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Giant Vesicles and Their Use in Assays for Assessing Membrane Phase State, Curvature, Mechanics, and Electrical Properties

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Keywords

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Abstract

Giant unilamellar vesicles represent a promising and extremely useful model biomembrane system for systematic measurements of mechanical, thermodynamic, electrical, and rheological properties of lipid bilayers as a function of membrane composition, surrounding media, and temperature. The most important advantage of giant vesicles over other model membrane systems is that the membrane responses to external factors such as ions, (macro)molecules, hydrodynamic flows, or electromagnetic fields can be directly observed under the microscope. Here, we briefly review approaches for giant vesicle preparation and describe several assays used for deducing the membrane phase state and measuring a number of material properties, with further emphasis on membrane reshaping and curvature.

Contents

1. INTRODUCTION

Cells typically have sizes in the range of 1 to 10 μm, which makes them visible with optical microscopy. The boundary ensuring their autonomy (i.e., the plasma membrane, which regulates a number of processes essential for the cell survival) has a comparable projected size. If we were able to empty the cell interior while controllably modulating the composition of the resulting capsule of lipids, membrane proteins, and other species, we would be able to better understand the biophysics of these membranes as well as the specific pathways that they govern, as these capsules would then be directly visible under a microscope and amenable to manipulation. We cannot do that (yet), but we can use giant vesicles, either synthetic (i.e., prepared from a starting well-defined mixture of molecules) or derived from the plasma membrane of cells. The former method represents a bottom-up approach of building a mimetic cell membrane, which can then be used to characterize the membrane properties or resolve the action of certain molecule(s) and environmental factors. This approach produces a well-controlled system because all players are known, but it is drastically limited in terms of complexity. The latter method, deriving the plasma membrane from cells, represents a top-down approach that reduces the cell membrane complexity by stripping it off from the adjacent cytoskeleton and isolates it from a large number of processes in

which it is involved during the cell life. Data collected on such complex systems exhibit strong noise owing to the enormous number of membrane components, which could be reacting simultaneously to perturbations. On the contrary, synthetic giant vesicles can be prepared from certain mixtures and thus provide the freedom to select specific components, where the role of each is of interest to the researcher. This review focuses on such synthetic giant unilamellar vesicles (GUVs) (43, 45, 48, 59, 123, 202), their use in resolving the material properties on cell membranes, and some of their applications in addressing membrane responses and functions.

Vesicles represent one of the many model membrane systems employed for resolving the membrane structure and its characterization. Examples of other model membranes include lipid monolayers at the air–water interface, solid supported bilayers, black lipid membranes, and bilayer stacks. Compared to these, vesicles or liposomes are the most natural system, because, shape- and structure-wise, they are closest to membranes of cells and cell organelles. Contrary to other model membrane systems, such as supported lipid bilayers or black lipid membranes, vesicles allow for control over the membrane tension [for giant vesicles, this control can be directly exercised by micropipette aspiration; osmotic inflation or deflation can also be used, but with lesser precision (81)]. Furthermore, black lipid membranes usually retain traces of the organic solvent used for their formation. The use of supported bilayers as model membranes typically raises concerns about steric hindrance or effects arising from the support. None of these disadvantages apply to vesicles.

Vesicles are typically classified according to multilamellarity (i.e., unilamellar and multilamellar), as well as according to size: Small unilamellar vesicles (SUVs) are a few tens of nanometers in size, and large unilamellar vesicles (LUVs) are in the hundred nanometer range. Compared to SUVs and LUVs, the GUVs are 10–1,000 times larger. GUVs employed for microscopy studies have sizes typically in the range of 10 to 100 μm, which makes them a handy biomimetic tool for directly displaying the response of the membrane on a cell-size scale. They can be observed under an optical microscope using various enhancement techniques such as phase contrast, differential interference contrast, or confocal and standard fluorescence microscopy, the latter two being particularly useful in, for example, distinguishing domains on multicomponent membranes (**Figure 1**). Higher-resolution techniques are less often applied to imaging GUVs as these are prone to hydrodynamic perturbations and require some kind of immobilization to allow imaging with precision in the nanometer range.

Since the first studies reporting observations on giant vesicles in 1969 (152), GUVs have developed into a rich research field (48). Because of the increasing interest and number of publications on giant vesicles in recent years, it is unavoidable that some studies are unintentionally omitted here. In this review, we briefly review methods for giant vesicle preparation and phase-state characterization. Then, we introduce several different approaches and techniques for assessing the membrane material properties. That is followed by a more extensive discussion on membrane reshaping and curvature generation.

2. THE MAKING OF

The development of methods for GUV preparation represents a particularly active area of research (for reviews, see 48, 139, 186, 194, 202). Methods are adjusted and optimized depending on the requirements for the GUV use and system properties. In some applications, the membrane composition is crucial, and traces of substances used for the preparation (e.g., organic phase) are undesirable, as they can strongly influence the membrane mechanical properties and phase state. In other applications, it is the size and solution composition inside and outside that are more important (e.g., when one aims to ensure conditions that are more physiologically relevant, or to

establish encapsulation of biomolecules). Finally, practical aspects such as time, yield, required materials, and setups, as well as cost, are also relevant factors for choosing a suitable method. A couple of approaches have been developed to establish control on the vesicle monodispersity (e.g., 101, 190); however, whether the GUV size distribution is preserved after harvesting the vesicles from the formation chambers is questionable.

2.1. One Classification of the Preparation Methods

The available protocols used for preparing GUVs have been previously organized in a number of different methods (202). Here, we conceptually divide them into vesicle swelling methods and methods based on the use of an oil/organic phase, as discussed in detail below. This classification is based on the following. (*a*) One starts by depositing lipids in organic solvent (e.g., chloroform) onto a solid or gel substrate, the solvent is evaporated, and then lipid hydration and swelling occur through the addition of an aqueous solution to form GUVs. Or (*b*) one takes advantage of the fact that GUVs are micrometer-sized (bilayer-encapsulated) aqueous droplets in a bulk aqueous media; to obtain the GUVs, one manipulates and combines two lipid monolayers at oil–water interfaces to form a bilayer enclosing quasi water-in-water droplets (i.e., GUVs) while removing the oil phase (most of the time, not completely) (see Section 2.3). In the following sections, we discuss in more detail each of these approaches. An alternative approach that does not seem to enter either of the above classifications relies on the deposition of the lipid bilayer onto the fluid substrate of a surfactant monolayer at the inner interface of an aqueous droplet in fluorinated oil, thus forming droplet-supported GUVs (205). The bilayer deposition is established similarly to how solid supported bilayers are formed—by spreading LUVs onto the substrate in the presence of divalent ions, but here, the substrate is a fluid interface. A combination of microfluidic manipulation and electroinjection allows the encapsulation of biomacromolecules and the building of cytoskeletal mimetics as well as the reconstitution of transmembrane proteins (205). Most importantly, the vesicles can also be successfully harvested from the droplets.

In all approaches, one should be aware of potential artifacts stemming from the preparation method and, depending on the application of the produced GUVs, decide whether to neglect these artifacts. For example, certain preparation conditions can lead to phospholipid degradation and inclusion of impurities in the vesicles deriving from the substrate or from the phase on which they were prepared, or even from the aqueous medium (28, 35, 95, 112, 126, 156, 192).

2.2. Vesicle Swelling

In this section, we focus on swelling that either occurs spontaneously or is enhanced by external forces such as electric fields, heat, and osmotic flow. More unconventional and less applicable methods also exist. For example, GUVs have also been prepared from preformed LUVs via fusion but at very high salt concentrations and under the application of freeze–thawing cycles (135).

2.2.1. Spontaneous swelling.
The simplest version of the vesicle swelling method is referred to as spontaneous swelling and was initially applied to egg yolk phosphatidylcholines or their mixtures with cholesterol (152). As summarized above, the dissolved lipids are deposited on the solid substrate (e.g., glass or Teflon surface), the organic solvent is evaporated, the swelling buffer is added, and vesicles are produced within approximately one day. For details and video material on one specific protocol, see Reference 98 (if a picture is worth a thousand words, a video is worth even more, especially as protocols are not described in sufficient detail in publications). Inclusion

of charged lipids was reported to improve the vesicle preparation in physiological conditions (3). The use of sugars already present in the deposited lipid film was also shown to be beneficial (193) as were a number of other factors such as the presence of divalent ions in the aqueous solution (4), localized heating (22), and pressure waves (141). Swelling of GUVs on glass beads as a support was also demonstrated to allow for encapsulation of a minimal gene expression system (134).

2.2.2. Electroswelling. The swelling of the vesicles can be enhanced and sped up (to ~1–2 h) through the application of an external alternating current (AC) electric field. This approach is also called vesicle electroformation (8), whereby the lipids are deposited on electrically conductive solid surfaces acting as electrodes. These can be semiconductive glass plates coated with indium tin oxide (ITO), platinum, titanium, or stainless steel wires as well as silicon substrates (e.g., as employed in 11, 101, 121, 124, 143, 148). For egg phosphatidylcholine, electroformation was also shown to be feasible with lipid deposition on nonconducive substrates immersed between electrodes (136). The method was also found applicable for the preparation of GUVs with reconstituted proteins (video material is available in 64, 164).

2.2.3. Gel-assisted swelling. Vesicle swelling in the absence of electric field is slow (~1–2 days) but can be dramatically sped up (~1–30 min) when one uses a polymer-based gel as a substrate. The vesicle growth in gel-assisted methods is enhanced by buffer influx from below the bilayers that are swelling on a porous polymer layer. The method was first demonstrated on agarose gels (85), but agarose molecules trapped inside during the vesicle formation were found to alter the physicochemical properties of the GUVs (112). An improved version of the gel-assisted method replaced agarose with polyvinyl alcohol (PVA), circumventing polymer encapsulation, as the lipids do not penetrate the PVA film (204). More recently, chemically cross-linked dextran–poly(ethylene glycol) was used as hydrogel substrate for GUV preparation under physiological ionic strength conditions (125).

2.3. Methods Based on the Use of an Oil Phase

These methods rely on the use of oil–water interfaces saturated with lipids, which are either dissolved in the organic phase or present as liposomes supplied in the aqueous phase. One of the approaches relies on the preparation of double-emulsion droplets followed by oil removal (40, 176). Another approach, the phase-transfer method (also referred to as droplet-transfer method or method of the inverted emulsion), represents a stepwise construction of vesicles in two separate stages (140). First, one forms a water-in-oil emulsion of droplets, which will represent the GUV interior with the inner membrane leaflet. These droplets are then forced (e.g., by gravity or centrifugation) to cross an oil–water interface that is also saturated with lipids (which will constitute the outer membrane leaflet), thus forming quasi water-in-water droplets with a bilayer interface (i.e., GUVs). The droplet interface crossing may also be facilitated by capillaries (1) or planar microfluidic channels (194). These approaches allow for facile encapsulation of material inside the GUVs (132, 145), as this material can be confined in the starting water-in-oil droplets. It also allows for building GUVs with asymmetric membrane composition (140).

An alternative approach employing lipids at oil–water interfaces relies on constructing an interface between two water phases across the oil (with the dissolved lipid) and thus forming a bilayer-spanning aperture, through which a water flow is jetted to inflate GUVs (63, 184), conceptually similar to blowing soap bubbles through a ring.

A potential disadvantage of these methods relates to the remaining organic-phase residues in the bilayer (192) even though attempts have been made to reduce the contaminations from the oil films (89, 183). Another one is the unknown final composition of the membrane when multicomponent mixtures are employed, as lipids might have different solubility in the organic phase, as well as different partition coefficients between the interface and the organic phase (23).

2.4. More Complex Membranes, Protein Reconstitution, and Biomolecule Encapsulation

Here, we briefly introduce a couple of approaches for obtaining vesicles of higher complexity. These can be either derived from cells or established in synthetic GUVs upon (bio-) macromolecule (e.g., protein) reconstitution. Giant vesicles can also be formed by electroformation from native membrane extracts purified from cells (121), although whether the initial membrane organization and protein activity are preserved is unclear.

2.4.1. Giant plasma membrane vesicles.
Giant plasma membrane vesicles (GPMVs) are also referred to as blebs and can be derived in a controlled way after subjecting cells to a cocktail of chemicals (18, 171). This approach stems from knowledge dating back to the 1960s that cultured cells shed giant vesicles from their plasma membranes upon chemical treatment (e.g., 169). The chemicals are deadly for the cells, but the resulting vesicles exhibit preserved plasma membrane lipidome and proteome (16, 104). Thus, this approach represents a poor man's way of reconstituting proteins ensuring the right conformation and orientation of the proteins, which most of the time is not as efficient in synthetic GUVs. GPMVs represent a promising system and have already proved suitable for studying phase separation in membranes, lipid–protein and lipid–lipid interactions, and adhesion (18, 189, 195, 211).

2.4.2. Protein reconstitution in synthetic giant unilamellar vesicles.
A number of methods have been recently developed for the reconstitution of membrane proteins in giant vesicles. Some of them rely on the use of proteoliposomes, whereby electroformation (2, 67) or gel-assisted swelling (64, 86) can be applied to dehydrated and rehydrated films of proteoliposome. Alternatively, membrane proteins can be also directly inserted or fused to preformed lipid GUVs in physiological buffers (41). In this case, the GUVs are formed in the presence of mild detergents at subsolubilizing concentrations. This is followed either by the addition of solubilized proteins, or, alternatively, purified native vesicles or proteo-SUVs are incorporated by detergent-mediated fusion. Finally, the detergent is removed with macroporous polymeric beads.

3. PHASE STATE OF MULTICOMPONENT MEMBRANES

The quest for rafts and phase separation processes in cell membranes has been on for almost two decades, and domain formation is still a matter of ongoing debate (69, 107, 137), with a number of experimental techniques put into action (92). One important result of this search was the identification of ternary lipid mixtures that undergo phase separation into liquid-disordered and liquid-ordered phases, which can be directly observed as fluid domains in GUVs (111) using fluorescence microscopy (42) (**Figure 1**). Gel phases can also be detected typically as irregular, faceted, static domains (e.g., 13, 58, 96). Contrary to gel domains, fluid domains (in fluid environment) adopt a

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GPMVs (giant plasma membrane vesicles): vesicles derived from cells with preserved lipidome and proteome of the plasma membrane; they enable the investigation of lipid phase separation in a system with native biological complexity under physiological conditions

Proteoliposomes: liposomes into which one or more proteins have been inserted, usually by artificial means and aided by detergents
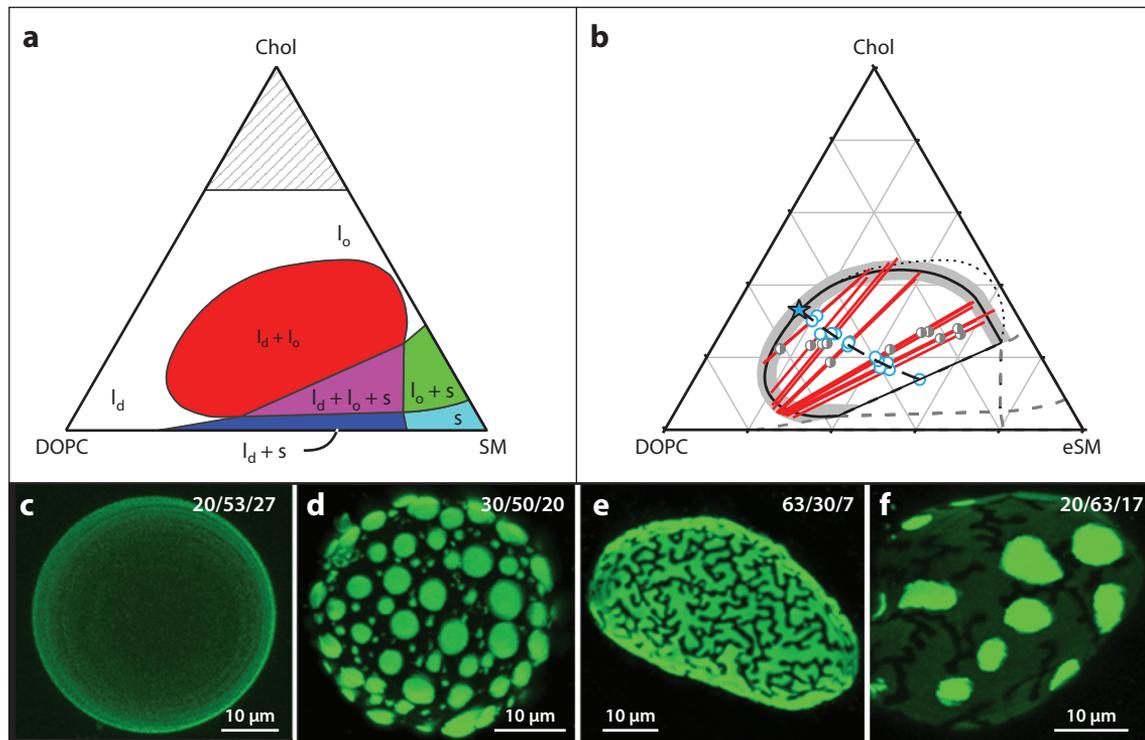
Figure 1

Phase diagrams can be deduced from fluorescence microscopy of giant vesicles. (*a*) Example of a phase diagram of a ternary lipid mixture of dioleoylphosphatidylcholine (DOPC), cholesterol (Chol), and sphingomyelin (SM) at ~23°C, illustrating the regions of coexistence of liquid-ordered (l_o), liquid-disordered (l_d), and solid (s) phases, which have been identified predominantly from microscopy observation of giant unilamellar vesicles. (*b*) The phase diagram of DOPC, Chol, and egg SM (eSM) with schematically outlined regions of phase coexistence, experimentally determined tie lines (*red*), and the tentative critical point (*star*) indicated. Panels *a* and *b* adapted from Reference 20 with permission from Elsevier. Three-dimensional projections from confocal images of vesicles with (*c*) homogeneous membrane or exhibiting coexistence of (*d*) liquid-ordered and liquid-disordered, (*e*) liquid and solid, and (*f*) liquid-ordered, liquid-disordered, and solid phases; notice the partitioning of the dye (*green*) with preference decreasing from liquid-disordered to liquid-ordered to solid domains. The membrane composition is indicated as molar fractions (dioleoylphosphatidylglycerol/eSM/Chol) in the upper-right corners of the images. Panels *c–f* adapted from Reference 138 with permission from Elsevier.

circular shape to minimize line tension and may coalesce upon contact as well as bud out as first theoretically predicted in Reference 108.

3.1. Phase Diagrams

The membrane thermodynamic state is reflected in phase diagrams. They show which phases are present at certain membrane compositions and temperatures (38, 70, 198). Phase diagrams (of membranes with a limited number of components) can be obtained from fluorescence microscopy of giant vesicles. For this, a small fraction of a fluorophore (less than 1 mol%) is incorporated in the membrane preferentially partitioning in the different phases (12, 19, 93, 172) (**Figure 1***c–f*). Conventional epifluorescence or confocal microscopy can be used to resolve the phase diagram (for examples of some ternary phase diagrams, see 20, 196–198, 210). Other imaging

techniques such as fluorescence lifetime imaging microscopy have also been employed (74, 117) as has imaging with environment-sensitive dyes such as Laurdan (14).

3.2. Critical Points and Tie Lines

Giant vesicles have been used to locate important components of phase diagrams such as the critical point and tie lines. At the miscibility critical point, micron-sized compositional fluctuations can be observed, and they were found to follow two-dimensional Ising behavior both for synthetic giant vesicles (82) and for the ones derived from plasma membranes (195). Observations on GPMVs also suggested that compositions of mammalian plasma membranes are tuned to reside near a miscibility critical point. Tie lines (**Figure 1***b*), and more specifically, their cross-points with the binodal, indicate the composition of domains in phase separated vesicles; the domain area fraction can be deduced using the lever rule. Tie lines are short in the immediate vicinity of the critical point (82–84, 195). Assessing them gives information about factors that drive phase separation, such as the difference in lipid acyl chain order parameters. Some examples of studies deducing tie lines from measurements on area fractions of domains in giant vesicles include References 20, 60, 87, and 196.

Tie line: a line drawn in the phase coexistence region of a phase diagram of a three-component membrane; the two end points of the line meeting the binodal (which shows the boundary of the phase-coexistence region) indicate the lipid composition of the two phases for membranes with compositions located along the tie line

3.3. Factors Affecting the Phase Diagrams of Lipid Mixtures

Lipid demixing that leads to phase separation in membranes is particularly relevant to protein functionality, with activity altered depending on the local lipid environment (e.g., 88, 103). However, proteins themselves can alter the phase diagram (138). To what extent the obtained phase diagrams of lipid mixtures with only a few components are biologically relevant is still not clear, as the majority of those deduced on giant vesicles are obtained in nonphysiological buffers such as pure water, sucrose, or low-salinity solution (24, 138, 197, 199, 210). Furthermore, a recent study has shown evidence for phase separation even in quasi-single-component membranes (95). This was caused by impurities present in the used sugars (note that the typical sugar concentration used in vesicle swelling, ∼100 mM, is orders of magnitude higher than the total lipid concentration in the sample, typically ranging from 1 to 100 μM, which is comparable to that of impurities from sugars). Studies on charged vesicles in low-salinity buffers indicated rather small effects of salts on the membrane phase state (24, 174, 199). In contrast, neutral membranes in salt-free conditions (20) and those exposed to saline buffers in the vesicle exterior (33) seem to differ in their phase behavior. The influence of buffer composition and transmembrane solution asymmetry on the membrane phase behavior have also been recently demonstrated (97). Whether the effect of salt and solution asymmetry on the phase behavior of membranes has implications for protein adsorption and domain repartitioning remains to be seen. Membrane tension is yet another factor affecting the membrane phase state that should be considered (81, 147).

4. SOME ASSAYS FOR VESICLE MANIPULATION AND ASSESSING THE MEMBRANE MECHANICAL AND ELECTRICAL PROPERTIES

Lipid membranes are characterized by a peculiar combination of elastic properties—namely, incompressibility and very low bending rigidity. Since the pioneering work of Helfrich (77, 78), a significant number of studies have been aimed at finding ways to assess the bilayer elastic properties (e.g., 26, 44, 118, 128) and the references therein. Below, we summarize some of the most popular approaches for membrane characterization and vesicle manipulation and give a special emphasis to approaches based on the use of electric fields. One of the membrane elastic properties, the

membrane bending rigidity, is extremely sensitive to changes both in the membrane composition [even to dissolved air gas in samples (102)] and to the presence of impurities. Because of this, the bending rigidity can be employed as a control parameter when comparing different systems (44).

4.1. Fluctuation Analysis

Experimentally, fluctuation spectroscopy is probably the least demanding technique for measuring the membrane bending rigidity. It involves collecting time sequences of optical microscopy snapshots of vesicles exhibiting visible fluctuations (which implies low membrane tension). The vesicle contours are extracted (**Figure 2a**). The thermally induced fluctuations around an equilibrium shape (e.g., a sphere or a prolate) are monitored, Fourier decomposition of the contours is applied, and the mean square values of shape deviations are determined. The method has been applied to tubular vesicles (170), fractions of a vesicle (127), prolate vesicles (52), and quasispherical vesicles (72, 79, 142, 165). An exhaustive overview on the development of this method can be found in Reference 122. More recently, a new approach accounting for the finite focal depth of the microscope was reported (149), but a consensus has not yet emerged on the proposed correction.

Fluctuation analysis has been employed to resolve the effect of various molecules on the membrane rigidity, which provides a mechanistic understanding of their action in processes that they participate in. For example, depending on the membrane composition and lipid architecture, cholesterol can decrease, increase, or show no effect on the bending rigidity (72), which dethroned the traditional understanding of cholesterol as a stiffening agent observed in other GUV-based studies (55, 56, 150, 160). The activity of bulky transmembrane proteins such as bacteriorhodopsin was shown to enhance membrane fluctuations without altering the membrane rigidity (116), while other proteins and peptides were shown to reduce it (e.g., 68, 173). Salts were also reported to decrease the membrane stiffness already at millimolar concentrations (26), while the inclusion of charged lipids was found to increase the membrane stiffness. The bending rigidity is a sensitive parameter and can be affected even by the buffer type used (27).

4.2. Micropipette Aspiration and Injection

The micropipette aspiration technique was introduced by Evans, Needham, and colleagues (55–57) and ever since has been applied to characterize the elastic properties of membranes of different composition (21, 61, 80, 150, 173). The mechanical tension is generated via a suction pressure in the aspirating glass capillary (**Figure 2b**) and can be estimated from the vesicle morphology and applied pressure. In the low-tension regime, membrane undulations are pulled out and the dependence of the projected vesicle area on the tension (in logarithmic scale) yields the bending rigidity. In the high-tension regime, typically above 0.1–0.5 mN/m, the membrane is stretched (the area per lipid is increased). Here, the vesicle area scales linearly with tension, whereby the proportionality coefficient yields the stretching elasticity modulus, typically in the range of 200 to 2,000 mN/m, depending on the membrane composition and phase state (150, 151).

Micropipettes are also ubiquitously used as a manipulation technique providing a readout of the membrane tension and vesicle area (e.g., 105, 106, 191), as well as for vesicle–vesicle adhesion (54) (**Figure 2c**). Apart from holding the vesicles, micropipettes can be used to locally introduce solutions in the vesicle vicinity; even a three-pipette setup has been employed to alternate the solutions introduced around the vesicle (129). Micropipette manipulation was also recently employed in establishing a challenging system of whole-GUV patch clamping, which allowed measurement of the membrane capacitance and the activity of ion channels reconstituted in the membrane (65). Finally, sharp micropipettes (microneedles) can be employed to inject various substances such as proteins, enzymes, or DNA as aqueous solution (30) (**Figure 2d**).
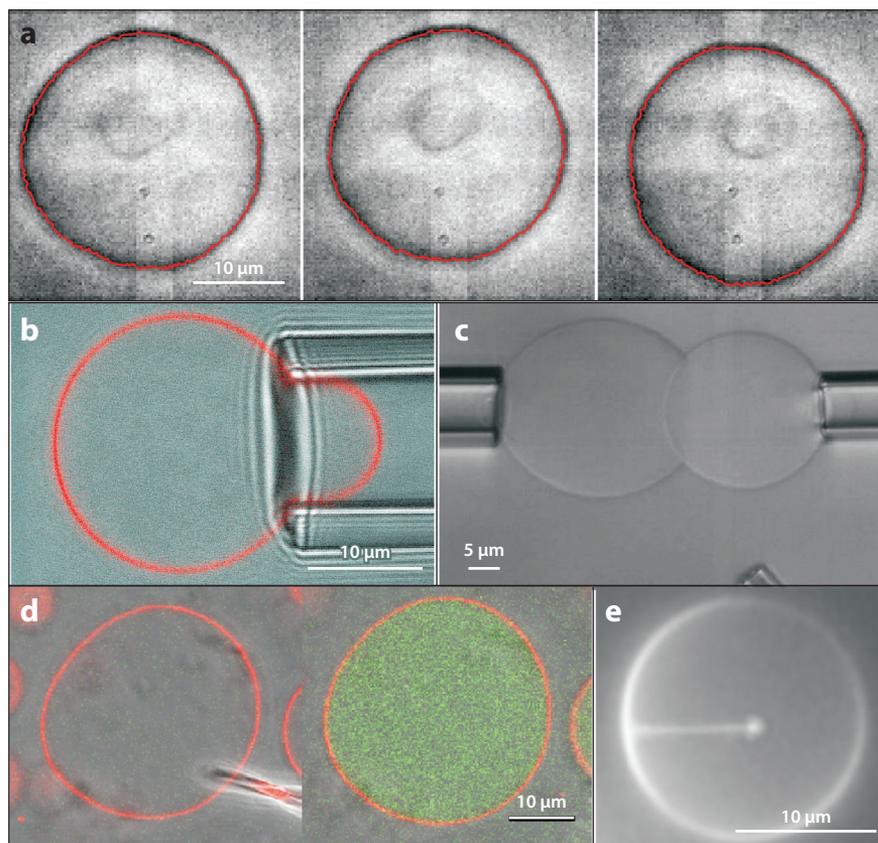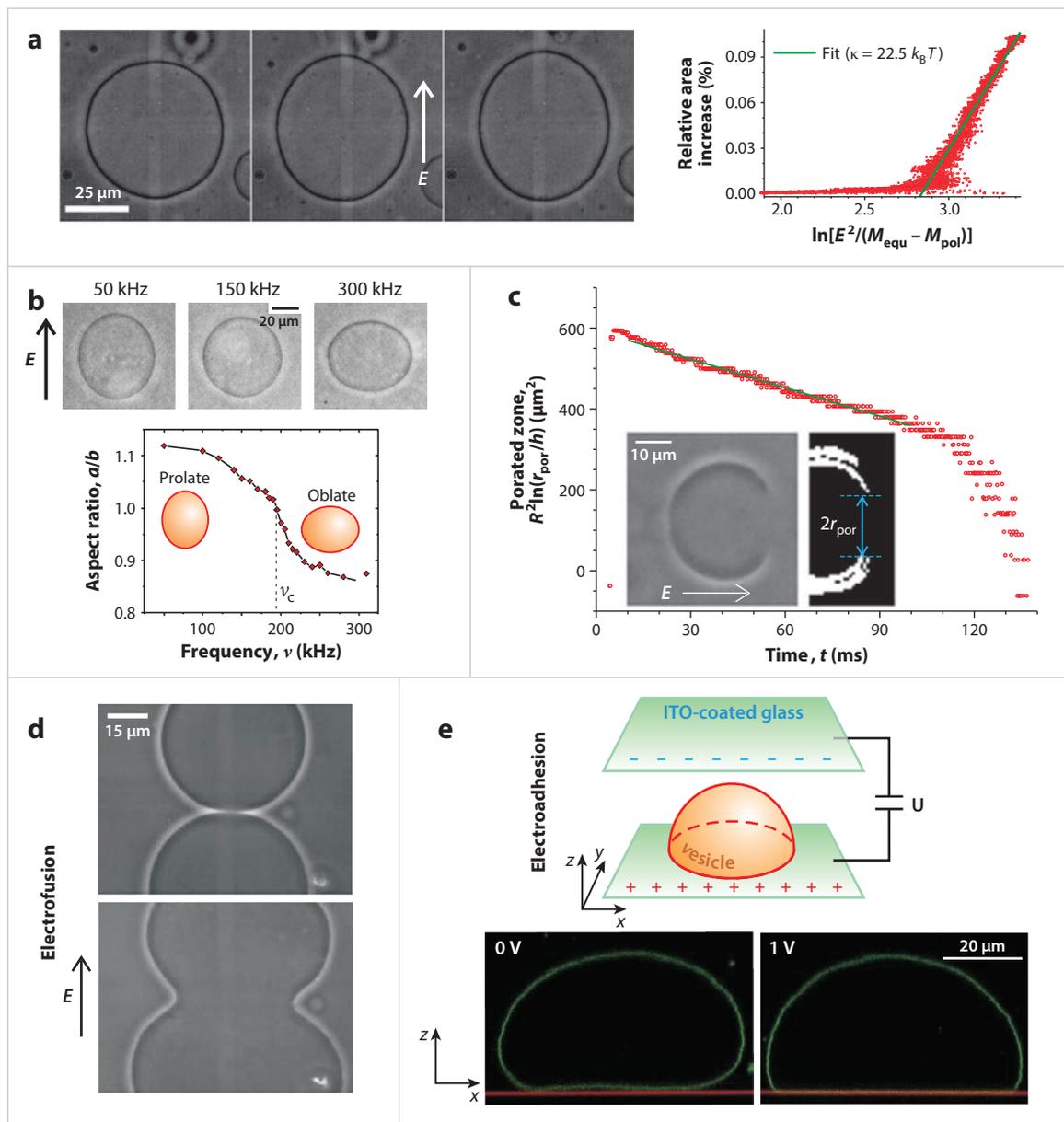
Figure 2

Techniques for assessing the membrane mechanics and for vesicle manipulation. (*a*) Fluctuation analysis. Phase-contrast images of a vesicle exhibiting shape fluctuations used for assessing the membrane bending rigidity. The detected vesicle contour is marked in red. The time lapse between the snapshots is several seconds. Panel *a* adapted from Reference 44 with permission from Elsevier. (*b*) Micropipette aspiration of a vesicle (an overlay of phase-contrast and confocal cross-section images): The membrane was fluorescently labeled (*false red color*). The inner diameter of the glass capillary is approximately 10 μm. Panel *b* adapted from Reference 44 with permission from Elsevier. (*c*) Two vesicles aspirated in micropipettes can be brought together to assess the membrane–membrane adhesion energy. Here, the adhesion is induced by a membrane–membrane bridging agent introduced via local injection through a third pipette, the tip of which is seen in the lower-right region of the snapshot. Image in panel *c* is courtesy of C. Haluska. (*d*) Femtoliter injection in giant unilamellar vesicles (an overlay of phase-contrast and confocal cross-section images): The vesicle (*red*) was immobilized on a gel substrate, and several femtoliters of a solution of low concentration of fluorescently labeled protein (*green*) were injected inside it. The tip of the injection pipette is seen in the lower-right corner of the left image. Images in panel *d* courtesy of Y. Avalos-Padilla. (*e*) In tube pulling experiments, a bead manipulated via optical tweezers is used to pull a tube out of a giant vesicle or inward as shown in this image. To pull the tube, the vesicle (i.e., the experimental chamber) is displaced relative to the position of the optical trap. Alternatively, it can be held by means of an aspiration pipette, as illustrated in **Figure 4f**. Panel *e* adapted from Reference 36.

4.3. Membrane Properties and Interactions Assessed from Exposing Giant Vesicles to Electric Fields

Exposing giant vesicles to electric fields offers access to versatile information about material characteristics of the membrane (46, 50). AC fields and direct current (DC) pulses can be used to deform and porate GUVs. From their response, as we briefly summarize below, one is able to deduce the membrane bending rigidity, stretching elasticity, shear surface viscosity, capacitance, and pore edge tension (**Figure 3**). Experimentally, these are undemanding measurements in terms of

(Caption appears on following page)

Figure 3 (*Figure appears on preceding page*)

Vesicles in electric fields: assessing membrane material properties and interactions. (*a*) Measuring the bending rigidity from vesicle electrodeformation at a fixed field frequency and varied field strength. From the slope of the vesicle area change as a function of electric field amplitude, E, and vesicle shape (M_{pol} and M_{equ} are the mean curvatures of the membrane at the vesicle poles and equator, respectively), one can obtain the bending rigidity. Panel *a* adapted from Reference 72 with permission from the Royal Society of Chemistry; data reproduced from Reference 62. (*b*) Membrane capacitance measurements. The shape response of a vesicle in a frequency sweep is used to determine the critical frequency ν_c of prolate–oblate transition, which depends solely on the membrane capacitance and the experimentally determined vesicle radius and solution conductivities. The vesicle shape is described in terms of the aspect ratio *a/b* of the long and short axis of the deformed vesicle. Panel *b* adapted from Reference 200 with permission from Elsevier. (*c*) The membrane edge tension can be estimated from the evolution of the porated region $R^2 \ln(r_{por}/b)$ as a function of time (note that to avoid plotting a dimensional value in the logarithmic term, we have introduced $b = 1$ μm; R is the vesicle radius). The solid line is a linear fit with slope that yields the edge tension. The inset shows a raw image (*left*) of a porated vesicle 50 ms after being exposed to an electric pulse with duration of 5 ms and amplitude of 50 kV/m. To the right is an enhanced and processed image of the vesicle half facing the cathode, showing the pore diameter, $2r_{por}$. Panel *c* adapted from Reference 146 with permission from Elsevier. (*d*) Fusion of two giant vesicles exposed to a direct current (DC) pulse of duration 15 μs and amplitude 3 kV/cm. The lower image shows the resulting fused vesicle 3 s after the application of the pulse. Panel *d* adapted from Reference 73, copyright 2006, National Academy of Sciences. (*e*) Adhesion of a giant unilamellar vesicle to a conductive substrate induced by the application of DC field of voltage U. In the absence of the field, the vesicle (*false green color*) rests on the electrode surface (*red*) and exhibits fluctuations (vertical *xz* confocal scans). Upon application of the field, the vesicle adheres to the substrate, exhibiting a finite contact angle. Abbreviation: ITO, indium tin oxide. Panel *e* adapted from Reference 187 with permission from Elsevier.

equipment, except if high-speed imaging is required to record fast response of the vesicles. Electric fields can be also used to manipulate vesicles, bring them together, and induce GUV–GUV fusion (**Figure 3d**). High-speed microscopy revealed that the opening of the fusion neck proceeds unprecedentedly fast (73). Adhesion of GUVs under exogenous electric field (analogous to electrowetting), whereby the adhesion strength could be smoothly regulated by turning the knob of the DC source (187), has also been explored (**Figure 3e**).

4.3.1. Measuring the membrane bending rigidity.
Vesicle deformation induced by electric fields as introduced by Helfrich and coworkers (99, 130) can be used to deduce the membrane bending rigidity by subjecting a GUV to an AC electric field of increasing strength and recording the induced shape deformation (72, 99) (**Figure 3a**). The tension of the deformed vesicle is obtained from the electric stresses (for details, see 62, 72, 201, 208). Typically, the conductivity conditions in these experiments are selected so that the vesicles attain prolate deformation (9, 46) with elongation along the field direction (**Figure 3**). This method has been employed for assessing changes in the bending rigidity associated with cholesterol content (72) or presence of the ganglioside GM1 in the membrane (62). The relaxation dynamics of vesicles exposed to DC pulses can also be employed for deducing the tension and bending rigidity (209).

4.3.2. Assessing the area of floppy vesicles.
Apart from measuring the membrane mechanics, vesicle electrodeformation can also be used to assess area and area changes in GUVs. Depending on the field frequency and conductivity ratio, the AC field can induce prolate and oblate deformation (9, 46). For estimating area changes, the prolate shape is preferred because of the smaller effect resulting from the proximity of the cover slip where the vesicle typically rests. Examples of the use of vesicle electrodeformation under AC fields include measurements of the area changes resulting from oxidative stress from photosensitizing molecules (154), detergent insertion (119), light-driven isomerization of molecules in the membrane (66), and vesicle fusion (113).

4.3.3. Measuring the membrane capacitance, edge tension, and viscosity.
As indicated above, deformation of GUVs in electric fields can be employed for assessing the membrane

bending rigidity and vesicle area. It turns out that this undemanding experimental approach of subjecting vesicles to electric fields (both AC fields and DC pulses) can also be employed to measure a number of other material properties of the membrane. The membrane capacitance can be estimated from measuring the field frequency at which the vesicle deformation switches from prolate to oblate (162, 163, 200) (**Figure 3b**). The membrane viscosity governs the vesicle relaxation when exposed to DC pulses and can be deduced from the shape relaxation dynamics (153, 163). Strong DC pulses can induce micron-sized pores (macropores) (153), the resealing dynamics of which can be used to measure the membrane edge tension (146) (**Figure 3c**).

4.4. All-Optical Assays for Measuring the Bending Rigidity, Membrane Fluidity, and Viscosity

Assays based on contactless probing of the membrane are not limited to fluctuation spectroscopy but also include manipulation via laser beams and are demanding in terms of laboratory equipment. One example is the so-called optical stretcher where a dual-beam laser setup is used to deform GUVs and extract their elastic properties (39, 179). Another example, optical dynamometry, is based on following the sedimentation of a bead while shearing the membrane (a minimal version of a Stokes viscometer) (49). The bead is brought to and stuck to the membrane of a GUV via manipulation with optical tweezers and then released to sediment along the bilayer, yielding the membrane shear viscosity.

The category of all-optical assays includes (but is not limited to) microscopy techniques (now conventionally available in confocal microscopes), such as fluorescence recovery after photobleaching (FRAP) ubiquitously employed on GUVs to assess the membrane fluidity. Similarly (but employed to a lesser extent), fluorescence correlation spectroscopy is also used to assess association of membrane proteins and diffusion coefficients of membrane probes as well as clustering of membrane-bound molecules. Both techniques typically require immobilization of the vesicles (for an example with agarose, see 114). Finally, Förster resonance energy transfer microscopy is well established in characterizing protein binding and the degree of lipid mixing occurring via fusion (see, e.g., 113).

4.5. Pulling Membrane Tubes

Tube pulling experiments on lipid membranes produce plenty of information about the membrane characteristics, including bending rigidity, shear surface viscosity, and intermonolayer slip coefficient (36, 76). The tube diameters typically vary between 10 and 500 nm. A tube can be extruded by subjecting the vesicle to fluid drag (36, 203) or by exerting a force via a membrane-attached bead sedimenting under gravity (25), or bead manipulation with an electromagnetic field (76), a pipette (175), or optical tweezers (34); for illustration of two of these approaches, see **Figures 2e** and **4f**. Molecular motors also have been shown to be able to pull tubes out of giant vesicles (159), while micropipette manipulation has been applied to create networks of membrane tubes connecting GUVs (91). Recently, it was demonstrated that inward tubes (pointing to the vesicle interior) can also be pulled (36) (**Figure 2e**), allowing for the direct assessment of membrane spontaneous curvature (37). The tube radius can be obtained from geometrical parameters, using the conservation of membrane area and total vesicle volume.

The tube pulling technique receives increasing popularity in measurements on membranes decorated with proteins (e.g., 17, 75, 160, 180), via which dynamic processes occurring in cell membranes can be directly studied, as reviewed in Reference 158. However, the analysis should be performed with caution and the tension associated with spontaneous curvature should be carefully

FRAP (fluorescence recovery after photobleaching): a method for measuring diffusion by observing the recovery of fluorescence in a small area or volume after photobleaching with short laser irradiation

Intermonolayer slip: describes the relative motion between the two leaflets of a bilayer; the ratio between the associated friction force per unit area and the slip velocity is known as the intermonolayer slip coefficient

considered (109). Studies based on tube pulling have demonstrated curvature-dependent sorting of lipids (160, 180) and proteins (17, 75), curvature-dependent protein binding (i.e., curvature sensing) (7, 17), fission (175), and polymerization (161) as well as curvature generation by proteins (94).

5. MEMBRANE CURVATURE AND VESICLE SHAPE

When GUVs are prepared, a number of vesicle morphologies can be observed in the sample—for example, spheres, prolates, oblates, and starfish (for a theoretical description, see 51, 110). In addition, very often, multilamellar vesicles, aggregates, and vesicles with lipid lumps are found and are typically labeled as "vesicles with defects." In this section, we focus on the membrane spontaneous curvature, which, together with the area and volume of the vesicle, determines its shape and is a governing factor in stabilizing highly curved structures typically observed as tubes and buds.
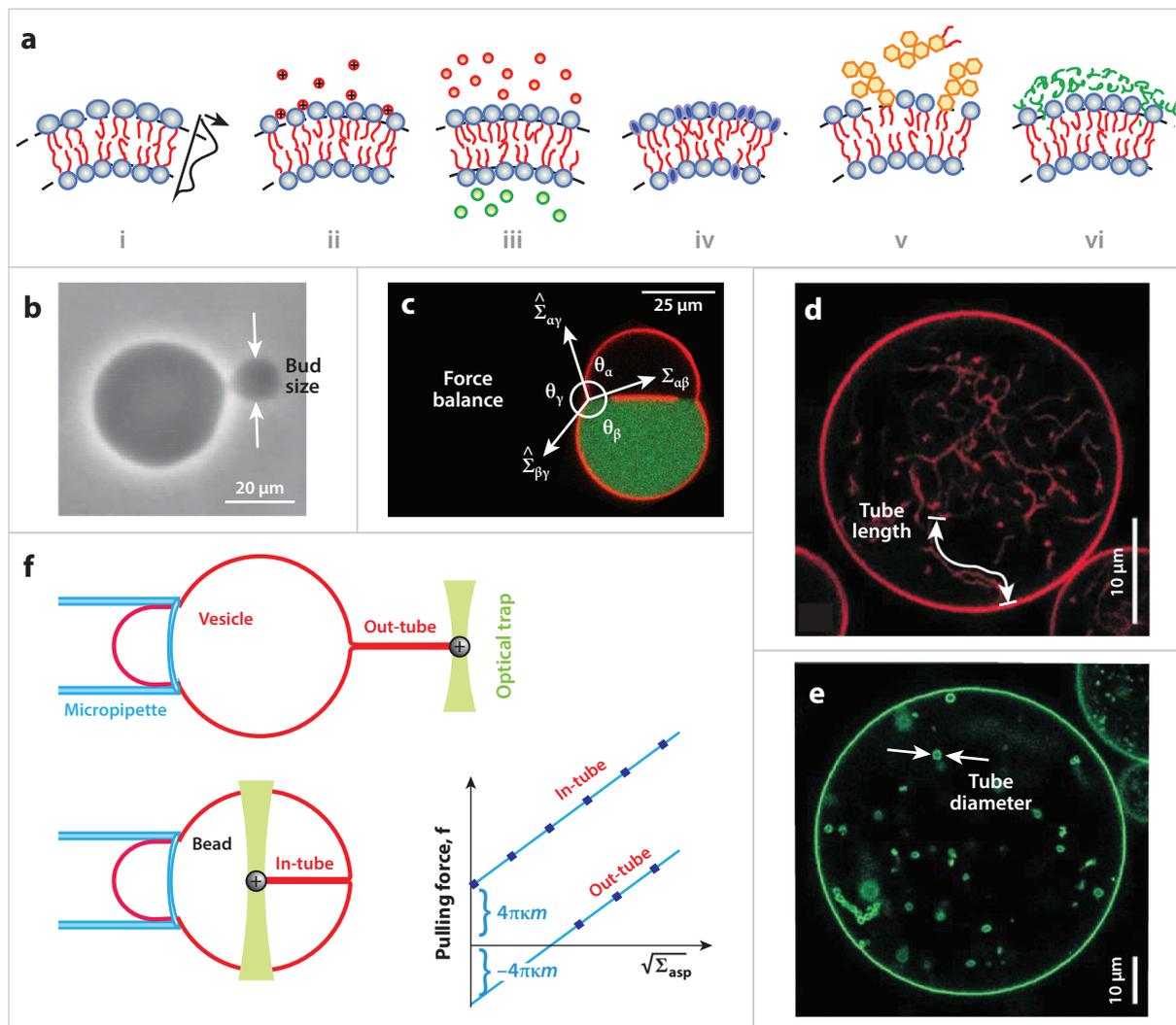
(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Examples for sources of nonzero membrane spontaneous curvature and experimental approaches for measuring it. (*a*) Nonzero spontaneous curvature can be generated from (*i*) differences in the effective head-group size (and respective molecular area) of the lipids in the bilayer (e.g., as a result of differences in hydration, pH, or molecular type); (*ii*) asymmetric ion distribution, leading either to condensing or expanding the lipids in one of the bilayer leaflets; (*iii*) asymmetric distribution of nonadsorbing particles or (bio)molecules of different sizes; (*iv*) amphiphilic molecules or lipid species asymmetrically distributed in the membrane; (*v*) partially water-soluble molecules (such as glycolipids or peripheral proteins) asymmetrically inserting in the membrane; and (*vi*) asymmetrically bound proteins with specific geometry. (*b*) The spontaneous curvature of a budded vesicle with a closed neck can be directly assessed from its geometry following $m = (M_{bud} + M_{ves})/2$, where M_{bud} and M_{ves} are the mean curvatures of the bud and the mother vesicle, respectively (66, 131). (*c*) Force balance at the three-phase contact line in vesicles enclosing two aqueous phases, α and β, yields a direct dependence of the spontaneous curvature $m = -\sqrt{\frac{\Sigma_{\alpha\beta}}{2\kappa} \frac{\sin\theta_\beta}{\sin\theta_\gamma}}$ on the geometric angles, the interfacial tension, $\Sigma_{\alpha\beta}$, and the membrane tensions $\hat{\Sigma}_{\alpha\gamma}$ and $\hat{\Sigma}_{\beta\gamma}$ (115). (*d*) The area stored in internal tubes and their length as measured from three-dimensional scans can be used to assess the tube diameter and thus the membrane spontaneous curvature (115). (*e*) Tube diameters can be directly measured when they are above the optical resolution. In the approaches in both panels *d* and *e*, the spontaneous curvature is $|m| = 1/R_{sph}$ for necklace tubes, where R_{sph} is the radius of the composing spheres or $|m| = 1/(2R_{cyl})$ for cylindrical tubes with radius R_{cyl}; the sign of *m* is negative for inward tubes and positive for outward ones. (*f*) Pulling outward and inward tubes of a vesicle held by a micropipette at tension Σ_{asp} and measuring the pulling force *f* applied by optical tweezers yields the spontaneous curvature $= \mp\frac{f}{4\pi\kappa}$, which is determined from the *y*-axis intercept of force data as sketched in the inset for membranes with positive spontaneous curvature (the membrane bending rigidity κ is assessed from the slopes of the data) (37, 109). Figure adapted from Reference 15.

5.1. Spontaneous Curvature of Membranes

The asymmetry across membranes, whether resulting from differences in the leaflets compositions or from differences in the solution composition across the membrane (**Figure 4***a*), gives rise to nonzero spontaneous curvature (i.e., the preferred membrane shape). In giant vesicles, nonzero membrane spontaneous curvature can be exhibited in outward or inward budding and/or tubulation. Thus, while one often looks for the "perfect" round vesicle with no inclusion and no internal or external structures, the "imperfect" GUVs may be the more representative ones (compared to the "clean" spherical vesicles), in particular for systems where the membrane and/or solution asymmetry is pronounced. Spontaneous tube formation in giant vesicles thus implies that the studied membrane exhibits spontaneous curvature, which can be positive (outward tubes/buds) or negative (inward structures). Examples of such spontaneous tubulation are provided in giant vesicles with asymmetric distribution of polyethyleneglycol in the solutions (106, 115), asymmetry in trans-leaflet distribution of the ganglioside GM1 (21, 37) or charged lipids (188), as well as protein–membrane interactions (182, 185).

5.2. Membrane Versus Monolayer Curvature

The membrane spontaneous curvature represents a material property of the membrane and should not be confused with what is sometimes referred to as molecular curvature or monolayer curvature of membrane inclusions or lipids. Indeed, the term molecular curvature is somewhat misleading, as one needs a surface to define curvature, and one molecule cannot make a surface. Even if many of the same molecule can make some type of structure (e.g., a micelle), the curvature of this shape is difficult to relate to the spontaneous curvature this molecule will generate when inserted into a membrane (see References 29 and 212 for the monolayer curvature of some lipids). This is because in the membrane, the intermolecular cohesion with the neighboring molecules is changed, and as a result, the molecule can even adopt a different geometry in space. In addition, the molecule distribution across the membrane matters. The spontaneous curvature of the membrane can change sign depending on the number of molecules in each of the leaflets, presumably irrespective of

the molecular shape. Spontaneous curvature of protein-doped membranes, as assessed from tube pulling experiments, are often interpreted in terms of some "effective spontaneous curvature of the protein" after taking into account its surface density (e.g., 181, p. 175). Note, however, that the obtained parameter represents, rather, a local curvature generated by the protein and is a material property that is not universal but depends on the molecule environment. Thus, reported values should not be generalized because they are characteristic for the explored system and the specific membrane composition.

To summarize, even though it is attractive to represent molecules as geometrical objects, cones, cylinders, or inverted cones, directly relating their geometry to the membrane spontaneous curvature is not straightforward unless the exact distribution across the membrane is known.

BAR domain (Bin, amphiphysin, Rvs domain): a domain found in a large family of proteins, which forms a banana-like dimer; it differentially binds to membranes of different curvatures and tubulates lipid membranes functioning as a scaffold

5.3. Membrane Remodeling by Proteins, Solutes, and Lipids

The membrane spontaneous curvature affects the preferred membrane shape and is therefore strongly implicated in the shape of membrane-bound organelles and cell protrusions (e.g., neurites). Indeed, the shapes of cellular organelles are highly conserved, suggesting that the morphology of their membrane is important for functionality. Membrane proteins are one of the most "popular" players considered in membrane reshaping. On the basis of their intrinsic shape [as for BAR (Bin, amphiphysin, Rvs) domains], proteins can act as curvature sensors (i.e., can bind to a membrane that is conveniently curved), but at high density, they can also induce curvature (177). Curvature effects are diverse: Both proteins and lipids can respond to curvature generation by sorting (31, 32) or can generate curvature themselves, if asymmetrically distributed across the membrane. Indeed, any type of substance, as long as it is asymmetrically distributed across a membrane, will affect the membrane spontaneous curvature. Such substances include ions (see, e.g., 90), particles, and water-soluble (macro)molecules, even those that are conventionally considered as inert to the membrane (e.g., polyethylene glycol). Only perfectly symmetric membrane leaflets and transmembrane solution compositions and asymmetric systems with perfectly balanced intermolecular interactions in both leaflets and solutions can result in a membrane of zero spontaneous curvature. This ideal case seems to be irrelevant because every biological membrane experiences asymmetry of various origins, as schematically represented in **Figure 4a**.

5.4. Measuring the Membrane Spontaneous Curvature

Knowing the membrane spontaneous curvature gives us an idea of the preferred shape the membrane will adopt when relaxed. One of the first attempts to measure the membrane spontaneous curvature, m, was applied to vesicles with sugar asymmetry across the membrane (53), yielding $m^{-1} \approx 10 \div 100$ μm as assessed from the vesicle fluctuation spectra. Nonzero spontaneous curvature generation can exhibit itself in spontaneous budding or tubulation of vesicles (109) (see also **Figure 4b–e**). Several approaches for deducing m are illustrated in **Figure 4b–f**. The spontaneous curvature can vary from several inverted microns (as in the abovementioned cases) to few tens of inverted nanometers ($m^{-1} \approx 20 \div 100$ nm), as is the case of BAR domain proteins (120). Intermediate values can be observed on membranes asymmetrically exposed to divalent ions (71) or polymers such as polyethylene glycol (115) ($m^{-1} \sim 0.1 \div 0.3$ μm). Measurements on systems with high spontaneous curvature typically rely on pulling lipid nanotubes out of GUVs (181) (see **Figure 4f** for one approach).

6. OUTLOOK

Giant vesicles are employed in a steeply growing number of applications, only a limited fraction of which were discussed in this review. There is no doubt that they are becoming an increasingly in-demand system, as evidenced even by the emergence of a company that provides commercial electroformation setup and a smartphone app for their production (5). How useful these enterprises are remains questionable owing to (*a*) the variable requirements for the encapsulated solutions, (*b*) the growing complexity of the membrane composition, and (*c*) new questions and fields addressed by means of exploiting giant vesicles.

GUVs have proved exceptionally instrumental in understanding the mechanistic action of certain proteins and peptides. A few examples include dynamin polymerization (161), Shiga toxin clustering (144, 157), the coupling of BAR domain density and membrane mechanics (177), membrane fission by protein crowding (178), curvature sorting based on steric protein interactions (182), and the membrane reshaping action of the ESCRT-III (endosomal sorting complex required for transport III) machinery (10, 207) and autophagy-related proteins (94). The mode of interaction of antimicrobial peptides with membranes has also been visualized on GUV-based assays (6).

Here, we discussed predominantly phospholipid GUVs, but studies on giant vesicles made of fatty acids have also brought understanding about processes related to the formation of protocells, such as growth of membranes, fission and fusion, and even self-reproduction of vesicles (206). Note, however, that contrary to phospholipid membranes, vesicles made of fatty acids are stable in a smaller pH range, they are more permeable, and the exchange kinetics of fatty acid molecules between the membrane and the solution as well as the flip-flop within the bilayer are faster.

As GUVs represent a simple and manageable cell-mimetic system, they are increasingly employed in synthetic biology and used in approaches toward building minimal cell- and organelle-like systems. The crowded cytoplasmic environment and ensuing phase separation were mimicked in GUVs encapsulating macromolecule solutions (47). Other examples include the establishment of a cell-free expression system encapsulated in the lumen of GUVs, where fluorescent protein expression could be followed optically (132). Because of the small volume of GUVs, substantial concentration of proteins can be reached for a short time in comparison to protein expression in bulk, where products become diluted. Limitations of current systems are outlined in Reference 100. Efforts are being made toward constructing individual modules reconstituting certain cell functionalities (168) that may lead to technological applications and pave a pathway in the far future toward building a synthetic cell (133, 155, 166, 167). Giant vesicles are already proving to be an essential component in this enterprise.

ESCRT-III (endosomal sorting complex required for transport III): a cytosolic protein complex that remodels the membrane; as a result, the membrane bends and buds away from the compartment containing the ESCRT complex, and membrane scission follows

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SUMMARY POINTS

1. Giant vesicles are becoming an increasingly in-demand system for applications in assessing material properties and interactions of biological membrane and organelle shape.

2. GUV preparation methods are constantly increasing in number, but the majority suffer from deficiencies. These should be carefully considered, depending on the target vesicle application. If the vesicles are to be used as containers, impurities in the membrane are not relevant, but if the membrane material properties are important, the presence of impurities can be crucial and the preparation method should be carefully chosen.

3. The membrane phase state can easily be influenced by environmental factors such as salinity. Thus, phase diagrams should not be considered as universal and should not be generalized.

4. Assays on GUVs for measuring membrane properties and interactions are numerous. Those based on the use of electric fields are experimentally undemanding and bring a wealth of information about the membrane elastic and electrical properties.

5. Biological membranes are asymmetric and thus characterized by a nonzero spontaneous curvature. The latter depends not only on the geometry of the constituting molecules but also on their distribution in the two leaflets.

FUTURE ISSUES

1. Increasing the complexity of the membrane of synthetic giant vesicles is still challenging and requires the development of new approaches for biomacromolecule reconstitution.

2. Preparing vesicles with size on demand using the swelling (non-oil-based) methods as well as controlled positioning of the vesicles after harvesting is still far from being established.

3. Being a simple and manageable cell-mimetic system, giant vesicles should be more intensively employed in synthetic biology applications to answer outstanding questions regarding processes like growth and division in cells and used toward building minimal cell- and organelle-like systems.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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