Lipid rafts: phase separation in lipid bilayers studied with atomic force microscopy

The lipid bilayers that form the surface of living cells have a rich structure, formed from many different lipids and proteins. The self-organisation of these membranes into different domains is critical in many cell processes, such as recognition, signalling, or endocytosis and exocytosis. The islands of particular lipids associating together are called lipid rafts [1], and these can move dynamically through the membrane [2]. Certain proteins also have a higher affinity for the raft lipids, so that the rafts can bring particular proteins together and raise their activity level.

Sphingolipids and cholesterol are both important in lipid segregation into rafts, so here a mixture of dioleoyl-phosphatadiylcholine (DOPC), sphingomyelin (SM) and cholesterol was used as a model raft-forming membrane. For most of the images here, an equimolar mixture of 1:1:1 DOPC:SM:cholesterol was used. This sample shows phase separation between a liquid-ordered state, enriched in sphingomyelin, and a liquid-disordered state, enriched in DOPC [3]. Such a phase separated bilayer is illustrated schematically in Figure 1.

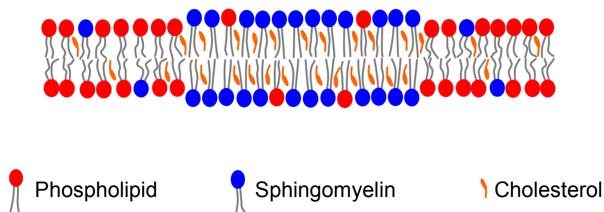

Fig 1. Diagram of a phase-separated lipid bilayer. The distribution of the lipids is only shown schematically, and is not according to exact proportions.

This report gives an introduction to the measurements that are possible with the AFM for characterising lipid phase behaviour. The AFM can show contrast between different lipid phases through differences in the height, friction, or mechanical properties of the phases. Subtle differences in lipid mixtures can be seen, and the phase behaviour studied over different conditions, by varying temperature or buffer composition, for example. The AFM is able to distinguish structures far below the optical resolution limit, without needing fluorescent markers. The resolution achievable is comparable to SEM, but imaging is possible under physiological conditions.

Height contrast between the lipid phases

Samples were prepared by dissolving the DOPC, SM and cholesterol in a 1:1:1 mixture in chloroform and drying to remove the chloroform. The lipids were then re-suspended by vortexing with aqueous buffer and sonicated at 55° C to form small unilamellar vesicles. The vesicles were incubated on freshly cleaved mica to form a supported lipid bilayer, which was rinsed to remove any free vesicles.

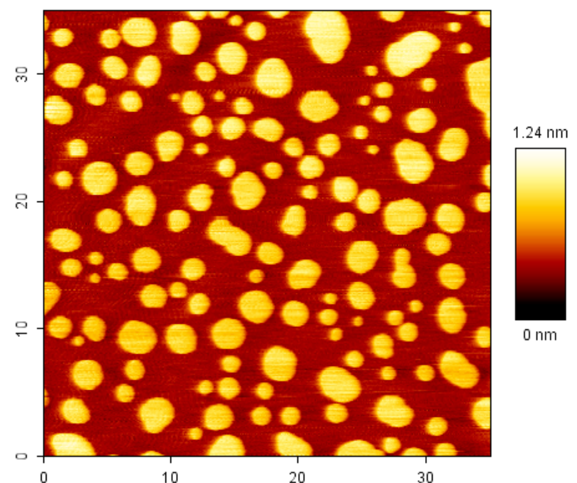

Fig 2. Height image (contact mode) of phase-separated lipid bilayer under buffer, 35 x 35 microns. The higher regions are the SM-rich liquid ordered phase, surrounded by the DOPC-rich liquid disordered phase.

AFM imaging was carried out on a NanoWizard® AFM from JPK Instruments, which was mounted on a Zeiss Axiovert 200 inverted microscope. The imaging was all performed in a buffer of 150mM NaCl, 10mM Hepes, 3mM NaN3 (pH 7.4), and the bilayer remained hydrated at all times during preparation and imaging. This was important, since dehydration causes irreversible damage to the bilayer and lipid phase separation [4].

The bilayer formed from this lipid mixture consists of a continuous phase in the liquid disordered state, with discrete patches of the liquid ordered raft-like phase [3].

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This phase separation can be seen clearly with the AFM, due to the height difference between the two phases. Figure 2 shows a 35 x 35 micron scan of a region of the lipid bilayer. The higher patches are discrete, rounded areas, uniform in height but varying in lateral dimensions.

The fluorescent marker DiD-C18 co-localises with the DOPC-rich phase, so fluorescent and AFM images of the same area were compared to confirm the composition of the higher and lower phases seen in the AFM images. The same fluorescent probe has previously been used to show that the two membrane leaflets are coupled, so the phase state is the same in both layers [3].

The height of the bilayer is around 5nm, as can be measured from occasional defects in the membrane. The lipids generally form a continuous layer, however, and the height differences generally seen are the 1nm height steps between the ordered and disordered phases. The image and line section in Figure 3 show typical height profiles through the domain structures.

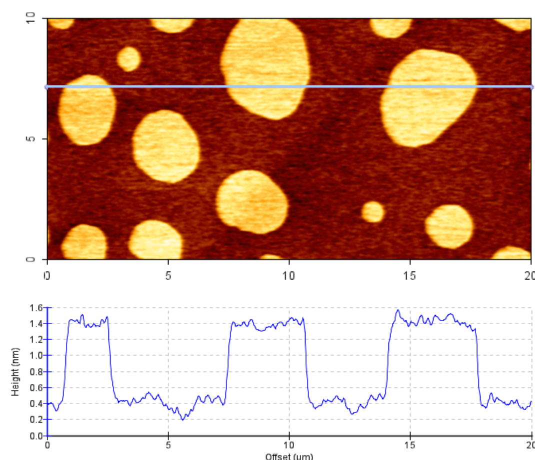

Fig 3. Height image and line section (for the scan line marked in the image). The height difference between the lipid ordered (higher) and liquid disordered (lower) phases is 1nm.

The size of the domains increases with time as they fuse, and the outline of some larger domains reflects the shape of two or more round domains merging. The slow domain rearrangement is probably due to the lower temperature compared with the preparation conditions.

Friction contrast between lipid phases

In contact mode, the vertical deflection is used to control the imaging height, but the lateral deflection of the cantilever can also be measured. This is a measure of the friction between the cantilever tip and the sample, and friction contrast can show differences in the chemical properties and fine structure of the sample.

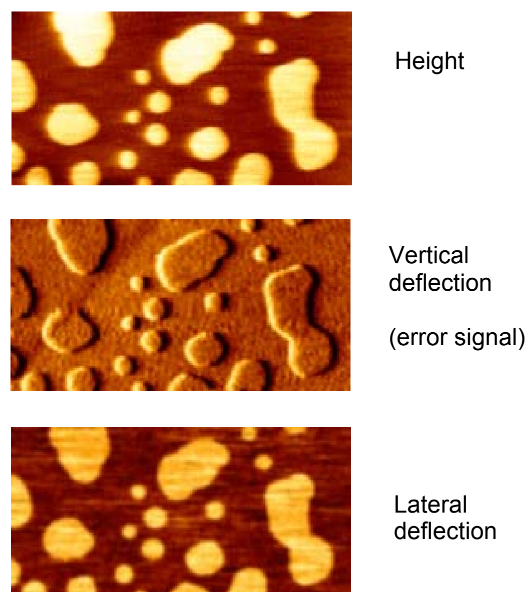

Fig 4. Friction contrast between the lipid phases. The images show a 20 x 10 micron area from the same scan in contact mode in liquid. The height (top) and lateral deflection (bottom) images show contrast between the lipid phases. The amplitude image (centre) shows contrast from the edges of the patches.

Figure 4 shows a 20 x 10 micron area of a contact mode scan of the lipid bilayer. The three images are the height and vertical and lateral deflection channels, which were collected simultaneously. The height (top image) and lateral deflection (bottom image) show similar contrast, with the domains appearing as flat areas. The vertical deflection signal, which is used in contact mode as the error signal to control the height, shows contrast only at the domain edges, where the height changes sharply.
The magnitude of the lateral deflection contrast depends on the setpoint used; as this is a frictional component, the difference generally depends on the normal force applied

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to the sample and also the scan speed. The contrast can be adjusted with the imaging parameters, but the regions of different lipid phases are constant.

AFM phase contrast between lipid phases

In intermittent contact mode, the phase of the cantilever oscillation can give further information about the properties of the sample. In this case, there was phase contrast between the two lipid phases. The lipids were imaged as before in buffer, and a relatively small amplitude was chosen for the cantilever oscillation.

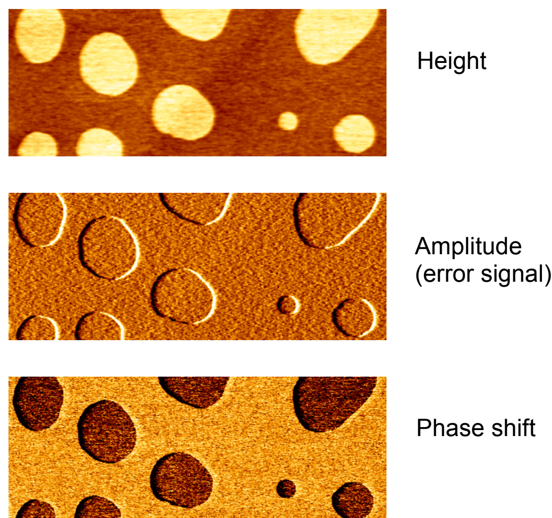

Fig 5. Lipid phase contrast from intermittent contact mode AFM in liquid, 18 x 7 micron area. The height (top) and phase (bottom) images show contrast between the lipid phases. The amplitude image (centre) shows contrast at the edges of the patches.

The three images in Figure 5 are simultaneously collected data channels from an 18 x 7 micron region of an intermittent contact mode scan. The height image (top) shows the arrangement of the patches, with a consistent height difference between the lipid phases. The amplitude signal (centre), which is the error signal used to control the imaging in intermittent contact mode, shows contrast only at the sides of the patches. This is as expected, since here the height changes abruptly. The phase image (bottom) shows a clear, stable phase contrast between the two lipid phases. The magnitude of the phase contrast depends on the setpoint chosen.

Domain rearrangement over time

The AFM can be used to follow the rearrangement of the domains over time as the membrane adjusts slowly to the conditions at room temperature. The general trend is for the domain size to increase over time. The changes happen slowly over hours, and the AFM imaging did not appear to affect the process. The domains were not very mobile at room temperature, and the same pattern could be imaged for many hours. It is unlikely that the domain rearrangement is caused by the AFM imaging, since samples left for hours without AFM imaging also showed domain fusion. Even after hours of imaging, the domains did not appear oriented or organised with any particular relation to the scan direction.

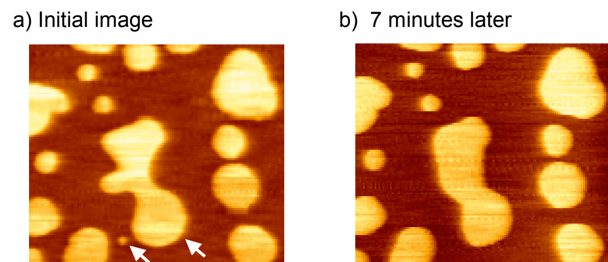

Fig 6. Height images of the same area of lipid bilayer showing domain reorganisation. 14 x 12 micron area.

Figure 6 shows two images of the same 14 x 12 micron area of the bilayer captured 7 minutes apart. In a), two domains are marked with white arrows. The small one disappears between image a) and b), and the larger domain rearranges its outline significantly between the two images. The likely explanation is that the large domain was recently formed from the fusion of two or more smaller domains, and the lipids then rearranged themselves to reduce the exposed edge of the new domain. The shape in the second image is more similar to the other domains in the sample, most are quite rounded and smooth-edged.

The gradual disappearance of the smaller domains can be observed over time with the AFM. Figure 7 shows a series of three images of the same 20 x 20 micron area of the sample and two areas are marked with circles to highlight the changes over time. It is clear that the smaller domains shrink and disappear, but it is not so obvious that the larger

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domains tend to grow with time. The percentage of the higher liquid ordered (lo) phase is marked on each image, as calculated from thresholding each image in a similar way. The apparent increase from 32.3 to 33.3 % is probably not a statistically significant change, but the numbers do confirm that the disappearance of the small domains is not due to conversion of the lo into the other phase, but domain fusion or growth of the larger lo domains.

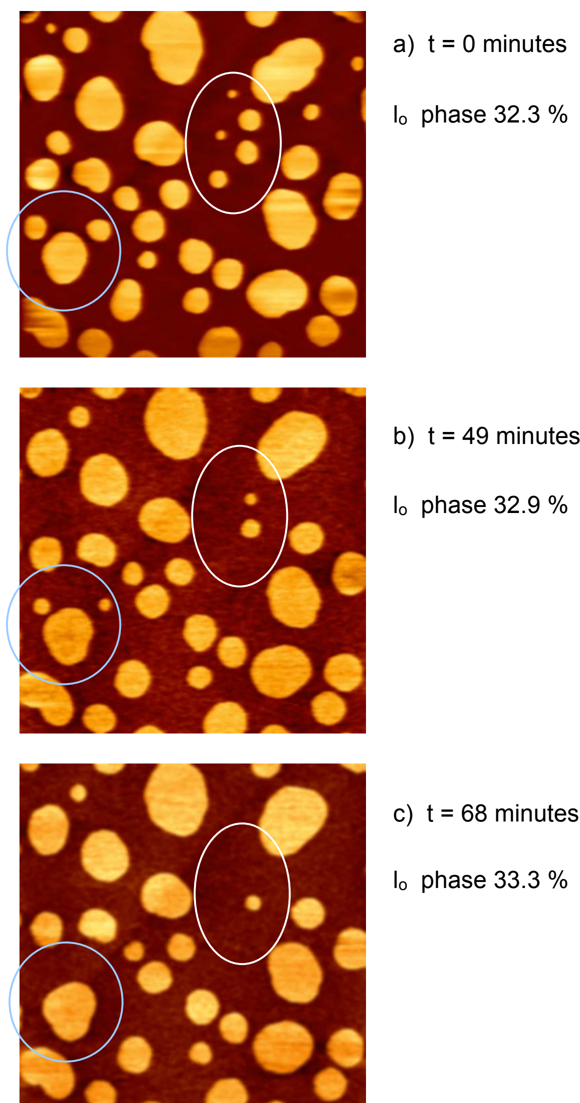

Fig 7. Height images of the same area of lipid bilayer showing domain reorganisation over time. 20 x 20 micron area.

Conclusions

This report has given an overview of some of the possibilities that AFM offers for characterising lipid phase behaviour and studying lipid rafts. Contact mode and intermittent contact mode offer different ways of observing contrast between lipid phases from friction or mechanical differences, as well as height difference. The AFM is well suited to in-situ measurements, and can also be combined with confocal fluorescence measurements to reveal extra information about the complex phase composition of lipid membranes. A further extension is to use the diffusion constant information from fluorescence correlation spectroscopy (FCS) to understand the phase behaviour [5,6]. Protein can also be added to see any partitioning of the proteins between the lipid phases.

AFM force spectroscopy can also be useful to directly measure the mechanical properties of lipid domains, as well as using the tip to manipulate lipid domains and study the dynamic response [5,6]. Force spectroscopy can also give useful results when proteins or peptides are added, for example in the case of pore-forming peptides that promote the formation of holes or defects in the bilayer [7].

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