

Review

**MECHANISMS FOR THE FORMATION OF MEMBRANOUS  
NANOSTRUCTURES IN CELL-TO-CELL COMMUNICATION #**KARIN SCHARA<sup>1,2</sup>, VID JANŠA<sup>1</sup>, VID ŠUŠTAR<sup>1</sup>, DRAGO DOLINAR<sup>1,2</sup>,  
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**Abstract:** Cells interact by exchanging material and information. Two methods of cell-to-cell communication are by means of microvesicles and by means of nanotubes. Both microvesicles and nanotubes derive from the cell membrane and are able to transport the contents of the inner solution. In this review, we describe two physical mechanisms involved in the formation of microvesicles and nanotubes: curvature-mediated lateral redistribution of membrane components with the formation of membrane nanodomains; and plasma-mediated attractive forces between membranes. These mechanisms are clinically relevant since they can be affected by drugs. In particular, the underlying mechanism of heparin's role as an anticoagulant and tumor suppressor is the suppression of microvesiculation due to plasma-mediated attractive interaction between membranes.

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Abbreviations used: cardiolipin – 1,1',2,2'-tetraoleoyl cardiolipin; FITC – fluorescein isothiocyanate; GPVs – giant phospholipid vesicles; HLA, -B, -C – human leukocyte antigens of class I; MHC – major histocompatibility complex; MVs – microvesicles; POPC – 1-palmitoyl-2-oleoyl-snglycero-3-phosphocholine

**Key words:** Membrane nanostructures, Cell-to-cell communication, Microvesicles, Nanotubes, Trousseau syndrome, Heparin

## INTRODUCTION

The main subject of this review is cell-to-cell communication mediated by the transport of microvesicles [1-4] that are free to travel to distal cells and fuse with them [5] or by nanotubes that connect neighboring cells [6-9]. Both microvesicles and nanotubes derive from the cell membrane and are able to transport membrane constituents and the contents of the inner solution. The nanotubes and microvesicles are small enough to be classified as nanostructures, with their radius ranging from fifty to a few hundred nm. An important feature of the formation mechanisms for both is that they are preceded by lateral reorganization of the membrane into rafts and nanodomains [10-15]. As this process is mediated by the local curvature of the membrane, which is in turn determined by the local composition of the constituents, it is the intrinsic shape of the constituents and their interactions with the neighboring molecules [9, 11, 15-20] that determine whether microvesiculation or tubulation of the membrane occurs [9, 10, 13-15]. In this review, we present some recent results on the formation of microvesicles and nanotubes, and interpret them in terms of curvature-mediated lateral redistribution of membrane nanodomains.

## MICROVESICULATION

Microvesiculation is a major yet underappreciated mechanism playing a vital role in the development of cells and organisms. Microvesicles (MVs) derived from cell membranes as the final products of the budding process (see also [4]) were found to have important roles in vascular haemostasis [21-23], the promotion of cancer [24], and inflammation [2, 25]. Increased levels of circulating MVs were found in patients suffering from cardiovascular disorders [26], cancer [28], infection [29] and autoimmune diseases [30-34], and were associated with an increased risk of thromboembolic events.

The abundance of MVs reflects the extent of the membrane pool available for budding and vesiculation, the efficiency of the clearance mechanisms, the properties of the solution surrounding the membrane, and those properties of the membrane that render it more or less likely to bud and vesiculate. The ions, soluble molecules and macromolecules (e.g. proteins, carbohydrates, lipids) in the solution not only enter into chemical reactions with the membrane-bound receptors, but may affect the budding and vesiculation process via different physical mechanisms [13, 35-40]. The majority of MVs isolated from the peripheral blood derive from the platelet membrane [41]; however, assuming that the essential physical properties of all biological membranes are the same [42], the relevant mechanisms can be studied in simpler systems, such as erythrocytes and giant phospholipid vesicles (GPVs), which can be observed live under an optical microscope.

Membranes can be induced to vesiculate by manipulating the solution that is in contact with the outer layer of the membrane (Fig. 1). In erythrocytes, there is no internal cytoskeleton, so the shape of the cell is determined by the properties of the membrane with its underlying dense fibrillar skeleton of spectrin and anchoring proteins. If detergents are added to a suspension of erythrocytes, minutes later, the membrane folds either outwards or inwards, depending on the type of detergent added [43].

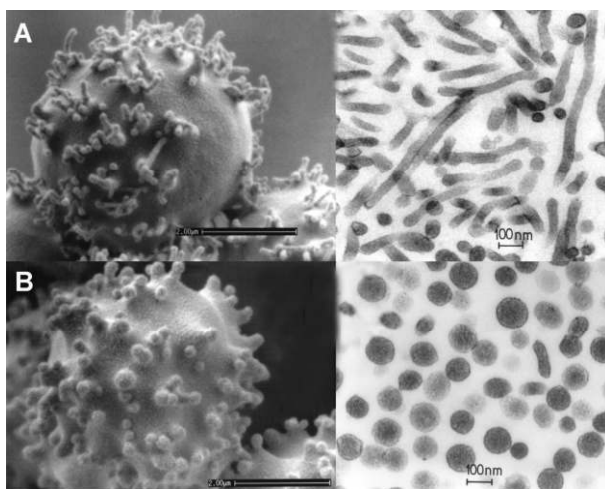


Fig. 1. The budding of the erythrocyte membrane (left), and isolated microvesicles (right). The budding and vesiculation process was induced by adding different detergents to a suspension of erythrocytes. Dodecylzwittergent induced predominantly tubular buds and microvesicles (A), while dodecylmaltoside induced predominantly spherical buds and microvesicles (B). Adapted from [46] with permission from Medicinski razgledi.

According to the bilayer couple model [35-37], this folding is caused by a change in the area difference between the outer and the inner membrane layers. If the substance added causes an increase of the outer membrane layer with respect to the inner membrane layer, the membrane folds outwards and the erythrocytes transform from a discocytic shape to an echinocytic shape. Further, buds devoid of membrane cytoskeleton form on top of the echinocyte spicules (Fig. 1, left), and are eventually pinched off the mother cell to become free microvesicles (Fig. 1, right). The shape of the buds (microvesicles) depends on the properties and interactions of the intercalated molecules and the membrane constituents [44]. It was suggested that such intercalated detergents induce the formation of membrane inclusions [45], which may be isotropic or anisotropic with respect to the axis perpendicular to the membrane normal. Isotropic inclusions give rise to spherical microexovesicles (Fig. 1B). In anisotropic inclusions, different orientations within the membrane plane are energetically non-equivalent, if the membrane is anisotropically curved (such as in tubules).

The orientational ordering of inclusions is energetically favorable, and therefore stabilizes tubular structures [45].

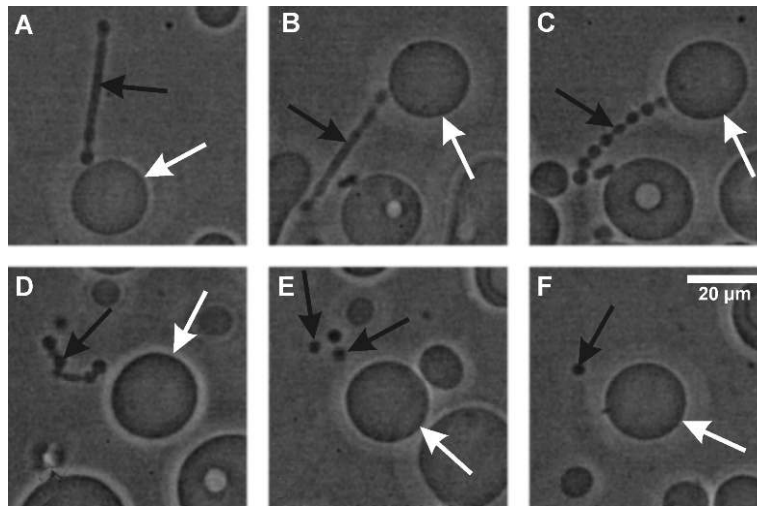


Fig. 2. The heat-induced budding of a giant phospholipid vesicle membrane composed of cardiolipin and POPC (A). Upon the addition of phosphate buffer saline, the protrusion became bead-like (B, C), eventually detached from the mother membrane (D), and decomposed into spherical vesicles (E), which were free to move away from the mother vesicle (F). The white arrows indicate the mother vesicle, the black arrows indicate the daughter vesicles. Bar = 20  $\mu\text{m}$ . Adapted from [38] with permission from Elsevier.

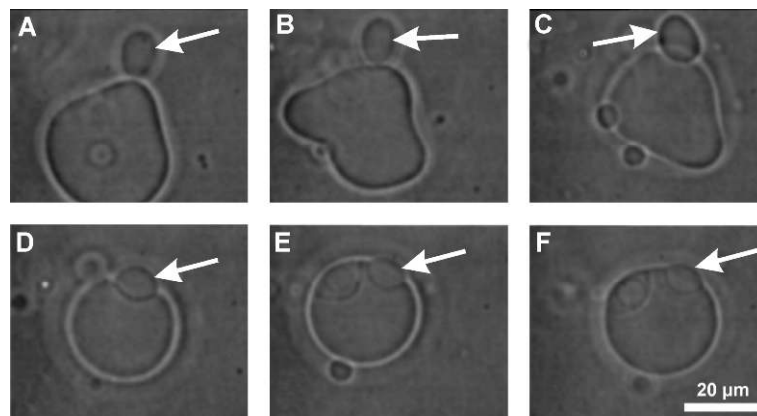


Fig. 3. The plasma protein  $\beta 2$  glycoprotein I was added to a solution with a budding GPV composed of cardiolipin and POPC (A-C). Thereafter, the bud adhered to the mother membrane (D-F). Vesiculation was prevented by protein-mediated interactions between the membranes of the bud and mother vesicle. The white arrows indicate the daughter vesicle. Bar = 20  $\mu\text{m}$ . Adapted from [38] with permission from Elsevier.

In studies with giant phospholipid vesicles, budding can be induced by increasing the temperature. Vesiculation may be also triggered by adding phosphate buffer saline to a suspension containing GPVs (Fig. 2). However, the presence of certain plasma proteins (in particular, beta2-glycoprotein I and antiphospholipid antibodies) in the suspension may cause buds to adhere to the mother membrane [38, 47] and thereby prevent them from becoming free vesicles (Fig. 3). The accompanying theoretical studies on protein-mediated interactions between membranes indicate that plasma proteins can indeed induce attractive interactions between membranes even when they are like-charged, due to the positional and orientational ordering of protein molecules with spatially distributed charges [47].

### MEMBRANE NANOTUBES

The results of some recent studies indicate that in many cases, vesicular transport between cells and between cell organelles over longer distances is not random, and that it takes place between specific surface regions of the cell organelles [6-9]. Such organized transport may be achieved via nanotube-directed transport involving carrier vesicles, or via direct transport through nanotubes.

It was recently found that cells can communicate with each other by direct interactions over distances of several cell diameters [7]. The connecting structures, called nanotubes, can bridge distances of more than 100  $\mu\text{m}$ , depending on the cell type [48, 49]. They are versatile in ultra-structure and formation, and consequently also in function [reviewed in 50-53]. Our recent results revealed that the nanotubes in epithelial cells can be divided into two distinct types with respect to their formation, stability and cytoskeletal content [9]. Type I nanotubes are shorter, usually not longer than 30  $\mu\text{m}$ , are more dynamic, and contain actin filaments. They are formed when a cell explores its surroundings through a tubular membrane protrusion extended from the cell surface in order to make contact with another cell. The same type of actin-containing nanotubes bridge cells at distances of less than 30  $\mu\text{m}$ , and are most likely derived from the adherence cell-cell contacts of cells that move apart. In terms of characteristics, the retraction fibers observed in migrating and mitotic cells most resemble the type I nanotubes, and thus can be included in this category [54].

Type II nanotubes can be longer, up to several 100  $\mu\text{m}$ , and are more stable and contain cytokeratin filaments. They are formed when two already connected cells start to move apart. Type II nanotubes differ from all previously found nanotubes: they have no actin filaments but have cytokeratin filaments, which are probably responsible for their stability [55] and longer life span. They do not seem to be involved in transport between cells, but rather in providing a positional effect to connected cells by sensing their direction of migration and enabling reverted movement along the intercellular tether [9].

Type I nanotubes largely outnumber type II nanotubes, which usually connect cells as single tethers. The two types of nanotubes also differ with respect to the proteins of the intercellular junctions that connect the cytoskeletal filaments of each kind between neighbouring cells (in preparation).

The protruding actin-containing nanotubes of type I start growing from the cell surface as filopodia [7], but continue until they reach the target cell. The actin filaments are very likely involved in the growth of the tubes [7, 48, 56]. If the growth of the nanotubes is related to the growth of the filopodia, it is expected that a bunch of actin-bundling proteins would be involved in this process, leading with fascin as the major actin-crosslinking protein responsible for the growth of the filopodia [57].

Another possibility for the growth of nanotubes is via the accumulation of flexible membrane nanodomains that prefer highly anisotropic curvature and that appear in the neck of a budding vesicle. If the separation of the budding vesicle from the mother membrane is somehow prevented, the neck can grow into a nanotube, pushing the vesicle at its tip. Such a situation can frequently be seen with a scanning electron microscope (Fig. 4). The growth of the nanotube by elongation of the neck can be supported by actin filaments or can be independent of the cytoskeleton. However, it is also possible for the tip vesicles to form after the nanotube has attached to the neighbouring membrane surface, and is subsequently detached or torn apart so that one end of the nanotube becomes free, as shown in Fig. 4. The possibility that the tip vesicles are attached to the substrate cannot be excluded either.

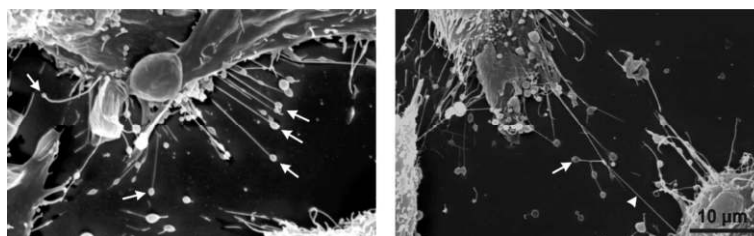


Fig. 4. A nanotube forming a continuous connections between neighbouring RT4 urothelial cells is indicated by the lone arrowhead. Some of the nanotubes which are not attached to the neighbouring cell surface have vesicles at their free tips, as indicated by the arrows. Bar = 10  $\mu\text{m}$ .

Fig. 5 shows that the brightness (originating from the ganglioside GM1-bound fluorescent label cholera toxin B-FITC) of the vesicles at the tips of the nanotubes that are not attached to neighbouring cells is more intense than the brightness of non-exvaginated regions of the cell membrane. This result supports the hypothesis that the vesicles at the tips of the nanotubes (having one free end) tend to accumulate ganglioside GM1 (Fig. 5), one of the characteristic components of lipid rafts. Lipid rafts are membrane micro-domains that accumulate several types of membrane proteins, like growth factor receptors and

enzymes [58, 59]. The appearance of lipid rafts in vesicular protrusions at the tips of the nanotubes might be crucial for the attachment of the nanotube to the target cell, because N-cadherins were found among the proteins included in the lipid raft domains [60]. These cadherins are responsible for making intercellular connections between mesenchymal cells and were also found in urothelial T24 cells [61] where nanotubes are frequently seen [9]. Differences in membrane composition between the tip vesicles and the nanotubes have also been suggested by other authors [10].

After the nanotubes reach the plasma membrane of an appropriate cell, they connect to it via an anchoring type of intercellular junction [7, 9, 62]. The formation of direct cytosolic continuity was proved by following the growth of actin filaments from cells into their neighbors, even though no exchange of membrane labels could be found between them [9].

Nanotubes are considered to be a new communication mechanism between two relatively close, but physically separated cells. They enable cells to exchange soluble messengers ( $\text{Ca}^{2+}$  mediated fluxes [56]), proteins (F-actin [7, 63]), membrane-bound receptors (HLA, -B, -C, class I MHC [56]), and cellular organelles (endosomes, lysosomes and mitochondria [7, 48, 63]). Nanotubes also help cells to function more efficiently in the maintenance of homeostasis and in defense against pathogens. On the other hand, nanotubes are a more efficient mechanism for pathogens to invade uninfected cells [49, 62, reviewed in 53].

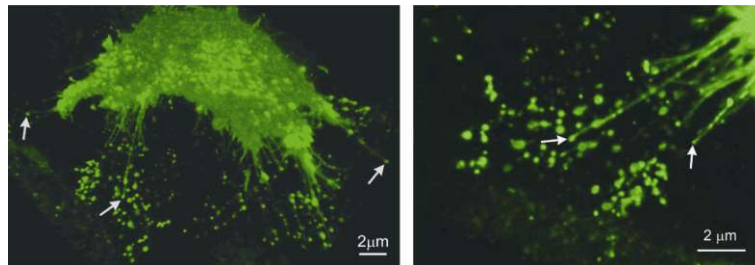


Fig. 5. Vesicular dilatations on nanotubes seem to have higher concentration of the ganglioside GM1, labelled with cholera toxin B-FITC (seen as brighter labeling; arrows show examples) in urothelial cell line T24. Bar = 2  $\mu\text{m}$ .

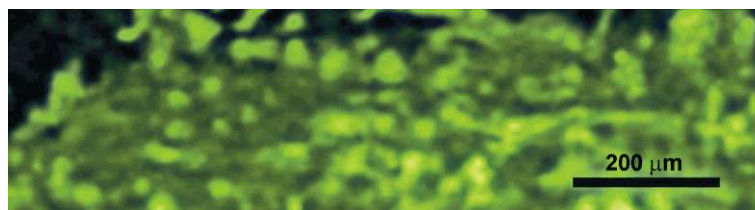


Fig. 6. A fluorescence microscope image of the urothelial RT4 cell membrane showing the higher concentration of the ganglioside GM1, labelled with cholera toxin B-FITC (seen as brighter labeling), on the membrane exvaginations (buds). Bar = 200  $\mu\text{m}$ . From [64] with permission from Elsevier.

**CURVATURE-MEDIATED LATERAL REDISTRIBUTION OF MEMBRANE CONSTITUENTS AS THE MECHANISM PRECEDING THE FORMATION OF MICROVESICLES AND NANOTUBES**

It was indicated that the clustering of small mobile raft elements into larger raft domains may be induced by a membrane bending and/or attractive forces between molecules [65 and references therein]. Interestingly, it was shown that overexpression of flotillin results in the induction of numerous thin tubular membrane protrusions [66]. Lipid-flotillin complex formation and flotillin oligomerization seem to play an important role in this process. Similarly, the accumulation of specific prominin rafts on highly curved membrane protrusions was recently indicated [67]. Therefore, it was suggested that prominin rafts play an important role in the stabilization of plasma membrane protrusions [18]. Since prominin does not directly interact with the actin-based cytoskeleton [18], the predominant localization of prominin in tubular membrane protrusions may be explained by the curvature-induced accumulation of prominin rafts, with the intrinsic shape of those prominin rafts as the main driving force [9, 14, 19]. The redistribution of prominin after mild cholesterol depletion from the protrusions indicates the importance of cholesterol [68] and other lipids as partners [53] in the formation of small rafts. Moreover, the non-homogeneous lateral distribution of flotillins and their accumulation during cytokinesis was recently observed [69], while the ganglioside GM1 was enriched in membrane exvaginations induced by cytosolic calcium and amphiphiles (Fig. 6). Based on these and many other experimental results and on theoretical considerations, it was suggested that laterally mobile nanodomains that detach from the membrane skeleton may sort into curved or flat membrane regions depending on their intrinsic shape and/or direct interactions between the nanodomains [9, 15, 70].

Much experimental and theoretical evidence indicates the importance of nanodomains in the process of membrane budding and microvesiculation [11, 15, 18, 72-74, and references therein]. It was shown that the membranes of  $\text{Ca}^{2+}$ -induced microvesicles and erythrocyte nanovesicles contain lipid rafts [74, 75], while the caveolae of  $\Omega$ -shaped membrane invaginations are enriched with cholesterol and sphingomyelin, known constituents of lipid rafts [11, 76-78]. Also, it was indicated that the assembly of cholesterol-based lipid microdomains is required for the biogenesis of secretory vesicles from the trans-Golgi network in the neuroendocrine cells [79, 80].

Considering the biological membrane as a mixture of different types of individual molecules with different intrinsic shapes and without explicitly taking into account the possibility of their self-assembly into energetically favourable heterogeneous membrane micro- and/or nanodomains, it would be possible to overestimate the role of the individual molecular intrinsic shapes in the mechanics of biological membranes and neglect the role of direct interactions between the membrane constituents. For example, membrane lipids, which comprise an impressively large number of molecular species with different



intrinsic shapes [81], may self-assemble into various micro- and nanodomains which have an average intrinsic shape (spontaneous curvature) that is very different from the intrinsic shapes of the lipids constituting the nanodomain (Fig. 7) [71, 72].

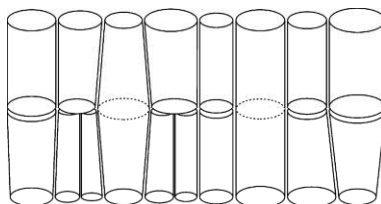


Fig. 7. The average intrinsic shape of the membrane nanodomain may be very different from the intrinsic shapes of the molecules (lipids and proteins) which constitute the domain. Adapted from [72] with permission from Elsevier.

Since the number of different types of molecules constituting the biological membrane is very large, it would be impossible to consider in the theoretical model all the molecules as building blocks and take into account their intrinsic shapes, as well as the direct interactions between them. Instead, in the theoretical approach applied in this review, we introduce the concept of flexible membrane nanodomains (Fig. 8) [9, 15, 72], which are defined as complexes of membrane molecules (lipids, proteins), and consider them as the membrane building blocks (see also [20] and references therein).

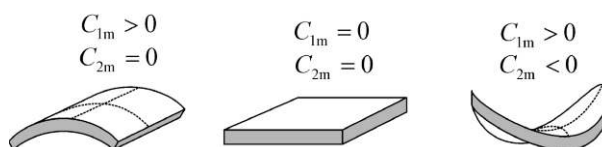


Fig. 8. A schematic representation of the various intrinsic shapes of flexible membrane nanodomains. The various intrinsic shapes are described by the two intrinsic principal curvatures,  $C_{1m}$  and  $C_{2m}$  [9, 72].

The intrinsic shape of a flexible membrane nanodomain can be described by two different intrinsic principal curvatures,  $C_{1m}$  and  $C_{2m}$  [9, 13, 15, 20, 64]. If  $C_{1m} = C_{2m}$ , the nanodomain is isotropic, while if  $C_{1m} \neq C_{2m}$ , the nanodomain is anisotropic.

The elastic energy of the flexible membrane nanodomain derives from the mismatch between the actual local curvature of the membrane and the intrinsic (spontaneous) curvature of the nanodomains (Fig. 8), which can be characterized by the mismatch tensor  $\underline{M} = \underline{R}\underline{C}_m\underline{R}^{-1} - \underline{C}$  [9, 13, 15, 20, 64]. Here, the tensor  $\underline{C}$  describes the actual curvature, while the tensor  $\underline{C}_m$  describes the intrinsic curvature of the nanodomains.

$$\underline{C} = \begin{bmatrix} C_1 & 0 \\ 0 & C_2 \end{bmatrix}, \underline{C}_m = \begin{bmatrix} C_{1m} & 0 \\ 0 & C_{2m} \end{bmatrix}, \quad (1)$$

where  $\underline{R}$  is the rotation matrix.

$$\underline{R} = \begin{bmatrix} \cos \omega & -\sin \omega \\ \sin \omega & \cos \omega \end{bmatrix}, \quad (2)$$

The angle  $\omega$  describes the orientation of the principal axes system of a single membrane nanodomain with respect to the local principal axes system of the membrane. In the respective principal systems, the matrices that represent the curvature tensors  $\underline{C}$  and  $\underline{C}_m$  include only the diagonal elements (for tensor  $\underline{C}$ , the principal curvatures  $C_1$  and  $C_2$ , and for tensor  $\underline{C}_m$ , the intrinsic principal curvatures  $C_{1m}$  and  $C_{2m}$  up to the second order in the components of  $\underline{M}$ ). The trace and the determinant of the tensor are taken as the set of invariants [13, 20, 45]:

$$f_i = \frac{K}{2} (\text{Tr} \underline{M})^2 + \bar{K} (\text{Det} \underline{M}), \quad (3)$$

where  $K$  and  $\bar{K}$  are constants. Taking into account the definition of the tensor  $\underline{M}$ , it follows from Eq. (3) that the elastic energy of the single membrane constituent can be written as [13, 20, 45]:

$$f_i = \left[ (2K + \bar{K})(H - H_m)^2 - \bar{K}(D^2 - 2DD_m \cos 2\omega + D_m^2) \right], \quad (4)$$

where  $H = (C_1 + C_2)/2$  and  $D = |(C_1 - C_2)|/2$  are the mean curvature and the curvature deviator of the membrane,  $H_m = (C_{1m} + C_{2m})/2$  is the intrinsic (spontaneous) mean curvature, and  $D_m = |C_{1m} - C_{2m}|/2$  is the intrinsic (spontaneous) curvature deviator. The constants  $K$  and  $\bar{K}$  are proportional to the area of the single membrane constituent. In the case of a simple flexible membrane nanodomain composed of a rigid core (protein) and the surrounding lipids, which are distorted in order to fit with the rigid core, the constants  $K$  and  $\bar{K}$  were estimated using a microscopic model [20]. The optimal values of the membrane mean curvature  $H$ , the curvature deviator  $D$  and the membrane constituent orientation angle  $\omega$  corresponding to the minimum of the function  $f_i$  can be calculated from the necessary and sufficient conditions for the extremum of  $f_i$  [72]:

$$H = H_m, \quad D = D_m, \quad \omega = 0, \pi, 2\pi, \quad (5)$$

where  $\omega = 0$  and  $\omega = 2\pi$  describe the same orientation and where  $K > \bar{K}/2$ ,  $\bar{K} < 0$ .

Fig. 9 shows the accumulation of isotropic membrane nanodomains (having  $C_{1m} = C_{2m}$ ), which favor high isotropic curvature, on a small bud. The fraction of the area covered by nanodomains increases towards the tip of the bud. As the neck becomes narrower, the distribution approaches a step function, while the free energy of the nanodomains lowers the free energy of the membrane. The proposed mechanism of coupling of the local membrane shape and the lateral density of the membrane constituents (nanodomains) may therefore be one of the mechanisms which is relevant for the formation and stabilization of highly curved spherical membrane shapes (such as membrane buds) with increased concentrations of particular membrane constituents [13, 15, 45, 82].

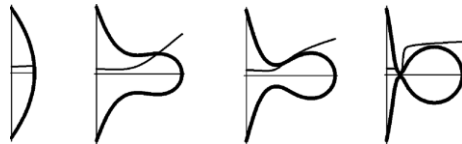


Fig. 9. The accumulation of membrane constituents (nanodomains) in the budding region. The gray line represents the relative concentration of membrane constituents (nanodomains) characterized by positive intrinsic curvatures ( $C_{1m} = C_{2m} > 0$ ) and attractive direct interactions during the budding/vesiculation of the membrane. Adapted from [15] with permission from Taylor & Francis.

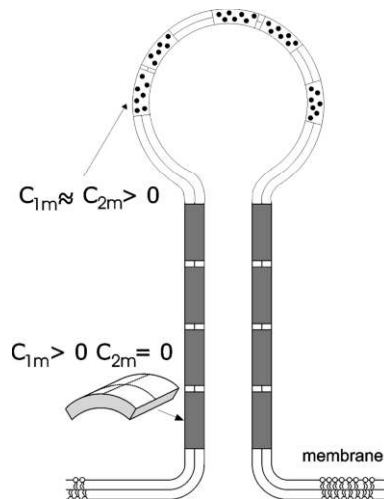


Fig. 10. A schematic illustration of the stabilization of Type I nanotubular membrane protrusions via the accumulation of anisotropic membrane nanodomains in the tubular region.

In considering the process of nanotube growth, it is assumed that the growing actin filaments push the membrane outwards. The tubular membrane protrusion is additionally stabilized by accumulated anisotropic nanodomains having  $C_{1m} > 0$  and  $C_{2m} \cong 0$  (see Fig. 10). These favour an anisotropic cylindrical geometry of the membrane [9, 72, 82]. Prominin-containing nanodomains might be candidates for such anisotropic membrane nanodomains [9, 14, 18, 72]. Once assembled in the membrane region of a nanotubular membrane protrusion, the cylindrical anisotropic membrane domains keep the protrusion mechanically stable even if the cytoskeletal components (actin filaments) are disintegrated by cytochalasin D [9]. The spherical vesicle at the tip of the nanotube is assumed to be stabilized by isotropic nanodomains characterized by positive  $C_{1m} = C_{2m} > 0$ , while the nanotube is assumed to be formed from the initial saddle-like neck connecting the vesicle and the parent cell membrane.

Also, it was shown [83] that the lipid components in a mixture may redistribute due to anisotropic membrane curvature, such as in the neck connecting the bud and the mother cell. Theoretical studies on the budding process [13] show that the anisotropic properties of laterally mobile membrane constituents promote the formation of the neck due to the orientational ordering of the anisotropic membrane constituents in the neck, while experimental and theoretical studies [15] show that the composition of the buds is also reflected in the composition of the MVs. This indicates that the intrinsic shape and lateral redistribution of the membrane components play an important role in the budding and vesiculation [64].

#### **DETERMINATION OF THE MICROVESICULATION LEVEL AS A POSSIBLE INDICATOR OF CLINICAL STATUS**

It was recently suggested that plasma protein-mediated attractive interaction between membranes may prevent the release of cell-derived MVs into circulation [38, 39]. A hypothesis was put forward that in subjects with plasma which induces a more pronounced adhesion between membranes, the number of MVs in the peripheral blood is smaller [38, 39]. The ability of plasma to mediate interaction between membranes was measured by adding plasma to a suspension of giant phospholipid vesicles (GPVs; Fig. 11). The proposed trend was confirmed in a pilot study including three healthy subjects [84] and a population study involving patients with gastrointestinal diseases [28].

In the latter study, a negative, statistically significant correlation (Pearson coefficient = -0.50,  $p = 0.031$ ) was found between the number of MVs in the peripheral blood and the ability of the plasma to induce coalescence between membranes, represented by the average effective angle of contact between the adhered GPVs (Fig. 12A). The statistical significance of the correlation was even higher if the number of MVs was calculated with respect to the number of platelets (Pearson coefficient = -0.64,  $p = 0.003$ ; Fig. 12B).

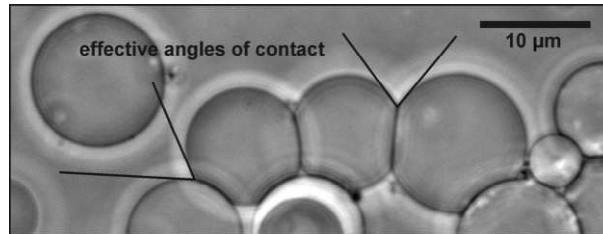


Fig. 11. The adhesion of GPVs after the addition of plasma. The angles of contact between GPVs are indicated. Adapted from [84] with permission from Elsevier.

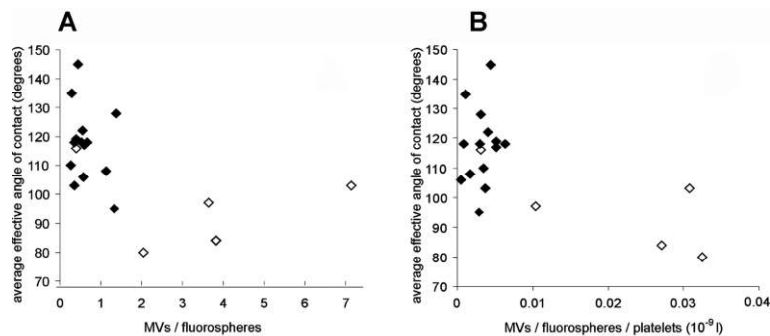


Fig. 12. The correlations between the average effective angle of contact between adhered GPVs and the number of MVs in the peripheral blood (A) and between the average effective angle of contact between the adhered GPVs and the number of MVs per number of platelets (parameter  $f$ ), for 19 patients with gastrointestinal diseases. The empty diamonds correspond to patients from group A (patients diagnosed with gastrointestinal cancer), and the full diamonds correspond to patients from group B (patients with other gastrointestinal diseases) Adapted from [28] with permission from Elsevier.

Furthermore, by comparing patients diagnosed with cancer with patients diagnosed with other gastrointestinal diseases, we found a large (140%) and statistically significant ( $p = 0.033$ ) difference between groups A and B regarding the number of MVs in peripheral blood [28].

Microvesiculation could also play an important role in Trousseau syndrome. In its strictest sense, Trousseau syndrome is defined retrospectively when a thromboembolic event precedes a diagnosis of cancer, while in a broader context, it includes all hypercoagulable states connected to cancer [85]. As microvesicles were found to be both procoagulant and tumor promoting, according to the hypothesis, the compounds that mediate the short-ranged attractive interaction between membranes are anticoagulant and tumor suppressing. The widely used anticoagulant heparin was indeed found to be successful not only in preventing thromboembolic events but also in slowing down the development of some types of cancer [86]. As microvesiculation of

membranes is enhanced both in hypercoagulable states and in cancer, there is a hypothesis of the anticoagulant and tumor suppressing effects of heparin. According to it, heparin mediates an attractive interaction between membranes and therefore suppresses microvesiculation via the adhesion of buds to the mother membrane. Indeed, adding heparin to a solution of giant phospholipid vesicles causes the vesicles to adhere to each other (Fig. 13).

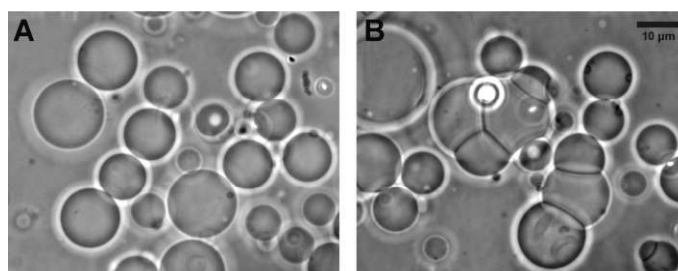


Fig. 13. Giant phospholipid vesicles composed of POPC (80%) and cholesterol (20%) before (A) and after (B) the addition of low molecular weight heparin. The vesicles were prepared by electroformation in 300 mosm saccharose solution as described in Urbanija *et al.* [38], and left to sediment in a gravitation field for one day. For the experiment, 36  $\mu$ l of a solution containing vesicles was placed in the observation chamber and 4  $\mu$ l of Fraxiparine Forte-nadroparine calcium (Sanofi Winthrop, France), dissolved in 300 mosm glucose solution in the proportion 1:80, was added. The image (panel B) was taken 15 min after the addition of heparin to the GPVs. Adhesion between vesicles indicates that heparin mediates the attractive interaction between membranes. Bar = 10  $\mu$ m.

In a pilot clinical study, the number of microvesicles isolated from the peripheral blood was determined in a population of patients who received low molecular weight heparin as a prophylaxis due to orthopaedic surgery, and compared with of the number in blood from healthy donors. Patients that underwent surgery at the Department of Orthopaedic Surgery, University Clinical Centre, Ljubljana, received low molecular weight heparin (clexane or fraxiparine) starting prior to and ending 7 or more days after the surgery. After the surgery, a 1.6-ml sample of venous blood was taken from the patients into vactubes containing sodium citrate. All the participants gave a written consent to the study. Microvesicles were isolated from the blood according to the protocol of Diamant *et al.* [41] and processed as described in Janša *et al.* [28]. In the population of patients who underwent orthopaedic surgery and received heparin, it was found that the average number of MVs divided by the number of fluorospheres (0.71, STD = 0.44) was higher than in the population of healthy donors (0.56, STD = 0.21). However, the difference (23%) was small and statistically insignificant. In this preliminary study, the populations were not age- and gender-matched, and the population of patients was heterogeneous regarding other diseases, the amount of blood received by transfusion, and the administration of drugs. It could be expected that the surgery would cause an increase in the number of

microvesicles due to stress, inflammation and blood transfusion, but it can be seen from the obtained results that the difference between the average values of the two populations was not so large considered that several-fold larger numbers were obtained for patients with gastrointestinal cancer using the same protocol [28]. Moreover, in 12 out of 24 patients, the number of MVs was lower than the average value of the healthy group. To yield more decisive results on the mechanisms underlying the anticoagulant and tumor suppressing effect of heparin, further studies based on more detailed and refined analyses involving larger groups of patients are needed. Using microvesicle analysis as a diagnostic tool remains a challenge that will very probably be addressed in the future.

## CONCLUSIONS

In the past, membranous nanostructures remained largely obscure due to their small dimensions and fragility. Therefore, the membrane was considered to be almost flat two-dimensional liquid. More recent experimental data revealed a possibility of an important pool of membrane contained in nanostructures. Microvesiculation and the formation of the nanotubes that connect cells are important processes in cell life, and they have recently become the focus of increasing interest, especially since they can be manipulated by drugs. It is indicated that the Singer-Nicolson model of the fluid mosaic and the Simons-Ikonen-Brown-London model of lateral nanodomains should be upgraded with the concept of average orientational ordering of membrane constituents (nanodomains, molecules) in strongly anisotropically curved membrane regions, since it provides the mechanism that stabilizes membranous nanostructures. Membranous nanostructures must be explored in greater detail.

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