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Budding and Fission of Vesicles

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ABSTRACT We report on budding *and* fission of protein-free vesicles swollen from a natural lipid mixture of bovine brain sphingomyelins. Budding was induced by increasing the area-to-volume ratio through heating. Morphological changes were monitored by phase contrast microscopy and correlated with the thermal behavior of the bilayer by differential scanning calorimetry. Freeze fracture electron microscopy revealed that budding and fission are not restricted to giant vesicles but also occur on length scales relevant for cellular processes. We also observed osmotically induced budding and fission in mixtures of dimyristoyl phosphatidylcholine with cholesterol. We find that these shape transitions are driven by liquid/gel domain formation and/or coupling of the spontaneous curvature of the membrane to the local lipid composition. Our results provide evidence that coat proteins are not necessary for budding and fission of vesicles. The physics of the lipid bilayer is rich enough to explain the observed behavior.

INTRODUCTION

Physiological processes in cells rely on the compartmentalization of the cell interior into various membrane bound organelles and the cytoplasm. These compartments are not static entities but incessantly exchange material via molecular transfer or vesicular transport. The latter process involves budding and fission of protein-coated and noncoated vesicles from internal organelles or the plasma membrane and subsequent fusion of the vesicles with a specific target membrane (Alberts et al., 1989; Brodsky, 1988; Sackmann, 1990). Whereas much effort has been focused on the physics of the fusion process (Siegel, 1993), there has been much less attention paid to budding and fission of vesicles in the cell (Oster et al., 1989). These vesicular carriers are now well characterized both morphologically and functionally (Pfeffer and Rothman, 1987; Pearse and Robinson, 1990) in the biochemical literature, but not much is known about the actual physical mechanisms involved in their creation.

Budding transitions are quite common in synthetic single component vesicles above the chain melting transition (Evans and Rawicz, 1990; Berndl et al., 1990; Käs and Sackmann, 1991; Wiese et al., 1992), but the resulting small vesicles remain attached to the parent vesicle by a narrow neck. The main purpose of the present work is to show that budding followed by fission of small vesicles can and does occur in lipid mixtures (Farge and Devaux, 1992). Parallel electron microscopy and calorimetric studies provide evidence that fission is related to lateral phase separation within the vesicles (Sackmann, 1990). We will discuss our findings within the concept of the bending energy of lipid bilayers (Canham, 1970; Helfrich, 1973, 1974; Evans, 1974, 1980; Svetina and Žekš, 1989) as well as domain formation (Wu and McConnel, 1975; Gebhardt et al., 1977; Markin, 1981; Lipowsky, 1992). It is important to note that our vesicles were protein free, so the observed budding and fission are physical properties of the lipid bilayer alone. Real biomembranes are much more complex systems than are investigated in this work. However, we will give theoretical evidence that the lipid matrix is likely to play an important role in morphological transitions in the cell as well.

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EXPERIMENTAL PROCEDURES

Materials

Samples were prepared from bovine brain sphingomyelin (SPM) obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). The composition (lot BSM-58) of the amide-linked chain was approximately 2% 16:0, 58% 18:0, 6% 20:0, 9% 22:0, 7% 24:0, 15% 24:1, according to Dr. S. W. Burgess (Avanti). The dimyristoyl phosphatidylcholine (DMPC) preparation contained 40% cholesterol (Chol). All phospholipids were obtained from Avanti Polar Lipids. Cholesterol was purchased from Sigma (Deisenhofen, Germany). The purity of the materials used was greater than 99%.

Preparation

SPM vesicles were observed after swelling for 30 min at 42°C. The hydration solution was either 250 mOsm sucrose or Millipore water. Vesicles of mixtures of DMPC with Chol were swollen for 12 h at 42°C in 200 mOsm sucrose solution. To enhance contrast the DMPC:Chol vesicle solution was mixed with 200 mOsm glucose solution in a 1:3 ratio. The details of the swelling procedure and the measuring chamber have been described previously (Käs and Sackmann, 1991).

Light Microscopy

In all but the DMPC:Chol system budding was induced by raising the temperature in order to increase the area-to-volume ratio of the vesicles. The thermal expansivity of the membrane is much larger than that of the enclosed water and thus an increase in temperature produces excess area. In the case of the DMPC:Chol vesicles shape changes and fission were induced by osmotically reducing the enclosed volume at a constant temperature of 23°C. For this purpose solution was allowed to concentrate by natural evaporation from the open measuring chamber, leading to a flow of water out of the vesicle to equilibrate the concentration difference.

Vesicles were observed by phase contrast microscopy using a Zeiss Axiovert 10 inverted microscope equipped with a phase contrast air objective (Zeiss 40 × Ph2 nA = 0.75). For documentation the microscope was connected to a CCD camera (HR 480; Aqua TV, Kempten, Germany). Images

were recorded on videotape by an S-VHS recorder (Panasonic) and analyzed on a Macintosh IIfx supplemented with a fast frame grabber (PixelPipeline; Perceptics, Knoxville, TN). Image analysis was carried out with a modified version of the image processing software Image 1.44 (Wayne Rasband, National Institutes of Health).

Differential Scanning Calorimetry

DSC scans were taken with a MC-2 microcalorimeter (Microcal, Amherst, MA) using samples with a concentration of 10 mg/ml of Millipore water. Scanning rates were 15 and 30 K/h. No dependence on heating rate was found. The peak form and the transition temperature were reproducible within one lot.

Freeze Fracture Electron Microscopy

Vesicles (10 mg/ml) were swollen at 70 and 56°C. Freeze fracture samples were prepared by quenching from these temperatures in a water vapor saturated environment 0.5 and 1 h after the beginning of swelling. Further preparation was done using standard techniques described in (Krbecek, 1979). No signs of lysis products from storage at high temperatures were found by high-performance liquid chromatography and film balance scans.

RESULTS

Phase Transition and Fission of SPM

Fig. 1 shows the heat capacity curve for a suspension of SPM vesicles. Scans were run 30 min after the beginning of swelling. They were repeated on the same sample after storage at 42 or 70°C for 8 h to exclude a possible time dependence. Within instrumental resolution we found the spectrum to be constant and reproducible.

The DSC curve exhibits a pronounced peak with a maximum at 41°C and a broad base. It extends from 20 to 65°C and reflects the multicomponent mixture of SPM chains. Interestingly, the temperature range of this gel-to-fluid phase transition (Bruzik and Tsai, 1987) includes physiological temperatures. A high temperature shoulder was noticeable, but we were not able to resolve two separate peaks.

Observations of SPM vesicles using light microscopy were started in the gel state. The radii of the vesicles were about 10 μm. The vesicles began to fluctuate strongly at about 41°C, a temperature within the gel-fluid coexistence region. As can be seen in Fig. 2, repeated budding and fission of small vesicles from the parent vesicle occurred as the temperature increased. The radii of the buds ($r \simeq 1$–2 μm) were approximately constant. It appears that once enough excess area had been generated to form a bud it was readily and spontaneously pinched off. The complete separation of the small vesicles was verified by monitoring the freely floating buds in solution. We observed this repeated budding and fission in 10 vesicles. Only in one vesicle did a bud stay topologically connected and could be pulled back by cooling.

An interesting fission event is shown in Fig. 3. A chain of vesicles encountered in solution was monitored while the temperature increased. The beads stayed connected until, at

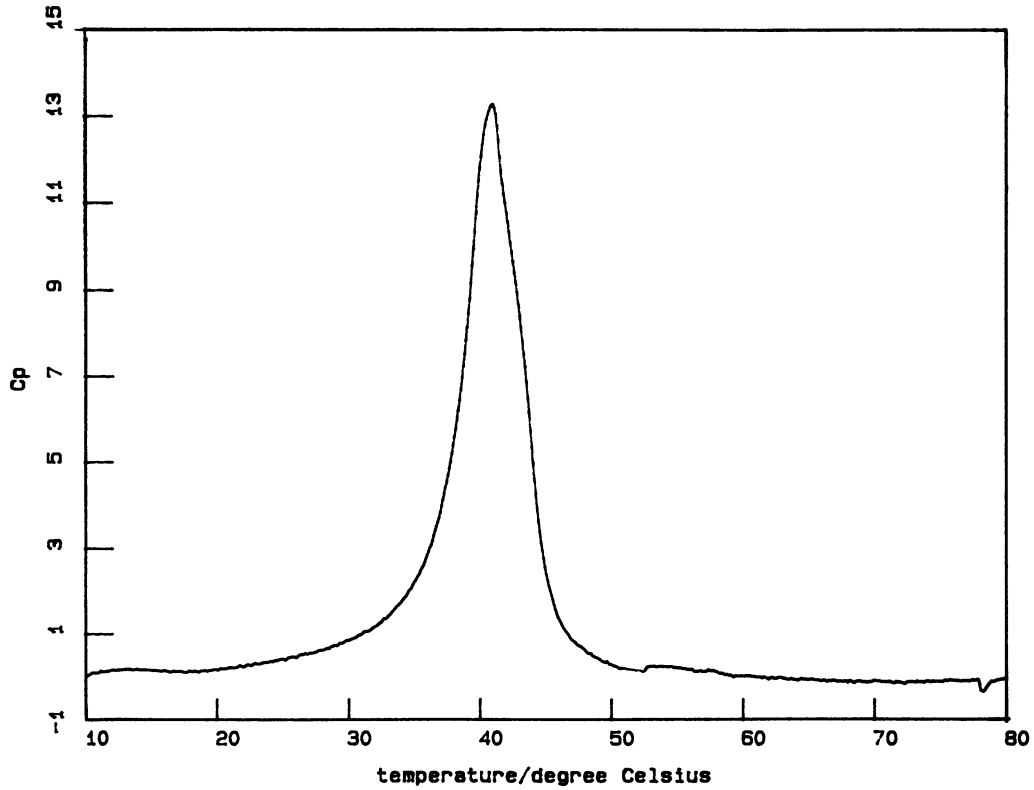


FIGURE 1 Heat capacity C_p versus temperature T plot of SPM vesicles suspension (10 mg/ml in Millipore-water, 30 min after begin of swelling at 42°C). The plot shows a pronounced peak at 41°C with a broad asymmetric base.

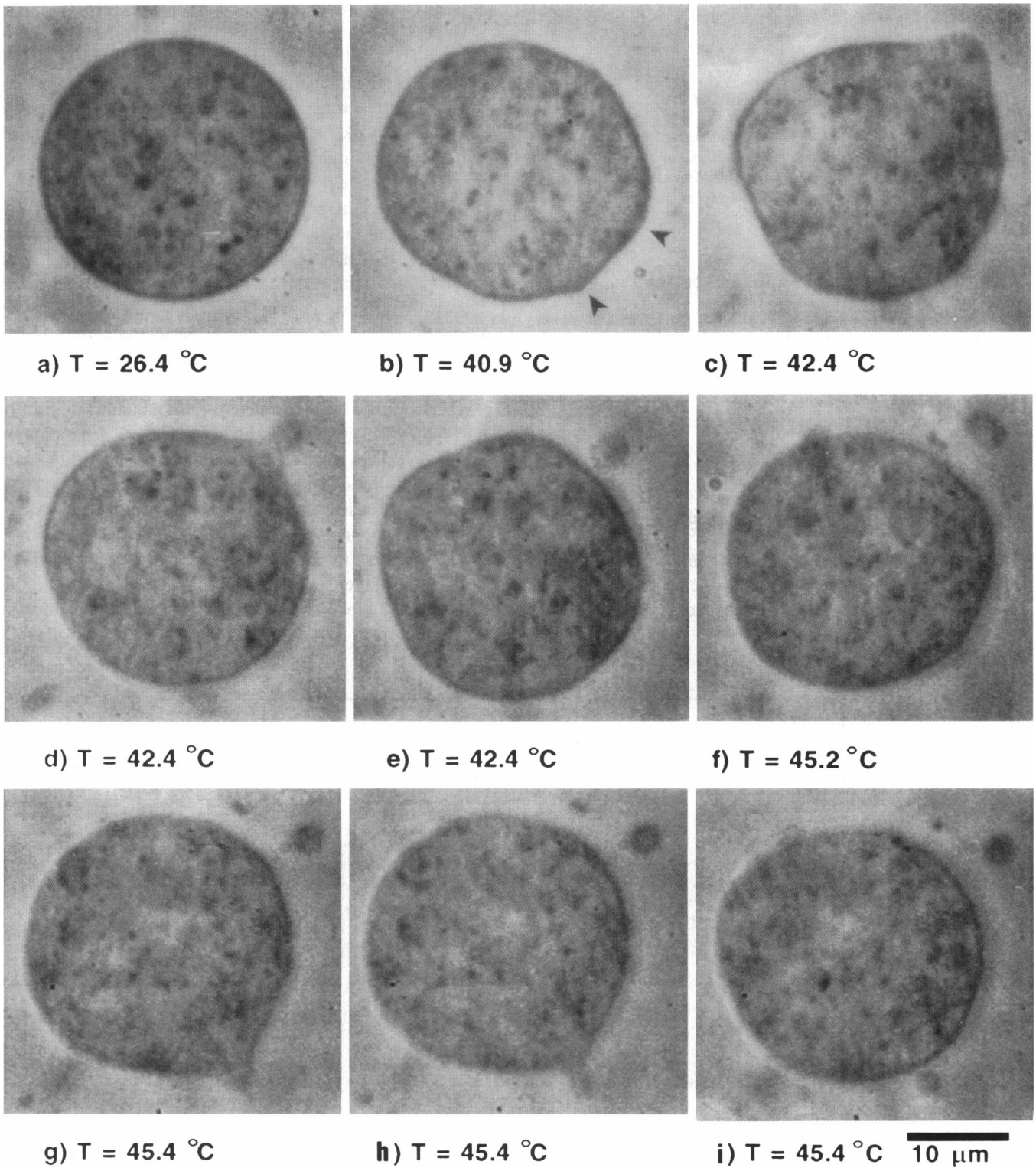


FIGURE 2 Budding fission sequence of SPM vesicles. At low temperatures the membrane was in the gel state and the vesicle was spherical and rigid. It was filled with small vesicles which were in fast Brownian motion (*a*). Raising temperature created membrane area, and, once the membrane became partially fluid, the vesicle began to fluctuate strongly around a quasispherical or elliptical shape with clearly visible facets and edges (see *arrows* (*b*)). Increasing the temperature even further resulted in a sudden shedding of a small child vesicle (*c–d*), which immediately separated from the mother vesicle (*e–f*). This shedding process was repeating itself as more membrane area was created by further heating (*g–i*).

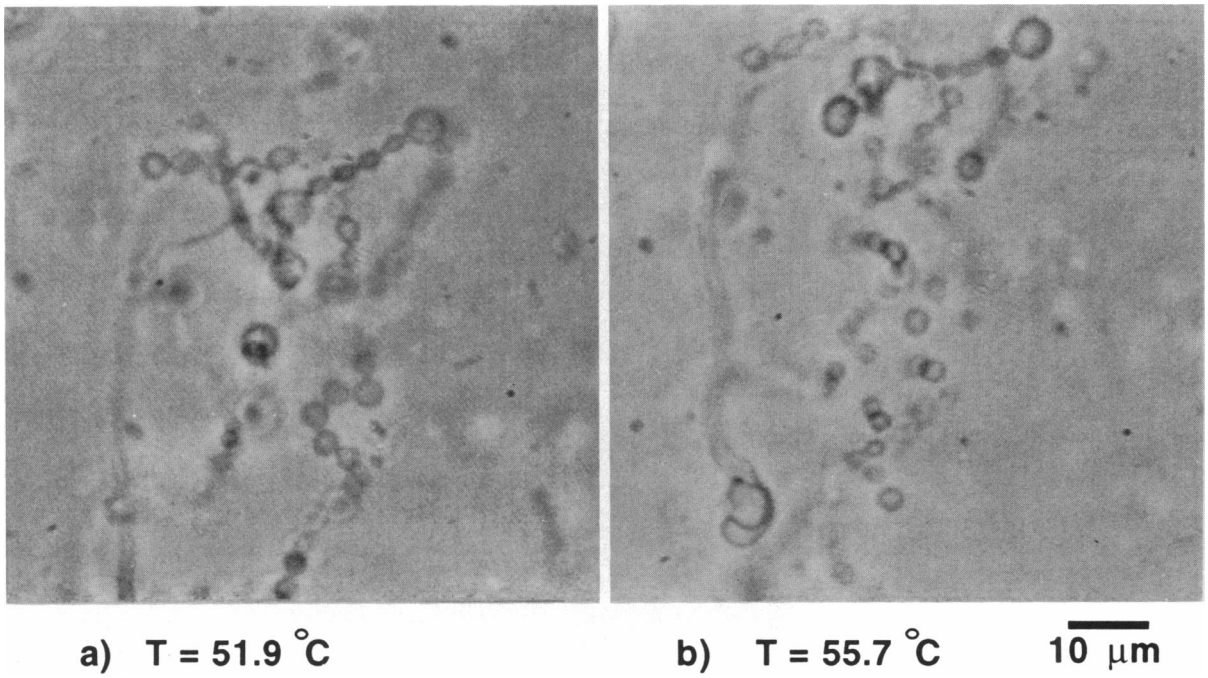


FIGURE 3 Chain fission of SPM vesicles. Several connected vesicle beads could be seen at 51.9°C (*a*). At 55.7°C they suddenly disintegrated and floated apart within a few seconds (*b*).

a temperature within the transition region, they suddenly disintegrated and formed separate vesicles, which drifted apart.

Comparison with the DSC curve shows that the vesicle membrane still was within the phase coexistence region when budding and fission took place. This holds for all samples examined. The membrane is expected to exhibit a domain structure of solid and fluid patches in this region. Such a picture is strongly suggested by the clearly visible facets and edges of the flickering vesicle contour (indicated by *arrows* in Fig. 2). These edges became less pronounced as the temperature was raised.

The domain structure is also demonstrated by the freeze fracture electron micrograph of a SPM vesicle shown in Fig. 4. The vesicle was immediately frozen from 56°C. Child vesicles in the process of budding and already pinched-off buds can be seen at various places (see *arrows*). This demonstrates that the expulsion mechanism is not restricted to giant vesicles but is also effective on cellular length scales. The buds are found to have diameters from 200 to 400 nm. The surface texture of the parent vesicle exhibits a wave-like domain structure in the coexistence region, while the buds on the vesicle and the already released child vesicle show the smoother surface characteristic of fluid membranes. Even the SPM vesicles frozen at 70°C show this wave-like structure, which could be explained by the broad base of the phase transition and by precooling effects before the sample was completely frozen.

In order to find other lipid mixtures exhibiting the described phenomenon, we performed experiments with mixtures of saturated and symmetric-chain phosphatidylcholines mimicking the chain length distribution of SPM (data not shown). Spontaneous budding was also found in these systems, however fission of the buds was absent.

Osmotically induced fission

Attempts to induce fission in an artificial DMPC:Chol mixture by thermally creating excess area were not successful. However, by reducing the enclosed volume osmotically we found the remarkable cascade process shown in Fig. 5. The vesicle repeatedly divided into smaller vesicles at a constant temperature of 23°C. The first fission event for this vesicle appeared after a decrease of volume by 30% starting from a sphere. We have observed this scenario in eight vesicles.

DISCUSSION

The geometrical changes during the budding transition can be described by a sequence of equilibrium shapes determined by the membrane configuration which minimizes the total energy (Wiese and Helfrich, 1990; Miao et al., 1991; Seifert et al., 1991; Lipowsky, 1991; Wortis et al., 1993). There are several contributions which will be discussed in turn.

The two main contributions to the elastic energy for fluid bilayers are the local bending energy and a nonlocal term resulting from relative stretching and compression of the individual monolayers (Seifert et al., 1992). The total elastic energy is thus a functional of the shape $\mathcal{S}$ of the vesicle and depends on several parameters:

$$E_{\text{elastic}} = E(\mathcal{S}; A, V, C_0, \Delta A_0, \alpha) \quad (1)$$

The area A and the volume V are geometrical quantities, C_0 is the preferred spontaneous curvature of the membrane, ΔA_0 is the equilibrium area difference between two monolayers, and $\alpha = \kappa/\tilde{\kappa}$ is the ratio between the local and nonlocal bending rigidity. The spontaneous curvature is given by the additive contributions of the two monolayers and their in-

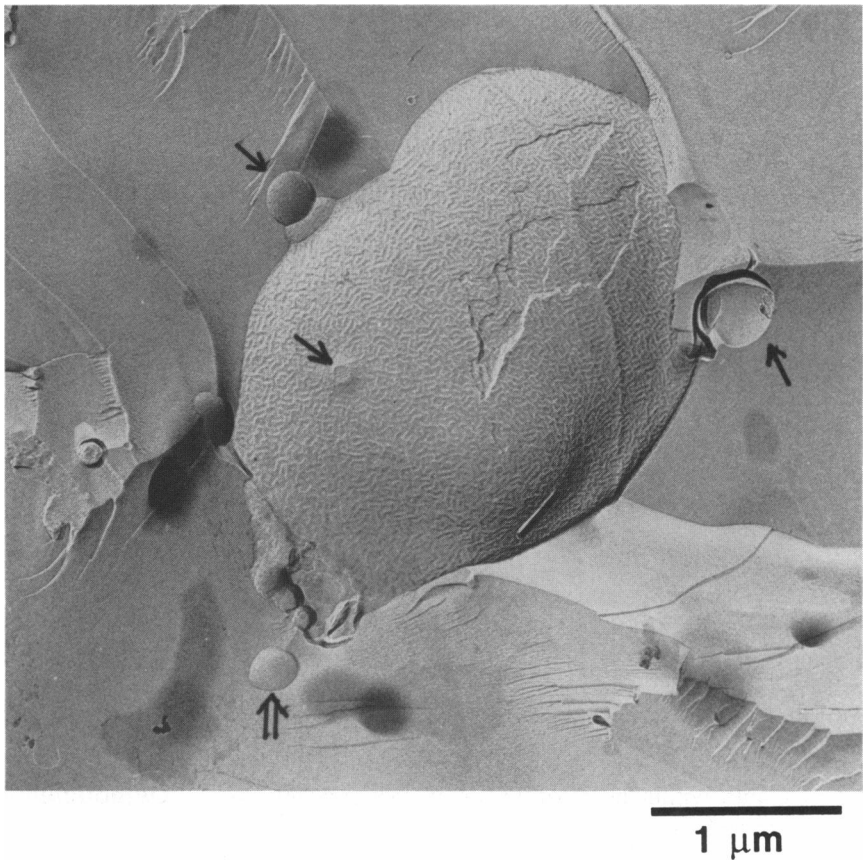


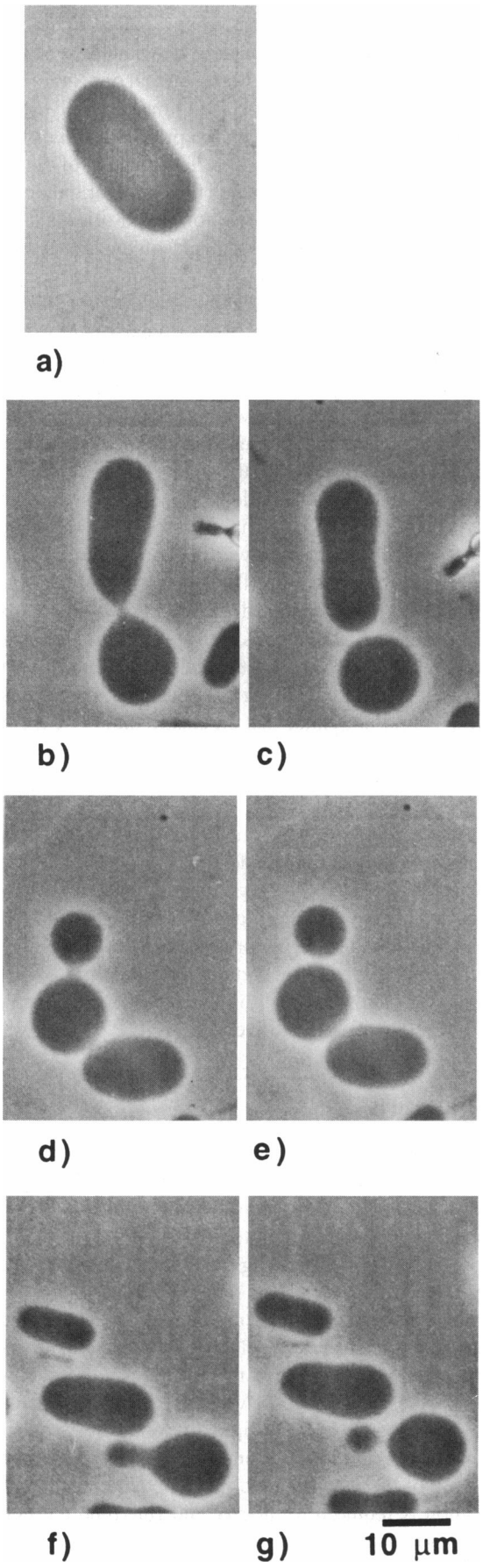
FIGURE 4 Freeze fracture electron micrograph of a SPM vesicle frozen from 56°C. Note the wave-like domain structure attributed to fluid-gel phase separation. Both small vesicles in the process of budding (→) and already pinched-off ones (⇒) are clearly visible.

teraction with the adjacent fluids. The equilibrium area per molecule in general is not the same for the inner and the outer monolayers. Thus a preferred global mean curvature, proportional to the equilibrium area difference, will result from the requirement that the vesicles form closed membranes.

For single component membranes the spontaneous curvature C_0 is uniform over the surface of the vesicle. However, lipid mixtures lead to a coupling between curvature and local lipid composition, and the spontaneous curvature becomes non uniform (Wu and McConnel, 1975; Gebhardt et al., 1977). This coupling can be understood in a simple way as resulting from molecular shape factors. A nonzero spontaneous curvature has its origin in an asymmetric lipid distribution across the membrane, since for a symmetric membrane the contributions from the individual monolayers would cancel each other.

In order to find equilibrium shapes for a multicomponent vesicle one has to minimize the bending energy as well as the free energy of the mixture, taking into account the dependence of the elastic parameters on composition. There are two principal cases possible: The components may either mix or phase separate laterally within each monolayer. Both scenarios lead to budding and have been recently investigated theoretically (Lipowsky, 1992; Jülicher and Lipowsky, 1993; Seifert 1993). We refer the reader thereto for a formal discussion and further references. For the SPM vesicles one expects lateral phase separation within the gel-fluid coexistence region. Indeed, evidence for domains is provided both by light and freeze fracture electron microscopy. The lipids which favor higher curvature will be enriched in the bud. We tacitly assume here that lateral phase separation is coupled to an asymmetric transversal distribution of lipids in the bilayer. It is this coupling of composition to morphology which we believe is part of the driving force for the expulsion of the bud.

Another important contribution to the expulsion mechanism comes from domain boundaries. The formation of a boundary in the membrane between regions of different lipid compositions and thermodynamic phases is energetically unfavorable and results in a line tension tending to diminish the circumference of the domain. This leads to a new and different budding mechanism, which was proposed recently (Lipowsky, 1992). In a planar geometry the domain will form a circle and reduction in the circumference invariably is accompanied by a reduction in area as well. However, taking advantage of the third dimension and forming a bud decreases the edge length without decreasing the area by placing the boundary in the neck of the bud. Bending of the domain costs elastic energy, and thus it is the competition between the elastic energy and the line tension which controls the budding mechanism. The relevant crossover between bending energy and line tension dominated regimes occurs at a vesicle size on the order of 1 μm. One thus expects the formation of buds from large vesicles to be more and more dominated by the latter mechanism. On the other hand it is shown in (Jülicher and Lipowsky, 1993) that there is a critical vesicle size below which budding cannot be induced by domain boundaries. We have therefore identified two re-



gimes which were investigated in this work by different techniques: Electron microscopy revealed that budding can be induced on a cellular lengthscale and is most likely driven by the spontaneous curvature of the domain being pinched off. On a more macroscopic length scale we have monitored budding and fission of vesicles using video-enhanced phase contrast microscopy and found domain boundaries to be more important in this regime.

A lot of open questions remain and experiments with well defined artificial mixtures are necessary to obtain more quantitative results. If the domain is larger than the available excess area it is geometrically impossible to form a bud and pinch off the domain. This frustration between line tension and geometric constraints may explain the strong fluctuations of the SPM vesicles we have observed prior to budding. Also for a full understanding one has to consider the dynamics of the system. The most relevant timescales are those for domain growth, the damping of thermal shape fluctuations (Lipowsky, 1992), and excess area production by thermal expansion. A detailed analysis of these processes and their interrelations is beyond the scope of this paper. All shape calculations for the budding transition in the literature have been performed for a completely fluid membrane. In our system the membrane is not completely fluid, and one has to admit a finite shear elasticity of the membrane to account for solid patches. However, we do not expect a qualitative change in behavior when this feature is included. The proposed mechanisms for the expulsion of the buds, coupling composition to curvature and line tension of domain boundaries, will still work. We should also point out that budding is of course possible in pure lipid systems, as has been shown both theoretically and experimentally. However, the budding process in mixtures is greatly enhanced and facilitated.

The concepts of bending energy and domain formation alone are not sufficient to describe the disruption of the bilayer at the narrow neck of the bud. It appears, however, that lipid mixtures and phase separation are necessary. It is particularly interesting to note that the domain boundary is located in the neck region after formation of the bud. This energetically unfavorable rim acts as a line defect and may facilitate fission (Lipowsky, 1992). Considering the fact that we did not find fission in the mixtures of phosphatidylcholines mimicking the chain length distribution of SPM, it becomes clear that lipid composition is a relevant factor in triggering the separation of the bud.

A further hint that domain boundaries play an important role comes from the finding that fission in the DMPC:Chol system could only be induced osmotically. At the cholesterol composition chosen the membrane is in the liquid ordered phase (Tampé et al., 1991; Thewalt and Bloom, 1992) and no lateral phase separation was induced by heating. On the other

FIGURE 5 Osmotically induced fission of DMPC:Chol vesicles (60:40). Initially the vesicle had a volume of 4900 μm^3 (*a*). After decreasing the volume to 3500 μm^3 at a constant temperature of 23°C, fission occurred (*b*, *c*). A further increase in the osmolarity of the surrounding solution led to repeated fission (*d–g*).

hand osmotic deflation drives water through the membrane and leads to a greatly enhanced flip-flop rate by coupling to the water flow (Boroske et al., 1981). Because cholesterol has a larger intrinsic flip-flop rate than other more polar lipids (Lange, 1992), this could lead to an increase in cholesterol concentration in the outer monolayer and to subsequent phase separation into liquid-ordered and liquid-disordered domains, promoting fission. This shows that budding and fission induced by phase separation is not restricted to fluid-solid demixing but can also be triggered by fluid-fluid phase separation, as probably seen in the DMPC:Chol mixture.

ROLE OF COAT PROTEINS IN BUD FORMATION

The preceding results showed that budding and fission of vesicles can and does occur in membranes composed only of mixtures of lipidic amphiphiles. Real biomembranes are much more complex systems. They include and are associated with various types of different proteins. The natural question is, "What kind of mechanism is responsible for budding and fission in the cell?" Is it the lipid bilayer which drives the morphological transitions, or are special proteins involved, with the lipid matrix playing only a passive role as an embedding medium?

Budding regions of the Golgi and the plasma membrane have been identified and found to be covered on the cytosolic side with a special coat protein, clathrin. It seems to be a widely accepted view that clathrin provides the driving force for membrane invagination (McKinley, 1983; Alberts et al., 1989). However, there have been emerging doubts about the universal role of this protein (Pfeffer and Rothman, 1987; Brodsky, 1988). Other coat proteins have been found and Golgi-associated vesicles have been characterized which seem not to be coated with protein (Orci et al., 1989).

To tackle the question posed above let us look at the energy scales involved in bilayer bending and coat formation. We can estimate the energy of polymerization from the chemical energy necessary to remove the protein. It is known that three ATP molecules per triskelion of clathrin are needed to shed the coat (Rothman and Schmid, 1986). With typical $n = 100$ triskelions per vesicle (Heuser and Kirchhausen, 1985) and $\kappa = 25kT$ one obtains

$$\frac{E_{\text{polymerization}}}{E_{\text{bilayer}}} = \frac{3n\Delta G_{\text{ATP}}}{8\pi\kappa} = 5 \text{ to } 10. \qquad (2)$$

This simple estimate shows that the energy of polymerization of the clathrin triskelions is comparable to the bending energy of the pure lipid bilayer. At first sight it may seem surprising that the bending energy, which has its origin in relatively weak intermolecular forces, is within an order of magnitude of chemical energies! The reason for this is the scale invariance of the bending energy, i.e., the total bending energy of a vesicle is independent of its size. Therefore a crossover length scale exists at which the two corresponding energy densities are equal. Nature found it convenient to set it at cellular dimensions.

The polymerization energy we have estimated is only an upper bound for the energy actually required to deform the coat. The energy scale for bending deformations of the clathrin coat is probably much lower than that of coat formation: The clathrin heavy chains are only noncovalently bound at the vertex and the link between the proximal and distal segments presumably provides a certain degree of flexibility (Morris et al., 1989). It may thus turn out that the dominant driving force for coated vesicle formation originates in the lipid bilayer, whereas the main role of the coat would be to provide a structural motif for interaction with receptors in the membrane mediated by adaptor proteins (Pfeffer and Rothman, 1987; Morris et al., 1989; Pearse and Robinson, 1990; Brodsky et al., 1991). Of course the properties of the lipid matrix will be modified by the presence of the protein coat. One has to consider the whole composite membrane. In particular, it seems likely that the spontaneous curvature of the bilayer is naturally set to the right value to promote budding by lipid-protein interactions. It is in this sense that one may regard proteins as a "driving force" for budding. Also the coat proteins themselves will in general introduce a nonzero spontaneous curvature through their molecular architecture. The differently seized clathrin cages with their varying ratio of penta- to hexagons are a specific example (Heuser and Kirchhausen, 1985). The precise way in which these cages are assembled during the budding process is not known. We do not claim that an elastic theory of the membrane solves the puzzle, but, as the above estimate clearly shows, it should be incorporated in a full description of coated bud formation.

We have focussed here on the role of the spontaneous curvature C_0 in inducing budding of vesicles. The equilibrium area difference ΔA_0, however, is of equal importance in this respect. It is sensible to changes in molecular areas by interaction with the specific proteins present and in fact may even be actively tuned by certain protein pumps, which are capable of translocating lipids across the membrane (Devaux, 1991; Farge and Devaux, 1992).

CONCLUSION

In summary, we have shown that budding and fission of vesicles are naturally occurring phenomena which can be found in a number of systems. Most interestingly special "fissogens" are not required to disrupt the bilayer and separate a vesicle from its budding site. Moreover, we have given a theoretical estimate that on cellular length scales the lipid matrix is likely to play the dominant role in the budding process, with coat-proteins playing only a secondary, albeit important, role. It seems that the mechanism of budding and fission of cellular transport vesicles can be understood as physical properties of the lipid bilayer. The expulsion is driven by domain formation and coupling of the spontaneous curvature of the membrane to its composition. Biochemical regulation is required to modify and fine-tune the material properties of the membrane and make budding and fission a more reliable and functionally directed process.

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