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Electron microscopic investigations on free-standing mixed lipid Langmuir-Blodgett-Kuhn monolayers: phase separation and aging process

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Abstract

Lipid monolayers were prepared by the Langmuir-Blodgett-Kuhn technique (LBK) as free-standing films spanning a diameter of up to 1 μm. These films were investigated by electron spectroscopic imaging (ESI) and electron energy loss spectroscopy (EELS). The free-standing monolayer is shown to be in a transient state in which an aging process is proceeding: after storage for two weeks in air at room temperature the films tear off the edges of the perforated supporting film. Ca^{2+} ions induced lateral phase separation in these films prepared from a 50:50 mixture of lecithin/glycerol could be visualized by means of ESI, i.e., by comparing micrographs below and above the Ca absorption edge in the EEL spectrum. The domain sizes of the demixed phases were determined to vary between 30 and 60 nm. In addition it was shown that the counter ion of the negatively charged glycerol in these films is Ca^{2+} and not Na^{+}.

Key words: Langmuir-Blodgett-Kuhn monolayer; Phase separation; Aging; Lipid monolayer; Calcium ion; EELS; Electron microscopy

1. Introduction

It was shown that bimolecular lipid membranes (BLM) allow for the study of phase separation phenomena in binary mixed membranes [1] and that it is thus possible to elucidate how lateral organization of two lipid components can influence the performance of integrated model proteins [2–4].

The phase diagram of a BLM of lecithin and negatively charged glycerol of which the charge was compensated by Ca^{2+} ions was investigated previously by conductivity fluctuations of gramicidin channels [4,5]. These investigations confirmed phase separation at distinct concentrations. Although this method is appropriate to measure the concentration dependence of the demixing the domain sizes of the demixed phases cannot be investigated.

In order to measure these domain sizes a fluorescence-microscope for BLMs was constructed which was able to measure the gramicidin channel fluctuations and simultaneously the domain sizes under controlled demixing conditions. A variety of amphiphilic fluorescence dyes (1 mol% in the lipid mixture) was not successful in introducing a contrast between both demixed phases. Because of these difficulties a simpler system was chosen where Eklund et al. [6] had been successful with the differentely charged PC/PA-system. The 50:50 PC/PG lipid mixture and a fluorescence dye were spread as a monolayer on a Langmuir trough equipped with a fluoresence microscope for the investigation of domain sizes in this 'semi BLM' [7]. But also in these monolayer experiments domains were not seen.

Only two explanations of this behaviour have to be considered:

(1) all kinds of dye molecules applied are homogeneously distributed among both different phases. There

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is therefore no reason for any discriminating contrast; or

(2) the domain sizes are too small to be detected by optical microscopy.

With the aim to profit from the higher lateral resolution attainable in an transmission electron microscope we transferred free-standing lipid monolayers onto perforated films (micro grids) by the Langmuir-Blodgett-Kuhn (LBK) technique. Profiting from an electron energy loss spectrometer integrated in the column of the electron microscope discrimination between the demixed phases was possible by exploitation of the elemental specific contrast owing to the distribution of calcium ions over the LBK film. The presence of Ca^{2+} ions could then be assigned unambiguously to one of the expected phases. Electron energy loss spectroscopy (EELS) was applied in addition to check the presence of Ca^{2+} ions ions and to examine whether Na^{+} ions, which were present in the subphase as well, were transferred onto the LBK film.

This technique is shown to be appropriate for the problem of domain sizes after a peculiar problem of preparation has been solved. In the course of the investigation an aging process of the free-standing ultrathin films could also be observed.

2. Sample preparation and measurement

In very good agreement with surface pressure values of BLMs [8] the area-pressure diagram of the 50:50 PC/PG mixture shows that at a surface pressure of about 30 mN/m each lipid molecule needs a surface of 60 $Å^2$. On the base of this value a predetermined amount of a $5 \cdot 10^{-4}$ M chloroform solution of a 50:50 mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (PC) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol (PG) (Avanti, Birmingham, AL, USA) was spread with a Hamilton syringe to prepare a monolayer on the air/electrolyte interface of a glass trough of fixed area. The electrolyte, a 10^{-2} M NaCl and 10^{-2} M $CaCl_2$ solution had pH 6. The surface pressure was not controlled. Samples were prepared at room temperature a few minutes after spreading the lipid solution [9]. All materials were used without further purification.

The preparation of hydrophilic perforated films was achieved in several steps: carbon was evaporated onto 300-mesh copper grids coated with a perforated formvar film. The formvar was subsequently dissolved by washing the grids in chloroform leaving a hydrophobic perforated carbon film. These grids were then hydrophilized by SiO_x evaporation under residual oxygen pressure. The monolayer was transferred onto the hydrophilic grids by scooping the monolayer out of the subphase with the freshly prepared micro grid giving no possibility for a second layer to fold over as is expected when Schaefer's technique [10] is applied to electron microscopic supporting grids. The use of micro grids instead of a continuous supporting film has the advantage that the investigation can be concentrated to the isolated monolayer. Otherwise the signal of a roughly 2.5 nm thick monolayer would be superposed by signals of a much thicker supporting film. A carbon film could hardly be prepared thinner than about 8 nm and its mass thickness increases significantly by the SiO_2 coating necessary to make the film hydrophilic.

Electron microscopical observation and registration of EEL spectra were performed in a Zeiss EM 902 transmission electron microscope at a high voltage of 80 kV. The width of the energy selector slit was 30 eV for imaging and about 1 eV for spectra registration. For the interpretation of electron micrographs one has to realize that thickness contrasts are inverted in the inelastic imaging mode of the instrument with respect to the conventional elastic mode: a hole in the perforated film which is not covered by lipid film looks dark. Electron micrographs were taken photographically at energy losses below and above the Ca $L_{2,3}$-edge (ΔE = 346 eV). The application of the method of electron spectroscopic imaging at the Ca $L_{2,3}$-absorption edge does not allow discrimination between atoms and ions. In the discussion of electron microscopical results we do not therefore differentiate between charged and uncharged calcium. The so called 'white line' of Ca superposes a steep slope of the EEL spectrum behind the carbon K-edge at $\Delta E = 284$ eV. High primary intensity of the electron beam necessary for inelasic imaging and the small scattering volume of a monolayer restrict the maximum magnification for photographic registration of images to a value of about 30000.

3. Results and discussion

Although lipid monolayers are in general very fragile, freshly prepared free-standing LBK films, however, are able to cover holes in the perforated supporting film. This is demonstrated in Fig. 1 where it is obvious that holes up to diameters of roughly 0.3 μm are homogeneously coated with the lipid monolayer. Most of the larger holes remain uncovered. But stable monolayers of freshly prepared samples were also found occasionally up to hole diameters of 1 μm. But there is an increasing tendency that free-standing monolayers display pinholes with increasing hole diameter. In order to investigate the monolayers by electron microscopy we found it necessary to inspect the films immediately after the film preparation. The micrograph of Fig. 1 was taken at en electron energy loss of

ΔE = 325 eV (below the Ca absorption edge) showing only mass thickness contrast. The bars of the carbon/SiO_x supporting film have the highest mass thickness and appear white. Empty holes are black because unscattered electrons are cut by the energy selector slit of the spectrometer and cannot contribute to the image. The monolayer appears grey because the chosen energy loss is located amidst the slope of the carbon K-edge towards higher energy losses. The contrast between only one monolayer of a thickness between 2 and 2.5 nm and an empty hole is much higher in an inelastic than in a conventional elastic image.

Fig. 2 displays the EEL spectrum between 250 and 500 eV of an area of the monolayer inside a hole of the microgrid (registration parameters: high voltage 80 kV, beam current 12 μA, magnification 30000 ×, 30 μm objective aperture). The lower curve extending from 250 to 400 eV shows both the carbon K- and the

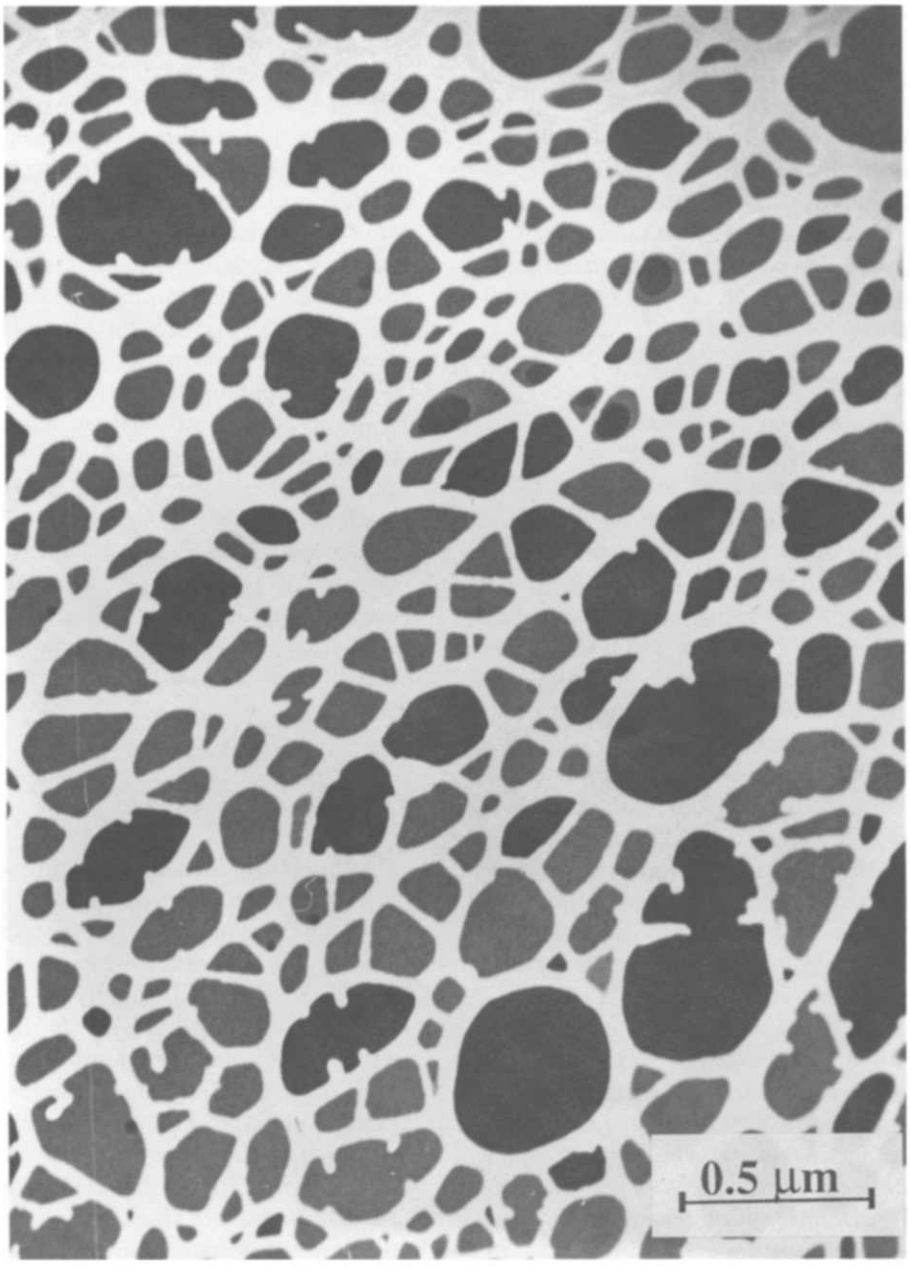


Fig. 1. Inelastic image (ΔE = 325 eV) of a perforated carbon film (micro grid) coated with a free-standing lipid LBK monolayer (white: bars of the perforated carbon film, grey: lipid monolayer, black: holes).

calcium L edges. The spectrum on top extending from 320 to 500 eV is registered at an higher amplification showing again the Ca $L_{2,3}$-edge; in addition, at 401 eV the nitrogen K-edge can be seen weakly superposing the slope of the carbon K-edge (without any background subtraction). It originates from the nitrogen in the zwitterionic PC. The detection of nitrogen in the spectrum as one out of 103 atoms (not taking hydrogen atoms into account) in a 50:50 mixture of PC and PG demonstrates the sensitivity of the integrated EEL spectrometer for an organic sample of such a minute thickness.

Fig. 3 shows an area of the same sample after storage for two weeks in air at room temperature. In contrast to the freshly prepared sample the aged free-standing monolayer has torn off the carbon edges. Obviously stress has relaxed in the unsupported parts of the lipid film. Although pinholes occur also occasionally in the freshly prepared film near the edges of the perforated supporting film they are here the dominant feature. Fragments of the monolayers are now wrinkled like a macroscopic foil and thicken the sample near the margins. There are still undestroyed parts of the monolayer, but thickness and film architecture may have changed.

Figs. 4a and b display a pair of electron micrographs taken at electron energy losses of $\Delta E = 325$ eV (below the Ca absorption edge) and at $\Delta E = 365$ eV (above the Ca absorption edge), respectively. The width of the energy selector slit of the EEL spectrometer amounts to 30 eV and is centred round the given values of energy loss. The diameter of the bridged hole in the supporting film has the exceptional size of about 1 μm. The picture at the lower energy loss (Fig. 4a) shows only mass thickness contrast. In Fig. 4b spots already seen in Fig. 4a by their higher mass thickness now shine very bright labelling the distribution of Ca^{2+} ions containing domains of 30–60 nm in diameter. This contrast enhancement enables us to discriminate unambiguously between the PC rich and the PG rich phase. Only the PG rich phase is negatively charged and needs Ca^{2+} as counter ions for charge compensation. PG is the dispersed phase in a matrix of PC with low or none Ca concentration because Coulomb attraction between the neutral PC and Ca^{2+} is lacking.

The same domain structure is also observed in much weaker contrast below the Ca edge. This observation makes it evident that the disperse phase has a higher mass thickness. With the experimental means used we cannot discriminate between the contribution of thickness and the contribution of the higher density of the Ca^{2+}-containing PG rich phase. We therefore do not know whether the PG phase exceeds the thickness of the PC matrix. Only elemental mapping would be appropriate to separate definitely the contribution of the element Ca to the local image brightness from the influence of varying mass thickness in Fig. 4b. But the excess brightness owing to the structure sensitive contrast [11] at the Ca absorption edge indicates the presence of Ca^{2+} ions as a label for PG. Its domain size of about 30 nm is indeed too small to be detected by fluorescence microscopy. However, one should keep in mind that the object under study was a monolayer which showed this feature of phase separation only on several sites. The domain size of the demixed BLM may be in the same order of magnitude; in any case it cannot be orders of magnitudes larger, because otherwise the fluorescence microscopy had given some indication. In a domain of 30 nm in diameter the gramicidin molecule would be surrounded by about $1.2 \cdot 10^9$ lipid molecules which is a reasonable size to be a polypeptide working independently from the other phase. But it can be speculated that the mutual interaction of both halves of the BLM could have an influence on the domain size. The measurement at the monolayer can only serve as a rough estimate for the situation in BLMs.

Nevertheless, it can be clearly demonstrated by electron-loss-spectroscopy that the counter ion for the negatively charged PG is Ca^{2+} and not Na^+ because the corresponding sodium edge was not detected. Even in the case of a mixed lipid monolayer prepared without $CaCl_2$ but with EDTA instead, no Na was detected in

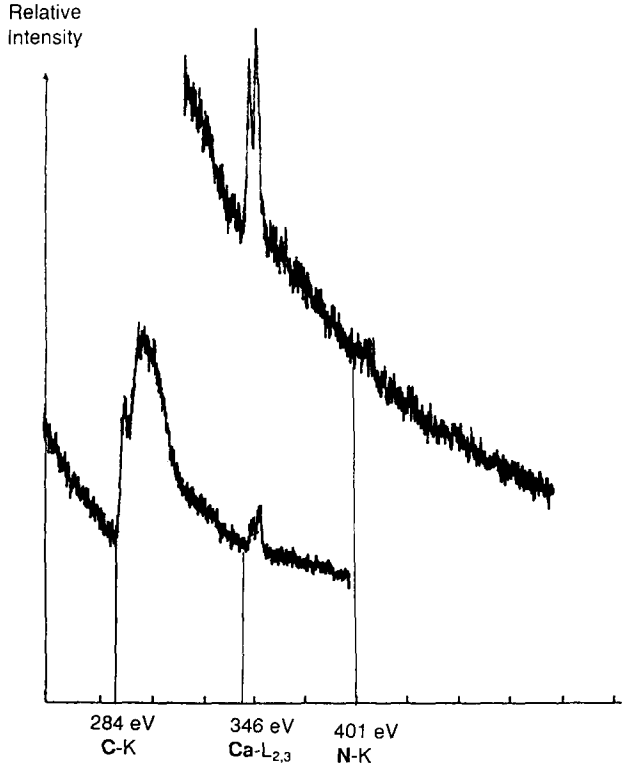


Fig. 2. EELS of a LBK monolayer of a 50:50 mixture of PC and PG.

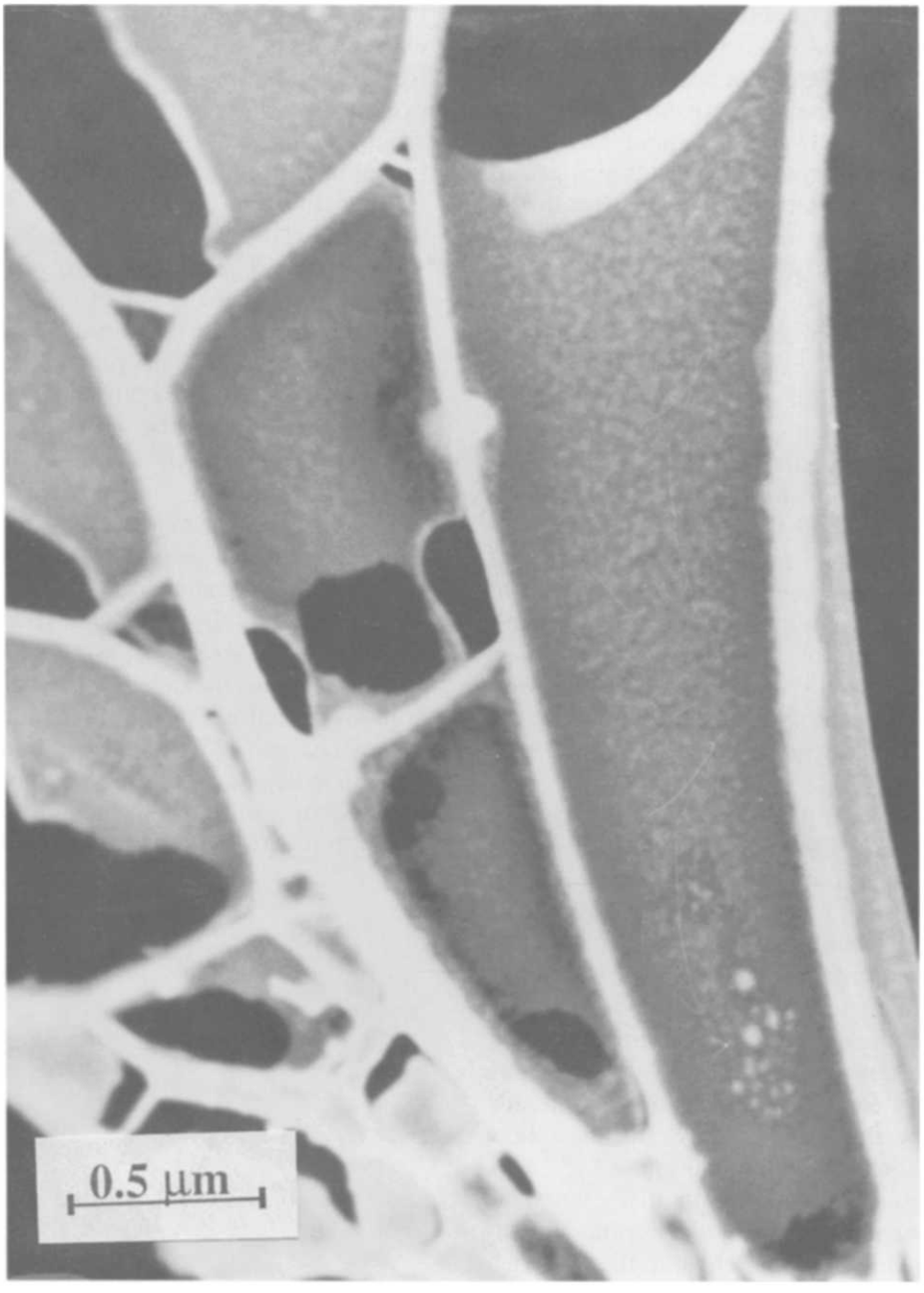
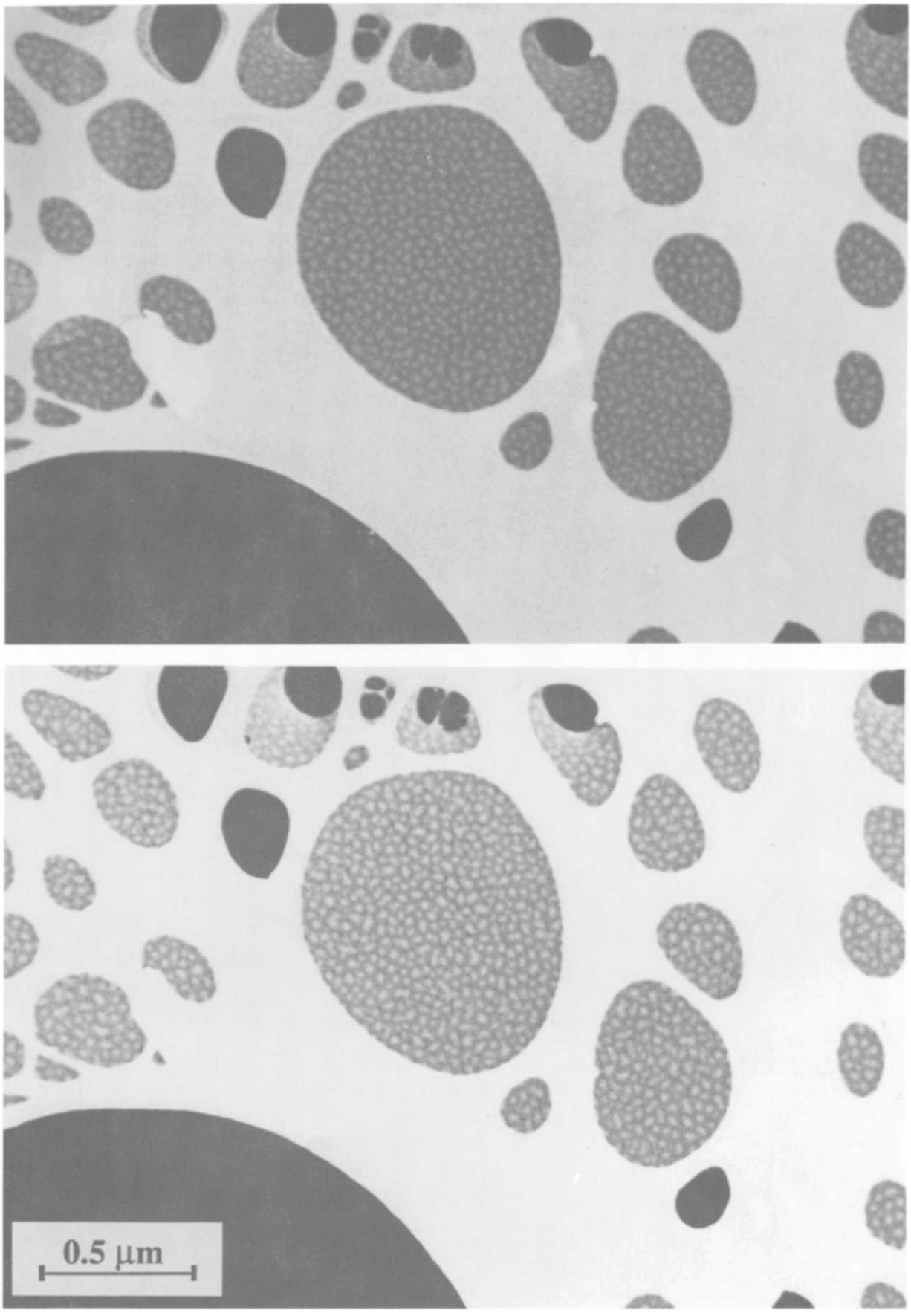


Fig. 3. Inelastic image (ΔE = 325 eV) of an aged lipid LBK monolayer torn off the carbon margin.

the electron-loss-spectra. This is in perfect agreement with data of Macdonald et al. [12] and Marra et al. [13].

An EEL spectrum registered at the site which is imaged in Fig. 4 shows beside both the carbon K- and the Ca $L_{2,3}$-edge as in Fig. 1 also the O K-edge at 532 eV. A signal of residual sodium ions from the subphase (Na K-edge at 1072 eV) could not be observed within the limits of error.

Fig. 4. Mixed lipid LBK monolayer separated in two phases. The disperse phase contains Ca^{2+} ions. (a) ΔE = 325 eV (below the Ca L-absorption edge): mass thickness contrast. (b) ΔE = 365 eV (above the Ca L-absorption edge): Ca structure sensitive contrast.


0.5 µm

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