CHAPTER SIX

Membrane Microvesiculation and its Suppression

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Abstract

Membrane microvesiculation is a common process in cells. Membrane constituents undergo lateral redistribution coupled to the change in local membrane curvature. Thin necks that are formed in this process can be torn by mechanical stress and membrane-enclosed fragments that contain various biologically active molecules become more or less free to move with fluids. Released vesicles are small in size (micrometer down to tens of nanometers). They interact with distant cells and thereby present an intercellular communication system which plays important physiological role in organisms. Micro and nanovesicles (NVs) can be isolated from body fluids. It was found that the concentration of NVs is increased in isolates from blood of patients with different diseases (e.g., cancer, inflammation, infection, thromboembolic diseases) indicating an increased vesiculability of blood cells. Here, we present some mechanisms of microvesiculation of biological membranes and suggest a possible mechanism for suppression of microvesiculation by a mediated attractive interaction between membranes.

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1. MEMBRANE CURVATURE AND CELL SHAPE

Shapes of membrane-enclosed systems without internal structure (including cells without internal structure) have been thoroughly studied in the past 40 years. Progress has been made by implementation of knowledge on biological membranes, in particular, the fluid mosaic model of the cell membrane (Fig. 1) [1] and layered structure of the membrane [2]. Changes of shapes of red blood cells from discocyte to stomatocyte or echinocyte due to addition of exogenously added substances have been experimentally observed [3–10]. Assuming that the shape of such system is essentially determined by the membrane properties and considering the membrane as an laterally isotropic thin elastic shell [11] enabled the use of the theory of elastic continuum in describing the shapes that were mildly curved all over the surface. The equilibrium shapes were determined by the minimization of the membrane free energy with relevant geometrical (or other) constraints imposed upon the system [11]. Taken that the area of the erythrocyte is determined at its genesis, this theory explained well

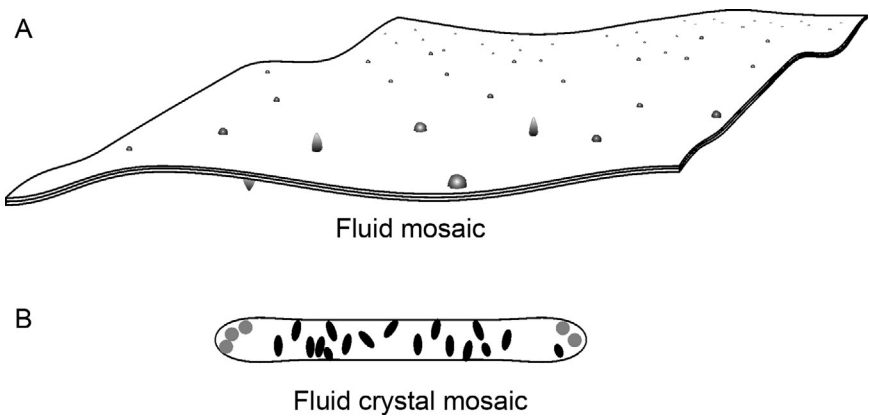

Figure 1 Illustration of the fluid mosaic model of the cell membrane considering almost flat membrane regions (A) and fluid crystal mosaic model of the cell membrane considering also membranous nanostructures (B). In the fluid mosaic model, the membrane is described as a lipid bilayer with embedded large molecules or complexes. The embedded molecules are uniformly distributed over the membrane with curvature radii much larger than the membrane thickness. In the fluid crystal mosaic model, membrane is described as composed of constituents characterized with intrinsic principal curvatures. If the intrinsic principal curvatures differ one from the other, the single-constituent energy depends on the orientation of the constituent in a given site. This effect is notable on strongly anisotropically curved membrane regions found in membranous nanostructures.

the equilibrium shape of erythrocyte in *in vitro* physiological conditions (the discocyte shape) and shape changes due to a decrease or an increase of the volume/area ratio and the spontaneous membrane curvature [12,13].

Related systems convenient for the study of the properties of the biological membranes are giant unilamellar phospholipid vesicles (GPVs) composed of phospholipid bilayers [14–17]. The theory used for description of the shape of erythrocytes applies also to these systems. The theoretical description based on the relevant elastic energy of the laterally isotropic continuum [18] was further developed [19] and experiments to study the effects of various substances on the biological membranes were also performed in systems with GPVs [20–27].

2. MEMBRANOUS NANOSTRUCTURES AND THE FLUID CRYSTAL MOSAIC MODEL

In the above studies, the focus was mostly on the biological membranes as envisaged by the fluid mosaic model (i.e., the cell plasma membrane and its artificial counterpart—the membrane of giant phospholipid vesicles). Developments in theoretical and experimental physics have however revealed spontaneously stable membranous nanostructures in experimental systems [28–36]. Such structures are, for example, membrane buds [37,38], tunneling nanotubules [39–46], nanovesicles [47–52], and narrow necks [53–58]. Small dimensions of these structures (their dimensions are of the order of membrane bilayer thickness which is around 5 nm) imply considerably higher curvatures than curvatures involved in determination of discocyte and stomatocyte shapes of red blood cells and giant phospholipid vesicles. Theoretical description of biological membrane as a laterally isotropic fluid mosaic was found unable to explain stable nanotubular protrusions on echinocyte spicules and respective tubular nanoexovesicles [37].

Theory and views upon the biological membrane therefore required upgrade in order to include the description of membranous nanostructures. The theoretical base of the generalized description was taken from the statistical mechanical description of electric double layer [59]. Electric double layer is created when electrolyte solution containing negatively and positively charged ions is in contact with a surface bearing the electrical charge [60,61]. Due to electrostatic forces, counterions (ions with charges of opposite sign with respect to the surface) accumulate near the charged surface, while coions (ions with charges of the same sign as the surface) are depleted. Entropic effects prevent collapse of counterions onto the surface. In that theory [59], the system is composed of constituents and the single-constituent

energy in the external field was proposed. Infinitesimal volume elements were distinguished with all fields constant within the element and with a very large number of constituents in each element. The variational problem was stated and solved by seeking field distributions within the system which yield minimal free energy of the system corresponding to thermodynamic equilibrium. In the case of electric double layer, the solution of the variational problem was a self-consistent set of quantities: distribution functions of counterions and coions, the electric field, and the equilibrium free energy. The description of the biological membrane was done analogously, with curvature taking the role of the electric field [62]. The membrane was considered as composed of constituents with intrinsic shapes, which however constitute the membrane and therefore are subject to local curvature. To attain the minimum of the free energy of the entire system obeying given constraints, all constituents cannot be located at sites with optimal (intrinsic) curvatures. The mismatch between the actual curvature and the intrinsic curvature is a source of the single-constituent energy [57]. The statistical mechanical description then followed the method developed for the electric double-layer system. The essence of the generalization of the description of biological membranes is the assumption that the energy of a membrane constituent depends on the orientation of the constituent with respect to the axis normal to the membrane surface. This gives the system an additional degree of freedom, i.e., orientation of the constituents in the curvature field may decrease the free energy of the membrane and thereby stabilize a particular configuration of the system [62]. In contrast to the electric double layer, the membrane does not have a fixed geometry. The orientation is opposed by the thermal motion of the constituents; therefore, collective ordering that would affect the shape of the membrane will take place only if strongly anisotropic molecules are found in strongly anisotropic membrane regions. It turned out [35,37,38,54,55,57,62] that such regions are membranous nanostructures. Orientational ordering and accumulation of membrane constituents with particular intrinsic shapes at strongly anisotropically curved regions is a possible mechanism that explained stability of erythrocyte spherical and tubular nanovesicles [37], and narrow necks [57]. Furthermore, orientational ordering was found to explain stability of nanostructures composed of pure phospholipid membranes, e.g., nanotubules [37,58], hexagonal structures [35], and narrow necks [53,54]. The theoretical description was further developed by considering membrane nanodomains as elements of anisotropic elasticity [63]. Generalization of fluid mosaic model was described as the fluid crystal mosaic model to point to the orientational ordering that is characteristic for liquid crystal systems [62].

Cell membrane nanotubules remained long time undiscovered due to their thinness and fragility. Improvements of microscopic techniques and indications derived from indirect observations [64] have led to their visualization. It was then found that cells exchange matter through tunneling nanotubules [39,41,45]. Cell nanovesicles were isolated from body fluids as well as from the media of cell cultures and it was revealed that they have physiological and pathophysiological roles [65]. Signalling by NVs may contribute to a variety of biological processes (e.g., spreading of inflammation [49,50], transport of infectious particles [66–68], and progression of tumor in cancer [69–72]). Membranous nanostructures were proven important for the function of cells and tissues since they constitute a cell to cell communication system. Cancer cell NVs-borne oncoproteins, lipids, and nucleic acids (DNA, mRNA, microRNA) may be transferred to other cells and thereby affect tumor progression, immunotolerance, invasion, angiogenesis, and metastasis [72], while cancer cell NVs-borne tissue factor is involved in coagulopathy leading to an increased risk for clot formation in blood vessels [73]. As the same NVs may carry molecules that are involved both in cancer progression and in thromboembolic disorders, it was suggested that they could play an important role in coagulopathies in cancer described as the Trousseau syndrome [74]. Clinical studies have shown that the concentration of NVs isolated from blood in patients with different diseases is changed with respect to healthy subjects. For example, the concentration of NVs in isolates from blood was found to be increased in patients with lung cancer [75], dermatofibrosarcoma protuberans [76], carcinoma of the oral cavity [77], ovarian cancer [78], and gastrointestinal cancer [79,80]. It was recently suggested that the material isolated from blood contains both, NVs and residual cells, and that residual cells, mostly platelets, are the origin of the majority of NVs found in isolates—as an artifact of the isolation procedure [81]. However, since clinical studies show differences between concentrations of NVs isolated from blood of patients with different diseases and of healthy subjects, it is indicated that the properties of blood cells and plasma which determine the state of the isolate in patients and in healthy subjects differ from each other.

3. MECHANISMS OF MICRO AND NANOVESICULATION

The buds may be pinched off the mother membrane and released into the surrounding solution to become free vesicles in different ways. Suggested mechanisms are shedding of buds formed at the tips of evaginations, releasing the exosomes formed inside the cell, fragmentation of cells during apoptosis, and fragmentation of cells due to mechanical impact.

3.1 Budding of Plasma Membrane

Shedding of vesicles in the final stage of the budding process is preceded by accumulation of membrane constituents that favor strongly curved membrane regions on the buds (Fig. 2). These processes were observed in erythrocytes treated with exogenously added molecules such as detergents [5]. Budding and vesiculation of the plasma membrane is common in all cells.

3.2 Budding of Internal Cell Membranes

Based on studies of sheep reticulocytes, it was suggested that budding and vesiculation of the membrane may take place also in the fluid pools inside the cell [82]. It was proposed [82] that these NVs can be released into the extracellular solution (Fig. 3). Internally shed and then released nanovesicles were called exosomes [83]. Similar mechanism was later suggested in white blood cells [84]. It is now considered that exosomes are secreted by most cell types and are thought to play important roles in intercellular communications [85].

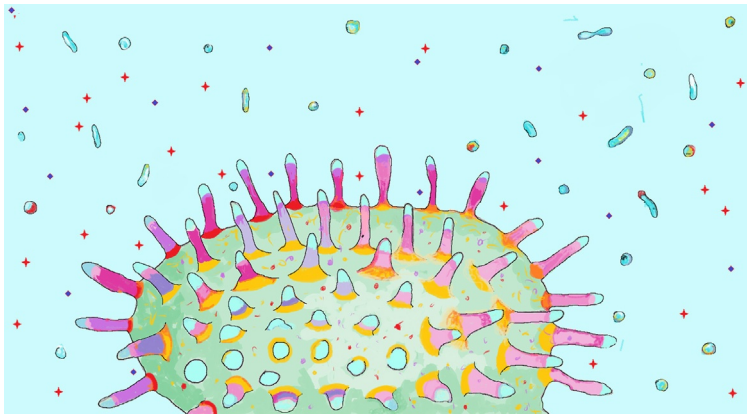

Figure 2 Illustration of the redistribution of membrane constituents on membrane nanobuds of a cell. Different shading illustrates constituents that favor (i) strongly isotropically curved regions (such as small spheres), (ii) cylindrical regions with small radii, (iii) almost flat regions, and (iv) saddle-shaped regions. The pinched-off extracellular vesicles are mostly light, indicating that they are formed from tips of the tubular buds. Point-like elements in the solution illustrate complexes of molecules in the extracellular solution.

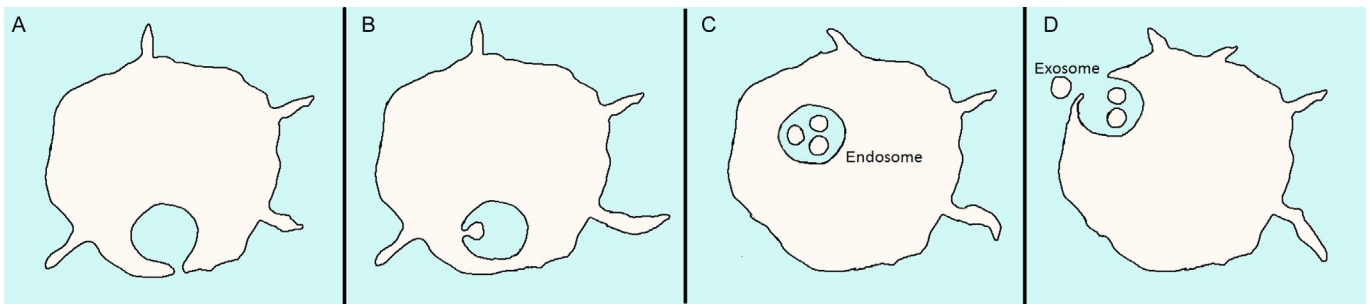

Figure 3 Formation of exosomes. Plasma membrane is internalized to form endosome (A). Buds are formed (B) and pinch off into the endosome (C). Pinched-off vesicles can be released into the extracellular solution by emptying the contents of the endosome (D).

3.3 Fragmentation of Cell During Apoptosis

During apoptosis the cell disintegrates. Fragments organize into irregularly shaped globular bodies that are heterogeneous with respect to composition and size. These fragments contain proteins and nucleic acids and are free to move with body fluids.

3.4 Fragmentation in the Shear Stress

Cells and their fragments are *in vivo* exposed to mechanical stresses, especially during the flow of body fluids. Also, they are exposed to mechanical stresses during the processing of samples in experiments, such as during flow through the needle, during centrifugation and during flow through nanostructured materials. Since the observation of nanovesicles implies processing of samples, these causes are necessarily present in all data, but are rarely taken into account (Fig. 4).

4. OBSERVATION OF MEMBRANE VESICULATION ON GIANT PHOSPHOLIPID VESICLES

To obtain insight into the processes taking place during the budding and pinching off of the vesicles, studies of giant phospholipid vesicles which are large enough to be observed directly by phase-contrast microscopy were undertaken.

Figure 5 illustrates the effect of the composition of the surrounding solution on the budding of the GPV membrane. Tubular budding was induced by raising the temperature of the sample (Fig. 5A). When the growing tube was of sufficient length, heating was discontinued. Upon addition of phosphate-buffered saline (with higher osmolarity than the GPV suspension) the protrusion became undulated (Fig. 5B) and underwent substantial movement followed by its detachment from the mother vesicle (Fig. 5C). Finally, the protrusion decomposed into spherical vesicles which migrated away from the mother vesicle (Fig. 5D) [86]. If the molecules which mediate attractive interaction between membranes (specific proteins, in particular, beta 2 glycoprotein I) were present in the added solution, the bud (Fig. 5E) was attracted back to the mother membrane (F) and remained bound to the surface of the mother vesicle (Fig. 5G and H) [86].

Although the GPV was composed of a mixture of palmitoyloleoylphosphatidylcholine and phosphatidylserine which was at neutral pH in the solution negatively charged, the bud adhered to the

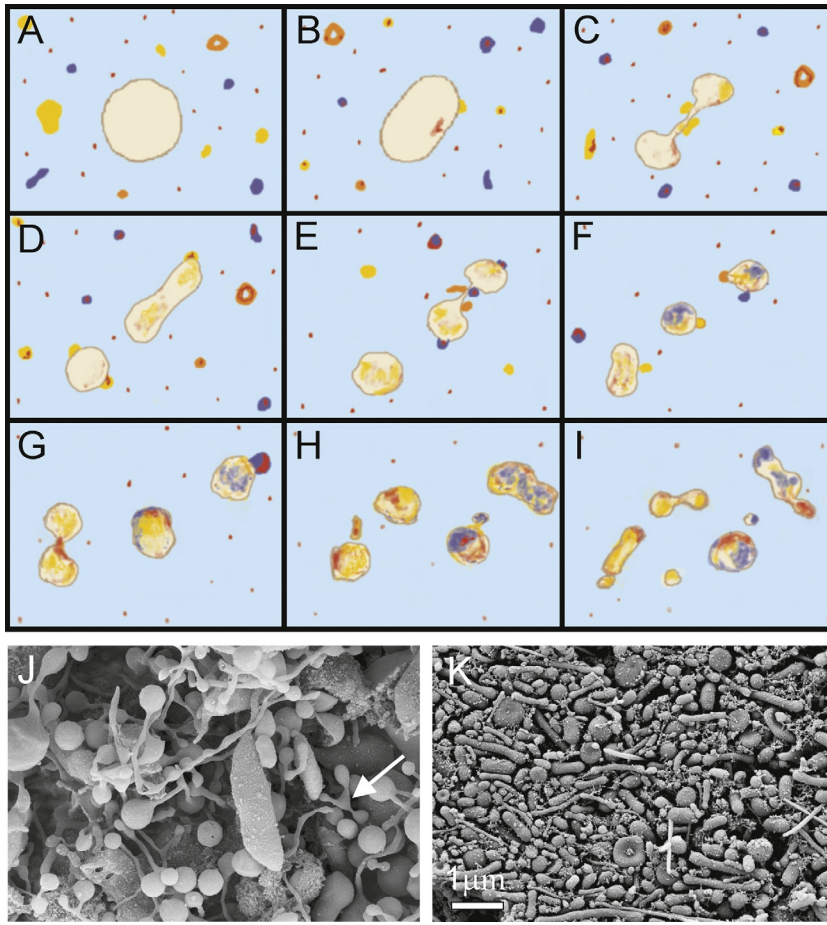

Figure 4 Fragmentation of a cell in the shear stress. (A) Unstressed cell in the solution containing NVs of various origin, self-assemblies (such as cholesterol complexes), antibodies, and other molecules. (B) Under mechanical stress, the cell shape and concomitantly, the local membrane curvature undergo changes, thereby increasing the probability of adhesion and integration of specific elements. (C) The probability for fusion of NVs with the cell increases at long tubular regions as they exhibit high curvature. (D) Thin necks are prone to tearing to create cell fragments. (E) Fragments, being smaller than the cell, exhibit larger local curvature which increases probability for integration of highly curved NVs. (F) With accumulation of particular NVs, the composition of the fragments changes and the constituents distribute according to their preferred curvature and interactions. (G) Due to redistribution of membrane components, the membrane undergoes budding and vesiculation. (H) Ultimately, the solution contains numerous fragments heterogeneous in shape and size. (I) Fragmentation of blood cells is preceded by formation of thin tubular structures and thin necks. (J) Elongated and oriented cell fragments in the isolate from blood indicate that the fragments are affected by the shear stress during the centrifugation of the sample (K). *Panels (J) and (K) from Ref. [81].*

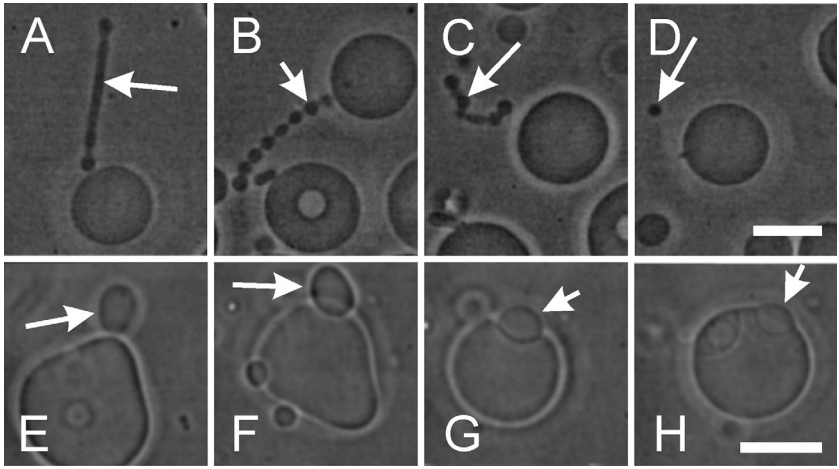

Figure 5 Vesiculation of a giant phospholipid vesicle (GPV). To a vesicle with a long tubular protrusion (A) a phosphate-buffered saline with higher osmolarity was added. This caused the tubular protrusion to exhibit undulations (B). Vigorous movements of the protrusion led to tearing of the membrane at the thin necks connecting the bead-like undulations (C). The released spherical vesicles were free to move away from the mother vesicle (D). Suppression of the vesiculation of the GPV. To a GPV with a globular bud (E) proteins which mediate attractive interaction between membranes (beta 2 glycoprotein I dissolved in phosphate-buffered saline) were added. Instead of pinching off from the mother vesicle, the bud was attracted to the mother vesicle (F) and adhered to it (G and H). White arrows point to the protrusion and its remnants. Bars = 10 μm. *From Ref. [86].*

mother vesicle (Fig. 5E–H), while in the case of a vesicle with long bead-like protrusion, the beads adhered to each other (Fig. 6). This effect was attributed to the mediating effect of the added molecules beta 2 glycoprotein I. In the control experiment where phosphate-buffered saline alone was added to the vesicles, the necks connecting the beads to each other and the neck connecting the protrusion to the mother vesicle were torn to yield small spherical daughter vesicles which were free to move away from the mother vesicle (not shown). As tearing of the necks was preceded by vigorous movements of the protrusion (most probably due to concentration gradient caused by adding the sample), it was interpreted that the reason for the tearing was mechanical in nature [86].

Also it was observed in concentrated suspensions of GPVs that added substances (in particular, plasma protein beta 2 glycoprotein I and antiphospholipid antibodies) may cause adhesion between membranes; adhesion took place also when both membranes were negatively charged

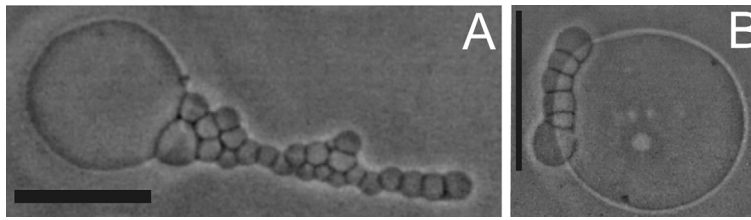

Figure 6 Attractive interaction mediated by beta 2 glycoprotein I caused adhesion of "beads" to each other (A and B) and to the membrane of the mother giant phospholipid vesicle (B). Bars = 10 μm. *Panel (A) is from Ref. [86].*

[87]. It was interpreted that added substances may mediate attractive interaction between membranes [86]. A model describing the adhesion due to the presence of mediating molecules was proposed, suggesting that the mediated attraction derives from a decrease of the free energy of the system due to orientational ordering of mediating molecules with spatially distributed charge [88]. The interaction turned out to be short-ranged [88]. Adhesion will likely take place if the distance between membranes is smaller than few nanometers [88]. For a bud, these conditions are fulfilled when it is connected to the mother vesicle by a short and thin but stable neck.

5. ATTRACTIVE MEDIATED INTERACTION BETWEEN MEMBRANES IS SUBJECT TO BRIDGING MECHANISM AND ORIENTATIONAL ORDERING OF MEDIATING MOLECULES

Beta 2 glycoprotein I is a J-shaped molecule composed of five domains. The fifth and the first domains are predominantly positively charged. Besides, there is a hydrophobic loop on the fifth domain. If the membrane is negatively charged, the fifth domain likely binds to the membrane surface due to electrostaticatic attraction [89]. The origin of attractive interactions between two negatively charged membrane surfaces is therefore the electrostatic attraction between the positively charged domains on the membrane-bound beta 2 glycoprotein I and negatively charged membrane surfaces (Fig. 7A). However, beta 2 glycoprotein I also mediates attraction between neutral membranes albeit the attractive interaction is weaker than in charged membranes [87]. This indicates that also the charge–dipole and dipole–dipole electrostatic interactions [90] between the lipid headgroup electric dipole moment and beta 2 glycoprotein I may contribute [87] (Fig. 7B). Also, it was found that beta 2 glycoprotein I binds to phospholipid

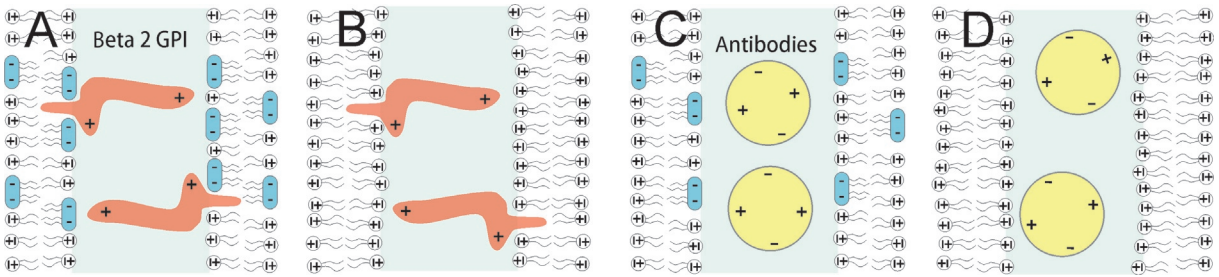

Figure 7 Scheme of the mediated interaction between membranes. Bridging interaction due to intercalation of a positively charged domain of beta 2 glycoprotein I into the negatively charged (A) and neutral (B) lipid layer. Interaction mediated by orientational ordering of mediating molecules (e.g., antibodies) in negatively charged (C) and neutral (D) lipid bilayer.

layers by hydrophobic interaction [91]. It is energetically advantageous that the hydrophobic domain is inserted into one membrane while the positively charged domains interact with the other membrane to form a "bridge" [86,88]. However, as the membrane headgroup interface is a source of electric field (in the case of charged or multi-polar headgroups) and screening of this field takes place due to the presence of ions in the adjacent solution, a gradient of electric field is created close to the phospholipid headgroup interface. In the solution, molecules with internally distributed charge will therefore orient in this gradient as to minimize their energy (Fig. 7C and D). The decrease of the free energy of the system is the greatest when the two interacting membranes are separated by a small distance within which the mediating molecules are orientationally ordered [88]. The attractive interaction would take place if the free energy minimum was deep enough to overcome thermal motion. Dimeric structure of large molecules (such as in antibodies) contributes to the significance of this effect [86,88].

6. STABILITY OF NARROW NECKS

The above described adhesion of the bud to the mother membrane would however take place only if the necks connecting the compartments were an energetically favorable structure. It is therefore of interest to understand the stability of the neck(s). As the bending energy of the harmonic modes of a flaccid membrane is comparable to the thermal energy, the vesicle shape spontaneously fluctuates. We have observed this feature during the development of thermal fluctuations of a mother GPV in a process where the necks were formed in a myelin-like protrusion which integrated into the mother GPV. In phospholipid systems, the existence of network of nanotubes was indicated in an experiment [64] which showed rapid transport of fluorescent label within the membrane between the GPVs prepared by electroformation [14]. The remnants of the network in the form of tubular protrusions (that are attached to the mother globule) became visible under the phase-contrast microscope (Fig. 8A) and underwent a slow spontaneous shape transformation in which the average mean curvature of the vesicle decreased causing the protrusion to become shorter and thicker (Fig. 8B–D). Thin necks between the "beads" were formed in the last stages of this process (Fig. 8E). The shape transformation continued by diminishing the number of beads (Fig. 8E–I). Finally, the neck connecting the protrusion to the mother vesicle opened and the protrusion was integrated into the mother vesicle (not shown) [93].

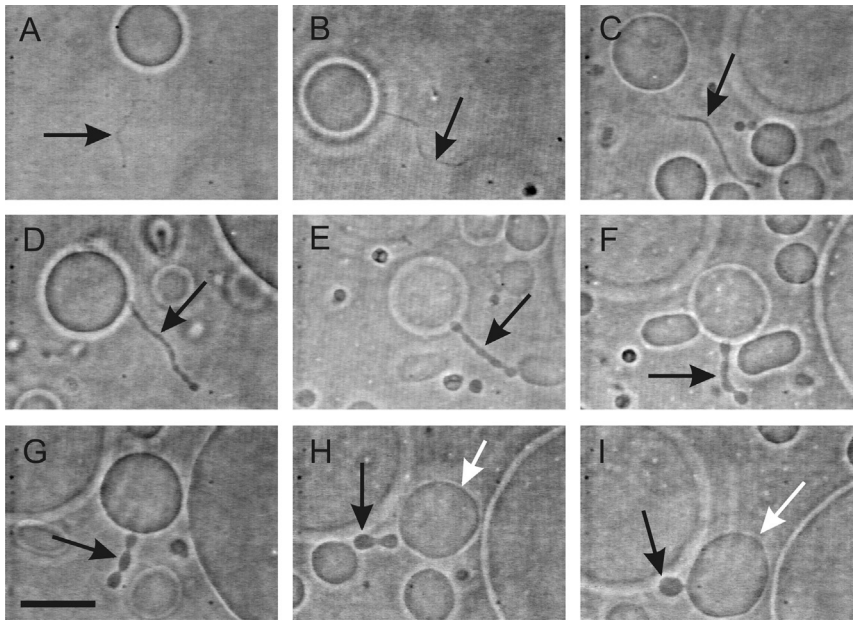

Figure 8 Integration of the tubular protrusion into the mother giant phospholipid vesicle. The protrusion (A) spontaneously shortened and thickened (B–D) and eventually exhibited a bead-like bud (E) which further transformed by diminishing the number of beads (E–I). Finally, the neck connecting the protrusion to the mother vesicle opened and the protrusion was integrated into the mother vesicle. *From Ref. [92].*

The stability of the neck was studied by analyzing the transformation of the shape of the almost spherical mother vesicle [92]. The shape of the mother vesicle was expressed by using the expansion into spherical harmonics,

$$R(\theta,\phi) = R_s\left(1+\sum_{\ell=0}^{\ell max}\sum_{m=-\ell}^{m=\ell} u_{\ell m} Y_{\ell m}(\theta,\phi)\right), \tag{1}$$

where $R(\theta, \varphi)$ is the distance from the contour center to the membrane, R_s is the effective radius of the mother globule, $u_{\ell m}$ are the Fourier coefficients, and $Y_{\ell m}$ are the normalized spherical harmonics,

$$Y_{\ell m}(\theta,\phi) = N_{\ell m}(\theta,\phi)P_{\ell m}(\cos\theta)\exp(\mathrm{i}m\phi), \tag{2}$$

$P_{\ell m}(\cos\theta)$ are the associated Legendre functions and $N_{\ell m}(\theta,\phi)$ are the normalization factors,

$$N_{\ell m} = \sqrt{\frac{(2\ell+1)(\ell-|m|)}{4\pi(\ell+|m|)}}. \tag{3}$$

The effective radius R_s is introduced in such a way that all the Fourier coefficients $u_{\ell m}$ are small. The shape of the cross-section of the vesicle is obtained from by taking $\theta = \pi/2$,

$$R(\theta = \pi/2, \phi) = R_s \left(1 + \sum_{-\ell max}^{\ell max} u_m \exp(\mathrm{i}m\phi)\right). \tag{4}$$

The corresponding Fourier coefficients are

$$u_m = \sum_{\ell=|m|}^{\ell max} u_{\ell m} N_{\ell m} P_{\ell m}(0), \tag{5}$$

where

$$P_{\ell m}(0) = \frac{2^m}{\pi} \cos\left(\frac{\pi}{2}(\ell+m)\right) \frac{\Gamma(\ell/2+|m|/2+1/2)}{\Gamma(\ell/2-|m|/2+1)}. \tag{6}$$

Figure 9 shows the time dependence of the averaged square of the Fourier coefficients with $m \geq 2$ normalized by the square of the effective radius (R_s) (A) and the time dependence of R_s (B), corresponding to the last stages of the slow spontaneous shortening of the myelin-like protrusion and its integration with the mother vesicle. The effective radius of the mother vesicle R_s on the average increased (Fig. 9B). However, the increase of R_s was not monotonous. Rather, a peculiar stepwise pattern could be observed [92]. The abrupt increase of the effective radius corresponds to a transformation of the protrusion into the elongated shape with one bead less [92]. The duration of steps increased so that the protrusion with three beads was less persistent than the protrusion with two beads and the latter was less persistent than the protrusion with one bead [92] (Fig. 9B).

However, the peculiar stepwise pattern of the time-course of the effective radius is in agreement with stepwise pattern of the time-course of the width of the protrusion necks [94]. The necks connecting four beads were wider than the necks connecting three beads and these were wider than the neck that connects a single bead to the mother vesicle [94]. The narrower the neck, the longer the persistence of the given number of beads (Fig. 9B). It was therefore concluded that the narrow neck tends to stabilize the shape of the entire GPV [92,94]. This effect is not limited to the neck that connects

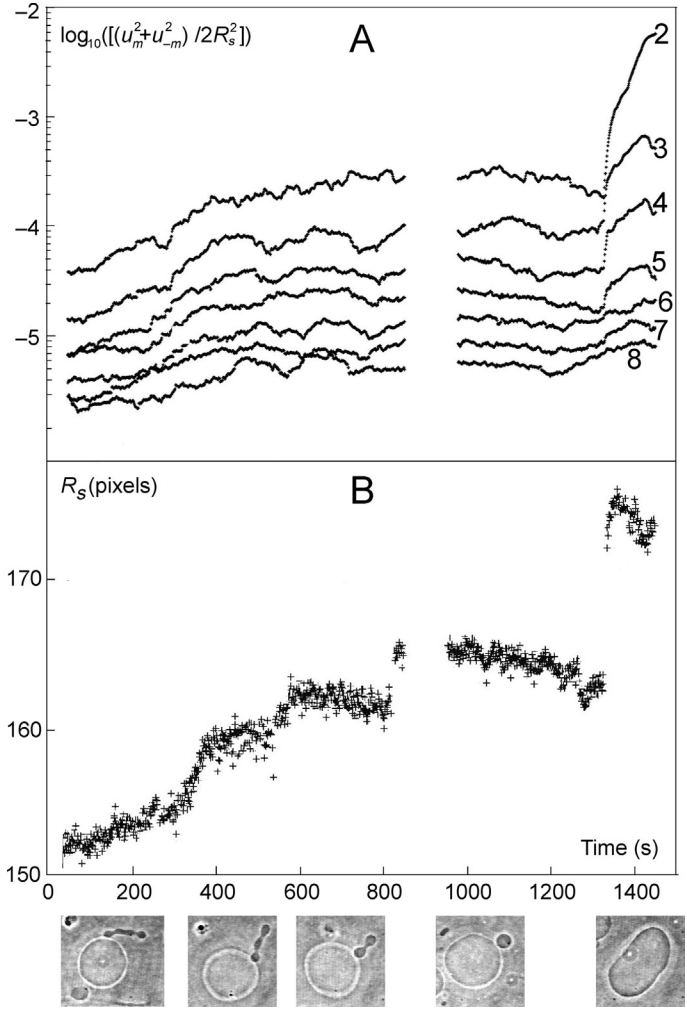

Figure 9 (A) The time dependence of the square of the Fourier coefficients normalized by the square of the effective radius. Moving averages over 100 s are presented. (B) The effective radius of the mother vesicle. The shapes of the vesicle corresponding to the times indicated are also shown. Measurement in the time interval between 830 and 860 s was interrupted due to technical issues. *From Ref. [92].*

the protrusion with the mother globule but is also present in the shapes with protrusions with two or three (wider) necks, although it is not so strong [92]. The Fourier coefficients u_m were however averaged over consecutive points meaning that each point was calculated as the average over the interval of 100 points centered at the given point. Averaging over time blurs the stepwise time dependence of Fourier coefficients.

Within the last two "steps" (times between 650–800 s and 800–1300 s in Fig. 9) the effective radius of the mother globule decreased (Fig. 9B). However, the effective radius decreased also after the protrusion was completely incorporated into the mother vesicle (times larger than 1300 s). It can be expected that the fluctuations immediately after the integration of a larger amount of material into the globular part were not spherically symmetric as the inflow appeared at a certain place where the protrusion was joined with the globular part, and after some time, the spherically symmetric mode was more or less restored.

The contribution of the Fourier coefficient with $m = 2$ was the largest, however, also the coefficients with higher m can be noted (Fig. 9A). On the average all the coefficients increased with time. The increase was especially large at the end of the sequence when the globular shape of the vesicle was reached. At this point, the effective radius of the mother vesicle and the Fourier coefficients abruptly increased [92] (Fig. 9A and B).

Before the opening of the neck connecting the globular part and the spherical daughter vesicle, oscillations of the neck width on the timescale of a minute were observed, indicating that the vicinity of a shape phase transition reflects a phase transition within the bilayer membrane. It was suggested that this phase transition could be based on in-plane orientational ordering of phospholipid molecules [92]. Based on the above described effect that anisotropic inclusions within the phospholipid bilayer membrane may become in-plane orientationally ordered in those regions which exhibit strongly different main curvatures, it was suggested that the free energy of the equilibrium vesicle shapes that are continuously transformed from a prolate shape to the pear shape and further to the shape with a spherical protrusion connected to the mother vesicle by a thin neck, exhibits a deep minimum. This minimum corresponds to a shape in which the mother vesicle and the daughter vesicle are connected by a thin, but finite neck [57]. In the neck, the inclusions exhibit orientational ordering which causes a decrease of the free energy [57]. Due to various reasons (e.g., equilibration of osmotic pressure, presence in the solution of molecules with particular properties, preferential intercalation of molecules into one of the two layers), the shape of the GPV may change. This change can be such that in some area(s) (e.g., necks) the curvature may become stronger and anisotropic. In order to constitute the membrane at that region, a phospholipid molecule may undergo a conformational change so that that the shape of the molecule becomes strongly anisotropic (in the sense that not all in-plane orientations are energetically equivalent). Such molecule may be considered as a seed for

an anisotropic inclusion. If the curvature relaxes, the conformational change relaxes too. It was suggested that such inclusion is transient [92]. However, if the vesicle fluctuates around the shape with an anisotropic region (e.g., neck), the phospholipid molecule spends more time in a highly anisotropic state. Due to the interaction between the phospholipid molecules, clusters of highly anisotropic molecules may be formed which in turn constitute the membrane and impose the local curvature [92,95]. Inclusions become orientationally ordered while the formation of the neck is promoted. The observed critical fluctuations may therefore indicate the vicinity of the phase transition in which a pool of phospholipid molecules that are strongly anisotropic and orientationally ordered is localized around a narrow but finite neck. The change of the average mean curvature (presumably due to the change of the number of the molecules in the outer membrane layer) is however important as to drive the shape over the prolate–pear transition where the probability of the proposed mechanism becomes high [92].

7. SUPPRESSION OF MEMBRANE VESICULATION IN CELLS

After observing suppression of vesiculation of the membrane due to attractive mediated interaction between the vesicle parts (Figs. 5E–H, 6) [86,95], it was suggested that a similar effect would take place in cells [79]. It was then shown that adhesion of buds to the mother membrane took place in erythrocytes (Fig. 10B and F), in platelets (Fig. 10C), and in leukocytes (Fig. 10E).

We indeed observed adhesion of the buds to the mother membrane in erythrocytes [92]. Figure 10 shows budding of biological membranes and

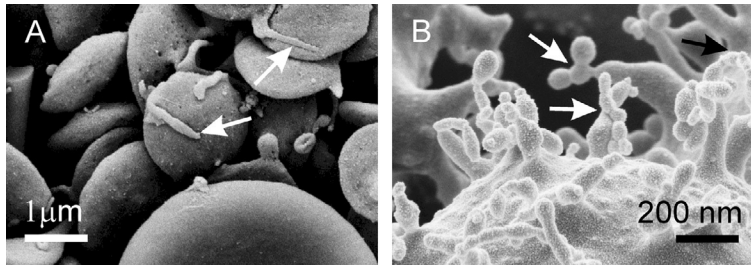

Figure 10 Adhesion of buds to the cell membrane. Tubular buds of the platelet membrane adhered to the mother cell (A). Erythrocytes treated with calcium ionophore A21387 underwent echinocytosis and budding at the tips of echinocyte spicules. The buds adhered to the mother cell (B). Arrows point to protrusions. *From Ref. [92].*

adhesion of buds to the mother membrane. Figure 10A shows adhesion of the tubular protrusion to the platelet. Adding ionophore to the suspension of erythrocytes caused a discocyte–echinocyte transformation (Fig. 10B). Budding of the membrane took place at the tips of the echinocyte spicules and the units adhered to each other (Fig. 10B).

However, there is an important issue that should be taken into account; namely, the outer layer of the plasma membrane contains glycolipids [96]. The sugar coating prevents adjacent membranes to approach each other to a distance that could be subject to attractive mediated interaction. It was suggested [92] that the self-adhesion of nanosized buds could occur if the membrane around the neck becomes depleted or nude with respect to the sugar coat, and if the appropriate mediating molecules are present in the solution. The favorable composition of membrane in the neck is attained by curvature-sorting of the membrane constituents [97–101]. Glycolipids with extensive parts sticking from the outer membrane layer will not likely accumulate in strongly negatively and anisotropically curved region of the neck, which enables the suggested process to take place. It can be interpreted that the particular curvature of the neck provides the field for appropriate sorting of membrane constituents in the neck (Fig. 11).

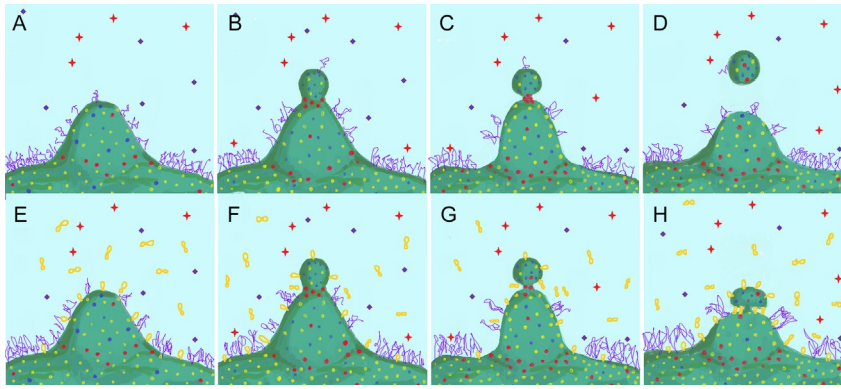

Figure 11 Illustration of microvesiculation-suppression mechanism. At the top of the echinocyte spicule (A), the bud is formed (B) with concomitant redistribution of membrane constituents. The neck narrows (C) and the bud is eventually pinched off (D). In the presence of the molecules that mediate attractive interaction between membranes, the process of budding at the top of echinocyte spicule (E–G) leads to the adhesion of the bud to the mother cell (H). Point-like elements in the solution illustrate molecules and complexes in the extracellular solution while elements in the shape of 8 in (E)–(H) illustrate the mediating molecules. It is suggested that the glycolipid coat is depleted in the buds and especially at the highly curved tips and saddle regions, as illustrated.

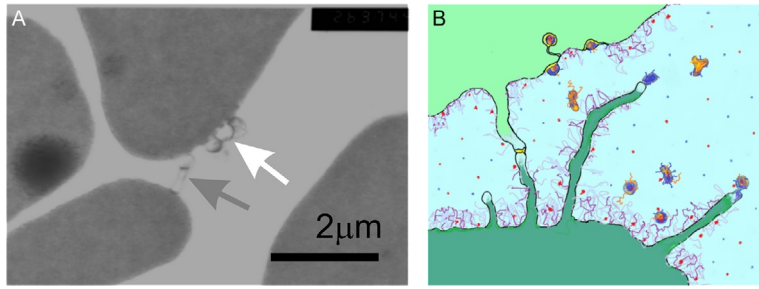

Figure 12 Adhesion of nanostructures to membrane. (A) Nanovesicles probably of leukocyte origin adhered to erythrocyte membrane as revealed by TEM. White arrow points to a group of spherical vesicles and gray arrow points to tubular vesicles adhered to two adjacent erythrocytes. The tubular vesicles interact at their tips where the membrane curvature is high. (B) A scheme illustrating interaction of membranous nanostructures at the sites of high curvature. The tips of tubular protrusions are depleted of glycan coat, thereby enabling mobile nanostructures in solution to approach the "nude" membrane and interact. *Panel (A): Adapted from Ref. [92].*

Adhesion of nanostructures is however not limited to the mother cell. NVs can adhere to any host cell provided that it can approach the membrane to a very small distance and that the mediating molecules are available. We have observed in samples of blood cells that NVs adhered to erythrocyte membrane (Fig. 12A). TEM micrograph reveals dark gray shadow of erythrocytes due to the presence of hemoglobin. The adhered vesicles are light, similar to leukocytes that were present in the sample. Also the erythrocyte membrane appears smooth and it is unlikely that the adhered structures were developed from buds of the host cell. Most interesting is the connection between two adjacent erythrocytes by interaction of adhered vesicles (Fig. 12A, gray arrow). The vesicles are in contact at the tips where their curvatures are large and matching.

8. CLINICAL IMPLICATIONS OF MEMBRANE BUDDING SUPPRESSION

Attractive interaction between membranes mediated by plasma proteins proved a mechanism that suppresses membrane vesiculation (Fig. 11). Since excessive vesiculation was observed in blood samples of patients with cancer, thromboembolic disorders, inflammation, and autoimmune disorders [75–80], it was suggested that that molecules which mediate attractive interaction between membranes have both anticoagulant and antimetastatic effect [62]. Blood plasma mediates attractive interaction

[95] indicating that molecules with the required properties are present in blood. Heparin (a common choice of anticoagulant prophylaxis and treatment) induces adhesion between phospholipid vesicles [74]. Also, heparin is known to have an antimetastatic effect in some types of cancer [102–106] which supports the hypothesis of the anticoagulant and antimetastatic effect of plasma constituents based on suppression of nanovesiculation [74].

A method for determination of the extent of plasma–induced adhesion between membranes was proposed by assessing the average effective angle of contact between GPVs which adhered due to the addition of plasma to the suspension of GPVs [79,95]. A group of patients with gastrointestinal cancer was compared to the group of patients with other gastrointestinal diseases [79] assuming that larger average effective angle of contact corresponded to a more pronounced adhesion [79,95]. The clearly visible effective angles of contact were measured in a representative micrograph and the average for each patient was determined. Also, the concentration of NVs was measured in the isolates from peripheral blood of these patients [79]. Differences between GPV-plasma samples pertaining to different patients were observed, such as the presence of "debris" in some samples and larger differences between refraction indexes of the vesicles and of the surrounding solution as exhibited in the halo effect. A negative, statistically significant correlation (Pearson coefficient $= -0.50$, $p = 0.031$) was found between the number of NVs in peripheral blood and the ability of plasma to induce coalescence between membranes—represented by the average effective angle of contact between adhered GPVs [79]. Statistical significance of the correlation was even higher if the number of NVs was calculated with respect to the number of platelets (Pearson coefficient $= -0.64$, $p = 0.003$). By comparing patients diagnosed with cancer (group A) with patients having other gastrointestinal diseases (group B), a large (140%) and statistically significant ($p = 0.033$) difference between groups A and B regarding the number of NVs in isolates from peripheral blood while the difference between the two groups regarding the average effective angles of contact between GPVs was smaller than the difference in NVs, but still considerable (20%) and statistically significant ($p = 0.013$) [79]. Further statistical analysis yielded power 100% for NVs at $\alpha = 0.05$, while for the average effective angles of contact, the power at $\alpha = 0.05$ was 90%. On the basis of these results, it was concluded that considerable and statistically significant differences in the number of NVs in isolates from blood and in the ability of plasma to cause adhesion of membranes existed between the two groups [79]. That

study presented the evidence in favor of the hypothesis that plasma which mediates attractive interaction between membranes may cause suppression of microvesiculation which resulted in a smaller number of NVs shed from the vesiculating pool [79]. These results agree with the results of Kim *et al.* [107] who found an increased number of NVs in peripheral blood of patients with gastric cancer with respect to normal controls, the number of NVs increasing with the advanced stage of the disease. Suppression of processes leading to the release of NVs into circulation may therefore be beneficial as to prevent or slow down the development of the above pathological processes.

Natural and artificial suppressors of microvesiculation could act simultaneously as anticoagulants and cancer deccelerators. It would therefore be of interest to establish which plasma constituents can mediate the attractive interaction between membranes. Such constituents were found to be plasma protein beta 2 glycoprotein I [88] and heparin [74]. In the future, the list of possible candidates could be expanded and their clinical relevance assessed.

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