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Morphology and Formation Mechanisms of Cellular Vesicles Harvested from Blood

Veronika Kralj-Iglič, Gabriella Pocsfalvi and Aleš Iglič

Abstract

Theoretical and experimental evidence on cellular vesicles (CVs) isolated from blood is presented. It is suggested that comparison of the observed shapes with theoretical shapes obtained by minimization of membrane-free energy in combination with electron microscopy is key in the assessment of CV identity. We found that shapes of CVs isolated from blood by repetitive centrifugation (up to 20.000 g) and washing, and observed by scanning electron microscopy (SEM) agreed well with theoretically observed shapes. It is indicated that these CVs are colloids deriving from residual blood cells, mostly platelets. SEM images of washed erythrocytes undergoing budding and transmission electron microscopy (TEM) images of isolated erythrocyte microvesicles likewise showed smooth shapes that we described as characteristic for colloidal CVs. Besides these, the CV isolates may contain other small particles, such as exosomes and viruses, as observed in isolates from tomato homogenate, however, we could not identify such particles in isolates from healthy human blood. Theory of deviatoric elasticity underlying minimization of the membrane free energy and simulated two-component vesicles with the orientational ordering of anisotropic constituents are presented to indicate the interdependence of curvature—sorting of membrane constituents and their orientational ordering in strongly anisotropically curved regions.

Keywords: extracellular vesicles, erythrocyte microvesicles, extracellular vesicle shape, scanning electron images of extracellular vesicles; cryo-TEM images of extracellular vesicles

1. Introduction

The great success of the double helix molecular model of DNA [1] has been based mostly on revealing specific, chemical mechanisms. This model has enabled profound advances in technology; however, it has not completely revealed the causes of common and debilitating physiological and pathophysiological mechanisms. However, the dynamics of the genetic material depend on its interaction with membranous systems which has hitherto not been given adequate attention. Moreover, nanostructures composed of- and enclosed by- biological membranes (e.g., vesicles and nanotubes) were long overlooked [2] due to their small size and fragility. Recently,

submicron-sized membrane-enclosed cellular vesicles (CVs) that have been formed in a process in which membrane plays a key role have become a subject of increasing attention. By being released into the cell exterior, they can move more or less freely in the surrounding medium. Extensive studies and empirical knowledge indicate that these tiny particles may have a great impact on living systems, in particular, because they present an intercellular communication system that connects different kingdoms of life. CVs include microexovesicles, exosomes, enveloped viruses, and cellular membrane endovesicles.

The structure of a membrane-enclosed entity that carries a specific cargo presents a foundation stone of life and dwells also on its border. Namely, the physical properties of membrane-enclosed entities are shared with any small particles that attain their configuration according to the minimal energy of the system (referred here as colloid systems). While consideration of biological nanostructures needs the support of a rigorous physical description, new evidence regarding nanoscale features needs interpretation by the development of new physical models, specific to these materials. We believe that the research of nanoscale systems at the cellular level requires the intertwining of existing fields of physics, chemistry, biology, and medicine in the course of their growth. By addressing the physical properties, methods of theoretical physics can be used to describe the system, interpret experimental data, and predict the behavior of the system.

In order to be studied, CVs should be harvested from their natural environment. Presently, integration of different methods is recommended [3], however, new technically advanced solutions are sought. The most commonly used method for CV harvesting involves differential centrifugation [4], which can be followed by using for example sucrose or iodixanol gradient [5]. As this technique is time-consuming and of limited access, alternative techniques were proposed. Ultrafiltration, flow field-flow fractionation, dialysis, size exclusion chromatography (SEC), microchip-based techniques, and precipitation-based methods are being developed to harvest CVs, alone or in combination with ultracentrifugation-based methods. Immunoaffinity-based isolation can also be applied to harvest CVs with particular surface protein composition [6]. Recently, a number of commercial kits are made available and have been widely reported in the literature for CV isolation. For example, ExoQuick (System Biosciences) and Total Exosome Isolation kits (TEI, Invitrogen) rely on polymer precipitation; qEV (Izon) is based on SEC; Millipore uses centrifugal filter devices for ultrafiltration; and exoEasy (Qiagen) is based on membrane affinity binding [7]. But different isolation methods were found to lead to different CV populations [8] due to mechanical and thermal stress and chemical reactions. Although the suggested methods are faster and easier to apply, a recent thorough comparison between isolates obtained from these methods and ultracentrifugation showed that ultracentrifugation is still the most appropriate method among those tested as regards purity [7].

According to their features, CVs are ideal candidates to serve as biomarkers, nanosized drug-delivery vehicles, and mediators for a variety of therapeutics in oncology, immune therapy, and regenerative medicine [9–11]. Thus, CVs have the potential for great clinical impact in nanomedicine. The dual potential of CVs as diagnostic tools and as therapeutic agents supports their use in “theranostics” [11–13]. This area of nanomedicine focuses on multidisciplinary research to set up new systems for various nanobiomedical applications, ranging from the medical use of nanoplatform-based diagnostic agents to therapeutic agents for possible future applications [14]. Furthermore, the theranostic “all-in-one approach” has great potential in the field of personalized medicine, as it enables the detection and monitoring of

disease in individual patients, possibly in early clinical stages, as well as targeted drug delivery at the site of the disease.

In order to manipulate cellular vesicles (CVs), the process of their formation should be better understood. Vesiculation of biological membranes was studied theoretically and experimentally. CVs were isolated from different biological samples, including blood [15–20]. In phospholipid vesicles, shape transformations involving evaginations were studied [20–23]. Budding and vesiculation of erythrocytes were also considered [24–29]. While erythrocytes shed vesicles in the final stage of the membrane budding, platelets undergo fragmentation in the shear stress [30].

Visualization of the samples is a prerequisite to identify CVs in samples. As they are very small, CVs are hidden within the organisms or cell assemblies and they cannot be directly observed in their natural environment. The methods used for their harvesting, observation, and assessment are to a large extent invasive enough to transform them to such extent that identification of their original nature is obscured.

We found that a large pool of submicron-sized particles in the isolates from blood was formed during the processing of samples [31–33]. This indicates that the formation of CVs in isolates can be influenced upon by changing the parameters of processing which is of advantage for the production of therapeutic preparations from biological samples.

In this review, we would like to point to some common properties of CVs that we characterize by the key role of the membrane in shape determination and refer to as colloid CVs. Also, we will address other types of cellular particles that can be present in the samples, for example, viruses.

2. Theoretical prediction of the CV shape

Theoretical and experimental studies on (artificial) phospholipid vesicles have shown that the shape of the bilayer membrane-enclosed compartments can be theoretically well described by the properties of the membrane [34]. By considering that membrane is composed of many constituents, methods of statistical physics were used for its description [35, 36]. The key feature in the expression of the energy of a single constituent is the mismatch of the local curvature and the constituent intrinsic curvature (the one fitting the shape of the membrane constituent) [36]. In order to compose the membrane, the constituent attains the local membrane shape that usually differs from its intrinsic shape. Moreover, if the constituent is not symmetric with respect to the axis perpendicular to the membrane surface, the principal axes of the membrane and the constituent are in general rotated by an in-plane angle, meaning that the constituents can attain different in-plane orientations in the membrane which correspond to different energies. The consequence of the mismatch in curvature and orientation is that certain energy is required to insert the constituent at the site, this energy being higher if the mismatch is greater. The thermal motion opposes the complete orientational ordering in the direction with the lowest energy but the constituents will spend on average more time in the orientation with lower energy. As constituents are more or less free to move laterally in the membrane, they can redistribute in a way to minimize the mismatch between the intrinsic and the actual shape. Summing up the contributions of all the constituents and considering the entropic effects due to lateral and orientational ordering yields the expression for the free energy of the membrane F in terms of the mean curvature of the membrane surface H and the curvature deviator D , both composed of the two principal curvatures C_1 and C_2 [34],

$$H = (C_1 + C_2)/2, \quad (1)$$

$$D = (C_1 - C_2)/2, \quad (2)$$

$$F = -kT \int \sum_i m_i \ln (q_i^0 / 2 \cosh (d_{i,\text{eff}})) dA + k_B T \int \sum_i m_i \ln (m_i/m) dA, \quad (3)$$

$$q_i^0 = \exp \left(-\frac{\xi_i (H - H_{i,m})^2}{2k_B T} - \frac{(\xi_i + \xi_i^*) (D^2 + D_{i,m}^2)}{4k_B T} \right), \quad (4)$$

$$d_{i,\text{eff}} = (\xi_i + \xi_i^*) D D_{i,m} / k_B T, \quad (5)$$

where ξ_i and ξ_i^* are constants, k_B is the Boltzmann constant, T is temperature, m_i is the local area number density of the i -th kind of constituents and m is the number density taking all constituents over all membrane area. Integration is performed over membrane surface A . The summation accounts for all types of constituents that are characterized by index i . The intrinsic mean curvature of the membrane surface H_m and the intrinsic curvature deviator D_m refer to respective principal curvatures intrinsic to the shape of the constituent [34].

Free energy given by Eq. (3) consists of two terms—the first one deriving from the single-constituent energy and entropy of orientational ordering, and the second one deriving from lateral distribution of constituents. Eqs. (3) and (4) can be rewritten in the form [34].

$$F = W_B - \int 2mk_B T \cosh (d_{i,\text{eff}}) dA + k_B T \int \sum_i m_i \ln (m_i/m) dA \quad (6)$$

where W_B has the form of the bending energy of a laterally isotropic membrane [34].

A basic physical principle that the system will attain the shape corresponding to minimal free energy [34]

$$F = \min, \quad (7)$$

at relevant constraints to the system is taken into account such as the requirement of fixed membrane area A and fixed enclosed volume V [37]. In the absence of net external forces acting on the membrane, a convenient geometrical parameter is the relative volume v which represents the volume to area ratio largely determined by the osmotic equilibrium [34]

$$v = \sqrt{V^2 / 36\pi A^3}. \quad (8)$$

Also, other constraints can be imposed upon the system, for example, constant average mean curvature [34]

$$\langle H \rangle = \int H dA / \int dA. \quad (9)$$

To find the free energy minimum, the above formalism [Eqs. (1)–(5)] is used to state and solve the so-called variational problem in which the principal curvatures are expressed in terms of convenient coordinates. Dimensionless parameters are used for clarity: $c_j = C_j R$, $j = 1, 2$ are the principal curvatures normalized with respect to the radius of the sphere with the surface area A , $R = \sqrt{A/4\pi}$, $h = HR$, and $d = DR$ are the

normalized mean curvature and curvature deviator of the membrane, respectively, and $h_{i,m} = H_{i,m}R$ and $d_{i,m} = D_{i,m}R$ are the intrinsic mean curvature and the intrinsic curvature deviator of the i -th type of constituents, respectively. The area element is normalized with respect to the area A , $da = dA/4\pi R^2$. The constants ξ_i and ξ_i^* are taken to be equal for simplicity. The free energy is normalized with respect to the free energy of the sphere composed of chosen constituents, $8\pi m\xi_i$.

Consistently related distributions of the constituents, their in-plane orientations (if relevant), and the membrane shape are determined simultaneously in a mathematical procedure. Several methods have been developed for this purpose—for example, ansatz [37], numeric solution of differential equation [38], surface evolver [39], or finite element method [40]. The simplest method is the ansatz approach in which the space of possible solutions is assumed within a family of shapes with adjustable parameters. Such a solution can be analytical throughout (depending on the sophistication of the ansatz) and therefore transparent. The advantage of transparent methods is that with some basic mathematical skills the procedures can be repeated and used accordingly. Differential equations expressing minimization of free energy are derived by applying the Euler–Lagrange method [36] in a convenient parametrization. Considering a multicomponent system, the constituents are free to move laterally over the membrane which may present singularities that have not yet been fully explored [36]. Such solutions require numerical procedures that are implemented in respective customized software which normally requires manipulation by a skilled researcher. However, the set of possible solutions is considerably larger than in the ansatz case, as for some classes of shapes the relevant ansatz does not exist. With more freedom in finding a solution, the achieved energy attains lower values. The advantage of the rigorous solution of the system of differential equations is that the lowest possible energy can in principle be found, however, the method is rather demanding for shapes that are not axisymmetric which limits the set of possible solutions.

The theory could be refined by considering particular experimental evidence. For example, it was found that cellular vesicles may go through a process of active and passive solutes' permeation which may cause cyclical expansion and contraction [41].

3. Visualization of CVs

Isolation of CVs from blood first requires removal of blood cells, in particular erythrocytes. Namely, to their prevailing abundance in blood of healthy humans, they present an obstacle in observation of the effects of other blood components. Usually, this is performed by centrifugation at a relatively low speed. Upon centrifugal force particles in the blood are inclined to move toward the bottom of the tube. The motion is roughly determined by the centrifugal force, buoyancy, and resistance force approximated by the Stokes law. It can be seen by equilibrating the forces that the speed of particles is proportional to the square of the particle radius and proportional to its density which means that larger particles will move faster. Besides being the most abundant, erythrocytes are also relatively large. If approaching each other close enough they form rouleaux which effectively speeds up their sedimentation and also creates channels in which smaller particles (platelets and CVs) are pushed out. This creates a counter-flow due to which platelets and CVs accumulate in the plasma above erythrocytes [32, 42]. However, this effect is temporary, as platelets and CVs in the erythrocyte-poor plasma reverse their flow and sediment as well. Plasma obtained by relatively low-speed centrifugation contains platelets as well as residual erythrocytes

Figure 1. A: Blood plasma observed by SEM. B: CVs isolated from blood as observed on the inner wall of the tube by SEM. A: From [31]. B: From [47].

and leukocytes (**Figure 1A**). Furthermore, some blood cells shed CVs during the processing. For further elaboration of the sample, different protocols have been proposed, differing in the amount of the required isolate and its purpose. Centrifugation is still the most widely used method as it does not induce changes in the chemical composition of the sample, is relatively simple, time effective and low cost, and enables the simultaneous elaboration of multiple samples. Different methods were suggested for the isolation of CVs from blood [43]. Centrifugation protocols may differ in time and speed of centrifugation as well as in other parameters (e.g., temperature, the type of laboratory material used, up-gradation by technologically advanced procedures) [44–46].

Figure 1B shows the interface of the isolate with the tube wall. The tube was cut and the sample was prepared directly on the piece of the tube for imaging with the scanning electron microscope (SEM). Smooth shapes of the particles in the isolate can be noted.

CV isolates from blood shown in this chapter were obtained by repetitive centrifugation and washing of samples with phosphate- and citrate- buffered saline (PBS). We used different centrifugation protocols. Unilamellar phospholipid vesicles were prepared by electroformation in sugar solution and rinsed into the observation chamber by solution of a lighter sugar with the same osmolarity.

Giant phospholipid vesicles exceed in size several micrometers and can therefore be observed live by optical microscope. Therefore, the comparison between theoretically predicted and experimentally observed shapes is straightforward. For submicron-sized CVs light microscopy does not provide sufficient resolution. The samples can be observed by electron microscope which requires more or less aggressive processing. To observe them by scanning electron microscope, samples are dried and sputtered with heavy metal. For cryo-electron microscopy, they are frozen in thin ice (about 100 nm of thickness) which deforms soft particles larger than this dimension and may cause their degradation. Interpretation of the images of processed samples is not always straightforward.

Figure 2 shows SEM images of CVs found in isolates from blood (a-d), an erythrocyte of a healthy human in physiological *ex vivo* conditions (e), and optical microscope images of a giant phospholipid vesicle (f-i). The corresponding theoretically obtained contours that were obtained by the solution of the variational problem are also given (a-i). A rigorous solution of the system of differential equations was sought. Two sequences of shapes are given, representing the transformation of vesicles

composed of a single type of constituents with fixed relative volume v and changing $\langle h \rangle$. The sequence a-d starts with a discocytic shape which with decreasing $\langle h \rangle$ transforms into a stomatocyte with a wide dimple. In continuation of the process, the dimple grows inwards while the neck at the top shrinks. The sequence f-i starts with a prolate shape which with increasing $\langle h \rangle$ transforms into a pear shape. The neck shrinks up to a point in which it becomes infinitesimal.

It can be seen that the shapes of the erythrocyte (**Figure 2e**) and the CV (**Figure 2a**) are the same although the size scale of the CVs is 10 times smaller. Mammalian erythrocytes have no nucleus and also no internal cytoskeleton and their shapes are likewise determined by the minimum of the free energy of the membrane (underlaid with membrane skeleton). Consideration of the membrane skeleton however requires additional assumptions which are important also in describing the formation of CVs.

Figure 3 shows calculated shapes representing the swelling and budding of a membrane-enclosed structure. The swelling was simulated by an increase of v and budding was simulated by an increase of average mean curvature $\langle h \rangle$. Vesicles were composed of one type of constituents. Calculation of the sequence is conveniently performed within a chosen class of shapes. For a membrane composed from a single type of constituents that favor flat shape, the pear shape sequence (**Figure 2f-i**) is energetically more favorable than the lemon shape sequence (**Figure 3B-D and E-G**) since the formation of the neck is energetically unfavorable for constituents that favor positive (evaginated) and flat regions. The geometrical constraints limit the power of the set of possible shapes. For relative volume 1, there is only one possible shape (e.g., sphere) and this is the shape that attains the biggest possible volume at the given surface area (v cannot exceed 1). With the decrease of v , the set of possible shapes increases. It can be seen that already small decrease of v (e.g., to 0.98 (**Figure 3B-D**))

Figure 2.

Experimental: a-d: SEM of CVs found in isolates from blood, e: SEM of a discocyte at physiological ex vivo conditions, f-i: Optical microscope images of giant phospholipid vesicles. Theoretical contours derive from the solution of the variational problem by rigorously solving a system of differential equations. Within sequences a-d and f-i, A and V were fixed while $\langle h \rangle$ was changing: The parameters of the calculated shapes were $h_m = d_m = 0$, (a, e): $v = 0.6$, $\langle h \rangle = 1.040$, $\langle d \rangle = 1.812$, (b, c): $v = 0.6$, $\langle h \rangle = 0.650$, $\langle d \rangle = 1.167$, (d): $v = 0.6$, $\langle h \rangle = 0.435$, $\langle d \rangle = 0.235$, (f): $v = 0.9$, $\langle h \rangle = 1.050$, $\langle d \rangle = 0.729$, (g): $v = 0.9$, $\langle h \rangle = 1.105$, $\langle d \rangle = 0.697$, (h): $v = 0.9$, $\langle h \rangle = 1.155$, $\langle d \rangle = 0.577$, (i): $v = 0.9$, $\langle h \rangle = 1.240$, $\langle d \rangle = 0.163$. Adapted from [34].

Figure 3.

Shapes corresponding to the minimum of the membrane free energy calculated by rigorously solving a system of differential equations. The parameters of the calculated shapes are $h_m = d_m = 0$, A: $v = 1$, $\langle h \rangle = 1$, B: $v = 0.98$, $\langle h \rangle = 1.0088$, C: $v = 0.98$, $\langle h \rangle = 1.011$, D: $v = 0.98$, $\langle h \rangle = 1.0224$, E: $v = 0.90$, $\langle h \rangle = 1.05035$, F: $v = 0.9$, $\langle h \rangle = 1.0634$, G: $v = 0.9$, $\langle h \rangle = 1.354$.

can induce visible changes in shape with respect to the sphere ($v = 1$). If the vesicle loses 10% of its relative volume, the shape is visibly elongated (**Figure 3E-G**).

The particles that are essentially membrane-enclosed fluid interior deform and eventually undergo fragmentation at the thin necks. As the tearing area is minute the membrane is likely to seal. Smaller fragments are thus created. Fragmentation of residual cells takes place in particular at the interface with the tube wall where the shear force is the highest. Centrifugation at high centripetal accelerations of the rotor was shown to induce the formation of CV aggregates composed of a mixture of CVs highly heterogeneous in size and number of associated CVs [32].

Characteristics of membrane-enclosed vesicles composed of constituents that are not directly interacting are the smoothness of the shape. Good agreement between the calculated and the observed shapes indicates that the particles in the samples are vesicles (membrane-enclosed fluid interior). There are no additional methods needed. According to this principle, **Figure 3B** shows particles that can be identified as CVs deriving from blood cells. It is, however, not clear from this point what is the origin of the CVs, as the material may undergo formation and re-formation of vesicles during the processing, and the constituents of the CVs may come from different cells as well as from the surrounding solution. Such vesicles are colloidal in their nature and their identity depends on the properties of the cells as well as on the processing of the samples (e.g., centrifugation parameters, the composition of the suspension,

temperature). They can be considered an artifact; however, this artifact can have clinical significance [30].

4. Shape determination of CVs with complex composition

If we take into account that the membrane is composed of more than one kind of constituents, the set of possible shapes with minimal energy is further expanded as the system has additional freedom by which it can adjust its configuration. The constituents may move to the regions of favorable curvature and additionally adjust their orientation. Allowing the system more freedom effectively increases its stability. To demonstrate the effect of the lateral distribution of constituents, **Figure 4B** shows two views on the shape with minimal free energy determined by the Monte Carlo simulation method. Two different isotropic constituents (favoring strong positive curvature and minute curvature, respectively) were considered. Red color denotes prevalence of the constituents that favor positive curvature and blue color denotes prevalence of the constituents that favor minute curvature; white color denotes the mixture of both kinds. Buds and undulated protrusions have formed largely from the constituents that favor strong positive curvature while the constituents that favor minute curvature organized themselves into a globule where the membrane is almost flat with respect to its thickness. Multiple buds recruit the constituents that favor strong positive curvature and span the almost flat parts to further minimize the energy. Agreement between the shapes observed in budding erythrocytes (**Figure 4A**) and shapes composed of two kinds of constituents does not reach the level evident in **Figure 1**. Budding erythrocyte undergoes detachment of the membrane skeleton at the top of the echinocyte spicules which is not taken into account in the theoretical consideration used to determine the shape shown in **Figure 4B**. Nevertheless, some aspects of the shape (i.e., multiple protrusions and rounded shape of the bud tips) can be noted.

Orientational ordering of the anisotropic constituents becomes evident in strongly anisotropically curved membrane parts such as in the necks and on the tubular parts (**Figure 5**). The variational problem of free energy minimization was solved by the expansion of the shape contour into the Fourier series [48]. Two kinds of constituents were considered—strongly anisotropically curved constituents and isotropic constituents that favor minute curvature. To enhance the formation of strongly anisotropically curved regions, a rod-like structure was imagined inside the vesicle by requiring a fixed length of the shape while the minimization took place. Longer rods induced

Figure 4.
A: Budding erythrocytes. B: Shape of a vesicle with a two-component membrane, calculated by Monte Carlo simulation. Membrane nanodomains with high intrinsic curvature (red color) are accumulated in undulated membrane protrusions. A: From [34]. B: From [48].

Figure 5. Shapes of vesicles that are composed of two types of constituents (anisotropic (red) and isotropic (blue)), calculated for different lengths of the rod-like structure inside the vesicle. Panels (a) - (e) show the effect of different length of the rod-like structure inside the vesicle as indicated in the figure. The color scale shows the proportion of the two kinds of constituents Φ . The average Φ over all vesicle membrane was 0.25. Orientation of the anisotropic constituents is denoted by short gray lines on the protrusions. From [48].

longer and thinner tubular protrusions with well-ordered anisotropic constituents (Figure 5). The constituents redistributed into almost separate parts. Red color denotes prevalence of anisotropic constituents, blue color denotes prevalence of isotropic constituents while the rainbow between these two colors denotes a mixture of both kinds of constituents. It can be seen in Figure 5 that the anisotropic constituents accumulated in the protrusion and underwent orientational ordering. While in thicker (conical or cylindrical) parts, the constituents ordered circumferentially (Figure 5a-c) the direction became tilted in thinner cylindrical parts toward the top of the shape (Figure 5b) and was almost uniform on longer tubular parts (Figure 5d-f).

Results shown in Figures 4 and 5 indicate that the curvature induces sorting of membrane constituents to different extents determined by factors such as geometrical constraints and the intrinsic shape of the constituents.

5. Microvesiculation of erythrocytes and fragmentation of platelets

It was found that in *ex vivo* conditions washed erythrocytes undergo transformation into echinocytes (**Figure 6A**) and eventually budding takes place on the top of echinocyte spicules (**Figure 6B and C**) [25, 26, 34]. Vesiculation was accelerated by the addition of amphiphilic molecules into the suspension of washed erythrocytes [25]. The shape of the buds as well as of the vesicles found in the isolates depended on the type of amphiphilic molecules added (**Figure 6B–E**). Vesicles matching in size and shape could be found in the isolate (**Figure 6D and E**) [34]. It was assumed that the amphiphilic molecules intercalate into the erythrocyte membrane and change the identity of the membrane constituents which in turn causes shape transformation. While dodecylzwittergent induced budding of globular structures and globular shape of isolated CVs (**Figure 6B and D**), dodecylmaltoside induced tubular shape of the buds and the CVs (**Figure 6C and E**). Dodecylmaltoside is composed of a carbohydrate tail and a bulky multipolar headgroup. The orientational ordering of the constituents involving dodecylmaltoside can explain the stable shape of tubular buds and vesicles that were observed in experiments. Budding erythrocytes were found also in CV isolates from blood [30] indicating that a part of CVs harvested from blood could be erythrocyte microvesicles.

Figure 6.
 A: Echinocytes, B: Spheroechinocyte with globular buds induced by dodecyl-zwittergent, C: Spheroechinocyte with tubular buds induced by dodecylmaltoside, as observed with SEM; D and E: Respective isolated spherical and tubular CVs as observed by TEM. From [34].

The relevance of the model lies in agreement with experiments. The model of isotropic bending describes well the discocyte-stomatocyte transformation of erythrocytes, but cannot distinguish between tubular and spherical budding of the vesicle and therefore the formation of tubular/spherical vesicles. To our best knowledge, the presented model is by now the only model that explained the stability of different nanostructures with strongly anisotropically curved membranes (tubes, thin necks, hexagonal and cubic stacks).

Observations of isolates from blood indicate the presence of a major pool of CVs which shape corresponds to the membrane free energy and can be described as colloidal CVs. It was found [30] that the size of the colloidal CVs in blood isolates was different for different isolation protocols indicating a transient identity of colloidal CVs. In contrast, erythrocyte microvesicles shed from washed erythrocytes were uniform in size and were sensitive to intrinsic curvatures of the membrane constituents. The identity of CVs depends on the processing of samples. As stated above, the most commonly used method for CV isolation involves (differential) centrifugation/ultracentrifugation, which can be followed by ultracentrifugation on a sucrose or iodixanol gradient [4, 5, 49]. Ultrafiltration, dialysis, and size exclusion chromatography are used to harvest fractions of EVs, and immunoaffinity isolation and precipitation methods are used to harvest CVs with particular compositions [50, 51]. But transformation of the material may occur during any of these harvesting procedures, due to chemical/mechanical/thermal stress, as the nanostructures are fragile and prone to interact. Furthermore, the same principle applies to assessment methods. Commonly used methods are flow cytometry, SEM, TEM, atomic force microscopy, light scattering, fluorescence microscopy with analysis of Brownian motion (nanoparticle tracking analysis), and immunoblotting. Also, CV contents are analyzed for high-resolution molecular profiling of protein, microRNA, and lipid content (reviewed in [51]).

Besides colloidal CVs, other types of particles can be expected in biological samples, such as lipoproteins [52] and viruses [53]. Recently, besides the colloidal CVs, rod-like viruses were identified in CV isolates from tomato homogenate. The CVs were isolated by differential centrifugation and by size exclusion chromatography and observed by SEM [53] and by cryo-TEM (**Figure 7**). Three different fractions of the isolate obtained by using iodixanol gradient are shown (denoted by A, B, and C, respectively). The fraction observed in Panel A contained mostly colloidal CVs (black arrow); the fraction observed in Panel B contained colloidal CVs (black arrow) and rod-like virions (white arrow); the fraction observed in Panel C contained mostly

Figure 7. Cryo-TEM images of three fractions of CV isolate from homogenate of tomato infected by the viruses. Panels A - C show three different fractions of the EV isolate that were separated by iodixanol gradient ultracentrifugation. Black arrows point to CVs, white arrows point to virions. Adapted from [53].

virions (white arrow). It was found by analyzing proteome with mass spectrometry that the samples contained capsid proteins of three tomato viruses [53].

6. Conclusions

Methods for CV characterization such as nanovesicle tracking analysis, flow cytometry, and light scattering can estimate the size and abundance of small particles in samples but are not suitable for their identification. To distinguish viruses or other cell-engineered particles from colloidal vesicles, imaging of samples is crucial, in particular when complemented with the identification of the molecular composition and with the results of the modeling. In constructing a model for CVs, we have implemented a theory based on statistical physics, that was previously used to describe the electric double layer [54]. We made a link to the theory of elasticity and found that cylindrical or saddle shapes that were observed in experiments can be stabilized by constituent redistribution and orientational ordering of anisotropic constituents. Inclusion of the orientational ordering of membrane constituents on strongly anisotropically curved regions is necessary for the description of the formation of CVs within the nanoscale. The so-called deviatoric elasticity of the membrane has been previously introduced [55–57], albeit not originating from statistical physics. We refer to the CVs that are formed due to minimization of the membrane free energy as the colloidal CVs, to distinguish them from cell-engineered CVs, such as viruses. Mechanisms of CV formation and transformation are fundamental and vital and there are prospects that they will in the future contribute to improved solutions in surface functionalization, diagnosis, and theranostics.

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Conflict of interest

The authors declare no conflict of interest.

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