Biochimica et Biophysica Acta 921 (1987) 411–414
Elsevier

BBA 50211

BBA Report

Detection of protein-free lipoprotein analogues with an apolar lipid core by freeze-etch electron microscopy

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(Received 27 May 1987)

Key words: Lipoprotein; Freeze etching; Phosphatidylcholine

Freeze-fracture electron microscopy of protein-free lipoprotein analogues consisting of polar phospholipids with neutral lipid cores shows that these systems can exhibit novel, smooth crossfracture faces. Resolution of these features can be enhanced by etching techniques. This novel particle morphology provides a new procedure to characterize microemulsions composed of polar and neutral lipids.

Protein-free analogues of plasma lipoproteins can be prepared as microemulsions consisting of particles with an apolar core of neutral lipids surrounded by a monolayer of polar phospholipids. Such microemulsions can be produced by extensive sonication of mixtures of polar and neutral lipids [1]. These preparations usually contain a variable fraction of small unilamellar vesicles (SUVs). Particles containing apolar lipid cores (model lipoproteins) can be separated from the bilayer vesicles on the basis of density. A number of techniques [1,2], including negative stain electron microscopy, differential scanning calorimetry, X-ray diffraction/scattering, and NMR spectroscopy have been employed to characterize such microemulsions.

We report here that freeze-fracture and etching electron microscopy can be employed as an alternative and graphic method of detecting micellar systems with apolar lipid cores. The technique depends on the observation that model lipoprotein particles can exhibit a novel smooth crossfracture face with little or no shadowing effects. In contrast, no such smooth crossfracture face is present in liposomes. These either show the usual shadowing associated with curved bilayer systems or crossfracture patterns characteristic of multilamellar systems. The results obtained are supported by ^{31}P-NMR studies.

A mixture of PC/cholesterol/cholesteryl linoleate/triolein (1 : 0.5 : 1 : 2.5, molar ratio) containing 250 μmol total lipid in 6 ml of 20 mM Hepes buffer (pH 7.1) was sonicated using a probe tip Biosonic Sonicator for 3.5 h at 40–45 °C. [^{3}H]DPPC and [^{14}C]triolein were added in the mixture in trace amounts for quantitation purposes (Table I).

The sample was centrifuged in the Hepes buffer using a Ti75 rotor in a Model L575 Beckman Ultracentrifuge spun for 24 h at 42 000 rpm. This resulted in two fractions. The low-density fraction (top) was obtained by slicing the tube just below the band. The high-density fraction (bottom) was isolated as a pellet which was re-suspended in the Hepes buffer for freeze-fracture and ^{31}P-NMR studies.

Abbreviations: PC, egg phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DPPC, dipalmitoylphosphatidylcholine.

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Egg phosphatidylcholine was obtained from Avanti; cholesterol was obtained from American Research Products; cholesteryl linoleate, triolein and Hepes were from Sigma; [^{3}H]DPPC and [^{14}C]triolein were from New England Nuclear. All materials were used without further purification.

The freeze-fracture and etching was performed on a Balzers Freeze-Etching System BAF 400D. The micrographs were obtained using a JEOL Model JEM-1200 EX Electron Microscope. The samples were cryofixed in the presence of 25% glycerol by plunging into liquid freon 22. In non-etched samples, the fractured surface was shadowed unidirectionally with platinum and coated with carbon immediately after fracturing at 163 K, 10^{-6}–10^{-7} torr, while etched systems were etched for 2 min at 183 K after fracturing at 163 K before being shadowed (10^{-6}–10^{-7} torr).

Figs. 1a–f are the freeze-fracture and etching electron micrographs of uncentrifuged (a and d), as well as the low-density (b and e) and high-density (c and f) fractions. The solid arrow in Figs. 1a and b points to a distinctive particle having a smooth crossfracture face. Many such faint 'ghost' particles can be seen in these micrographs. These particles become much more visible when the fractured surface is etched before shadowing as seen in Figs. 1d and e. This may be interpreted as due to sublimation of the surrounding ice but not the apolar lipid core. Thus, the crossfracture of such a particle remains smooth. The particle size of the systems with apolar cores appears quite heterogeneous, but this is not unequivocal as the location of the crossfracture planes is not known. Etching also further enhances the resolution of the smaller particles, making them much more discernible (see Figs. 1d and e). The arrowhead in Fig. 1a indicates a system with the characteristic shadowing of a bilayer vesicle (cf. Figs. 1c and f). However, the observation of shadowing effects normally associated with curved bilayer systems in the micrographs does not appear to necessarily indicate the presence of bilayer vesicles. For example, for the low-density fraction (Fig. 1b) a system exhibiting the shadowing characteristics of bilayer vesicles can be observed (see outlined arrows). These are unlikely to be bilayer vesicles; rather, they are most probably systems with apolar cores that did not crossfracture. The various possibilities are presented in Fig. 2. The most commonly occurring particle morphology is that depicted in Fig. 2b, which gives rise to smooth crossfracture faces. The less common situations are those depicted in Figs. 2a and c, which give rise to various shadowing effects of curved surfaces. Fig. 2c depicts an interesting situation in which the neutral lipid core fractures along a plane parallel to the fractured surface resulting in a 'depressed' smooth crossfracture face as shown in Fig. 1b (see open arrow). Finally, freeze-fracture micrographs of the pellet obtained after centrifugation revealed the presence

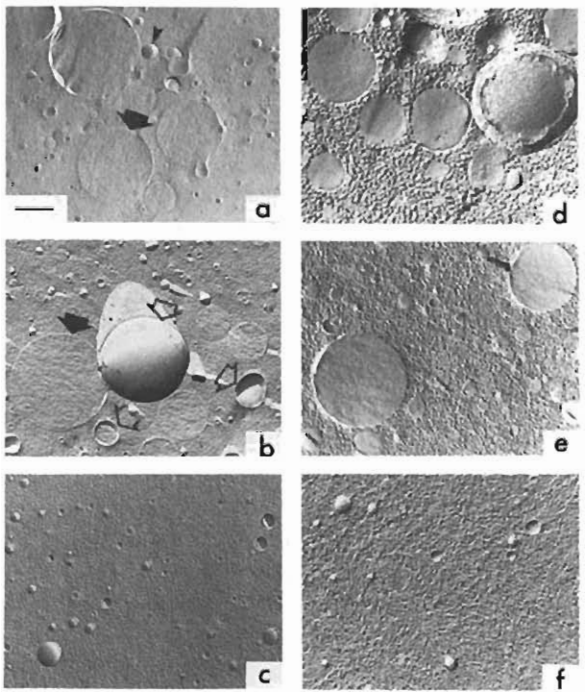

Fig. 1. Freeze-fracture electron micrographs of a sonicated mixture of PC/cholesterol/cholesteryl linoleate/triolein (1:0.5:1:2.5 molar ratio) in 20 mM Hepes buffer (pH 7.1). The samples were cryofixed in the presence of 25% glycerol by plunging into liquid freon 22 and fracturing at 163 K, 10^{-6}–10^{-7} torr on a Balzers Freeze-Etching Systems BAF 400D. a and d, uncentrifuged; b and e, low-density fraction; c and f, high-density fraction. a, b and c were shadowed immediately after fracturing. d, e and f were etched for 2 min at 183 K, 10^{-6}–10^{-7} torr before being shadowed unidirectionally with platinum and coated with carbon. In a and b the solid arrow indicates a model lipoprotein particle with a smooth crossfracture face (cf. d and e), while the arrowhead shows the presence of a bilayer vesicle (cf. c and f). The outlined arrows in b point to uncrossfractured particles (see a). The bar represents a distance of 200 nm.

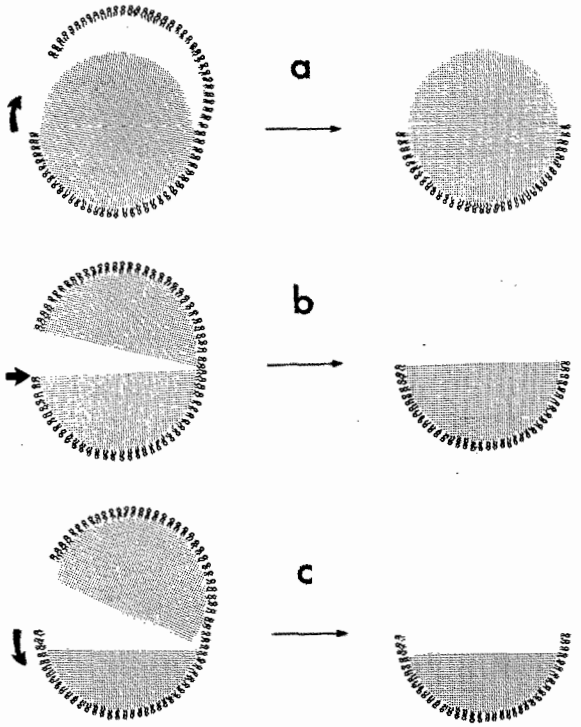

Fig. 2. Schematic representation of the freeze-fracture of a model lipoprotein particle at the molecular level. (a) Fracture along the interface between the polar phospholipid monolayer and the neutral lipid core giving rise to a 'dome' (as shown) on the fractured surface, or conversely, a 'trough' (see outlined arrows in Fig. 1b). (b) Crossfracture along the plane of the fractured surface (see solid arrow in Figs. 1a and b). (c) Crossfracture parallel to the plane of the fractured surface (see open arrow in Fig. 1b).

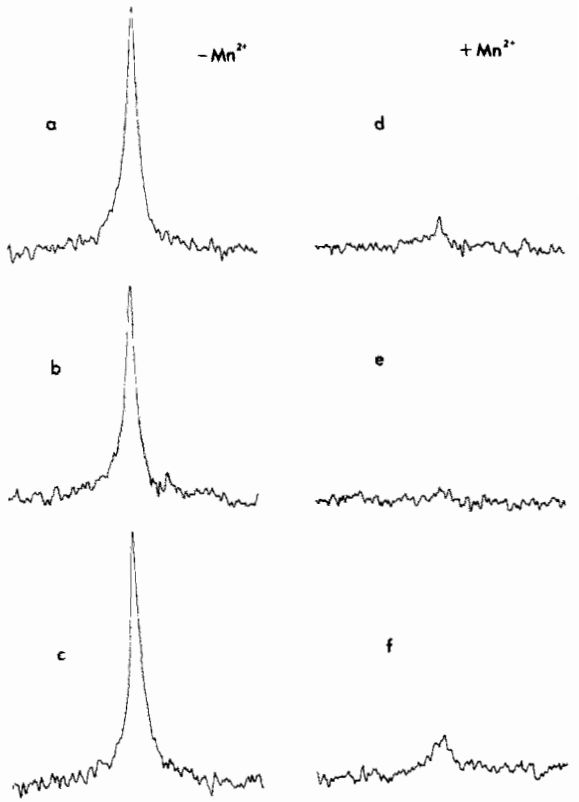

Fig. 3. Proton-decoupled ^{31}P-NMR spectra (81.0 MHz) of a sonicated mixture of PC/cholesterol/cholesteryl linoleate/triiolein (1 : 0.5 : 1 : 2.5 molar ratio) in 20 mM Hepes buffer (pH 7.1) to show the effect of Mn^{2+} quenching. a–c, unquenched; d–f, quenched with 5 mM Mn^{2+}. a and d, uncentrifuged mixture; b and e, low-density fraction; c and f, high-density fraction. Plot width is 2500 Hz.

of small unilamellar vesicles (Figs. 1c and f).

NMR spectra were also obtained for the same samples. Fig. 3a is a ^{31}P-NMR spectrum of the uncentrifuged sonicated mixture, while Figs. 3b and c are the spectra for the low- and high-density fractions, respectively. Figs. 3d–f are the spectra obtained for samples a–c, respectively, in the presence of 5 mM $MnCl_2$. In the uncentrifuged sample only approx. 87% of the ^{31}P-NMR signal (see Figs. 3a and d) is accessible to Mn^{2+}, indicating the presence of a fraction of closed bilayer systems. The low-density fraction has virtually no signal intensity left (see Figs. 3b and e) after addition of Mn^{2+}. This would be consistent with essentially all the particles in this fraction being emulsions of neutral lipids surrounded by a monolayer of phospholipids. This interpretation is also supported by the increase in the PC/neutral lipid ratio of 1 : 3.8 in the uncentrifuged sonicated mixture to a ratio of 1 : 5.7 in the low-density fraction (Table I). The high-density fraction has approx. 69% of the signal quenched (see Figs. 3c and f),

TABLE I

RATIOS OF PHOSPHOLIPIDS TO NEUTRAL LIPIDS
CL, cholesteryl linoleate; TO, triolein.

Component	PC/CL+TO (by wt.)
Uncentrifuged sonicated mixture	1 : 3.81
Low-density fraction (top)	1 : 5.71
High-density fraction (bottom)	1 : 0.65

suggesting that the majority of the particles are SUVs which can exhibit outside-inside ratios of 2:1 or higher [3]. However, the PC/neutral lipid ratio of 1:0.65 exceeds the amount of neutral lipid (5%) that can be accommodated in SUVs (Table I). Hence, it is likely that very small solid particles with neutral lipid cores are also present in the bottom fraction. However, these are not evident in the freeze-fracture micrographs (Figs. 1c and f). It may be that these particles are too small to be detected.

In conclusion, the results presented here demonstrate the use of freeze-fracture electron microscopy for detecting lipoprotein analogues with an apolar lipid core. This procedure provides enhanced resolution of detailed structural morphology over other techniques (such as negative stain electron microscopy) commonly utilized to visualize lipoprotein analogues. This is clearly of utility for the characterization of microemulsions composed of polar and non-polar lipids.

This research was supported by the B.C. Heart Foundation. P.R.C. is an MRC Scientist.

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