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Na,K-ATPase and carboxyfluorescein distinctly alter vesicle formation in vitro

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Abstract

The mechanism of vesicle formation as well as the precise reasons for their stability are not known. Thus, it is necessary to simulate the process in vitro for studying its mechanism. If phospholipids are suspended in physiological solution by means of cholate and the detergent is then removed by dialysis, the phospholipids self-assemble to form unilamellar vesicles. We report here that the addition of Na,K-ATPase (an integral membrane protein) to the phospholipids changes the vesicle structure, they become larger and a multilamellar population appears. By contrast, carboxyfluorescein, a compound commonly used for labelling the aqueous vesicle compartment, produces an unexpected effect on vesicle structure by inducing complex, tore-like intravesicular multilayer formations associated with a 5-fold increase in diameter. Thus, the presence of a protein in the membrane phase or of a compound in the water phase can influence and direct vesicle formation in vitro; these model systems might give some clues to possible physicochemical or biological factors governing the formation of natural membrane structures.

Key words: Vesicle self-assembly in vitro; Cholate dialysis; Ultra thin-section; Homogeneous single-walled vesicle; Size increase; ATPase, Na^+/K^+-; Multilayer; Carboxyfluorescein

1. Introduction

In their role as carriers for neurotransmitters and hormones, intracellular vesicles undergo intense intracellular traffic [1,2]. Despite their mobility and their passage across various intracellular compartments, their diameter and their structure are remarkably stable [3] reflecting a thermodynamic state of minimal energy. Yet, the molecular mechanisms of vesicle formation and the physicochemical and thermodynamic reasons for their stability are not well understood [4,5], mostly because such precise parameters are difficult to assess experimentally in a cell or with isolated natural vesicles which are already formed. Therefore, it seemed interesting to define some factors which could influence the vesicle structure during their formation in vitro using a self-organizing model system. For this purpose, phospholipid micelles are suspended in a buffered salt solution by means of cholate to constitute a lipid/water interface [6]; during detergent removal by dialysis, the micelles assemble to spontaneously form homogeneous vesicles [7,8] indicating that we are in presence of a self-organizing system. We report here that the presence of an integral membrane protein during vesicle self-assembly increases their diameter from about 60 to 100 nm. On the other hand, the addition of a hydrophilic compound (carboxyfluorescein), a commonly used fluorescent vesicle marker, during their preparation had a totally unexpected effect: formation of homogeneous multilayered vesicles of an average diameter of about 320 nm. By contrast, the addition of 20% fluorescein-phosphatidylethanolamine to the lipid phase had no structural effects. To work out these differences in a reliable way, four distinct types of vesicles were formed repeatedly in identical conditions: (i) pure phosphatidylcholine-vesicles (PC-vesicles), (ii) PC-vesicles plus functional Na,K-ATPase (NKA-vesicles), (iii) PC-vesicles plus fluorescein-phosphatidylethanolamine (Fluo-vesicles), and (iv) PC-vesicles plus carboxyfluorescein (CF-vesicles).

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Abbreviations: PC, phosphatidylcholine; Fluo, fluorescein-phosphatidylethanolamine; CF, 5,6-carboxyfluorescein; NKA, Na/K-ATPase.

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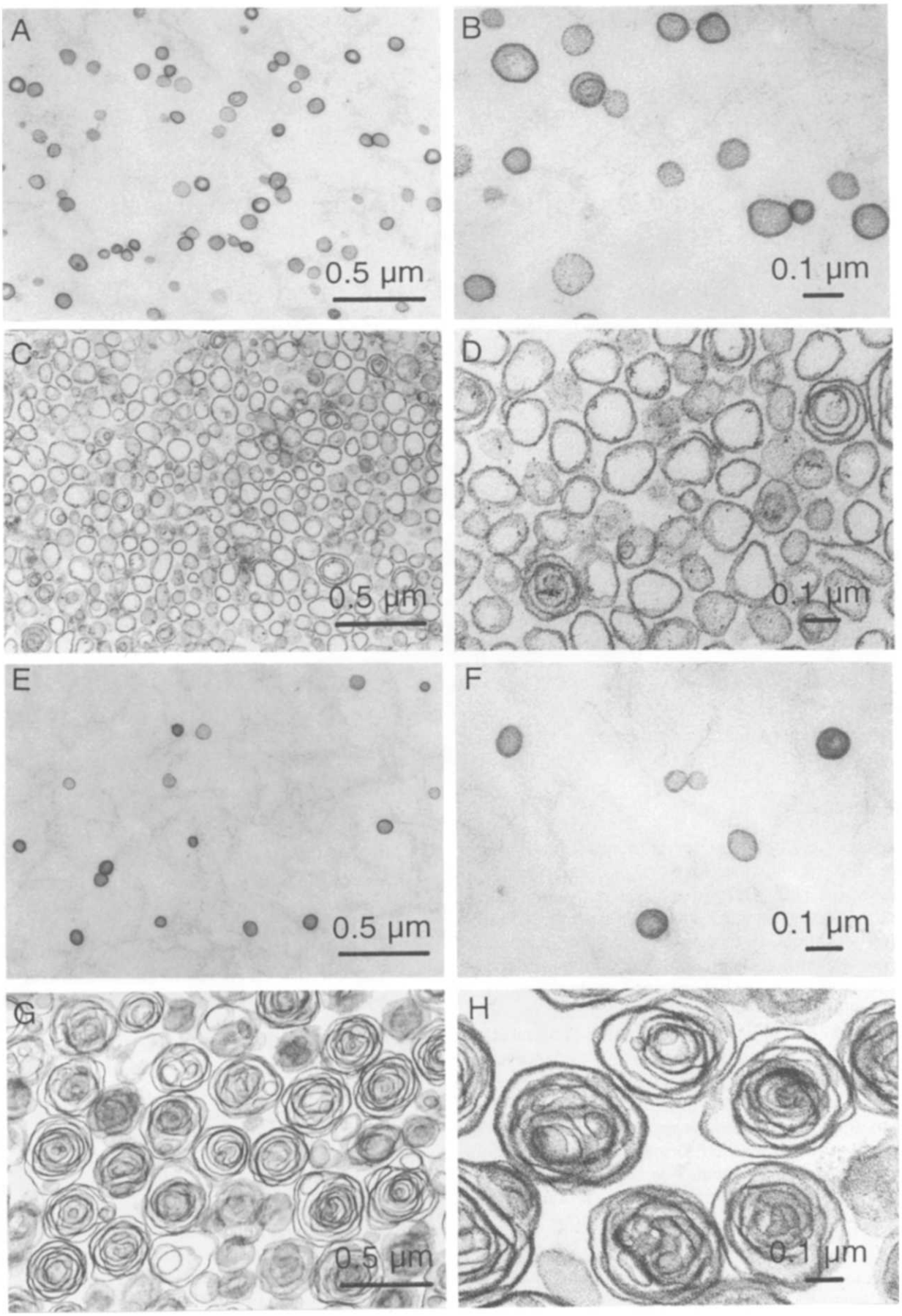
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2. Materials and methods

PC-vesicles were prepared by detergent-dialysis as described in detail previously [9]. Briefly, 60 mg PC grade I or IIa (Lipid Products, Nutfield, UK) was dried in a 50 ml round-bottomed flask fixed to a rotary evaporator apparatus under a stream of ultrapure nitrogen, redissolved three times with diethyl ether and dried finally to a very thin film in a water bath at 25°C followed by 2 min at 37°C. To the lipid film 3 ml of a solution containing 1 mM EDTA, 30 mM L-histidine, 5 mM $MgCl_2$ and 23 mM cholate (Merck) (pH 7.2) was added and the flask rotated until a clear solution was obtained. The lipid-cholate solution (solution A) was stored at −70°C in 100-μl portions. To form pure PC-vesicles (Figs. 1A, B), a 100 μl aliquot of solution A was mixed with an equal volume which contained in addition 50 mM NaCl, 50 mM KCl, 30 mM histidine, 5 mM $MgCl_2$, 1 mM EDTA (pH 7.2) (solution B); the solution was then added to sterile EDTA-treated dialysis tubing of 8 mm diameter flat (Union Carbide) and dialysed at 20°C for 15 h in 10 ml of the solution B devoid of cholate. The same vesicles were seen when dialysis temperature was 0°C. To form NKA-vesicles (Figs. 1C, D), functional, transport-active NKA was isolated from the renal outer medulla of lamb kidney to a specific activity of about 1200 μmol P_i/mg protein per h by the dodecyl sulfate extraction method [10]; 800 μg protein were sedimented at 100000 × g in a Beckman Airfuge and resuspended in 100 μl of a solution (solution C) containing 50 mM Na_2ATP (Boehringer), 5 mM $MgCl_2$, 30 mM histidine, 1 mM EDTA and 23 mM cholate (pH 7.2) as described [11]. The resulting turbid enzyme suspension was again centrifuged at 100000 × g for 15 min and the supernatant which contained about 50% dissolved but active NKA protein was added to an equal volume of lipid solution (A). The specific NKA activity was monitored throughout the whole procedure to assure preservation of activity [9]. Dialysis was performed as described for PC-vesicles (A, B), except that the dialysis took place in 5 ml solution C containing 500 mg EDTA-washed cholestyramine-resin (Sigma) and the temperature was kept at 0°C to prevent ATP hydrolysis [11]. The external ATP was removed by two 75 min centrifugations at 100000 × g (0°C) in an ATP-free solution D (100 mM NaCl, 30 mM histidine, 5 mM $MgCl_2$, 1 mM EDTA (pH 7.2)). Fluo-vesicles (Figs. 1E, F) were prepared as PC-vesicles except for the replacement of 20% PC by fluorescein-phosphatidylethanolamine (Molecular Probes, US), a marker of the lipid phase. Finally, CF-vesicles (Figs. 1G, H) were prepared identically to PC-vesicles (A, B) except for the presence of 200 mM 5,6-carboxyfluorescein (Fluka, Switzerland) added prior to dialysis at 20°C; the pH was adjusted to 7.2 by adding 1.5 ml of 2.5 M NaOH to 9 ml of solution B; thus, 357 mM supplementary Na ions were added to solution B and the final concentrations were: 400 mM Na ions (43 mM added as Cl salt), 43 mM KCl, 4.3 mM $MgCl_2$, 0.86 mM EDTA, 25.7 mM histidine (solution E). To remove the external CF after dialysis, the vesicles were centrifuged four times for 60 min at 100000 × g in CF-free solution B.

Table 1
Na,K-ATPase-liposomes (Preparation No. 596) containing about 1 mg protein and 10 mg PC per ml were thin-sectioned processed for electron microscopy as described in legend of Figs. 1C, D

Micrograph No.	Vesicles counted No.	Ratio Multi-/unilamellar
8812	503	0.044
8812	368	0.045
8813	399	0.036

360–500 vesicles were counted on the micrograph and determined as uni- or multilamellar. The ratio of multi- to unilamellar vesicles was then calculated.

3. Results and discussion

Fig. 1 shows electron micrographs of the four thin-sectioned vesicle types at two distinct magnifications, 32500 × (Figs. 1A, C, E, G) and 70000 × (Figs. 1B, D, F, H). The PC-vesicles (Figs. 1A, B) are single-walled, homogeneous, with an average diameter of 60 to 70 nm determined on the thin-sectioned preparations as well as by laser light scattering [12] and by previous statistical analysis of freeze-fractured preparations that had been prepared in identical conditions [8].

By contrast, when isolated NKA was present during vesicle assembly, their average diameter increased from about 60 to 100 nm as shown in Figs. 1C, D in agreement with previous laser light scattering measurements [12] and measurements of identical NKA-vesicles by freeze-fracture [8]. In ultra thin-sections it can be seen that a certain number of NKA-vesicles became multilamellar. Table 1 shows that the occurrence of the multilayered population was uniformly distributed

Fig. 1. Electron micrographs of artificial phosphatidylcholine (PC)-vesicles formed once only with PC, PC-vesicles (A, B), once in the presence of NKA, NKA-vesicles (C, D), once in the presence of fluorescein-phosphatidylethanolamine, Fluo-vesicles (E, F) and, finally, in the presence of carboxyfluorescein, CF-vesicles (G, H). Vesicles were prepared by detergent-dialysis as described in the text. For electron microscopy, vesicles were sedimented at 100000 × g, the resulting pellets prefixed in 2% glutaraldehyde in cacodylate buffer, postfixed in 2% OsO_4-collidine, dehydrated in ethanol and embedded in Epon. Ultrathin sections stained in uranyl acetate and lead citrate were examined in a Phillips 400 electron microscope. For each vesicle-type a representative preparation is shown. Magnifications: A, C, E, G, 32500 ×; B, D, F, H, 70000 ×.

among the single-walled vesicles as determined on a population of 1270 vesicles and was near 4%. The formation of multilayers must be caused by the NKA which is composed by a catalytic 110 kDa α-subunit responsible for energy transduction, conformational change and ion-transport, a 50–60 kDa β-glycoprotein serving as membrane anchor for the α-subunit [13] as well as adhesion molecule [14], lipids and cholesterol [12]. Zampighi et al. [15] also observed that purified NKA in isolated membrane sheets was able to induce coupling of adjacent membranes, presumably by interaction of the glycomoities of the β-subunit. A similar mechanism may lead to the formation of multilayered vesicles if the NKA density in the artificial membrane reaches a critical level since multilayered structures were rare or absent in vesicles prepared with about 4-fold less NKA-protein [16].

Fluo-vesicles were single-layered and not different from pure PC-vesicles. By contrast, the presence of CF produced an unexpected and drastic effect, shown in Figs. 1G, H: the vesicles became large and multilayered in a strikingly homogeneous manner. The number of lamellae after thin-section, varied from 5 to 10. The homogeneity of the vesicle size was confirmed by laser light scattering as shown by a typical recording (Fig. 2A) in comparison to a control experiment performed whit calibrated micro spheres of 300 nm (Fig. 2B). Statistical analysis of the size measurements confirmed the narrow distribution of the diameter in two different preparations (Table 2). To our knowledge, such artificially formed homogeneous multilayered vesicles have not been described before.

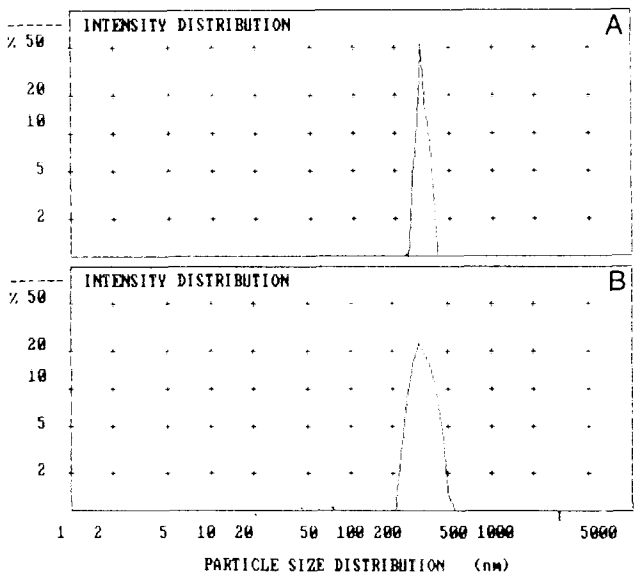

Fig. 2. (A) Size measurements of CF-liposomes by laser light scattering: 20 μl of CF-liposomes (preparation No. F23) was diluted in 2–3 ml of filtered buffered saline solution as described in legend to Table 2 and their size was determined by laser light scattering in comparison to polystyrene spheres calibrated at 300 nm shown in (B).

Table 2
Size measurements of CF-liposomes by laser light scattering

Liposome preparation No.	Diameter (nm) (mean ± S.D.)
F23	309.2 ± 5.4
F24	335.3 ± 6.6
F24	331.5 ± 15.4

20 μl of two different CF-liposome preparations (F23, F24) were diluted in 2–3 ml of filtered buffered saline solution (PBS) solution (mM: 137 NaCl, 2.68 KCl, 8.1 Na_2HPO_4, 1.47 K_2HPO_4 (pH 7.25–7.35)) and their size was determined by laser light scattering.

In summary, pure lipid vesicles become always single-walled when prepared by cholate dialysis, whereas their structure is drastically and distinctly changed when either NKA is present in the membrane phase or CF in the aqueous phase. The structural change is not chaotic but ordered: in the first case larger vesicles form and 4% of them become multilayered (2 to 3 layers) whereas in the second case large vesicles containing intravesicular tore-like structures. It can be calculated that the presence of these intracellular layers displace about 20% of the aqueous volume, that is, less solution is entrapped as predicted from their diameter assuming unilamellar vesicles.

Since the physicochemical parameters guiding the formation of homogeneous and structurally identical vesicles of fixed diameter, uni- or multilamellar or containing tore-like structures, are not known only tentative interpretations of our observations can be proposed. To form the vesicles, cholate is removed from the mixed phospholipid-cholate or NKA-phospholipid-cholate micelles by dialysis in a solution without cholate. While the detergent is being removed, discs fuse. At a certain point, the detergent can no longer shield the borders of the discs which tend to bend and fuse to form spheres. The presence of a transmembrane protein could increase the vesicle size by mobility restriction of the adjacent lipids and overall reduction of the lipid bilayer motion leading to more rigid lipid discs and hence reduce the curvature of the final vesicle [17,18]. It can be calculated that 60 nm PC-vesicles are composed of about 28300 PC molecules brought together by fusion of 1400 disc-shaped micelles containing each about 170 PC molecules [13].

On the other hand, 100-nm proteo-liposomes containing 4 pump molecules, for instance, are composed of about 82900 PC extrinsic plus 520 intrinsic (NKA-associated) phospholipid molecules coming from the fusion of 4140 PC-discs and 4 NKA-containing discs [13]. When vesicles are prepared with increasing NKA concentrations, the vesicle size augments from 60 to 120 nm when the number of pumps per vesicle is

increased from 2 to 6 and levels off around 120 nm when the vesicle contains 6 and 10 NKA molecules as determined by extensive numerical and statistical analyses of 4250 concave and 4250 convex freeze-fractured vesicle halves combined with determination of the distribution and number of intramembrane particles indicating each the presence of a reconstituted sodium pump molecule [8,13]. Na,K-ATPase is an ubiquitous, primary transport system of eukaryotic cells and the receptor for cardioactive steroids [19,20]; our description of its vesicle-structuring property suggests that this most abundant integral membrane protein might have also roles for modulation of lipid fluidity and mobility.

It is evident that the structural constancy must be due to a thermodynamically optimal self-assembly process leading to a final state with minimal energy. Hence, the size and structure attained in each situation must correspond to a state with minimal structural constraints, minimal energy and hence maximal resistance to mechanical strain. In fact, the different vesicle preparations were subjected to repeated centrifugation at $100\,000 \times g$ centrifugal force without alteration of structure; similarly, their structure does not change within at least 10 days at 4°C (data not shown).

Interestingly, the structure of the NKA-vesicles corresponds to the aspect of the natural intracellular vesicles seen in cells; they are composed also predominantly by PC, diverse other lipids and, generally, transmembrane ATPases [21]. Thus, the ATPase-vesicles which self-assemble in vitro as shown herein are virtually indistinguishable form natural intracellular vesicles. Even cells are unable to distinguish them: when isolated human lymphocytes and monocytes are incubated with our artificially formed NKA-vesicles, they take up a large number of the artificial vesicles without loss of viability [22]; the internalized NKA-vesicles are indistinguishable from natural intracellular vesicles (data not shown) The use of natural cholate, PC and NKA for the preparation of vesicles in vitro might be the main reason why they become so similar to naturally formed vesicles.

On the other hand, CF, a non-physiological, inert hydrophilic compound, added during the process of vesicle formation, creates tore-like intravesicular structures. The simultaneous presence of 50 mM ATP and 220 mM Na, a condition used currently for the preparation of ATP-filled NKA-liposomes, do not modify the vesicle structure (data not shown). However, to set the pH at 7.2, 357 mM NaOH are added to the 200 mM CF yielding 400 mM final Na and 43 mM K ions. When the corresponding concentration of Na and K ions (solution E) were added as Cl salts without CF, very heterogenous liposomes were formed ranging on the average around 0.5 μm (data not shown). That high salt favors contacts between small micellar structures has been described [23]; such networks could be the starting point for multilayer formation. However, since the CF-free pure salt liposomes are chaotic the hydrophilic organic anion appears to contribute to the homogenous size and the regular rose-like aspect of the CF-liposomes. CF could interfere directly in the process of vesicle formation when they close in the dialysis bag; it must exert such an effect on the lipid bilayer that the phospholipid-detergent discs may tend to be very close together so that when they fuse, they spontaneously form tore-like multilamellar structures.

4. Conclusion

Taken together, the results presented herein illustrate that phospholipids are self-organizing systems leading to formation of homogeneous vesicles with well defined diameters indicating a minimal energy state. The self-assembly process is influenced importantly by Na,K-ATPase a fundamental ubiquitous membrane transport system which produces 100 nm vesicles of size and aspect equal to the natural vesicles. Finally, we describe the induction of vesicles filled with complex tore-like multi-layers by the presence of a commonly used hydrophilic and biologically inert fluorescent marker, carboxyfluorescein. Regardless of the precise physicochemical mechanisms involved, our results show striking effects of a membrane-embedded protein as well as of the water phase composition on vesicle structure. It will be useful to investigate further the molecular mechanism causing such profound structural alterations in vitro for better understanding biogenesis of natural vesicles.

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