Budding and Tubulation in Highly Oblate Vesicles by Anchored Amphiphilic Molecules

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(Received 13 October 2002; published 25 September 2003)

We studied local budding and tubulation induced in highly oblate lipid vesicles by the anchoring of either polymers having a hydrophilic backbone and grafted hydrophobic anchor groups, or by oleoyl-coenzyme A, an amphiphilic molecule important in lipid metabolism. The dynamics of bud formation, shrinkage, and readsorption is consistent with an induced spontaneous curvature coupled with local amphiphile diffusion on the membrane. We report a novel metastable state prior to bud readsorption.

DOI: 10.1103/PhysRevLett.91.138102 PACS numbers: 87.16.Dg, 68.15.+e

The formation of vesicles by budding and tubulation is essential in a variety of cellular processes. Notable examples include transport in the Golgi apparatus and endoplasmic reticulum [1], signaling in neuronal synapses [2], and mitochondrial structure [3]. Both budding and fission in cells are promoted by the recruitment of specific proteins to a membrane and proceed through well-defined pathways, some of which have been reproduced in *in vitro* experiments. In this Letter we show that budding and tubulation can be induced in a biomimetic system consisting of single-component phospholipid vesicles of highly oblate shape, and *anchoring* amphiphilic molecules [4]. The latter anchor on vesicle bilayers by the penetration of one or more hydrophobic groups. Our system mimics in the simplest fashion the budding observed in complex biological systems such as in a Golgi cisterna, the anchoring amphiphiles playing the role of curvature-inducing proteins.

Anchoring polymers can induce striking changes in vesicle morphology [4–6] as well as in membrane bending modulii. An analysis of the relaxational dynamics of a pearling instability [5] has provided evidence indicating that the polymers induce curvature both by increasing the area of the monolayer into which the anchors sink [area-difference elasticity (ADE)] and by a local deformation of the bilayer [spontaneous curvature (SC)] [7–9]. A budding instability has been observed in vesicles using anchored polymers with a collapsible backbone (N-isopropyl-acrylamide), but only by varying the temperature, thereby triggering backbone collapse [10]. Budding can also be induced by changing the area to volume ratio of a vesicle upon a modification of the temperature and/or osmolarity of the medium [11].

In this work we show that anchoring amphiphiles induce a budding and tubulation instability in highly oblate vesicles. A novel feature of our system is the generation of long tubular extensions rather than the quasispherical buds observed in other systems [10–12] and predicted theoretically [7,13,14]. In our system, tubulation occurs without a directed force, and tension is negligible in our system, in contrast with previously reported cases [15,16]. Furthermore, we present quantitative measurements of the dynamics of the system, which reveal a surprising metastable state appearing during the final stages of bud readsorption. The dynamics of the instability suggests that the same conceptual framework used to explain polymer-induced pearling and coiling [5,17], i.e., spontaneous curvature, may account for many aspects characterizing amphiphile-induced tubulation. In addition, the results suggest that the concentration of the anchors is the dominant parameter in determining the amount of induced curvature.

Vesicles were made of stearoyl oleoyl phosphatidylcholine (SOPC) with C_{18} alkyl chains. Three different amphiphilic molecules were used: two polymers having hydrophilic backbones, one with 40 hydrophobic anchors per chain on average, and the other with 1 anchor per chain on average. The backbone is dextran [4], with a molecular weight of 162 000 g/mol, functionalized both with palmitoyl C_{16} alkyl chains as hydrophobic anchors and dodecanoic nitrobenzoxadiazole (NBD) as fluorescent markers. The anchors were distributed statistically along the backbone. In the case of 40 hydrophobic anchors per chain, there are about four persistence lengths between consecutive anchors on average. The third amphiphilic molecule used is oleoyl-coenzyme A (CoA). Long-chain acyl CoA derivatives are important intermediates in many reactions associated with lipid metabolism [18] and are essential ingredients in budding and fission of transport vesicles in Golgi cisternae [19].

Oleoyl CoA has a molecular weight of 1031 g/mol. Typically, several 0.5–1.0 μl droplets of SOPC in a 7:1 chloroform-methanol solution (10 mg/ml) were placed on a glass coverslip forming small lipid patches. The sample was prehydrated for 10–20 min under water-saturated nitrogen, then hydrated with either 0.01 or 0.1 mM potassium buffer at either *p*H 7.4 or *p*H 6.5, respectively. After hydration, a variety of self-assembled structures formed, including a large number of hollow tubes with one or more lamellae. Some of these tubes transformed spontaneously into highly oblate vesicles connected by a stem to the lipid patch, which constitutes

a reservoir with which the structures can exchange lipid molecules. Experiments were conducted at room temperature, ensuring that the membranes were in a fluidlike state. This allowed free diffusion of both lipids and anchored molecules along the bilayers. Events were observed by phase-contrast microscopy and recorded on video. For fluorescence imaging, the NBD markers attached to the anchors were excited with an argon laser (488 nm) and observed with a cooled charge-coupled device camera.

The amphiphiles were administered either *locally* or *globally*. In the first case, a micropipette containing the amphiphile solution was brought to within a few microns of a particular vesicle, and a small quantity ($\sim 10^{-4}$ μl) of solution was then injected. Typical polymer concentrations used were in the range of 20–100 μg/ml for the multianchor polymer and 1 mg/ml for the single-anchor polymer. In the case of CoA the concentration was 50 μg/ml. The pipette was then drawn out to ensure that no more amphiphile entered the chamber. In order to administer the amphiphile globally, a drop of solution of a given concentration c_p, in the range 20–100 μg/ml, was introduced through one of the chamber sides. Using this procedure, the amphiphile concentration *on* the membrane generally grows with time, as more and more molecules anchor from solution. In all our experiments, the amphiphile was added after vesicles were formed. Hence amphiphilic molecules anchor primarily on the outer monolayer.

A typical oblate vesicle is shown in Fig. 1(a). After adding multianchor polymer globally, buds emerge almost simultaneously all along the rim contour [Fig. 1(b)]. No effect is observed when purely hydrophilic dextran of the same molecular weight is used regardless of the concentration, as previously observed by others [20]. Both the typical number of buds and the time scale for their formation varies with concentration c_p. At the value of c_p used, buds form along the entire length of the rim almost simultaneously, while at low enough c_p only a single one forms. The time scale of formation ranges from seconds for large values of c_p to hours for small values of c_p. For high c_p, the ensuing tubes subsequently undergo pearling [Fig. 1(c)]. During the final stages of evolution [Fig. 1(d)], structures of progressively larger specific area and curvature form, which can accommodate larger amounts of anchoring chains. Note that only for high values of c_p tubes undergo pearling. For small concentrations tubes grow and grow instead, until they consume the vesicle from which they formed. In these experiments the chamber volume acts as a polymer reservoir, and more and more polymer molecules anchor from solution, driving further the evolution in the system.

Applying the polymers locally yields valuable insights about the mechanisms driving bud formation, shrinkage, and disappearance. After injection of multianchor polymer solution in the vicinity of a vesicle, the thermal fluctuations of its rim are strongly enhanced [Fig. 2(a)], and eventually a bud emerges [Fig. 2(b)], suppressing the fluctuations in the vicinity. If more polymer is applied slowly near its base, the bud grows into a tube whose length increases, as more polymer anchors [Fig. 2(c)]. Alternatively a number of tubes may form and grow, the bases displaying a clear tendency to cluster together, until they reach a nearly fixed distance apart. In order to estimate the spatial extent of the polymer cloud after

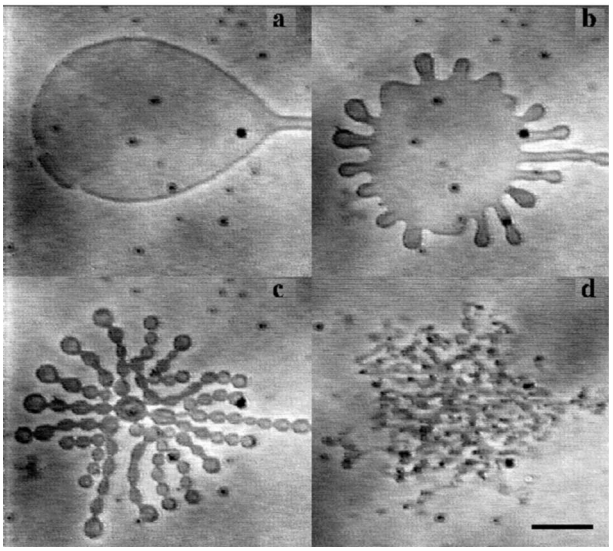

FIG. 1. Oblate vesicle before addition of polymer (a). After flushing the cell with multianchor polymer solution of large concentration ($c_p = 0.4$ mg/ml), buds form all along the rim (b). Buds grow into tubes which subsequently pearl (c). At high polymer concentrations on the membrane, small structures of high curvature result (d). The scale bar represents 10 μm.

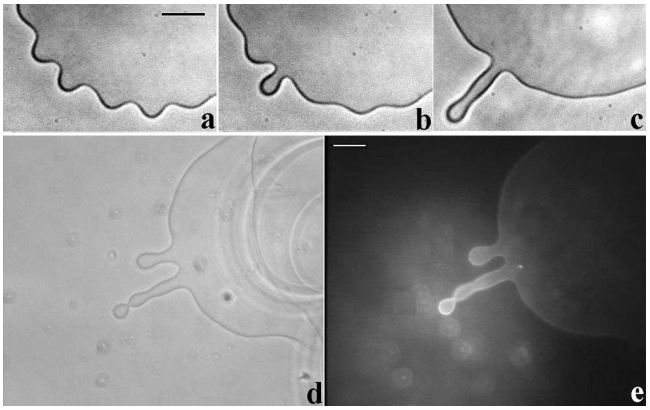

FIG. 2. Enhanced fluctuations of the rim after local injection of multianchor polymer in the vicinity of a vesicle (a). Nucleation of a bud suppresses rim fluctuations (b). The bud grows into a tube (c). Phase contrast (d) and fluorescence image (e) of the local polymer concentration around a bud induced by the local addition of polymer to one side of a vesicle. The scale bar represents 10 μm.

local injection, we excited the fluorescent NBD markers on the polymer, whose emission is considerably larger within the hydrophobic environment of the bilayer. Figures 2(d) and 2(e) show phase-contrast and fluorescence pictures of the resulting bud. The polymer visibly anchors on the membrane, and its concentration is clearly correlated with local membrane curvature. Over time, polymer diffuses along the membrane, so that the local polymer concentration on the buds decreases. If pure buffer without polymer is injected, no buds are formed.

If after local tube formation no more polymer is added, tubes shrink until they disappear into the vesicle's rim. We illustrate the dynamics of this decay process in the case of multianchor polymers in Fig. 3. A short time after local injection is stopped, the amount of polymer in the solution is negligible. Tubes which have grown to different lengths [Fig. 3(a)] start shrinking. Shrinking is not uniform: when the tube length L is about 3 times the diameter, tubes reach a metastable configuration [Fig. 3(b)] and finally disappear [Fig. 3(c)]. We plot the tube length L as a function of time t for different buds in Fig. 4. As this plot illustrates, there is no apparent correlation between the maximal length a tube reaches and the lifetime of the metastable state into which it decays.

Buds in their metastable state are characterized by scale-independent shapes. Measurements over a large number of buds show that the ratio between their length and width (0.32 ± 0.02) is independent of the nature of the amphiphile, its concentration, the number of times it was applied, and the total amount injected. In addition, Fig. 5 shows a plot of the spacing between buds as a function of bud diameter. The spacing between tubes is consistent with a linear dependence on the tube width. Finally, the spatial order at which the buds in a cluster decay from the metastable state does not follow any particular pattern [e.g., Fig. 3(c)].

To what extent do the phenomena illustrated in Figs. 1–3 depend on the multianchored nature of the polymer? To address this question, we carried out experiments with

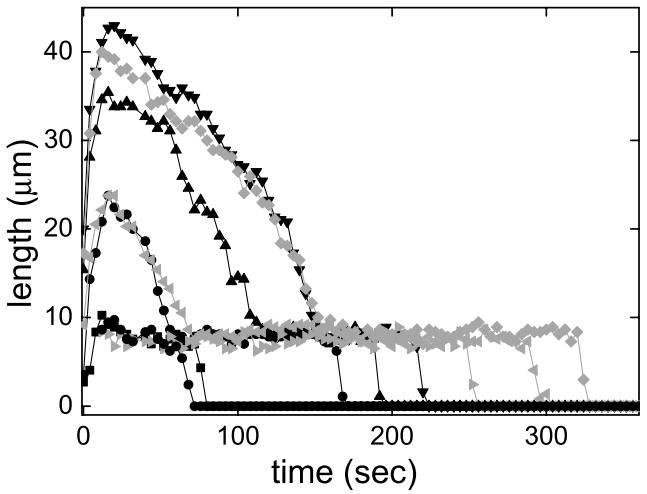

FIG. 4. Bud length L as a function of time t for various buds. L decreases linearly with t until a plateau corresponding to the metastable state is reached. Individual buds are then readsorbed completely.

polymers having only one anchor per chain, and with oleoyl-CoA, an amphiphile with a compact hydrophilic head. In contrast to the multianchor polymers which sit almost flat on the membrane [21], the backbone of polymers with one anchor can adopt mushroom configurations. We found that single-anchor polymers induce both tubulation and pearling [4,5] instabilities with similar characteristic time scales as multianchor polymers, but at concentrations about 40 times higher. Similar behavior is induced by oleoyl-CoA when its concentration in the pipette is comparable to the anchor concentration in the polymer experiments. These results suggest that local membrane curvature correlates well with the concentration of anchor groups embedded in it, in agreement with experimental results which show that the vesicle-to-micelle transitions induced by grafted diblock copolymers is independent of the polymer mass [22].

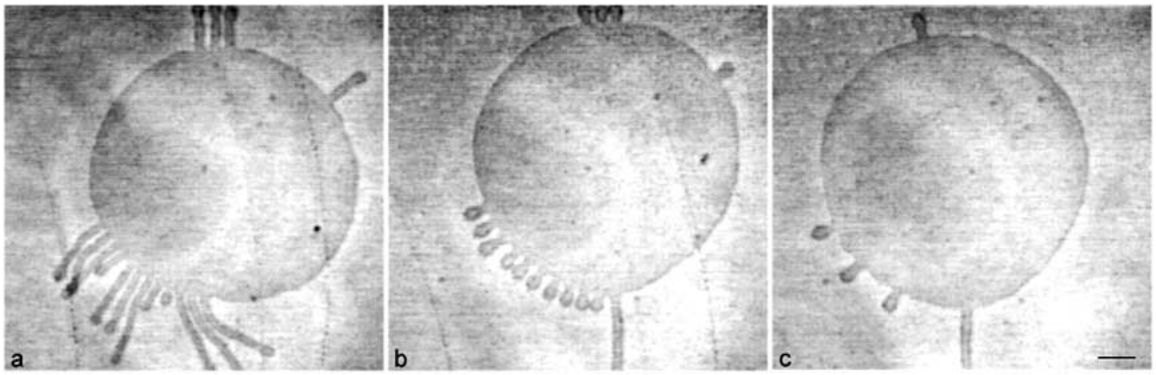

FIG. 3. Buds induced locally by the local addition of multianchor polymer with a micropipette and its subsequent removal (a). Buds decrease in length until a metastable state shown in (b) is encountered, from which they decay (c). The scale bar represents 10 μm.

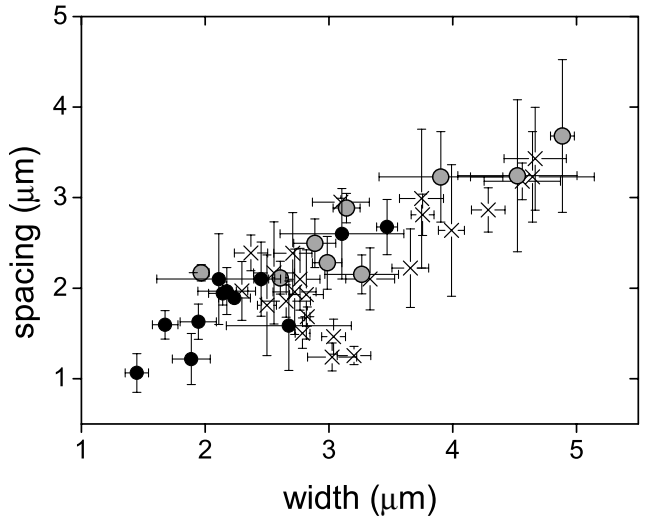

FIG. 5. Bud spacing as a function of bud diameter for multianchor polymer (black circles), unianchor polymer (grey circles), and oleoyl-CoA (crosses). Each point represents an average over buds emerging from a vesicle after an application of amphiphile. Error bars represent 1 standard deviation. Measurements were taken when all buds were near their metastable state (see text).

Both the geometry and characteristic time scales of the phenomena described above suggest that spontaneous curvature is the dominant driving mechanism, irrespective of the amphiphilic molecules used. First, tubes form only along the rim where most of the oblate vesicle's curvature is concentrated, and not in its flat upper portion (Figs. 1–3). Furthermore, the observation that all buds emanating out from the rim of a particular vesicle always have the same width at their base, together with the scale-independent bud shape, imply that the latter is controlled by the local geometry of the oblate vesicle's rim. Tube shrinkage takes typically several minutes (Fig. 4). By this time, both ADE and hydrodynamic processes have long since relaxed. Since tubes are quasi-one-dimensional objects connected to a quasi-two-dimensional oblate vesicle, it is difficult to solve the coupled diffusion problem. However, diffusion constants can be obtained from measurements of the relaxation dynamics of pearled structures [5]. The system exhibits diffusive behavior at early times with a diffusion constant D of 200 $\mu m^2/sec$ in the case of multianchor polymers, and 185 $\mu m^2/sec$ for oleoyl-CoA. At later times there is a sharp crossover to a much slower diffusive behavior with $D \sim 5$ $\mu m^2/sec$ and 15 $\mu m^2/sec$, respectively. These behaviors were attributed to ADE (or hydrodynamical effects) and SC stemming from diffusion of the amphiphile. The smaller diffusion coefficient agrees well with the rate of tube shrinkage (Fig. 4). Taken together, these findings suggest a coupling between the concentration of anchoring groups on the membrane and local membrane curvature as the driving mechanism [5,6].

Our findings leave three intriguing questions unanswered. (i) Why do tubes formed as a result of the tubulation instability in highly oblate vesicles not pearl readily while there is polymer in their vicinity, unlike the behavior of tubes observed in other experimental and theoretical investigations [5,20]? We point out that the fact that tubes do not pearl readily is not related to whether they are connected to a reservoir or not. However, if we inject polymer solution towards those tubes for sufficiently long times in high enough concentration, they can pearl. Pearling then relaxes leaving the unperturbed tube to evolve as described above. (ii) What is the nature of the metastable state preceeding final bud decay? (iii) What is the reason for the formation and stability of these highly oblate vesicles?

We thank Daniel Kandel for highly stimulating discussions. This work was supported by the Israel Science Foundation-Recanati and IDB Group.

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