Lipid vesicle formation: the transition from open disks to closed shells

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The relaxation of a lecithin suspension after sonication is studied by electron microscopy. It is shown that vesicles are formed in a two-stage process: Fragmentation into open sheets of bilayer under the action of sound and subsequent spontaneous closure of the planar disks into closed shells. A prerequisite of the discovery is a slowing down of the relaxation process by the addition of a moderate amount of cholate as an 'edge-actant'.

Vesicle *Lecithin* *Cholate* *Bilayer*

1. INTRODUCTION

The classical method for preparing unilamellar lipid vesicles is the sonication of a lipid dispersion [1]. A mechanism of vesiculation is not known for this procedure nor for any other [2,3].

A general theoretical framework of vesiculation has been proposed [4,5], such that in a first step open planar disks of bilayer are formed by some process of fragmentation or growth and that in a second step these disks close to vesicles spontaneously. Here, the existence of the transient disks is demonstrated. The experimental conditions are chosen to match conventional procedures as closely as possible: We use egg lecithin as a lipid [1], sonication for fragmentation [1,6], and electron microscopy with negative staining for visualization [7].

To catch transient disks, never observed hitherto, their presumed decay to vesicles must be slowed down. The disk/vesicle transition is governed by the 'vesiculation index' V_F [4] as defined by the radius R_D of the disks, by the elastic bending modulus k_c and the edge tension γ_M of the membrane according to eq. 1:

$$V_F = R_D \gamma_M / 4k_c \quad (1)$$

A disk is unstable with respect to closure if $V_F > 2$. Lowering V_F makes a disk metastable, such that near $V_F = 1.7$ its lifetime is of the order of 1 h.

A simple modulation of V_F is provided through the parameter γ_M: According to Gibbs'isotherm the intrinsic edge tension γ_o is lowered by additives which accumulate at the edge as expressed by eq. 2, where $\bar{c}_E$ is the number of binding sites per unit length, c_W is the free concentration of the 'edge-active' agent and K_{EW} is the dissociation constant from edge to water [4].

$$\gamma_M = \gamma_O - kT\bar{c}_E \ln (c_W/K_{EW} + 1) \quad (2)$$

As the free concentration c_W exceeds a certain value the edge-tension vanishes: The disks exist as thermodynamically stable micelles.

To consider cholate as an 'edge-actant' in the sense of the theory is obvious, taking into account its chemical structure [8], its ability to emulsify lipid to stable mixed micelles [8,9] and its application in various procedures of vesicle production [10–13]. The formation of the stable micelles – with $\gamma_M = 0$ – sets an upper limit in the search of a concentration which gives rise to metastable disks of sufficient lifetime after sonication.

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2. EXPERIMENTAL

2.1. *Suspension*

Appropriate volumes of methanolic solutions of egg lecithin (2.6 mM) and of taurochenodesoxycholate (TCDC, 3.84 mM) (both Sigma) are mixed, evaporated in a titanium vessel with dry N_2 and kept over P_2O_5 overnight. 2 ml of 150 mM NaCl are added. The sample is sonicated (Branson sonifier, level 3) for 4×5 min at 25°C and for 4×5 min at 0°C with intervals of 5 min. It is centrifuged at 0°C at $30\,000 \times g$ for 30 min. The supernatant is kept at 0°C.

2.2. *Electron micrographs*

Carbon films (5–10 nm thick) are deposited on copper grids (400 mesh) and exposed to a glow discharge within 2 h before use. At 0°C first a drop of the suspension then a drop of phosphotungstic acid (Merck, 1% solution, pH 6.9) is applied each for 30 s and sucked off. The grid is dried at 40°C. The electron micrographs are taken in a Philips EM 301.

2.3. *Evaluation*

Two types of objects can be distinguished uniquely on the micrographs: closed loops and straight lines – both isolated or in stacks (cf. figs 1 and 2). Their number and size is evaluated by projection onto a reversed plotter (Watanabe) where the patterns are probed by a microcomputer (Apple II). Objects which cannot be assigned uniquely as some multilamellar liposomes and blurred patterns are not considered.

3. RESULTS

For control purposes fig.1a shows an electron

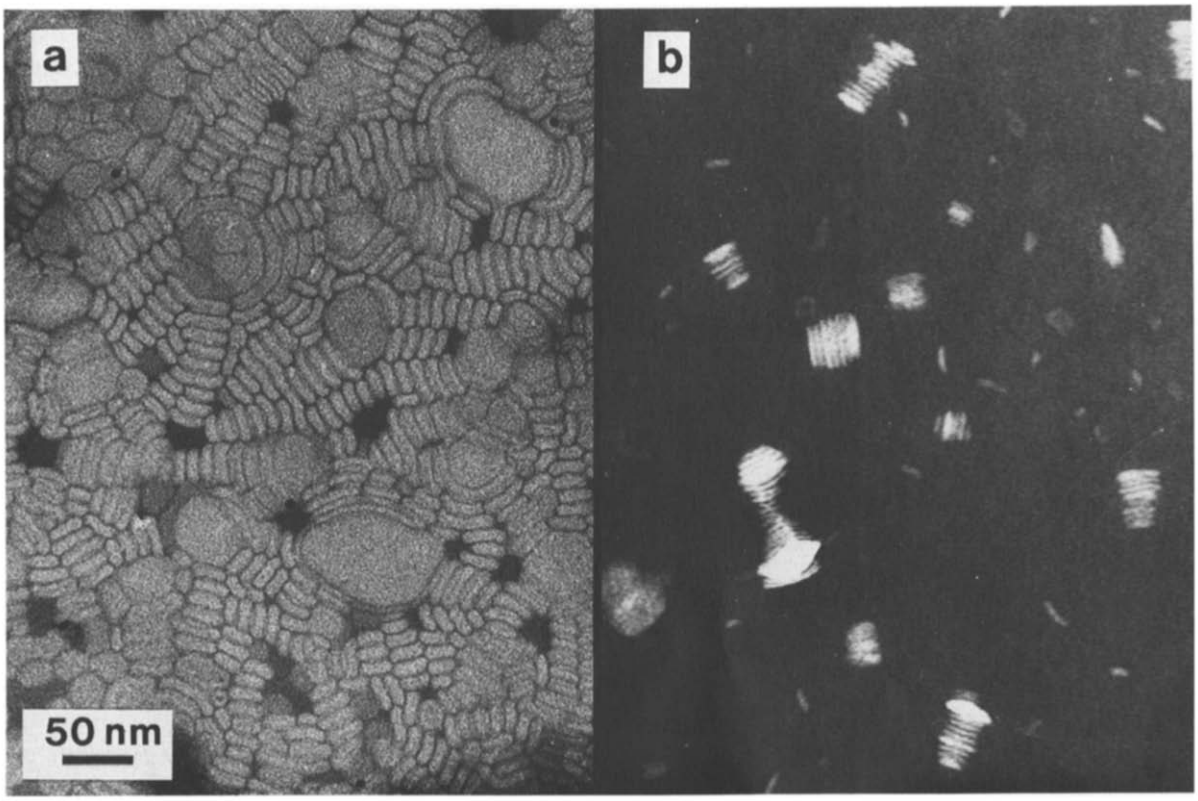

Fig.1. (a) Electron micrograph of a sonicated suspension of 2.5 mM egg lecithin in 150 mM NaCl (negative stain). The pattern is typical for unilamellar vesicles. (b) Micrograph of a suspension of 2.5 mM egg lecithin and 2.5 mM taurochenodesoxycholate in 150 mM NaCl incubated for 48 h at 0°C (negative stain). The pattern of stacked lines 3.3 nm wide is assigned to stacks of discoid mixed micelles viewed in profile.

micrograph of a suspension with 2.5 mM lecithin stained 3 min after sonication. The pattern of closed loops is typical for unilamellar vesicles [7]. The aggregation is induced by the stain.

For vanishing lecithin concentration the free concentration c_W of TCDC above which stable micelles exist ($\gamma_M = 0$, eq. 2) is $c_W^* = 0.9$ mM [14]. With 2.5 mM lecithin, the phase limit of micellization, expressed in terms of the total concentration c of TCDC, is shifted to about $c = 2$ mM, taking into account the molar ratio lecithin/cholate of 2 in the mixed micelles [9]. Fig.1b shows the micrograph of a suspension with 2.5 mM TCDC and 2.5 mM lecithin after an incubation of 48 h [9]. We assign the pattern of stacked lines being 3.3 nm wide to stacks of disk micelles embedded into the stain with their planes perpendicular to the plane of observation [15]. The aggregation is assigned to the stain. The length of the lines is about 26 nm, corresponding roughly to the diameter of the disks as indicated by light scattering [9].

Fig.2a shows a micrograph of a suspension with 0.8 mM TCDC and 2.5 mM lecithin 35 min after sonication. The pattern of stacked lines is most similar to the pattern assigned to the mixed micelles at high cholate concentration. Fig.2b shows a micrograph of the same suspension 180 min after sonication. The pattern of closed loops is typical for unilamellar vesicles.

Fig.2 reveals the primary formation of disk fragments by sonication and their subsequent relaxation to closed vesicles. The time course of relaxation is shown in fig.3a as obtained from an evaluation of 4 preparations with 19500 objects. Although the standard deviations are considerable, a half-life of about 1.5 h can be estimated. For two samples the size of the primary disks and that of the vesicles are evaluated as shown in fig.3b. The

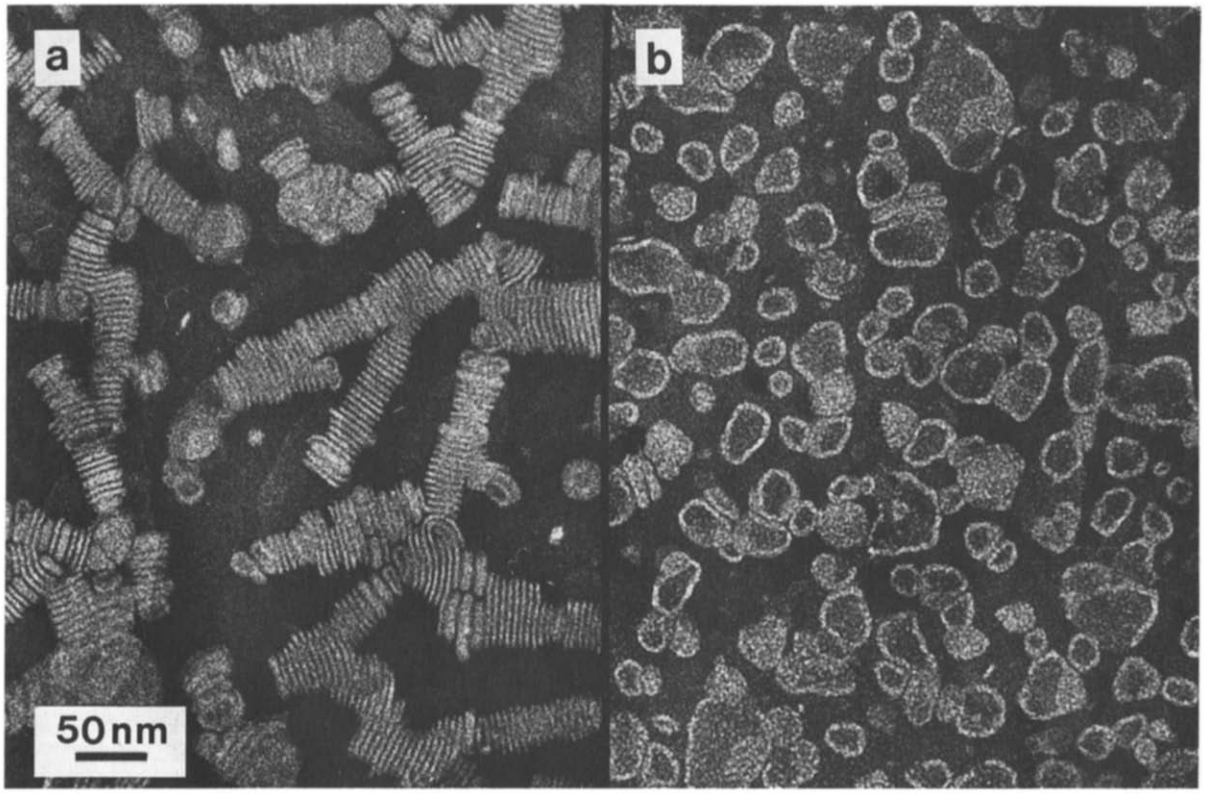

Fig.2. (a) Electron micrograph of a suspension of 2.5 mM egg lecithin with 0.8 mM taurochenodesoxycholate in 150 mM NaCl at 0°C prepared 35 min after sonication (negative stain). The dominating pattern of stacked lines 3.3 nm wide is assigned to stacks of disks of bilayer viewed in profile. (b) Micrograph of the same suspension 180 min after sonication. The pattern dominating now is typical for unilamellar vesicles.

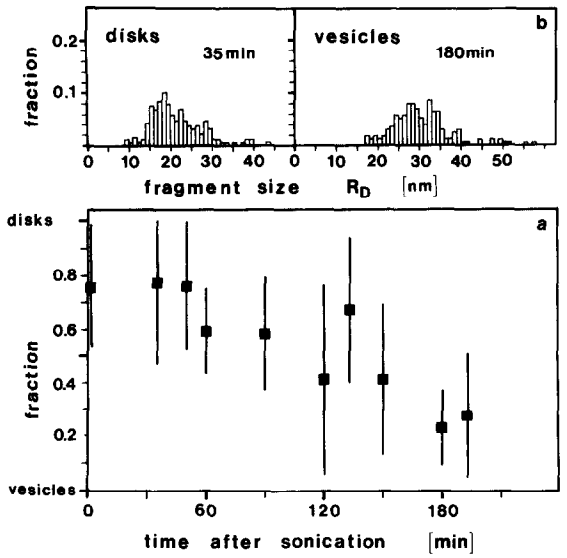

Fig.3. (a) Number fraction of disks and vesicles as a function of time after sonication of suspensions of 2.5 mM egg lecithin and 0.8 mM taurochenodesoxycholate in 150 mM NaCl at 0°C. Mean value and standard deviation are indicated (The first sample is taken before centrifugation). 19500 objects from 4 suspensions are evaluated. (b) Left: distribution of the radius R_D of disks as calculated from their lateral projections observed 35 min after sonication (cf. fig.2a). Right: distribution of the diameter of vesicles - equal to the radius R_D of corresponding disks - as estimated from their patterns observed 180 min after sonication (cf. fig.2b). (180 objects evaluated for each histogram)

vesicles appear to be somewhat larger than the original disks, although the significance of that difference is uncertain. The relaxation reflects the kinetics of the closure process itself or/and of a preceding growth process of the disks.

4. DISCUSSION

A study of the pathway of vesicle formation implies the characterization of objects of different shape and size. This task cannot be accomplished by light scattering. A perturbation of the system for electron microscopy is inevitable. In this first approach we have chosen the negative staining technique - although it is particularly prone to artefacts - because a unique assignment of patterns to vesicles and to disks was possible and because these patterns were suitable for a statistical evaluation.

The inspection of scores of micrographs as shown in fig.2 and the quantitative evaluation as shown in fig.3 reveal unambigously that the sonication of the lipid suspension creates open disks which close to vesicles in a second step without energization. The discovery of this pathway of vesiculation (cf. fig.4, inset) is due to the selection of a system where the rate of the second process is slowed down sufficiently as suggested by the theory.

To interpret this result we use the phase plane of fig.4 relating the size R_D of the fragments and the total concentration c of the edge-actant [4]. The limit of stable micelles is marked as c^*. For $c > c^*$ the decreasing size of mixed micelles is indicated [4,9] whereas for $c < c^*$ two curves of constant V_F are drawn with $V_F = 2$ marking the limit of

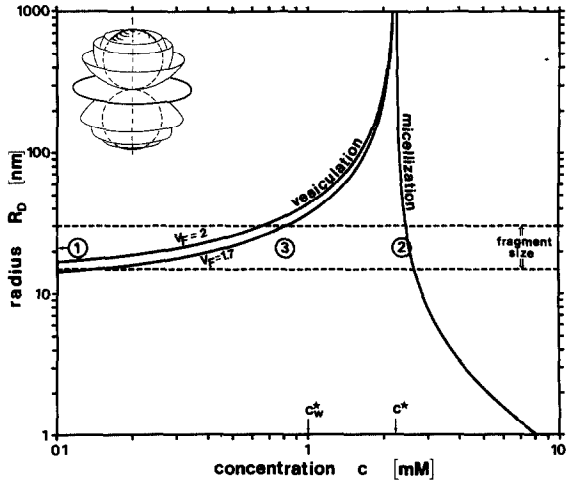

Fig.4. Schematic phase plane relating the radius R_D of planar disks (equal to the diameter of corresponding closed spherical shells) and the total concentration c of an edge-actant. The phase limit of stable micelles for vanishing lipid concentration is chosen as $c_w^* = 1$ mM to match approximately the value for TCDC. The phase limit c^* refers to 2.5 mM lecithin assuming a molar ratio of lecithin/cholate of 2 in the mixed micelles. For $c > c^*$ the decreasing radius of mixed micelles is drawn. For $c < c^*$ the lines $V_F = 2$ (limit of metastability of the disks with respect to vesiculation) and $V_F = 1.7$ (lifetime of the disks of 1 h) are shown. The size of fragments as obtained by sonication is marked. The numbers indicate the conditions of the experiments: (1) Instantaneous vesiculation with pure lecithin, (2) stable mixed micelles, (3) slow transition from disks to vesicles. Inset: transition (bifurcation) of a planar disk into a closed vesicle.

metastability of disks and with $V_F = 1.7$ marking a lifetime of about 1 h (cf. eqs 1 and 2 [4]). Sonication creates fragments with $R_D = 15$–30 nm (fig.3b). With pure lecithin (point 1 in fig.4) these disks are not detectable (cf. fig.1a) because $V_F > 2$: They are unstable with respect to vesiculation. At a TCDC concentration of $c = 2.5$ mM (point 2 in fig.4) the fragments form stable micelles (cf. fig.1b) because $c > c^*$. At a TCDC concentration of 0.8 mM (point 3 in fig.4) the fragments are visible as disks just after sonication (cf. fig.2a) because $V_F < 2$: They are metastable. They disappear as they overcome the activation barrier for closure directly or after approaching the stability limit by growth (cf. figs 2b and 3).

The future complete characterization of the disk-vesicle transition after sonication or in other procedures of vesiculation as dialysis [12] and dilution [13] will be based on the evaluation of various electron microscopic techniques as combined with the non-invasive techniques of light scattering, magnetic resonance and fluorescence. The effect of membrane proteins on the – symmetry breaking – vesiculation is to be studied in particular.

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