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Shapes of phosholipid vesicles with beadlike protrusions

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Abstract Giant phospholipid vesicles obtained by the method of electroformation were observed by the phase contrast microscope. Most of these vesicles contain a protrusion which shortens in a slow shape transformation process until it is absorbed into the main vesicle body. We are concerned with the last stages of this shape transformation process, where the protrusions attain a beadlike shape. The number of "beads" decreases one by one in consecutive steps, and it is demonstrated that each such step consists of two distinguishable phases. During the first phase the beadlike shape does not change and the necks connecting the "beads" are narrow. During the second phase the width of the protrusion necks increases. On the basis of the assumption that these shape transformations are driven by the decrease of the equilibrium difference between the outer and the inner membrane monolayers areas, the system behavior is analyzed in terms of the generalized bilayer couple model. The theoretical results confirm the observed time sequence: at a given number of "beads" the protrusion has in the first phase the shape that consists of spheres connected by infinitesimal necks, and during the second phase the protrusion is a single prolate unit with open necks. The discrepancies between the observed and the predicted widths of the necks are interpreted by the repulsive forces between the neighboring "beads" induced by the membrane thermal fluctuations. The analysis presented extends the existing catalog of vesicle shapes to the region of larger differences between the areas of membrane monolayers, and confirms the applicability of the generalized bilayer couple model to the description of the shape behavior of phospholipid vesicles containing beadlike protrusions.

Keywords Phospholipid vesicle shapes · Beadlike pro- trusions · Shape transitions

Introduction

Because of their amphiphilic nature, phospholipid molecules tend to associate into closed bilayer membranes which under certain conditions form unilamellar phospholipid vesicles of spherical topology. A unilamellar vesicle, if freely suspended, attains a shape which depends on the amount of the internal vesicle solution measured in relation to the membrane surface area, and on the mechanical properties of the membrane. The shape behavior of phospholipid vesicles has been the subject of an extensive experimental and theoretical research work (Lipowsky and Sackmann 1995). Most experimentally observed shapes of phospholipid vesicles seem now to be satisfactorily explained in terms of the so-called generalized bilayer couple model (Božič et al. 1992; Heinrich et al. 1993; Miao et al. 1994; reviewed in Svetina and Žekš 1996; Seifert 1997), which considers as the relevant mechanical properties of the membrane the local and the nonlocal bending energies. The important membrane parameters of this model are the local and the nonlocal bending moduli, membrane spontaneous curvature, and the number of molecules in the monolayers composing the bilayer membrane, which is represented in the model by the equilibrium difference between the two monolayer areas.

The predictions of the generalized bilayer couple model have been up to now systematically analyzed mostly with regard to the shape formation and shape transformations of vesicles with equilibrium area differences which do not differ appreciably from the values for a sphere (Döbereiner et al. 1997). However, certain preparation procedures yield phospholipid vesicles that exhibit more complex shape behavior, and accordingly, their equilibrium area differences can largely exceed the

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value for a sphere. Such are the vesicles that are obtained by the method of electroformation. They are composed of a relatively large parent body and an elongated protrusion that originates from a connecting network formed in the process of electroformation (Mathievet et al. 1996; Kralj-Iglič et al. 2001). The observed protrusions differ in their lengths and exhibit various forms, from cylinders with minor undulations to shapes composed of a series of "beads" connected by necks. Vesicles with protrusions are, due to high protrusion curvatures, characterized by relatively high local bending energies and relatively high differences between the monolayer areas. It is the aim of this work to test whether the shape behavior of these vesicles can also be described in terms of the generalized bilayer couple model. We shall consider only vesicles with the protrusion lengths up to the diameter of the parent body. An attempt will be made to identify the parameters of the generalized bilayer couple model that affect the protrusion behavior in the most significant manner.

In this work we shall study the behavior of vesicles obtained by the method of electroformation. The vesicles exhibit long protrusions that shorten by consecutively decreasing the number of "beads" in a slow process. This process in general terminates when the last "bead" and the parent vesicle body merge. The experimental results for such protrusion shape transformations will be presented and analyzed on the basis of the solutions of the shape equation obtained by the minimization of the membrane bending energy. The specific problem encountered with vesicles with protrusions are the narrow necks connecting different vesicle sections. In analyzing such shapes we shall follow the method of combining different solutions of the shape equation which has been used in an analogous problem of long phospholipid vesicles of spherical topology within the spontaneous curvature model employed by Miao et al. (1991).

The shapes of phospholipid vesicles with beadlike protrusions have theoretically not yet been described in a systematic manner. It is to be noted that similar protrusions also appear as parts of cells or cellular organelles (Lutz et al. 1977; Ochs et al. 1997; Kralj-Iglič et al. 1998; Blum et al. 2000), and therefore the analysis of the behavior of phospholipid vesicles involving protrusions may also improve our understanding of some aspects of the corresponding cellular processes.

Methods and materials

Giant phospholipid vesicles were made from the phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) by the method of electroformation (Dimitrov and Angelova 1988), modified as described previously (Kralj-Iglič et al. 2001). The POPC was purchased from Avanti Polar Lipids. The vesicles were prepared in an electroformation chamber containing 2 mL of 0.2 M sucrose solution. Immediately after preparation, the sucrose solution containing the vesicles and phospholipids was poured out of the chamber into a plastic beaker. Then the chamber was rinsed by 2 mL of 0.2 M glucose solution, which did not contain the phospholipids, and this solution was then added to the plastic beaker. The obtained vesicle suspension was afterwards gently mixed and in a time shorter than 15 min placed into the observation chamber that was made by a pair of cover glasses and sealed by the grease. All experiments were performed at constant pH (7.4) and at room temperature (23 °C). The vesicles were continuously observed under inverted microscope (Zeiss IM 35, Germany) with phase contrast optics, using a CCD black-and-white video camera (Sony SSC-M370CE, Japan), and the vesicle shape transformations were recorded on a video cassette recorder (Panasonic AG-7350, Japan).

As described in Kralj-Iglič et al. (2001), under the above conditions, long thin protrusions connected to nearly spherical parent bodies become visible after a certain period of time (usually up to half an hour). These protrusions appear as long thin cylinders, which slowly become shorter and broader. Later on, the undulations of the tubular protrusion become noticeable. The parent body with the protrusion that appears beadlike evolves with time. The shape of the vesicle exhibits thermal fluctuations. It is also noticeable that the protrusions with four or more "beads" exhibit necks that are more narrow at the beginning and at the end of the protrusion.

In this work we focus on the final steps of the protrusion shortening (Fig. 1). In the course of time the process of protrusion shortening continues by decreasing the number of protrusion's "beads" in steps. In Fig. 1A a vesicle with a globular parent body and with a protrusion with four "beads" is shown. Over a period of time it transforms into a protrusion with three (Fig. 1C), two (Fig. 1E), and one (Fig. 1G) "bead". During each step, two phases can be noted. In the first phase the shape of the protrusion with narrow necks between the "beads" is not essentially changing. In the second phase the protrusion undergoes a slow synchronous broadening of necks between the "beads". When the necks attain a certain width, the number of "beads" decreases by one and the necks become quickly narrow again. The second phase of consecutive steps in this slow shape transformation is exemplified by Fig. 1B, D, and F, where it is clearly visible that the necks are wider. The width of the necks in the course of the protrusion shortening is shown for the same vesicle in Fig. 2, where both phases are also noted: the protrusion shape with narrow necks between the "beads" with approximately constant width, followed by a synchronous neck broadening. Finally, the vesicle passes through the stage with a broad neck connecting the protrusion (the remaining "bead") and the parent body (Fig. 1H), and then the integration of the protrusion into the parent body is observed (Fig. 1I). It is to be noted that the shape transition in which the number of "beads" decreases by one does not happen instantaneously. Namely, during the transition the number of "beads" alternates typically three to four times in between the new and the previous value. The process of such an alternated protrusion shape lasts approximately for 10 s. The radi of the parent body and the small spheres of the beadlike protrusion change with the protrusion shortening.

The whole transformation sequence lasted for several hours. The phase of beadlike shape with narrow necks was on the average much longer than the phase of neck broadening (Table 1). Such shape transformations were observed on at least 20 different vesicles with protrusions. Most of vesicles that had, at the beginning, a long thin protrusion, sooner or later underwent the described shape transformations. However, there were also vesicles with the protrusion in which the transformation process was not fully completed, i.e. the vesicle shape transformation stopped with the protrusion still having some "beads" (Fig. 3). In this case the protrusion exhibited truly narrow necks between the "beads" and considerably smaller thermal fluctuations were observed.

Theory

Elastic properties of phospholipid vesicles with spherical topology

The shapes of vesicles, which are observed during the shape transformation, are described within the

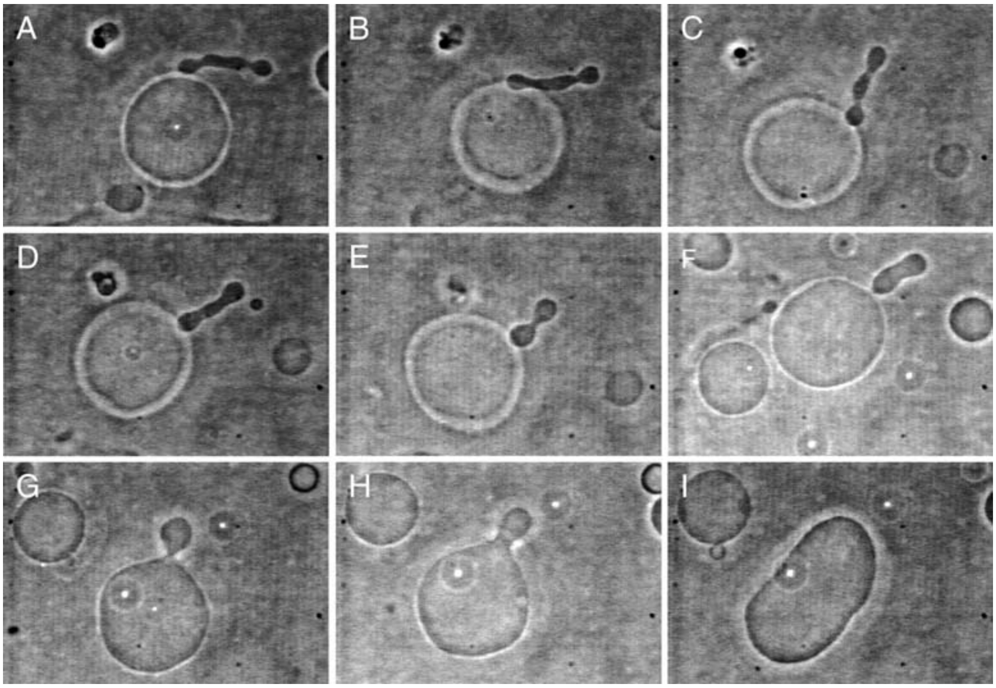

Fig. 1A–I The transformation of a vesicle with protrusion. It is characterized by a series of nine micrographs that were taken at 0 min (**A**), 3 min (**B**), 7 min 25 s (**C**), 16 min (**D**), 19 min 45 s (**E**), 43 min (**F**), 80 min (**G**), 87 min (**H**), and 88 min (**I**). The protrusion moves freely around the equilibrium axisymmetric shape, indicating that it is connected to the parent body only in one point. The diameter of the parent body is approximately 30 μm

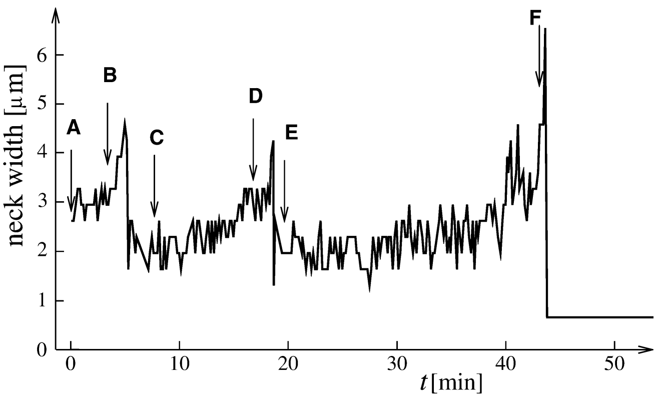

Fig. 2 The time dependence of the width of protrusion necks during the shape transformations. The neck width was determined for the vesicle in Fig. 1 in time intervals of approximately 10 s. The points representing these measurements are connected by *lines*. The time span below 5.2 min corresponds to the step with four protrusion "beads", the time span between 5.2 min and 18.6 min corresponds to the step with three protrusion "beads", the time span between 18.6 min and 43.6 min corresponds to the step with two protrusion "beads", whereas the time span above 43.6 min corresponds to the step with one protrusion "bead". It has to be noted that the neck width in the time longer than 43.6 min corresponds to the neck between the protrusion and the parent body. Its neck broadening is not shown. The width of protrusion neck was determined as the width of the middle neck for the protrusion with four "beads", and as the width of the neck closer to the parent body for the protrusion with three "beads". Letters *A*, *B*, *C*, *D*, *E* and *F* denote the respective times when the micrographs in Fig. 1 marked by the same letters were taken

generalized bilayer couple model that takes into consideration the bilayer structure of the vesicle membrane. While remaining in close contact, both monolayers can freely slide over each other in the lateral direction. The shapes of vesicles are governed by the elastic energy of the vesicle membrane (W_{el}) (Svetina and Žekš 1992). The monolayers can be bent and may undergo relative changes in their areas at constant area of the neutral surface of the bilayer. Thus, the elastic energy is the sum of the local (W_b) and nonlocal (W_r) bending term (Svetina et al. 1985; Miao et al. 1994), and can be expressed as:

$$W_{el} = W_b + W_r = \frac{k_c}{2}\oint (c_1 + c_2 - c_0)^2 \mathrm{d}A + \frac{k_r}{2Ah^2}(\Delta A - \Delta A_0)^2 \tag{1}$$

where k_c and k_r are the local and the nonlocal bending constants, respectively, c_1 and c_2 are the principal curvatures, c_0 is the spontaneous curvature, A is the membrane area, h is the distance between the neutral surfaces of the individual monolayers, ΔA is the difference between the areas of the two monolayers, and ΔA_0 is the corresponding equilibrium area difference.

The difference between the areas of the layers, which is given by the integral:

$$\Delta A = h \oint (c_1 + c_2)\mathrm{d}A \tag{2}$$

is characteristic of the vesicle shape. In the elongated conformations the area of the outer monolayer is much larger than the area of the inner monolayer, and therefore the shapes of vesicles with protrusion have much larger difference between the areas of the monolayers than the shapes of vesicles without protrusion. The dependence of the equilibrium difference between the areas of the two layers on the number of lipid molecules constituting the outer (N_2) and the inner (N_1) monolayer is given by (Svetina et al. 1985):

Table 1 Time table of transformation steps in protrusion shortening for four vesicles. The duration of the corresponding steps for protrusions with three, two, and one "bead", including the phase of narrow (constant) necks and the phase of neck broadening, are indicated by $F_{43}\rightarrow F_{32}$, $F_{32}\rightarrow F_{21}$, and $F_{21}\rightarrow F_{10}$. The duration of the neck broadening phase during each of these steps is given in parentheses. The entries $S_2\rightarrow F_{32}$ and $S_1\rightarrow F_{21}$ pertain to the protrusion with three and two "beads" in the neck broadening phase, whereas $L\rightarrow F_{10}$ pertains to the neck broadening connecting the one "bead" protrusion and the parent body, respectively. The times were determined by three independent observers; mean values ± s.d. are given for each transformation step. The fourth vesicle is presented in Fig. 1

Vesicle number	Transformation step (min)					
	Three "beads"		Two "beads"		One "bead"	
	$F_{43}\rightarrow F_{32}$	$(S_2\rightarrow F_{32})$	$F_{32}\rightarrow F_{21}$	$(S_1\rightarrow F_{21})$	$F_{21}\rightarrow F_{10}$	$(L\rightarrow F_{10})$
1	5.4 ± 0.1	(1.0 ± 0.3)	17.6 ± 0.1	(1.3 ± 0.3)	3.7 ± 0.2	(0.8 ± 0.1)
2	2.9 ± 0.2	(1.0 ± 0.3)	3.4 ± 0.2	(0.6 ± 0.3)	6.2 ± 0.1	(0.5 ± 0.3)
3	0.7 ± 0.2	(0.3 ± 0.2)	0.5 ± 0.1	(0.3 ± 0.3)	1.7 ± 0.1	(0.5 ± 0.1)
4	14.2 ± 0.4	(3.8 ± 3.5)	25.3 ± 0.2	(3.8 ± 1.5)	43.2 ± 0.3	(2.0 ± 1.0)

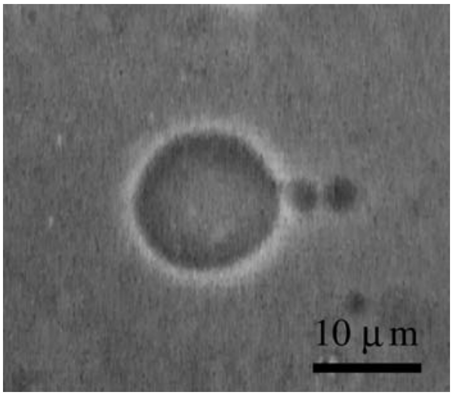

Fig. 3 The protrusion shape of the vesicle where the transformation process stopped when the protrusion shape had two "beads"

$$\Delta A_0 = N_2\bar{A}_{0,2} - N_1\bar{A}_{0,1} \tag{3}$$

where $\bar{A}_{0,2}$ is the equilibrium area per lipid molecule in the outer monolayer and $\bar{A}_{0,1}$ in the inner monolayer, respectively.

The nonzero values of the spontaneous curvature can arise from the chemical asymmetry between the external and the internal lipid monolayer of the membrane (Evans 1974) or from the difference between the outer and the inner solutions (Döbereiner et al. 1999). We only consider a symmetrical bilayer membrane, where the monolayers are composed of the same type of lipids. During the observation the outer and the inner solutions do not change; hence, the spontaneous curvature in the bending term should be constant. The elastic energy can be rewritten in the form where c_0 is incorporated in the nonlocal bending term by the transformation of the parameter ΔA_0 (Svetina et al. 1985; Döbereiner et al. 1997). Thus, for the sake of simplicity, we can take the value of c_0 to be zero.

Conformation of phospholipid vesicles at large area differences

The whole process of vesicle transformations is slow (compared to the relaxation time of mechanical equilibrium), so that all the observed shapes can be considered as stable, i.e. are the subject to the minimization of the membrane elastic energy (Canham 1970; Seifert 1997). If we denote by v the negative derivative of the local bending term with respect to the relative area difference:

$$v = -\frac{\mathrm{d}W_\mathrm{b}}{\mathrm{d}\Delta A} \tag{4}$$

the differentiation of the elastic energy (Eq. 1) with respect to ΔA leads to the relationship (Jarić et al. 1995; Božič et al. 1997):

$$v = \frac{k_\mathrm{r}(\Delta A - \Delta A_0)}{Ah^2} \tag{5}$$

It follows that v represents the difference between the lateral tensions of the membrane monolayers.

Both the parent body and the protrusion of the observed shapes appear axisymmetrical; therefore, the shape analysis can be limited to this kind of symmetry. The variation of the elastic energy with respect to axisymmetrical shape at given vesicle volume, membrane area, and difference between the areas of the layers gives the shape equation (Svetina and Žekš 1989, 1996):

$$\frac{R}{2(1-R^2C_\mathrm{p}^2)}\left[RC_\mathrm{p}\frac{\mathrm{d}C_\mathrm{p}}{\mathrm{d}R}(2C_\mathrm{p}+R\frac{\mathrm{d}C_\mathrm{p}}{\mathrm{d}R})-\frac{\mu}{k_\mathrm{c}}+\frac{2\lambda C_\mathrm{p}}{k_\mathrm{c}}+\frac{2vhC_\mathrm{p}^2}{k_\mathrm{c}}\right] + \frac{\mathrm{d}}{\mathrm{d}R}(2C_\mathrm{p}+R\frac{\mathrm{d}C_\mathrm{p}}{\mathrm{d}R}) = 0 \tag{6}$$

where μ is the pressure difference across the membrane, λ is the lateral tension in the membrane, C_p is the principal curvature along the parallels, and R is the distance between the symmetry axis and a point on the contour of the membrane. The effect of the relative area difference is considered in the shape equation through parameter v (cf. Eq. 5).

In order to describe the shape of the vesicle with protrusion in accordance with the experiment, the solution is sought within the classes of shapes that are composed of different axisymmetric units (Miao et al. 1991). The contour of each unit, which is the solution of the shape equation (Eq. 6), begins and ends at the symmetry axis. However, as the observed vesicles are single connected, the spherical topology of the composed shape must not change during the vesicle transformations. This restriction can be incorporated into the composed vesicle model by considering that the units are connected by the necks of infinitesimally small radius (Fourcade et al. 1994). Hence, the material is exchanged between the vesicle sections through the necks, so we can assume that the pressure difference (μ), the lateral tension (λ), and the difference between the lateral tensions (ν) are the same for all units.

There are values of μ, λ, and ν for which the solutions of Eq. (6) represent prolate shapes whose curvatures vary over the membrane area. When $\lambda^2 + 2\mu\nu h > 0$, two additional solutions corresponding to spheres of two different radii $R_{1,2}$ are also obtained (Miao et al. 1991):

$$R_{1,2} = \frac{\lambda \pm \sqrt{\lambda^2 + 2\mu\nu h}}{\mu} \tag{7}$$

Thus, the shape of the vesicle with the protrusion could be described by any combination of units where each unit must correspond to one of these three solutions of the shape equation (Eq. 6). However, the appropriate combination of these units should be chosen by using the criterion of the minimal elastic energy of all possible combinations at given conditions.

In practice, the shape of the vesicle for the composed model is obtained by applying several steps. In the first step a closed shape of the prolate unit with variable curvatures is calculated at certain values of the parameters μ, λ, and ν. Next, the radii of the spheres are determined by using Eq. (7) for the same values of parameters μ, λ, and ν. In order to obtain the shape of the vesicle with protrusion, different combinations of single units are composed. The larger radius obtained by Eq. (7) is reserved for the parent body. The shooting method is used to determine the values of μ, λ, and ν to fulfill the conditions of chosen vesicle volume, membrane area, and equilibrium area difference. The elastic energy is determined for different combinations of vesicle units. In the final step, the combination of units with the smallest elastic energy is chosen.

Theoretical results

According to the composed vesicle model, the vesicle with the protrusion is described as composed of units connected by infinitesimally narrow necks. Theoretically, any combination of the units corresponding to the three solutions of the shape equation obtained in the previous section could be employed. However, based on the experimentally observed shapes, we restricted ourselves to a single sphere with a larger radius, which represents the parent body, and a mirror-symmetric shape composed of either spheres with a smaller radius or a single prolate unit with variable curvatures, which represents the protrusion. This restriction is in agreement with the theoretical calculations. Considering the elastic energy of individual conformations, for example, the shape composed of a large sphere, a small sphere, and a prolate unit with one open neck has larger elastic energy than the shape composed of a large sphere and a prolate unit with two open necks.

As an example of the predictions of the composed vesicle model, we present the behavior of the vesicle of the relative volume:

$$v = V/(0.75\pi R_0^3) \tag{8}$$

equal to 0.9, where R_0 is the radius of the sphere with the same area as the vesicle. This relative volume corresponds to the vesicle presented in Fig. 1. The nonlocal bending constant is taken to be three times the value of the local bending constant ($k_r = 3k_c$), which corresponds to measured values (Waugh et al. 1992; Raphael and Waugh 1996; Svetina et al. 1998).

The results presented in Fig. 4 show how the relative area difference ($\Delta a = \Delta A/(8\pi h R_0)$), the relative width of the protrusion necks ($2R_{\text{neck}}/R_0$), and the relative elastic energy ($w_{\text{el}} = W_{\text{el}}/(8\pi k_c)$) depend on the relative equilibrium area difference ($\Delta a_0 = \Delta A_0/(8\pi h R_0)$). The corresponding theoretical shapes of the vesicle with the protrusion are also shown in Fig. 4a. The globally stable shapes are defined by the lowest elastic energy at a given Δa_0. In Fig. 4c the positions of discontinuous transitions are determined by the line intersections corresponding to the equality of the elastic energies of the two conformations.

Following the shape transformations due to a continuous decrease of Δa_0, it can be seen in Fig. 4a that, from $\Delta a_0 = 3.6$ down, moving along the thick dashed line, the vesicle with protrusion is composed of a single large sphere describing the parent body and three spheres with a smaller radius describing the protrusion. The spheres are connected by two infinitesimally narrow necks. At Δa_0 lower than 3.53, the necks in the protrusion open, i.e. the width of the necks becomes finite (Fig. 4b). In Fig. 4c this continuous (second-order) transition is indicated by S_2. At further decrease of Δa_0 the protrusion necks increase (Fig. 4b) and the area difference of the vesicle with the protrusion decreases (the thick solid line in Fig. 4a). Here, the shape is described by the large sphere connected by an infinitesimally narrow neck to the protrusion that is a single prolate unit with two open necks. When the value of Δa_0 is decreased below 3.40, the protrusion is transformed into two spheres connected by the infinitesimally narrow neck. In Fig. 4c the position of this discontinuous (first-order) transition is indicated by F_{32}. This transition is characterized by shifting the minimum-energy state from the vesicle with a protrusion having prolate unit to the vesicle with a protrusion composed of two spheres.

A similar behavior is predicted for further decrease of Δa_0 (Fig. 4), where the number of the "beads" is lowered by one. The dependence of w_{el} on Δa_0 (Fig. 4c)

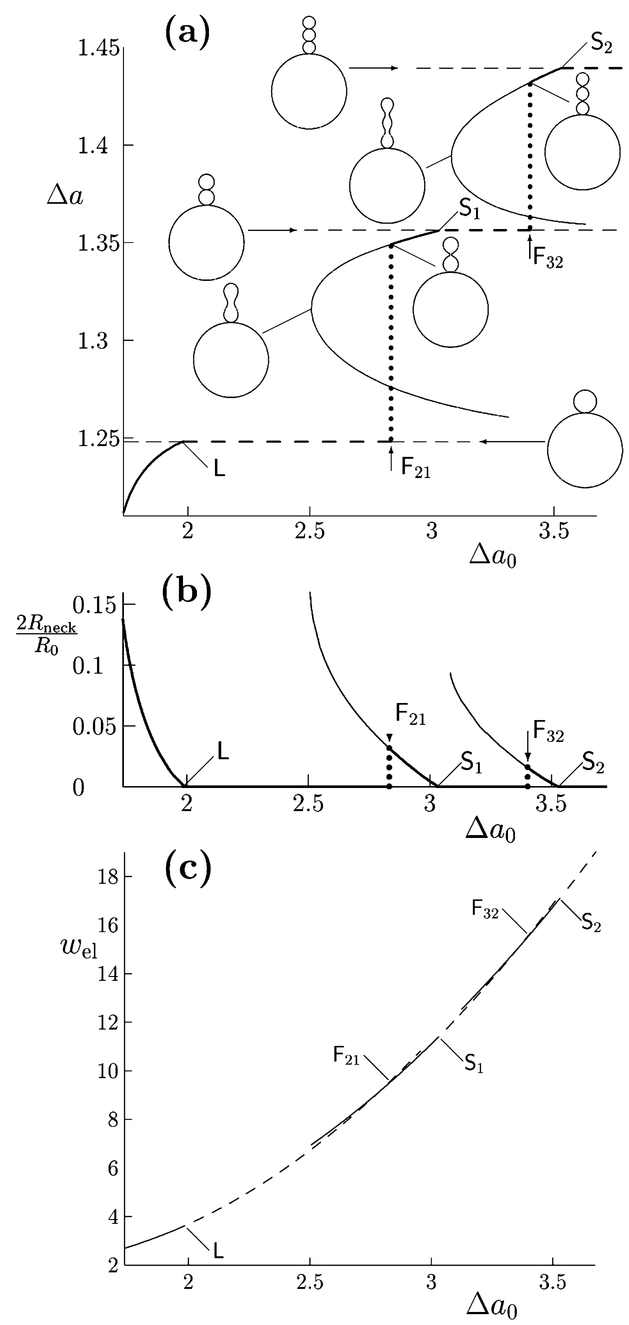

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Fig. 4a–c The dependences of vesicle shapes and their elastic energies on the dimensionless equilibrium difference between the areas of membrane monolayers (Δa_0) for the relative volume (v) equal to 0.9 and the ratio between the nonlocal and the local bending constant equal to 3. **a** The dimensionless difference between the areas of membrane monolayers (Δa) as a function of Δa_0. The *dashed lines* denote Δa of shapes with infinitesimally narrow necks. The *solid lines* denote Δa of shapes with open necks. The *thick line* sections denote dependences of stable configurations, whereas *thin line* sections denote the dependences of unstable configurations. The *dotted vertical lines* (marked by F_{32} and F_{21}) denote where the discontinuous shape transitions occur. The points marked by S_2 and S_1 show the locations where two and one infinitesimal necks between spheres of the protrusion open, respectively. The point marked by L denotes the final transition into the prolate "pear" shape that comprises a single unit. The axial cross-sections of shapes are given at the indicated positions. **b** The dependences of the relative width of the protrusion necks ($2R_{\mathrm{neck}}/R_0$) on Δa_0. To complete the figure, the dependence of the neck width between the parent body and the protrusion is also drawn. The different transition points are marked as in **a**. The *thin line* sections denote the dependences of metastable configurations. **c** The corresponding dependence of the relative elastic energy (w_{el}). The continuous transitions, which are characterized by the neck opening, occur where the dashed lines are followed by the solid lines (marked by S_2, S_1, and L). The discontinuous transitions are characterized by shifting the minimum-energy state from the solid lines to the dashed lines (marked by F_{32} and F_{21}) where the number of necks changes

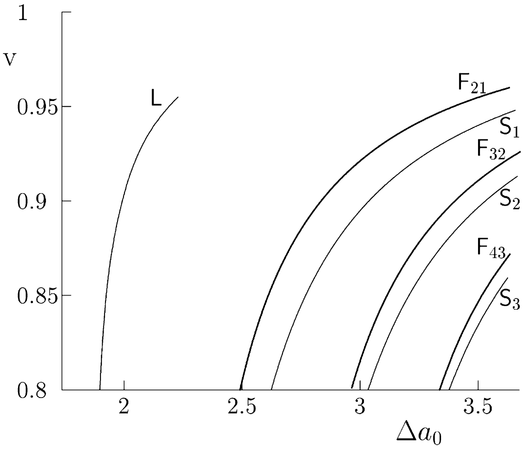

Fig. 5 The relative volume versus Δa_0 phase diagram for the vesicles with protrusion at $k_r/k_c = 3$. The region of prolate "pear" shapes is to the left of the curve L, the region with spherical protrusion is between the curves L and F_{21}, the region with the protrusion with one neck is between the curves F_{21} and F_{32}, the region with the protrusion with two necks is between the curves F_{32} and F_{43}, and in addition the region with the protrusion with three necks is to the right of the curve F_{43}. The boundary lines between the vesicle shapes of open and infinitesimal narrow necks are on the curves L, S_1, S_2, or S_3, respectively

demonstrates that the protrusions with the smaller number of "beads" are energetically more favorable at smaller Δa_0. Finally, at $\Delta a_0 = 1.98$ the last neck, i.e. the neck between the parent body (large sphere) and the protrusion (smaller sphere), opens (cf. Fig. 1), as has already been described by Käs et al. (1993).

In order to provide an insight into the vesicle behavior at different relative volumes, the stable conformations of vesicles with protrusion can be presented in the v versus Δa_0 phase diagram as a function of the relative volume (v) and the equilibrium difference between the areas of the outer and the inner monolayers (Δa_0) (Fig. 5). The curves divide the phase diagram into a zone that determines pear shapes (at low Δa_0), and alternating zones that determine either vesicles with protrusion composed of spheres or vesicles with a prolate unit with open necks. In accordance with Fig. 4, a discontinuous transition from the vesicle with a spherical protrusion to the vesicle with a prolate unit with one open neck occurs on the curve marked by F_{21} in Fig. 5 on increasing Δa_0. The discontinuous transitions also occur on the curve F_{32}, where the vesicle with the protrusion composed of two spheres is transformed into the vesicle with the prolate unit with two open necks, as well as on the curve F_{43}, where the vesicle with the protrusion composed of three spheres is transformed into the vesicle with the prolate unit with three open necks. Continuous transitions occur where one, two, three, or four necks become infinitesimally narrow, i.e. the protrusion becomes composed of spheres (marked by L, S_1, S_2, and S_3, respectively). The phase diagram presented shows that at constant Δa_0 the number of protrusion necks decreases on increasing in the relative volume (v).

The theoretical results show that for reasonable values of k_r/k_c the zones determining vesicles with protrusion composed of spheres are always broader than those determining the vesicles with a prolate unit having open necks. Therefore, when observing the protrusion transformations, it is expected that the vesicle will spend more time in the first conformation under the assumption that the decrease of Δa_0 is a linear function of time.

A question can also be posed considering the behavior at larger relative equilibrium differences between the areas of the monolayers (Δa_0) as well as different ratios between the elastic constants (k_r/k_c). The zones that determine either vesicles with protrusion composed of spheres or vesicles with a prolate unit having open necks are expected to alternate also at larger Δa_0. However, these zones become more narrow compared to those shown in Fig. 5, i.e. a faster exchange of the alternating zones is theoretically found. The theoretical model also predicts that these alternating zones narrow if the ratio k_r/k_c is increased.

Discussion

Previous systematic experimental and theoretical studies of the shape behavior of phospholipid vesicles based on the generalized bilayer couple model were mostly limited to differences between the areas of the lipid monolayers which are close to the corresponding values of the sphere. Here we extended such studies to the shape behavior of vesicles with protrusions. These vesicles have their relative volumes close to the volume of the sphere; however, their area differences are considerably larger.

In this analysis, phospholipid vesicles prepared by the method of electroformation were used as a suitable experimental system. In this preparation, most vesicles appear initially as being composed of a large parent spherical body and a protrusion. Then, in the course of time the protrusion shortens and eventually absorbs into the parent body. At the beginning of this process the protrusion looks as a long thin tube (Kralj-Iglič et al. 2001), but when it becomes shorter it is broader and appears like a necklace of "beads" (Fig. 1). The observations indicate that the neck connecting the parent body and the protrusion behaves differently from the necks between small "beads". The width of the latter necks varies during the protrusion shortening, whereas the neck connecting the parent body and the protrusion remains narrow until the protrusion is absorbed by the parent body.

The slow shape transitions of phospholipid vesicles with beadlike protrusions are interpreted on the basis of a monotonous decrease of equilibrium area difference (ΔA_0). In addition, the shape transitions are explained within the hypothesis that the characteristic time for the mechanical relaxation of vesicles is always much shorter than the time of the protrusion shortening (Kralj-Iglič et al. 2001). Therefore, at any given time the vesicle can be considered to be in a mechanical equilibrium. This assumption, which formed the basis of the analysis of our observations, is also supported by the experiments of Käs and Sackmann (1991), where the beadlike protrusions of the same shape were obtained by decreasing the temperature. The shapes of these structures were considered as stable in these experiments because of the slow rate of temperature decrease (0.2 K/min) (Käs and Sackmann 1991).

It is to be noted that in a different but related problem of long tubular vesicles, beadlike structures were induced by laser tweezers (Bar-Ziv and Moses 1994; Nelson et al. 1995). These beadlike structures (the pearls) were unstable and relaxed towards the equilibrium through slow shape transitions, which resemble our observations of protrusion shortening (Bar-Ziv and Moses 1994). The difference between the two systems seems to be the fact that the laser tweezers at high amplitudes induce a sudden tension in the tubular membrane, leading to unstable (excited) states (Goldstein et al. 1996), while the slow growth of vesicles in the electroformation chamber, on the contrary, leads to equilibrium shapes with respect to the volume, surface area, and equilibrium area difference of the vesicle.

In the process of protrusion shortening the radii of the parent body and of the "beads" change, which confirms our assumptions that the narrow necks that connect vesicle sections allow water and membrane material movement in between them and that the vesicle behaves as a single entity. However, it may also be considered that the narrow neck between the parent body and the protrusion hinders the flow of the material from one unit to the other. At this point it is not possible to predict the effect of narrow necks. The detailed geometry of narrow necks is difficult to assess because the curvatures in the necks may be comparable to the reciprocal width of the membrane and there are no adequate theories. Considering this, it is not possible to predict for which quantity (water solution, bilayer membrane as a whole, and/or two monolayers relative to each other) the neck behaves like a "bottleneck". Nevertheless, an explanation of the slow shape transitions, for instance at constant ΔA_0, which is caused by hindered flow through the narrow neck between the parent body and the protrusion, can be envisioned. In this case the transformations of a vesicle with the protrusion would be completed by attaining the shape that corresponds to the minimum of the elastic energy at a given initial ΔA_0. In a particular case when a given ΔA_0 is appropriately large, the final shape could have a long tubular or beadlike protrusion. However, the consistency of our theoretical predictions and experimental results indicates that our assumptions concerning the free passage through the narrow neck are justified.

The open question in the interpretation of our observations remains the mechanism for the decrease of the equilibrium area difference (ΔA_0), which seems to cause the protrusion shortening. There are several possibilities and it was speculated previously by Kralj-Iglič et al. (2001) that the most probable reason for the slow continuous decrease of ΔA_0 might be the loss of phospholipid molecules from the outer monolayer caused by the difference between their chemical potentials in the solution and the membrane. These chemical potentials are different at the beginning of the observations because during the preceding manipulations we diluted the vesicle bathing solution.

The analysis of the shapes of vesicles with a protrusion was restricted to the protrusion lengths up to the diameter of the parent vesicle body. The hypothesis was that the shape behavior of such vesicles can also be described on the basis of the generalized bilayer couple model, contrary to the case of longer, cylindrical protrusions where other contributions to the membrane energy may have to be taken into consideration (Kralj-Iglič et al. 2002).

Because the catalog of possible vesicle shapes has been in the past thoroughly explored mainly for the values of the parameter Δa close to the value of the sphere (Deuling and Helfrich 1976; Svetina and Žekš 1989, 1990; Seifert et al. 1991), it was necessary to extend the catalog of vesicle shapes into the parameter region covering also the treated protrusion lengths. Technically these determinations are difficult because the shapes of interest involve at least one narrow neck. A suitable approach for studies of such shapes has been introduced by Miao at al. (1991). It is based on the notion that the shapes that are composed of units which are the solutions of the shape equation, and connected by the infinitesimal necks, are also the exact solutions of the shape equation. There are many different combinations of units which are all solutions of the shape equation. However, we found, at the relevant values of the model parameters, as solutions with the lowest energy only the vesicles where the parent body represented by a large sphere is connected by an infinitesimally small neck to the protrusion that consists either of small equal spheres or of a mirror-symmetric prolate unit involving open necks. The former of these solutions is an example of the so-called limiting shapes (Svetina and Žekš 1989). Any pathway through the catalog of shapes due to monotonous changes of Δa has eventually to cross one or the other limiting shape. Considering the later solution, one cannot exclude the possibility that there is a solution of the shape equation with lower system energy in which the parent body is connected to the beadlike protrusion via the neck with the finite width. However, our initial attempts to find such a solution of the shape equation by the available numerical methods failed. Beadlike protrusion conformations were also studied by Koswada et al. (1999), but in these cases the studies did not include the parent body.

The comparison of the theoretical predictions (Fig. 4) with the experimental data shown in Fig. 1 demonstrates that the obtained subset of solutions of the shape equation with the lowest system energy is in accordance with the observed vesicle shapes. The observed protrusions with narrow necks between the "beads", which resemble the calculated protrusions composed of spheres, alternate with the protrusions involving the necks which are open. The theoretical analysis correctly predicts a synchronous broadening of the narrow necks, which can be observed during the transformation steps. The theoretical predictions also correspond to the observed timing of the transformation process. Namely, the duration of the protrusions of the prolate unit (neck broadening) is shorter than the duration of the protrusions composed of spheres (Table 1), a feature that can be predicted from the phase diagram (Fig. 5) under the assumption that the changes in ΔA_0 are monotonous. Moreover, the calculations of shapes with open protrusion necks predict that the necks are wider in the middle of the protrusion, which is in agreement with Fig. 1A and with previous observations (Kralj-Iglič et al. 2001).

A closer look into the resemblance of the calculated and observed protrusions shows a discrepancy in the magnitude of the effect. The observed necks (Fig. 1) are always broader than the theoretically predicted necks of stable configurations (Fig. 4a). The broadening of the neck can be the consequence of membrane thermal fluctuations, in analogy to the deviations of average vesicle shapes from the equilibrium (zero temperature) shapes as previously presented for the dumbbell shaped vesicles (Heinrich et al. 1997). A more specific view of the effect of membrane thermal fluctuations can be gained also on the basis of the repulsion forces between two opposing fluctuating membranes (Helfrich 1978). In the case of the described protrusions, a repulsive force is expected to act in between two neighboring "beads". The fact that the axial force widens the neck has been theoretically shown in a comparable situation where an axial force acting on the poles of a prolate pear vesicle with a narrow neck was considered (fig. 6 in Heinrich et al. 1999). It has to be noted that the two "beads" also attract each other owing to the van der Waals forces (Käs et al. 1993). It seems that in some situations such attractive forces prevail over the repulsive forces due to membrane thermal fluctuations, and the system stabilizes in the configuration of narrow necks, as it is the case with the shape presented in Fig. 3.

A barrier between two stable states could cause a shift of the position of the transition corresponding to equal energies of the states to lower Δa_0 (Döbereiner et al. 1995). In this case, the shapes would follow the metastable shapes beyond the theoretically predicted transition point, which are determined by the thin solid line in Fig. 4a. These metastable shapes are characterized by significantly broader necks. We are not able to determine exactly whether the shapes with open necks are stable or unstable since the necks observed are always broader than theoretically predicted. However, the alternating of the protrusion's shapes having one "bead" more or less during the observed transitions indicates a low barrier between two stable states comparable to the energy of fluctuations.

In conclusion, the present study offered an enlargement of the vesicle shape phase diagram towards higher values of the equilibrium area difference Δa_0. A theoretical model describing the shape transitions of vesicles with short beadlike protrusions has been proposed. It is supported by a good correlation with the corresponding experimental data.

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