structures which looks like filaments rolled into spirals made of α-helices [114]. AFM also contributed to the understanding of interactions between puroindolines, plant lipid binding proteins with antifungal properties with wheat galactolipids [115]. This also applies to the characterization of nanoscale film heterogeneity in models of pulmonary surfactant [94, 116, 117], with an elegant experiment performed directly on an air bubble coated with the surfactant [118]. By giving access to the nanoscale organization, AFM has played a particularly important role for the understanding of LB films made of ternary and complex mixtures of biological interests. Complementarity with other recent techniques including FTIR, PM-IRRAS and mass spectrometry imaging (TOF-SIMS) [119, 120]115] will be likely scientifically rewarding for many years to come.

III AFM characterization of supported bilayers.

IIIa) Formation of SLB

Besides being a widely used model for analysing fundamental properties of cell membranes, supported planar lipid bilayers (SLB) [121] also offer unique possibilities for the development of nanobiosensors, nanomotors and nanotools [55, 122-124]. Today, formation of (SLB) is achieved using three main classes of methods. The first is based on the use of a LB film for the proximal (inner) leaflet facing the solid support. Deposit of the distal (outer)-leaflet facing the bulk solution is done using either LB or LS technique. Formation of SLB from adsorption of unilamellar vesicles, SUV, LUV and GUV [43, 125, 126] regroups the second category of methods and the third uses spin coating [127, 128]. In addition, some

protocols belong to two different classes like the LB/vesicle fusion (VF) method [129]. SLB formation from direct adsorption of detergent-phospholipid micelles was also described [130, 131].

For each class of method, AFM has brought crucial information for establishing that differences in the experimental protocol, including the temperature, the nature and roughness of supports (quartz, glass, mica, gold, alumina, SiO_2, TiO_2, ,..) covered or not by a polymer layer, the use of water or various buffers, the lipid constituents etc, can influence the bilayer properties [43]. Thus, for LB-based protocols whose major interest is in the formation of asymmetric bilayers which mimic the situation found in cells plasma membranes, the earliest AFM investigations suggested the use of a LC phase, DPPE, DSPE or DPPC, which enhances the bilayer stability, as the proximal layer in building stable bilayers on mica from LB/LB deposition processes [91, 132 , 133, 134]. Even under these most favourable conditions and despite good transfer ratios during their formation, AFM examination revealed the presence of defects and holes in these SLB that escape optical detection [132 , 135-137]. According to the fluorescence interference contrast microscopy method (FLIC), extensive loss of transmembrane asymmetry occurs during the formation of SLB by the use of LB/LS protocol. Moreover, the use of tethered polymer support for the initial monolayer does not improve lipid asymmetry in the resulting bilayer which would be only preserved using the LB/VF method [32, 138]. Contrasting with these LB data on methods initially developed to insure SLB asymmetry, vesicles fusion expected to provide symmetrical bilayers can result in the formation of asymmetrical bilayers, as a function of the support, buffer, lipid composition, vesicles size and temperature conditions used [139 , 140-142]. Although not complete, the understanding of mechanisms and parameters involved in SLB formation from lipid vesicles has strongly benefited from AFM and its association with quartz crystal microbalance dissipation monitoring (QCM-D) [43, 143 , 144 , 145 , 146]. It is worth noting that, like for

LB-based methods, optically continuous SLB formed by vesicles fusion are most often pierced by holes, even for lipids in L_α or L_o phases (Fig.6) [44, 136, 145, 147 , 148, 149]. This imposes to probe different zones of each sample with large scans for interpretation of dynamic data. Finally, it is worth noting that the same three main classes of methods can be used to prepare supported double bilayers, [128, 150-152] examined by AFM only in a few occasions [153, 154].

IIIb) AFM imaging at room temperature of domains in SLB

Like LB films data, most AFM information collected from SLB has been based on the characterization, at room temperature, of samples topographical height differences between ordered and disordered phases which depend on the acyl chains length of glycerophospholipids and sphingolipids. Various binary mixtures under L_α / $L_{\beta'}$ phase separation were first characterized on asymmetrical SLB built by LB/LB transfers where the proximal leaflet facing the support was made of a single phospholipid in a single phase. Briefly, in this configuration, the topography of SLB is close to that of LB films having the same composition, *as long as transfers were performed at the same surface pressure*. This has been established for DOPE/DSPE mixtures were the size of LC domains in monolayers corresponded to that of gel domains in the DSPE supported DOPE/DSPE bilayer [155]. Thus, like for LB films examined in air, in these SLB nanodomains are expected to coexist with the larger domains which are observable by fluorescence microscopy. It is worth noting that height difference between fluid and gel phases lipids take a single value, generally consistent with the results of X-ray diffraction studies when using low scanning forces. Large scanning forces increase the thickness differences because of different elastic properties of the two phases [156]. For these highly asymmetrical samples, no evidence for time-dependent transmembrane migration of phospholipids has been reported, which suggests that these

LB/LB bilayers were more stable than LB/LS bilayers. Besides topography, direct visualization of the local surface charge in 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) /1,2 dimyristoyl-sn-glycero-3- phospho-L-serine (DMPS) phase separated distal leaflet was obtained in aqueous solution from the phase shift in oscillating mode [157]. Using phase-separated films for both proximal and distal leaflet can result in LB/LB bilayers locally presenting three quantized thickness levels (Fig.6), as reported for DPPC [70].

Imaging of SLB made by SUV or LUV fusion has demonstrated why their complete characterisation requires AFM, even for simple binary mixtures. Taking the DOPC/DPPC 1:1 mixture as an example, AFM imaging shows that, keeping the buffer composition, the fusion temperature and the nature of the support (mica) constant, large variations exist in the size, the spatial distribution of domains, the extent of support coverage, the presence of holes, pieces of double bilayers and non-fused vesicles still adsorbed (Fig. 6 A, B, C&D). Changing mica for glass significantly modifies the aspect of the surface and enhances the heterogeneity of the topography between different zones of the sample (Fig. 6E white and dark arrows). Consequently, whereas the single step in height between the gel and the fluid domains indicates they are superimposed in proximal and distal leaflet, i.e. that coupling between leaflets is complete, when using mica as a support, it is difficult to exclude the existence of a second step in height, corresponding to asymmetric fluid/gel phase superimposition, with the glass support (see the virtual section in E). With DLPC/1,2-distearoyl-sn-glycero-3-phosphocholine DSPC mixtures in SUV, such uncoupling between leaflets can be induced by modifying the temperature of vesicles during their formation and their incubation before deposit on mica surface [140]. In the same study, time lapse study of SLB topography demonstrated that mixed symmetry lipid bilayer can convert over a period of several hours to either an asymmetric or a symmetric SLB, likely via a flip-flop at the interface of domains. The rate constant for flipping event was estimated to be ~76 h^{-1} [46, 140]. Phase coupling

between leaflets determined by AFM for a variety of binary phospholipid mixtures under gel-fluid phase separation [158] was previously observed by FRAP on DMPC/DSPC multibilayers [159] and in GUV [64, 160]. Besides differences in the size of domains and their transmembrane coupling, the behaviour of phase-separated binary mixtures made of synthetic lipids leads to rather flat structures. This is not the case for mixtures of natural SM with DOPC or DPPC which frequently adopt a variety of mesoscopic morphologies including large stripes, tightly packed small globular structures, branched-filaments decorated domains and gel-gel phase-separated systems surrounded by a fluid phase [161]. The diversity of structures imaged strongly evokes composition fluctuations corresponding to spinodal decomposition process [162-166] and also makes clear that small changes in experimental conditions can lead to large changes in the domains morphology. Various morphologies also coexist within the same sample in fluid-L_o phase-separated GUV containing SM [167], indicating that vesicles-support interactions are probably not at the origin of the topography diversity in SLB. Although much smaller than in GUV, the average size of ordered microdomains in phospholipid binary mixtures examined by AFM remains much larger than that predicted from FRAP and ESR experiments on flat multibilayers or from Monte Carlo simulations. Using similar DMPC/DSPC mixtures, the estimated size of gel domains corresponded to ~250 to 1500 molecules, i.e. less than 30 nm in diameter [159, 168, 169]. On the other hand, earliest studies using freeze-fracture electron microscopy and electron diffraction described domains in the few-hundred nanometers to a few micrometer range for liposomes made of various phase-separated binary mixtures of phospholipids [170-172]. These differences could be at least partly explained considering that the size of domains is markedly affected by the thermal history of the samples, the composition of their bathing medium [158, 173, 174] and by lipid oxidation [45, 175]. Another unique contribution of AFM to the characterization of phase-separation in binary mixtures is the study of nucleation

and domain growth process which requires the use of high resolution imaging techniques in real time. This was first achieved on DOPC/DPPC mixtures after a rapid quench in temperature from 60 (miscibility state) to 23° (immiscibility region) [176]. More recently, using new facilities for controlling the sample temperature under the microscope, nucleation rates and growth of domains were determined on DOPC/DSPC and DOPC/GalCer symmetric and asymmetric bilayers and the analysis of data further allowed to determine the values of the interfacial line tension, of the activation energy barrier and of the lateral diffusion coefficient of lipid addition to a growing domain [177, 178]. Differential resistance of phase separated binary mixtures to detergent solubilisation can be explored in situ by AFM. This has been done, for example, on DOPC/DPPC mixtures treated with Triton X-100, a detergent frequently used for DRMs preparation [89, 179]. The behaviour of other binary mixtures made of phospholipids more particularly present in identified cellular organelles like mitochondria has also been characterized [95, 180]. The vertical sensitivity of AFM provided direct evidence for alcohol or halothane-induced formation of interdigitated domains in saturated phosphatidylcholine SLBs [181, 182]. Vesicle fusion method on mica with SUV and LUV made of a single saturated PC component or equimolar DMPC/DSPC mixtures often results in the formation of large patches of supported double bilayers [154, 183]. Such double bilayers are characterized by the presence of a ripple phase which forms at the pretransition temperature upon heating from the gel phase (Fig. 7). Ripple repeat distances corresponding to the stable (13-15 nm) and metastable (26-30 nm) ripples were directly measured from images taken in solution. In these samples, the surface of the first bilayer, closest to mica, can be flat and featureless or presents wavelike structures of low amplitude at room temperature, likely as a function of the thickness of the aqueous film between the mica surface and this first bilayer. These data strongly suggest that the early AFM observation of a buffer-induced ripple phase in diC15-PC bilayers [184] was due to the presence of double bilayers. Unfortunately,

to our knowledge, there is no data on the topography of phase separated domains in hydrated supported double bilayers made by the other techniques.

In biological membranes, because of the presence of Chol as a major constituent of membrane lipids, phase diagrams strongly suggest that microdomains formation results from L_o-L_d and, eventually, gel-L_o-L_d phase-separations [185, 186]. Uncertainties exist firstly because the distribution of Chol between the inner and outer membrane leaflets remains poorly known for the reasons previously mentioned and secondly because of the likely existence of different Chol pools which also might involve protein-lipid interactions [187, 188]. Behavior of ternary mixtures made of DOPC/SM or POPC/SM containing various amounts of cholesterol, taken as models for "rafts" biological membrane microdomains, has been examined by AFM [40, 79, 91, 189]. Again, as for simpler binary mixtures and probably further amplified by the SM domains polymorphism [190], various morphologies were described in accordance with the observation reported for GUVs [167]. For models of BBM exoplasmic membrane leaflet, it was established that in accordance with monolayer studies, gel-L_d phase separation exists in the absence of Chol and that there is a range of Chol concentration (~15-25 mol%) where gel/L_o domains connect over the sample surface before disconnecting again. Finally, contrasting with corresponding LB films, no more domains could be detected for 33 mol% Chol [79]. AFM also allowed following in real time the consequence of in situ manipulating Chol level on microdomains behaviour [191, 192]. Three remarks have to be done concerning all these experiments on ternary mixtures model microdomains. The first is that, in most cases, AFM can hardly discriminate a gel from a Lo phase only on the basis of their bilayer thickness. X-ray studies on SM/Chol mixtures established that thickness of the bilayer decreased only by 0.5 nm for temperatures below gel to fluid transition Tc upon addition of 50 mol% Chol [193]. Moreover, presence of Chol in the POPC fluid phase can increase the bilayer thickness by up to 0.4 nm for 30 mol% Chol

[194]. This explains why increasing Chol concentration in the bilayer reduced the height difference between the L_d phase and the gel-L_o/L_o phases [79]. In fact, coexistence of gel and L_o phases can be detected by the presence of straight and angular portions at the interface of domains protruding from the fluid L_d phase. As a consequence of this reduced difference between bilayer domains thicknesses, assessing the degree of membrane leaflet coupling becomes very difficult in these samples. The second remark concerns the temperature control of commercial AFMs sample stages which, due to the temperature sensitivity of scanners and tips, took time to be developed. Consequently, most of the AFM experiments on domains behaviour were done at room temperature, i.e. ~15°C below the physiological temperature. Finally, the third remark is also linked to a technical limitation i.e. that of AFM scan rate. Whereas the "freezing" of structures in LB films allowed to demonstrate the lateral heterogeneity of renal Brush Border Membrane containing 33 mol % Chol with the presence of small (20-70 nm) domains [111], such small domains were not detected in corresponding SLB. It can not be excluded that they escaped detection because they were diffusing in the fluid phase during AFM imaging. Going to quaternary mixtures of lipids brought original new data and added a supplementary level in the image interpretation complexity. Introducing ceramide (Cer), the second messenger involved in sphingolipid signal transduction which can account up to 10% of membrane lipids [195-197], into model rafts under conditions where L_d -L_o [198] or L_d –L_o-gel [199] phase separations occur results in the detection of three topographic levels. In both cases, this third level was also induced by in situ sphingomyelinase treatment suggesting it resulted from the displacement of a part of Chol from the L_o/ L_o-gel domains to the L_d phase [198, 199]. To our knowledge, there is no published AFM images of SLB made of lipid mixtures modelling the cytoplamic membrane leaflet composition, likely because they do not show phase separation [32, 200].

Unfortunately there is also no AFM report of the domain coupling induced by models of outer leaflet [32].

IIIc) AFM imaging of lipid- peptides and lipid-protein interaction in SLB

The interest of using simple membrane model systems like SLBs to better understand interactions between proteins and lipids has been recognized for long [201]. These model systems enable detailed analysis of how the properties of lipids influence the structure and the dynamics of proteins and in reverse how proteins and peptides affect the lipid bilayer behaviour. The pioneer work of the Shao's group, revealing the exquisite supramolecular organization adopted by gramicidin A in DPPC bilayer [147], has been followed by numerous AFM studies on lipid-peptide and peptide-peptide interactions which all gave fundamental information that could not have been obtained by other techniques. For example, this was the case of WALP, KALP, HALP [202, 203], primary amphipathic [204], and fusogenic tilted peptides [205], all forming microdomains of supramolecular structures only visible at the nanoscale in SLB. Because they are mostly associated with membrane domains enriched in SL and Chol, the exoplasmic proteins class constituted by glycosylphosphatidylinositol-anchored (GPI) proteins was a good candidate for AFM studies. Direct evidence for an insertion of a GPI in the most ordered domains of binary or ternary model rafts mixtures was obtained taking intestinal (BIAP) or placental (PLAP) alkaline phosphatases (AP-GPI) as models [189 , 206]. AFM has also allowed to get direct information on the associated transfer of lipids between phases [207] and on the effect of temperature on GPI distribution [208]. Indeed, the origin of proteins, the SLB composition, the experimental conditions chosen for protein insertion, the imaging temperature, are all parameters capable to influence the imaged enzyme distribution (see [209] for a recent review). It must be kept in mind that the time required to acquire such images (between ~ 1 and 5 min for commercial equipments) actually

precludes the quantitative determination of fluid ordered partition coefficient for diluted, rapidly diffusing molecules [210]. Interactions of amyloids [211-214], of annexins [215, 216] with SLB and of a carrier peptide, with or without its cargo, with phase-separated model rafts [217] constitute other examples of the interest of AFM in characterizing lipid-protein interactions.

IIId) Temperature-dependence of AFM membrane domains imaging. Interaction SLB-support.

The first, relatively recent, AFM studies where the temperature was varied in situ in order to characterize the SLB phase behaviour and thermotropic properties were performed on DMPC [218] and DMPC/DSPC samples [148]. Since that time, various laboratories have investigated the thermal behaviour of SLB, essentially that of disaturated PC species. The results are presented in table 2 which also includes the transition temperature (T_m) determined by other techniques. The first observation, common to all AFM determinations, is the increase in the transition half-width, as compared to DSC determination on multilamellar vesicles [219]. The T_m itself was reported to be unchanged [220] or to be increased from ~ 4 to 16°C depending on the acyl chain length and the leaflet considered, proximal or distal. For example, the upper end of DPPC melting was reported to be as high as 52°C for the proximal and 60 °C for the distal leaflets of DPPC, [221, 222]. Moreover, in these studies the melting of the proximal leaflet started only after the distal leaflet gel to L_d phase completion, indicating a complete decoupling of the two leaflets. These data strongly differ from those obtained by DSC, also on mica, showing a slight T_m shift of 2°C for the proximal and of 3°C for the distal leaflet [223], associated with a modest increase in Tm half-width. They also differ from those reported using FRAP for LB/LS DMPC and DPPC SLB on oxidized silicon where no difference with liposomes data were observed [121] and from the literature on beads

and nanoparticles-supported SLB which most often indicated a slight decrease in T_m (Table 2). Studies of thermotropic and lyotropic mesorphism of saturated diacylphosphatidylcholines have demonstrated that the larger shifts recorded for the proximal leaflet would be equivalent to a marked (>50%) dehydration of the polar head groups [224, 225]. Comparing with DSC on multilamellar vesicles, the marked widening of the transition is associated with an important decrease of the transition cooperativity and can hardly be accounted for by a classical first-order process [218, 226]. Taking into account AFM experiments on supported double bilayers [154], the results presented in Table 2 strongly suggest that the shifts in gel-L_d transition temperature of lipids present in the proximal leaflet are markedly dependent on the nature of the support, the thickness and composition of the aqueous sandwiched layer, in particular the presence or not of divalent cations, the experimental procedure followed to prepare the SLB and, as shown by the correspondence between thermotropic behaviour expected from DSC and temperature-dependent AFM imaging of model rafts [209], the lipid composition. Determination of the aqueous layer thickness by various techniques like NMR, neutron diffraction, *X-ray reflectivity,* FLIC, gives values between ~0 and 4 nm [121, 138, 142, 151, 227-229]. This is also the case of the thicknesses estimated by AFM from the distance between the bilayer surface and the support, see for example [79, 181 , 184 , 222] and Fig.6. The thinner the aqueous film, the higher the T_m shift. Why is the T_m of the distal leaflet of SLB made by vesicles fusion so much affected while it is unchanged when using LB/LS transfers [121] remains unexplained.

IV AFM recent developments and perspectives.

The capacity to image surface topography from the nano to the micro scale, in air and under liquid, has imposed AFM as a major tool in the characterization of complex surfaces

like microdomains in model membranes. The possibility to follow in situ, at the nanoscale, membrane modifications upon addition or insertion of drugs, peptides, proteins, has also provided direct structural information inaccessible to other techniques. *Comparison of LB and SLB AFM data has one more time emphasized the interest of each model to characterize the membrane behaviour of complex lipid mixtures containing several components. Thus, besides being excellent model for studying pulmonary surfactant, monolayer films allow to determine the physico-chemical properties of one isolated membrane leaflet, its ordering in two dimensions and surface interaction with peptides, proteins or drugs introduced in the subphase as a function of the surface pressure, keeping in mind the limitation associated with film deposition on solid substrates. As discussed above, nanometer scale details of supramolecular arrangements revealed by AFM are obtained in air on films where lateral diffusion is blocked and might be actually inaccessible for freely diffusing structures at the surface of membranes under liquid buffer. The inverted AFM system equipped with a tip approaching the liquid-air interface from the subphase developed at the ETH Zurich [230], combined with grazing-incidence x-ray diffraction and x-ray reflectivity techniques[231], could provide direct access to the nanoscale organization of Langmuir films. SLB gave such information for the membrane surface exposed to the buffer. Local uncoupling of leaflets and time-dependent reorganization of membrane surface associated with the loss of asymmetry or with the addition of compounds that insert differently in mono and bilayers due to hydrophobic matching are among numerous examples of events where LB and SLB studies give complementary information.*

While new imaging AC modes like phase modulation AFM [232], higher harmonic AFM [233] and bimodal AFM [234] are expected to lead to improved resolution of topographical features and local mechanical properties, increasing the application field of the AFM technique requires to widen the nature of the information collected, to address the

question of the membrane-support relationships and, last but not least, to decrease by at least two or three orders of magnitude the image capture time.

In what concerns the first requirement, fluorescence-based imaging techniques are among the most powerful approaches for examining structure-function relationships in biology. Following the AFM coupling with far field fluorescence imaging [70], the use of fluorescence correlation spectroscopy (FCS) has confirmed the compositional/structural heterogeneity of ordered domains in a phase-separated DOPC/DPPC binary mixture [235] and has shown that the diffusion coefficients for fluorescent lipids and for two membrane proteins in model rafts SLB were comparable with diffusion in free-standing membranes [210]. The first images recently obtained on DOPC/DSPC/Chol mixtures using a combined Polarized total internal reflection fluorescence/AFM gave access to the order parameter in the same SLB domains characterized by AFM [236]. It can be predicted these fluorescence/AFM couplings will be helpful in the understanding of domains behaviour and influence of fluorescent probes on the existence and size of domains [175, 237 , 238, 239]. Raman-AFM [240], association of AFM with high–resolution secondary ion mass spectrometry (Nano SIMS) which also demonstrated the heterogeneity of local composition within a single domain and between domains in order-disordered phase separated DLPC/DSPC freeze-dried bilayer [241], will also provide the necessary complementary chemical analysis of domains for ternary and more complex mixtures. Recent progress in nanoscale magnetic resonance imaging, with resolution in the nanometer range [242], will also result in the development of a new powerful tool for the understanding of membranes structure.

There is good indication that solutions to the unpredictable effects of support on membrane properties are under way. From the use for AFM studies of SLB flat supports pierced by holes of various diameters like those described by Steltenkamp et al. [243], Böcker et al. [244], and by Goncalves et al. [245], one can expect to get direct information on the

parameters involved in the support influence on lipid bilayers and lipid domain properties. Moreover, filling these holes by gels having cytoplasmic-like properties, which can also promote phases separations [246], would also offer SLB models closer to the biomembranes situation.

Finally, while it has the unique capability to image biological samples at a nanometer resolution in physiological solutions, the time required to acquire one image between 0.5 and 10 min. has until recently limited the application of commercial equipments either to the high resolution imaging of immobile structures or to the low resolution of fast diffusing structures or fast events. As recently reviewed [247], the pioneers studies in the Hansma [248], Ando [249] and Miles [250] groups for developing a high-speed AFM (HS-AFM) capable to work in liquid on soft matter have now succeeded in offering the capacity to film in real time the structural modification of a functioning single molecule like the GroEL-GroES interaction regulated by the ATPase [251]. As shown by the time lapse study illustrated by Fig. 8 and the corresponding film placed at http://www.s.kanazawa-u.ac.jp/phys/biophys/BBA/lipid.htm. , it is now possible to follow the SLB formation from a ternary phospholipid mixture with a sampling rate better than 1 image per sec. This opens a new field of applications for the study of model membranes including, for example, the early steps of bilayers formation and of domains nucleation, the diffusion of nanoscale domains or of slow diffusing proteins and lipids whose intra and inter-molecular motions remain important, even in the gel state [3, 252, 253].

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Legend for figures

Figure 1: Phase behaviour of phospholipids.

Upon hydration, a majority of phospholipids form lipid bilayers that can exist in two distinct physical states, gel (L_β and $L_{\beta'}$) and fluid (L_α, L_d), according to the temperature. In the gel phases, molecules are tightly packed in a quasi-hexagonal array. The acyl chains are extended and parallel to each other. Intra-and intermolecular motions are slow as compared to the fluid, liquid disordered phase where the acyl chains are highly mobile and the molecules undergo fast rotational and lateral (D) diffusion. For pure phospholipid species, the gel to fluid transition is characterized by a melting temperature, T_m, recorded as a sharp peak by differential scanning calorimetry. It is accompanied by a thinning of the bilayer. Addition of cholesterol induces a concentration dependent decrease in the sharp peak until it is suppressed and the formation of a new phase, the liquid ordered phase (L_o). In the L_o phase, the acyl chains are ordered and mostly extended but the molecules have a high rotational and lateral mobility (adapted from [42]).

Figure 2. **AFM Imaging of POPC, DPPC and SM LB films**

Langmuir film transfers were performed at 32 mN/m for POPC in LE (A), DPPC in LC (B) and SM (C) in LE+LC phases. (D) is a virtual section of (C). The (E) cartoon illustrates the sample positioning under the tip when imaging with an AFM in air. Blue headgroups correspond to LC, red to LE lipids. Bar: 500 nm

Figure 3. **Example of LE-LC phase separated binary mixture in LB film.**

DOPC/DPPC (1:1) films were transferred at 32 mN/m onto mica and examined in air with an AFM working in contact mode. A: low magnification height image: bar 5 µm, z scale 20 nm; B & C: samples from two other preparations, bar: 2µm, z scale 15 nm; D: height image at a higher magnification of A: bar 400 nm, z scale 7 nm. E & F, corresponding lateral force (friction) images in the forward and backward direction of the tip scanning , z : 0.2 V.

Figure 4. **Topography of DPPC/DOPC LB films containing 4% GM1**.
The film was transferred at 32 mN/m onto mica and examined in air in an AFM working in contact mode. bar: 250 nm, z scale: 5 nm; For more details see [88].

Figure 5. **Branching of SM enriched nanodomains by cholesterol addition**
A: height image of SM/POPC (3:1) LB film. B: SM/POPC (3:1) + 20 mol% Chol. Bar: 500 nm, z colour scale: 10 nm.

Figure 6. **Supported DOPC/DPPC 1:1 bilayers made by vesicle fusion. Influence of substrate.**
A, B, C & G: SLB on mica; D,E,F & H: SLB on glass. White arrows in (C) point at the presence of holes in the bilayer whereas dark arrows indicate the presence of aggregates (and/or non-fused liposomes). In D & E, white arrows show the presence of patches made of unconnected, tiny, pieces of bilayers while black arrows indicate glass zones not covered by the bilayer. The white arrow in H shows a zone of the bilayer with a different organization. Bars: A, B, C: 2 µm; D, E, F: 5, 1, 0.3 µm. vertical z colour scale: A, B, C: 20 nm; D, E, F: 30, 30, 10 nm, respectively. Note that the bilayer surface is > 8 nm above the substrate (G & H virtual sections).

Figure 7. **Imaging Ripple Phase in multibilayers**.

Multibilayers were formed from di-C15:0 PC LUV in PBS buffer and imaged in the same buffer at room temperature. A, B & C and D & E correspond to different experiments. A,B &E are deflection images. C & D: height images. Bar A, B, C: 300 nm; D & E: 250 nm. White arrow in A shows the absence of ripple on the first bilayer. The white arrow in B strongly suggests the presence of triple bilayers.

Figure 8. **Imaging SLB formation by High-speed AFM (HSAFM).**

Membranes were made of a ternary mixture made of DOPC/DOPS/biotin-cap-DPPE in buffer (10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4) and sonicated. The lipid suspension (0.5 mg/ml) was then directly diluted into the buffer present in the HSAFM liquid cell and the bilayer formation imaged in continuous at a 975 ms/frame scanning rate (scan size 800x800 nm). SLB was formed from tubular lipid membranes (arrows, approximately 20 nm in height) and unruptured liposomes (arrowheads, 7.5-30 nm in height). Z color scale: 25 nm. The corresponding film is placed at http://www.s.kanazawa-u.ac.jp/phys/biophys/BBA/lipid.htm

Table 1. **Lipid composition of DRMs and apical membranes of intestinal and renal epithelial cells (brush border membranes,BBM)**

Composition (mol%)		DRMs		Intest. BBM		Renal. BBM	
		Total[1]	Out.Leaf. (calculated)[3]	Total[2]	Out.Leaf. (calculated)[3]	Total[4]	Out.Leaf. (determined)[5]
Sphingolipids (SL)	GSL	22	~40	37	~70	<2 [6]	3
	SM	14	~30			23	47
Glycerophospholipids (GPL)		33	_	32	_	36	12
Cholesterol (Chol)		32	~30	31	~30	38	38
SL/GPL ratio		1.09		1.16		0.72	4.17
Chol/(SL+GPL) ratio		0.47	~0.43	0.45	~0.43	0.59	0.59
Chol/GPL ratio		1.03		0.98		0.62	

Total lipid composition of DRMs, intestinal and renal brush border membranes taken from literature. Foe each membrane, the second column gives the corresponding composition of the exoplasmic leaflet (Out.Leaf.) either calculated from the known sphingolipid asymmetry or determined experimentally.
From :[1] [17] ;[2] [254] ; [3] Estimation assuming that GSL and SM are localized on the external leaflet [1]; [4] [255];[5] [256, 257];[6][258].
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Table 2. **DMPC and DPPC Transition temperature (T_m) in liposomes and SLB**

Sample	Technique Used	DMPC	DPPC
		T_m (°C)	T_m (°C)
MLV [219]	DSC	23.9	41.4
SLB mica, [222]:	AFM		(42-52)/(53-60)*
SLB, mica, [221]	AFM	(23.6-30.3)/(31.3-37.5)*	(44.8-51.4)/(52.9-59.4)*
SLB, mica [259]:	AFM	(22-25)/(28-31)*	(41-45.5)/(46.5-49.0)*
SLB, mica, [220]	AFM		40.3-43.0
SLB, mica , [226]	AFM	(26-35)/(36-47)*	
SLB, mica [218]	AFM	28.2 (24-32)	
SLB, Au (111) [260]	AFM	22.5	
SLB, mica [223]	DSC		40.4, 42.4, 43.8
SLB silica bead, [261]:	DSC	~21	~38.4
SLB glass beads, [228]	NMR	25.4	
SLB, SiO2 nanoparticles, [262]	DSC	~ 22.9	39.4
SLB silica bead [263]	DSC		39.4
SLB, oxidized silicon [121]	FRAP		40

* leaflets uncoupling. Values given in brackets correspond to the lower and higher ends of the phospholipids melting temperature of uncoupled proximal/distal leaflets.

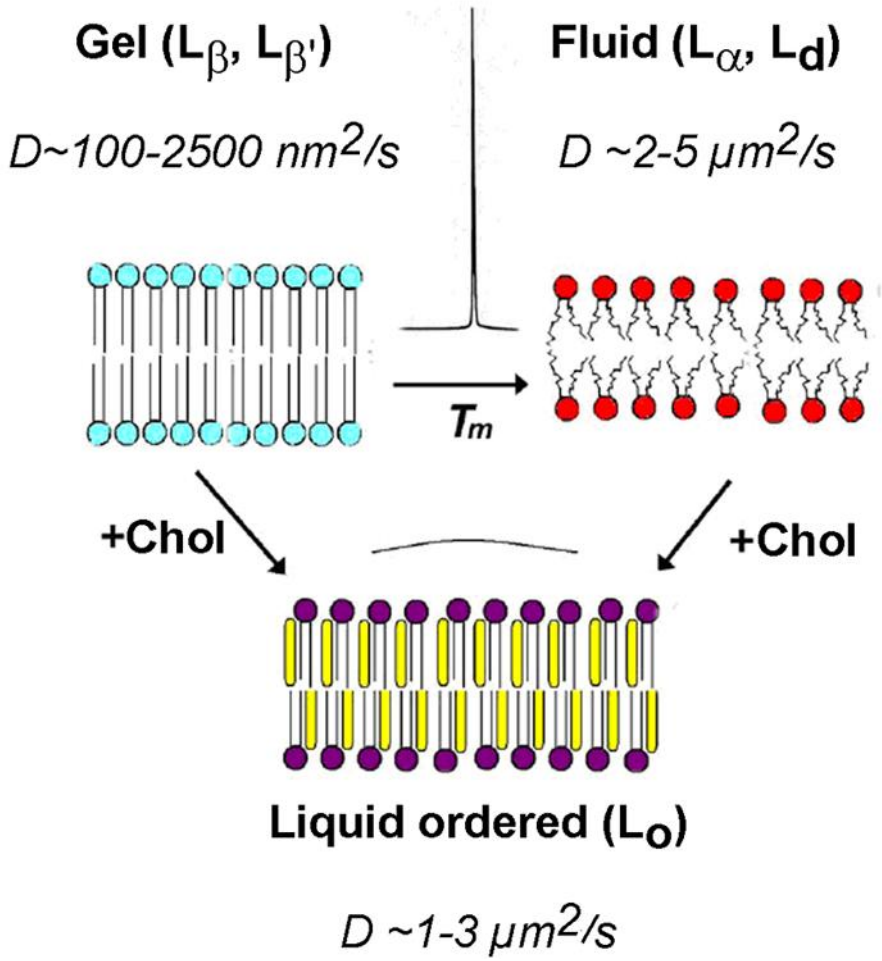
Gel (L_β, $L_{\beta'}$)
D~100-2500 nm^2/s
Fluid (L_α, L_d)
D ~2-5 $\mu m^2/s$
T_m
+Chol
+Chol
Liquid ordered (L_o)
D ~1-3 $\mu m^2/s$

Fig.1

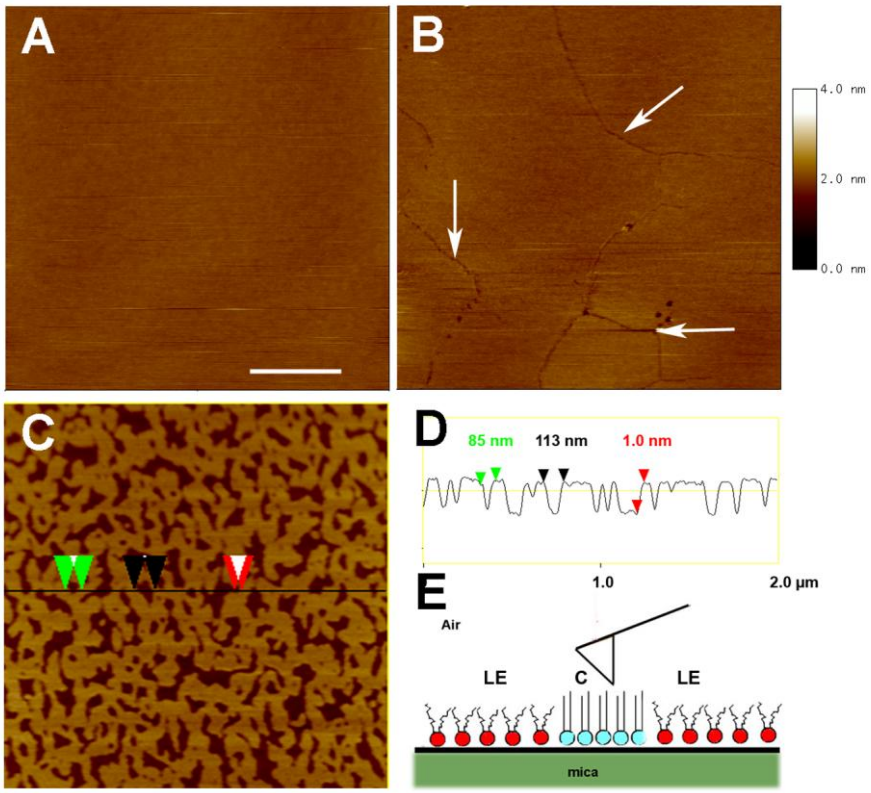

Fig.2

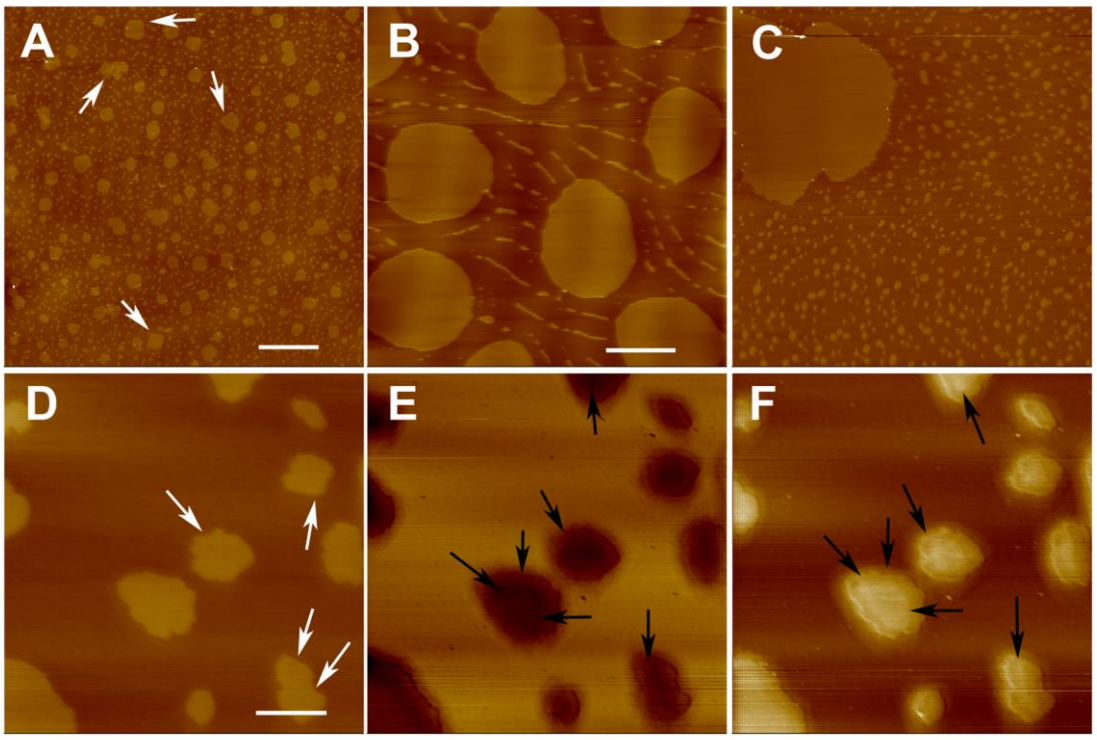

Fig.3

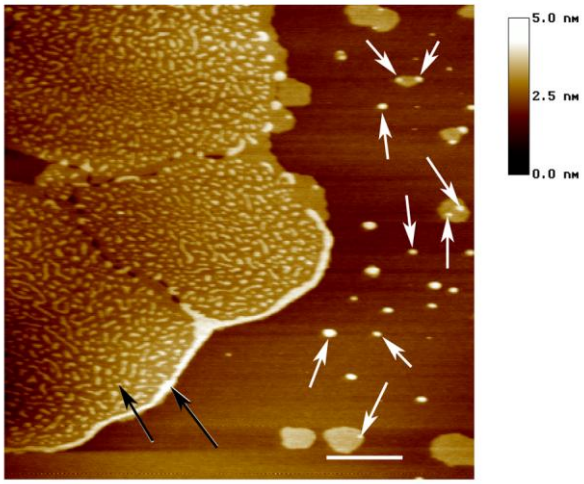

Fig.4

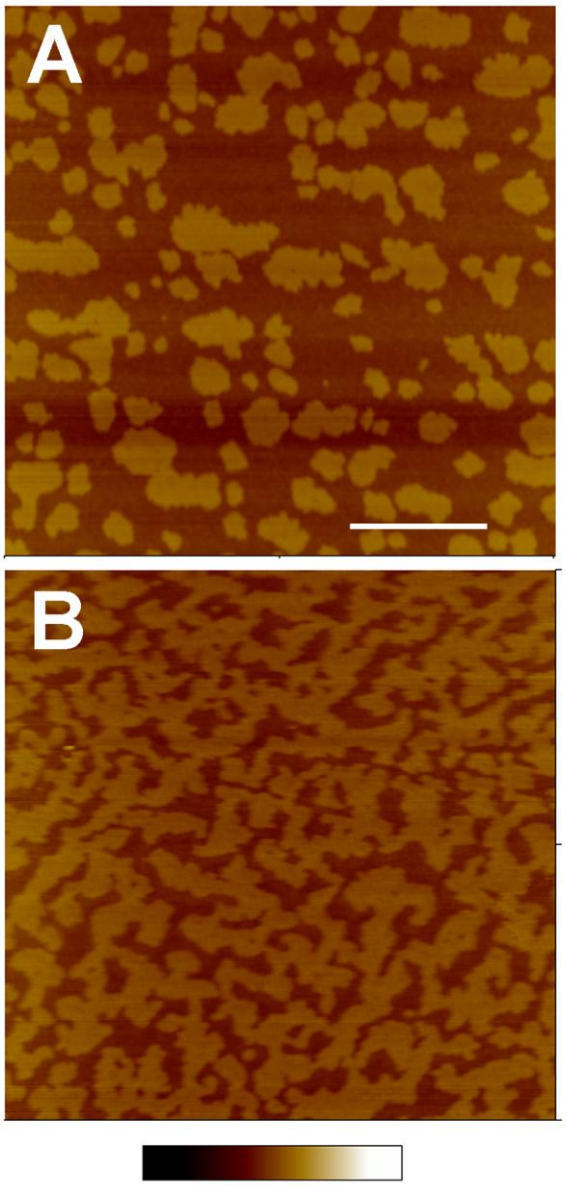

Fig.5

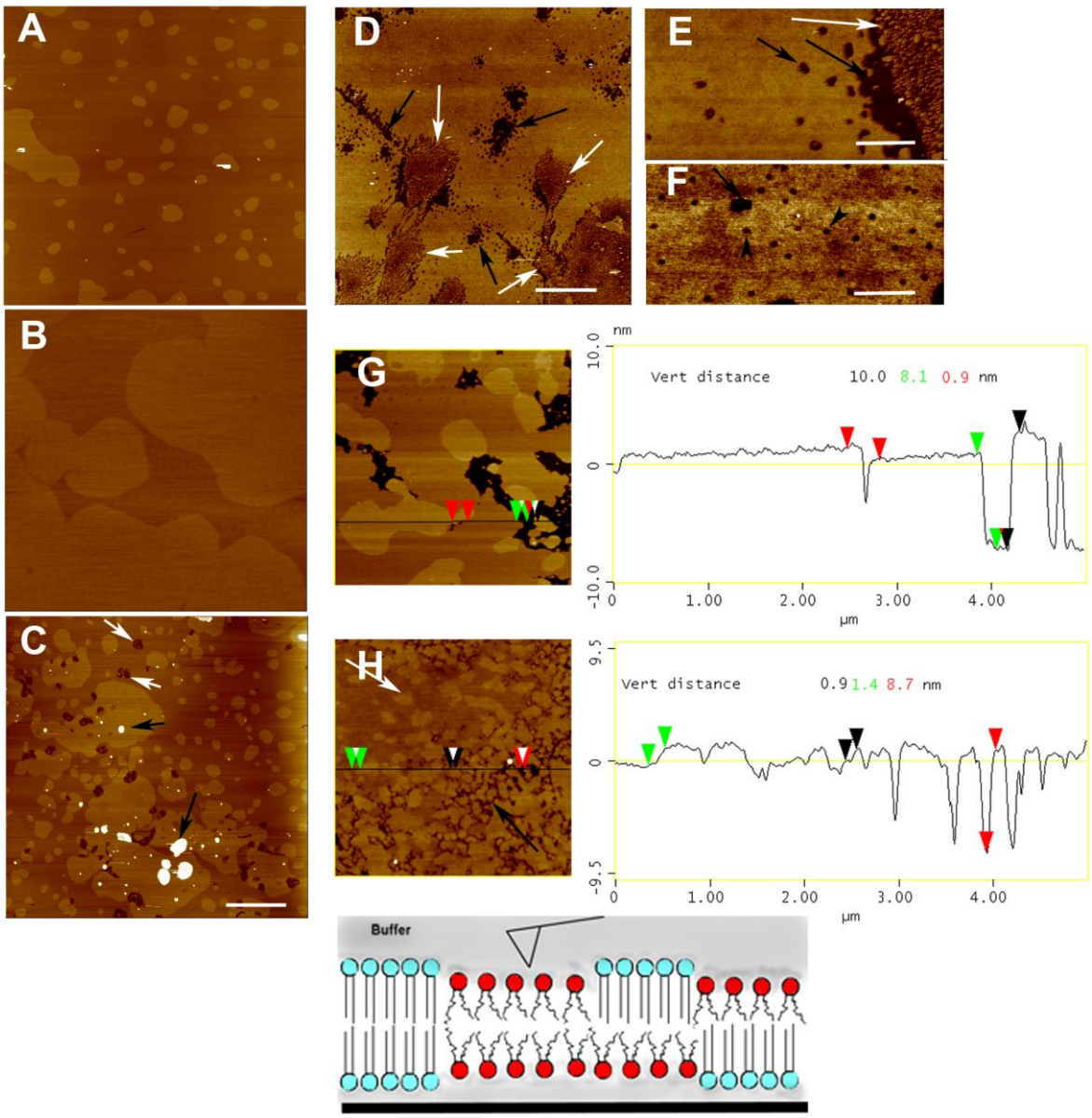
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Fig.6

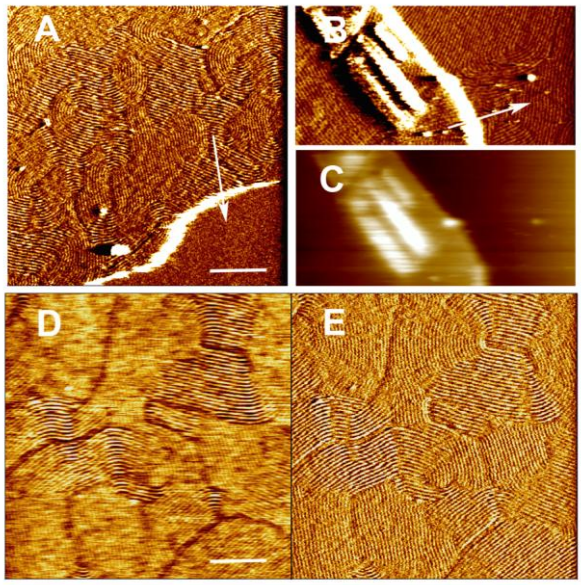

Fig.7.

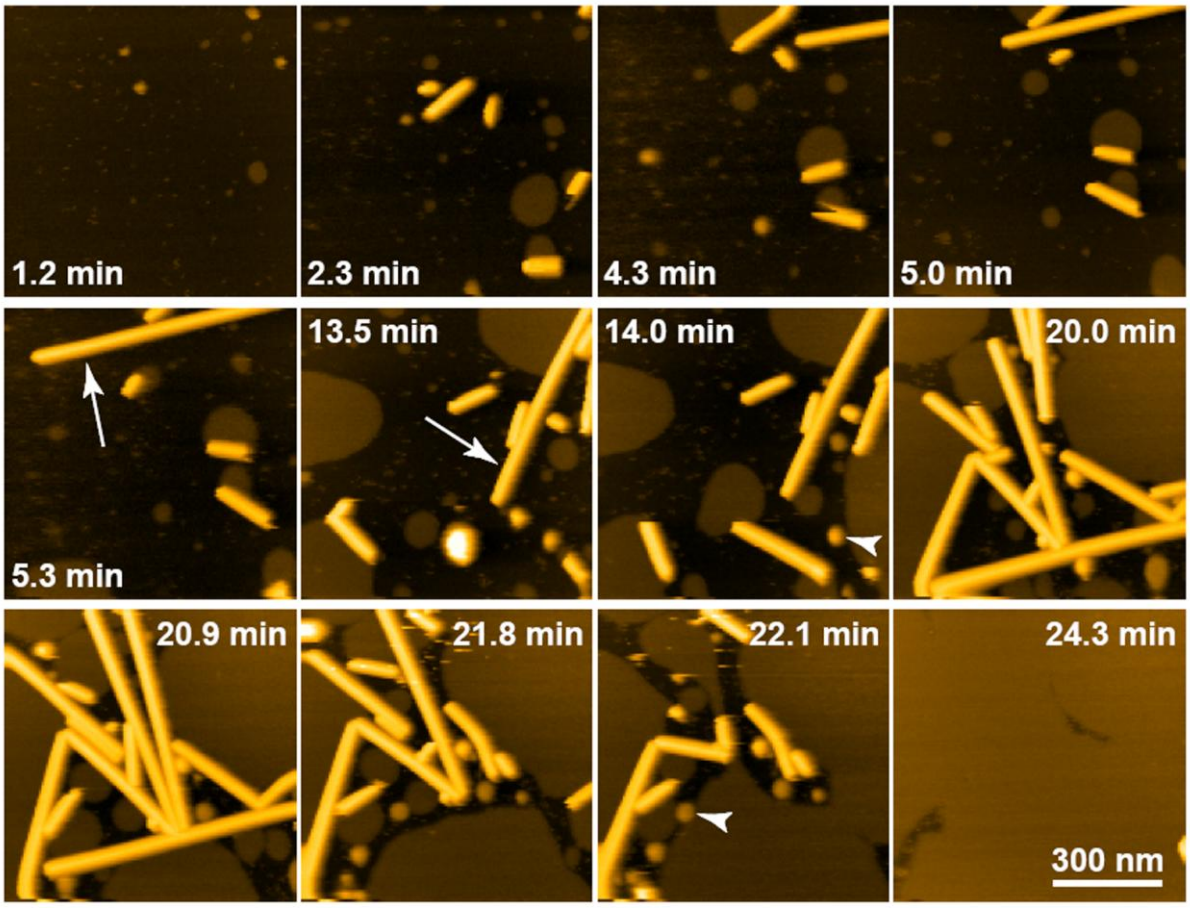
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Fig.8