Active probing of the mechanical properties of biological and synthetic vesicles

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Abstract

Background: The interest in mechanics of synthetic and biological vesicles has been continuously growing during the last decades. Liposomes serve as model systems for investigating fundamental membrane processes and properties. More recently, extracellular vesicles (EVs) have been investigated mechanically as well. EVs are widely studied in fundamental and applied sciences, but their material properties remained elusive until recently. Elucidating the mechanical properties of vesicles is essential to unveil the mechanisms behind a variety of biological processes, e.g. budding, vesiculation and cellular uptake mechanisms.

Scope of review: The importance of mechanobiology for studies of vesicles and membranes is discussed, as well as the different available techniques to probe their mechanical properties. In particular, the mechanics of vesicles and membranes as obtained by nanoindentation, micropipette aspiration, optical tweezers, electrodeformation and electroporation experiments is addressed.

Major conclusions: EVs and liposomes possess an astonishing rich, diverse behavior. To better understand their properties, and for optimization of their applications in nanotechnology, an improved understanding of their mechanical properties is needed. Depending on the size of the vesicles and the specific scientific question, different techniques can be chosen for their mechanical characterization.

General significance: Understanding the mechanical properties of vesicles is necessary to gain deeper insight in the fundamental biological mechanisms involved in vesicle generation and cellular uptake. This furthermore facilitates technological applications such as using vesicles as targeted drug delivery vehicles. Liposome studies provide insight into fundamental membrane processes and properties, whereas the role and functioning of EVs in biology and medicine are increasingly elucidated.

1. Introduction

The interest in the mechanical properties of membranes and membrane enclosed compartments such as cells and vesicles has grown significantly during the last decades. In particular techniques that actively probe these properties are gaining more attention [1,2], because they allow an investigation of the force response of the membranous compartments at the single entity level. Vesicles are membrane-enclosed compartments which occur naturally and can be produced synthetically. Natural vesicles contain a very rich composition of lipids, as well as integral and peripheral membrane proteins. When vesicles are artificially prepared using lipids, they are often referred to as liposomes, although they can also be formed using other amphiphiles such as amphiphilic polymers [3]. To facilitate the study of membrane properties, synthetic vesicles are often used as models since their structure and composition can be easily tuned and controlled. If a vesicle consists of a single lipid bilayer enclosing the fluid compartment, it is called unilamellar. More complex structures typically consist of concentric arranged bilayers (multilamellar vesicles) or consist of vesicles in vesicles (oligovesicular vesicles). However, unilamellarity is poorly understood.

Abbreviations: AC, alternating current; AFM, atomic force microscopy; ATP, adenosine triphosphate; CHMP2B, Charged Multivesicular Body Protein 2B; Cryo-EM, cryo-electron microscopy; cw, continuous wave; DC, direct current; DHPE, 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; DIC, differential interference contrast; EpCAM, epithelial cell adhesion molecule; ESCRT, endosomal sorting complexes required for transport; EV, extracellular vesicle; FDC, force distance curve; FLIM, fluorescence-lifetime imaging microscopy; GUV, giant unilamellar vesicle; LUV, large unilamellar vesicle; MPA, micropipette aspiration; MVB, multivesicular bodies; OT, optical tweezers; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; PEG, polyethylene glycol; RBC, red blood cell; SUV, small unilamellar vesicle; Syt1, Synaptotagmin-1

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the structure of most naturally occurring vesicles. Depending on their size and complexity, vesicles can be classified as small unilamellar vesicles (SUV, diameter < 200 nm), large unilamellar vesicles (LUV, 200 nm - 1 µm), and giant unilamellar vesicles (GUV, diameter > 1 µm). GUVs are easy to study membrane models due to their large size, permitting direct microscope observation and manipulation, whereas small vesicles usually require indirect techniques to study them [4].

Vesicles are formed as a result of the closure of initially flat lipid bilayers. Phospholipids and cholesterol are the major building blocks of bilayers. They are amphiphilic molecules containing a hydrophilic and a hydrophobic region, and phospholipids self-assemble into aggregates (i.e. vesicles) in water solutions. In order to avoid the exposure of the hydrophobic tails to water, lipids at the edges of the membranes would bend, with their headgroups facing the water solution due to the so-called hydrophobic effect [5]. This situation is energetically costly because it forces a conformation that is different to that preferred by bilayer-forming lipids. As a result, these membranes tend to coalesce by forming sealed (vesicular) structures without open edges [6]. This can only occur because lipid membranes are very flexible, and thus offer almost no resistance to bending. The manner and strength lipids laterally or axially (on the opposing leaflet) interact with other lipids or different molecules embedded in the membrane or dispersed in the medium define the physical properties of the bilayer. It is characterized by the variables viscosity, stretching, bending and shear. These are the basic material properties of lipid bilayers, which dictate the structure, stability, conformation, and ultimately, the behavior of membrane-enclosed structures, including cells. Such properties are equally important in synthetic and natural membranes.

Synthetic vesicles are often used as model membranes since their composition, and hence physical properties, can be finely tuned by simply changing their precursors during preparation. In this way, the wide range of properties shared by their biological counterparts can be mimicked. However, synthetic vesicles possess limited complexity; the variety of the lipid species is much lower and they typically lack important structural elements (i.e. membrane proteins). Nonetheless, the advantages of synthetic membrane vesicles being flexible and easy to work with, help researchers to better characterize the physical properties of natural membranes.

A class of natural vesicles that is not only biologically, but also clinically relevant, is extracellular vesicles (EVs). EVs are released by cells to serve as signaling system and facilitate the exchange of bioactive molecules between cells in close proximity of each other as well as at long-distance ranges [7,8]. The interest in EVs has grown enormously because of their impressive potential in nanomedicine, their physiological functions and their role in diseases [8,9]. Being involved in intercellular communication, EVs have the potential to serve as natural drug delivery systems for cells [10]. Whether studied in their role as a biological signaling platform or as a drug-delivery system, the mechanical properties of EVs have recently been recognized as an important aspect in their functional description [9,11].

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The mechanics of membranes are altered in some pathologies [12]. This could lead to a change in mechanics of vesicles that are secreted by the diseased cell, which could potentially be used in a diagnostic approach to identify pathologies such as cancer. Furthermore, the mechanics of synthetic vesicles seem to be a dictating factor in drug delivery. The potential use of liposomes and EVs as drug delivery vehicles [13], and the interest in nanoparticle mechanics in general [14], are pushing the field of vesicle mechanics forward. There are different ways of assessing membrane and vesicle mechanics, both at single and ensemble-averaged level. One way is observing their behavior passively by direct imaging [15] or by using nanoprobe probes [16] on membranes in thermal equilibrium. Alternatively, it is also possible to actively disturb the system and to measure its response. Here, we critically review techniques that actively probe the mechanics of synthetic and natural vesicles. During active probing a force/stimulus is applied to the vesicles and the subsequent response is detected. We discuss how these techniques help to better understand basic fundamental properties of membranes. Among the variety of microscopic/spectroscopic techniques used to investigate the mechanical properties, we will focus on micropipette aspiration, electrodeformation and electroporation, optical tweezers and atomic force microscopy nanoindentation.

2. Techniques to actively probe mechanical properties of biological and synthetic vesicles

2.1. Optical tweezers

Optical tweezers (OT) are used to trap and manipulate microscopic objects using light [17]. In 2018, the Physics Nobel-prize was awarded for the development of this technique. OT date back to the work of Arthur Ashkin in 1970, who discovered that beads of micrometer size were attracted towards the focus of a laser beam [17,18]. Further research was needed until in 1986 Ashkin et al. presented the first single trap optical tweezers in a liquid environment [19]. Only one year later, Ashkin used the tweezers to manipulate viruses, bacteria and cells by using infrared light [20,21]. Compared to trapping with visible light, infrared light is less absorbed by the biological sample, and thus optically-induced damage is reduced. The working principle of OT is based on the trapping of microscopic objects using a tightly focused laser beam. The laser beam exerts radiation pressure on the microscopic object which is sufficient to trap small objects within its focus [17]. By means of beam splitters the number of traps can be increased yielding different set-up configurations. Thus, it is also possible to trap multiple beads in an OT experiment. For instance, in a dual-trap set-up, two beads are trapped at different locations. Although OT is a sensitive technique that allows for precise measurements of the forces that are exerted on a trapped bead, it is also often used simply as a way to fix the position of the bead. Alternatively, a micropipette can also be used to trap a second bead, or to manipulate GUVs (see below). The use of OT in biology has steadily increased over the years and is now wide-spread [22-28]. Examples include the investigation of DNA-protein complexes, protein-protein interactions [25,29,30], the activity of individual motor proteins [25,28], cell membrane elastic properties [24] and protein folding [31]. Below, we discuss experiments performed on vesicles.

By means of dual-trap OT, hemifusion of vesicles supported by beads was induced by the presence of the calcium-sensor protein Doc2b [32]. Doc2b is a Ca2+-sensor protein involved in the secretion of neurotransmitters and endocrine substances. The used set-up consisted of two optical traps to catch beads coated with SNARE-free membranes. The beads were placed in a reservoir in which fragments of Doc2b could bind phospholipids in the membrane if Ca2+ was present. By bringing the two trapped beads in contact and separating them again, the effect of the Doc2b domain 2AB on the interaction of both membranes was investigated. An increase in force is an indication of interaction between the membranes, which however does not necessarily mean they have fused. By combining fluorescence microscopy and force spectroscopy in the OT set-up, it was possible to directly quantify and visualize fusion events simultaneously by imaging lipid exchange and the interaction forces, respectively. Thereby, hemifusion and full fusion events could be distinguished. The former is characterized by the occurrence of lipid mixing only, and the latter by lipid and content mixing [32]. Both of these processes depend on Ca2+ and are a function of Doc2b concentration. The dependency of stalk (tether) formation between the beads after retraction upon contact on phosphatidylyserine and Ca2+ and protein fragment was discussed. In presence of the phospholipid, calcium and Doc2b, a broad distribution of rupture forces together with an increased probability of stalk formation was observed while there was hardly any interaction when one of the components was missing.

In a follow-up study, Sorkin et al. [33] moved on to compare two calcium-sensor proteins – Doc2b and Synaptotagmin-1 (Syt1) – to elucidate the mechanism of Ca2+-induced fusion triggering. Using again SNARE-free synthetic membranes to coat the beads, it was found that
the C2AB domain of Syt1 induces membrane interactions that are 
Ca\(^{2+}\)- and protein-dependent. Comparing similar experimental conditions (e.g. same concentrations), a difference in the hemifusion probability was observed; for low protein concentrations, the probability of hemifusion was higher for Doc2b compared to Syt1. Furthermore, different configurations were investigated with this dual-trap set-up: ‘symmetric’ and ‘asymmetric’, referring respectively to protein being present on both or only on one of the membrane-covered beads. It was shown that Syt1 favors an asymmetrical configuration, and therefore protein-
membrane interactions, while Doc2b prefers a symmetrical configuration, and thus protein-protein interactions.

The examples above nicely illustrate the use of OT to unravel the effects of proteins in membrane fusion. However, the information about the physical properties of the membranes is limited. Another approach used a single-trap OT set-up to bring a trapped bead in contact with a GUV. By applying a controlled hydrodynamic flow dragging the GUV away from the trapped bead, a tether is formed [34]. Depending on the flow velocity, the maximum length and diameter of the tether varied;

![Image of micropipette aspiration](https://example.com/image.png)

**Fig. 1.** Micropipette aspiration enables obtaining material properties of membranes. A) A giant vesicle aspirated inside a micropipette. The values of applied tension are given. Note that higher tensions increase the aspirated projection of membrane inside the pipette. B) and C) The increase in apparent area as a function of applied tension. Fitting the data in the low and high-tension regime gives the membrane’s bending rigidity and stretching elasticity, respectively. Measurements for individual stearoyl phosphocholine C18:0/1 and dilinolenyl phosphocholine diC18:3 vesicles. D) A GUV with encapsulated phase-separated polymer solution forms membrane nanotubes that accumulate at the polymer-polymer interface (arrowheads and inset). These nanotubes can be reabsorbed into the main vesicles by applying an external tension with the help of micropipettes. Panels A to C reprinted from Biophysical Journal (ref. [44]) with permission; Panel D reprinted from Proceedings of the National Academy of Sciences USA (ref. [45]) with permission.
higher flow led to thinner tethers compared to reduced flow velocities. Interestingly, this approach cannot only be used to pull outward membrane tubes, but it is also applicable to form inward tethers within the vesicle. By analyzing the static and dynamic behavior of the tube pulling forces, and accounting for hydrodynamic drag, it was possible to extract fundamental mechanical properties such as bending rigidity, membrane tension and curvature. Relaxation upon inward or outward pulling of membrane tethers led to a comparable behavior, and the obtained values were similar to those reported by other methods [34].

In contrast to the above described studies using OT to trap beads, it is also possible to use light to deform vesicles. This technique is a variation of OT and is called the optical stretcher [35]. Here, a GUV is trapped based on a refractive index gradient generated by different solutions inside and outside of the GUV. For instance, glucose and sucrose solutions can be used to tune the refractive indices. At the start of such an experiment, the vesicle fluctuates because of the available excess area of membrane. The optical force induced by the increased laser power caused a deformation along the laser beam axis and simultaneously reduced fluctuations, resulting from the increase in area strain. The bending rigidity was determined by calculating the area strain as a function of lateral tension and turned out to be in good agreement with literature values [35].

2.2. Micropipette aspiration

Micropipette aspiration (MPA) is a versatile, well-established technique for mechanical manipulation of microscopic objects. It was first introduced in the 1960s [36,37] and later refined for the study of the mechanics of red blood cells and artificial lipid vesicles [38–40]. The method consists of using a glass capillary, the pipette, hydraulically connected to a water reservoir that controls the applied hydrostatic pressure through the pipette via vertical displacements of a water column (the reservoir). When the height (hres) of the reservoir is identical to that of the micropipette tip, the applied pressure (P) is zero, and hence the net flow is zero. This can be easily observed by placing the pipette tip near a light object, which should not move. Conversely, moving the reservoir up or down will result in a pressure difference (∆P), positive and negative, with the objects moving away or towards the pipette’s interior, respectively. In that case, ∆P = ρghres, where ρ and g are respectively the density of water and the gravitational pull. For aspiration, negative pressure is required, whereas for the injection of substances inside or in the surroundings of the vesicle, positive pressure is used.

As with most micromechanical manipulation techniques [1], MPA can be used either to detect the mechanical response of the aspirated objects, functioning as a mechanical sensor, as well as for actively probing membrane mechanics by inducing tension. In other words, it can both sense and apply tension. It also affords a fine control of the applied pressure and enables obtaining dynamical information by applying tensions at varying rates. Mechanical sensing and load application can be applied from very small areas of a few micrometers, to a large segment of the membrane covering most of the aspirated vesicle/cell, where local and global information on mechanics can be obtained.

In a typical experiment, a lipid vesicle (or a cell) is mechanically aspirated inside a glass micropipette through the application of negative pressure. In general, the experiments are performed under a microscope, using bright field, differential interference contrast (DIC), phase contrast, or fluorescence imaging. A typical DIC image of an aspirated vesicle is shown in Fig. 1A. As the technique relies on optical observation of the sample, it requires samples of micrometer size. The ∆P applied over the vesicle is related to the caliber of the micropipette and can be converted to membrane surface tension (σ) according to

\[ \sigma = \Delta P \frac{R_p}{2 \left(1 - \frac{R_p}{R_v}\right)} \]  

where \( R_p \) is the pipette radius and \( R_v \) is radius of the vesicle (or cell) [41]. Aspiration takes advantage of the fact that lipid bilayers are incompressible materials (the area between lipid molecules is virtually constant) and yet they are very soft, and thus very easy to deform. The applied pressure in non-supported membranes creates a local tension that is nearly instantaneously distributed across the whole surface within milliseconds [42], and it leads to an increase in the apparent area of deformation (ΔA), as

\[ \Delta A = 2\pi R_p \Delta P \left(1 - \frac{R_p}{R_v}\right) \]  

where ΔP is the change in the aspirated part of the membrane inside the pipette [43,44]. Due to its incompressible nature, the area that is pulled inside the pipette is not necessarily a result of an increase in actual area, but might simply be a result of the “usage” of the hidden excess membrane that was stored in membrane fluctuations [43,44]. Pulling out this excess of membrane requires only very low tensions and this is dominated by the membrane’s bending rigidity, \( \kappa\) [40,41]. Note in Fig. 1A that with the application of a low tension (0.2 mN/m), a segment of the vesicle is aspirated inside the pipette, and the length of this segment increases with an increase in the applied tension (to 6.2 mN/m).

Generally, experiments are performed with vesicles that had been slightly osmotically deflated, providing enough area that can be used for deformation, and ΔA is recorded for small increments in tension. Fig. 1B shows the typical increase in the apparent area (ΔA) as a function of surface tension for GUVs composed of stearoyl phosphocholine (C18:0/1), a lipid with a double bond in one of its hydrophobic tails, and dilinoleoyl phosphocholine (diC18:3), a lipid with three double bonds in one of its tails. Note that the tension axis is in a logarithmic scale. Fitting the data in this low-tension regime gives the membrane’s \( \kappa\). From the data, diC18:3 membranes are softer (lower \( \kappa\)) than C18:0/1 membranes, and thus increasing the number of double bonds softens the membranes [44]. In fact, more recent optical tweezers experiments showed that the low \( \kappa\) of membranes rich in lipids with highly unsaturated acyl chains facilitates membrane deformation and fission in cells [46].

Once the excess area is consumed, further increase in tension results in membrane stretching, and much higher tensions are required to produce smaller and linear increments in area (Fig. 1C for the same vesicles as in Fig. 1B). This transition from membrane bending to stretching occurs at a tension of ~0.5 mN/m [44]. In the high-tension regime, deformation is dominated by membrane cohesion, given by the stretching elasticity (Ks), and it is characterized by an actual increase in the area per lipid. Note that the effective area of lipids can only increase by ~3% [40,44]. Ks can be obtained by fitting ΔA vs. tension. Despite the large differences in \( \kappa\), these two membranes exhibit similar Ks. In fact, although membrane rigidity is very sensitive to the type of lipids in single component membranes [41], Ks is nearly constant [44]. In stark contrast, the inclusion of even small fractions of cholesterol increases both \( \kappa\) and Ks, and this increase scales with the cholesterol fraction in the membrane [40], although this behavior is not universal but rather depends on the lipid types [47,48].

If the vesicle is initially tense, none or only a minute area can be aspirated. In this case, the micropipette-induced additional pressure can lead to membrane lysis and the vesicle is completely aspirated inside the pipette. For a given lipid composition and aspiration rate, membrane rupture occurs at similar tensions, and this characterizes the membrane’s lysis tension (σlys). Thus, σlys can be easily measured by micropipette aspiration as the tension at which membrane ruptures. From Fig. 1C, C18:0/1 can stand higher tension before rupture, displayed by its higher σlys. In fact, lysis is related to membrane cohesion, given by Ks, and experiments with micropipettes have shown that σlys is a linear function of the membrane’s stretching elasticity [40]. Importantly, σlys is loading rate (tension/time) dependent [49]. For
example, from Fig. 1C, the lysis tension for C18:0/1 membranes is around 8 mN/m for very low loading rates, whereas with a load rate of 10 mN/m/s, it increases to ~30 mN/m [50].

The experiments above were performed with vesicles that presumably have zero spontaneous curvature (c), a parameter related to the equilibrium shape of the membrane in the absence of an external tension. Membranes displaying zero c will form (quasi-)spherical shapes, whereas c will lead to other shapes (i.e. buds and tubes) [51]. Because c has a mechanical origin, it can be measured by vesicle aspiration. Upon phase separation of polymers encapsulated inside the GUVs, caused by an increase in osmolarity of the medium, it was shown that the excess of membrane created was not used to induce membrane fluctuations, but rather to form lipid tubes [45]. Due to interfacial tension between the polymer media, the nanotubes accumulated at the polymer interface, as shown in Fig. 1D (see arrows and inset). If mechanical tension is applied, these tubes can reincorporate into the main vesicle (see Fig. 1D), and the process is reversible. An increase in the fraction of glycolipid GM1 in the outerleaflet increases c and decreases tube diameter. Previous micropipette measurements demonstrated that leaflet asymmetric distribution of GM1 decreases κ [52]. Unlike GM1, the presence of symmetric PEG polymers on both leaflets increases κ due to steric hindrance, and this scales with the molecular weight and molecular fraction of PEG, whereas κ_{R} is seemingly constant [53]. Interestingly, κ_{R} is also reduced in the presence of PEG, which helps to explain the long pore lifetime in membranes with anchored polymers [54]. Still a direct comparison is difficult since the polymers were symmetrically (in the case of PEG) [53], or asymmetrically distributed (in the case of GM1) in the membranes [52,54,55], and asymmetry is known to modulate membrane mechanics [56]. The examples above show that from a single MPA measurement, it is possible to determine a number of key membrane mechanical parameters, including κ, κ_{R}, κ_{syn} and c.

Another way of extracting mechanical information from micropipette-aspirated vesicles is by adding another mechanical point of contact to the system that can be used to pull a membrane tether. Membrane tethers are thin membrane tubes mechanically pulled from vesicles and cells and have been classically used to measure membrane mechanics [57–59]. Their mechanics are well understood [60,61]. These nanotubes are usually extracted by pulling a spherical bead that is strongly attached to the membrane (e.g. via biotin-streptavidin bonding) with the help of a second pipette or with optical tweezers. In addition to providing information about membrane mechanics, lipid tubes have more recently been recognized as a useful platform for the study of membrane curvature sensing and generation [61,62]. The radius of the nanotube (R_{t}) can be obtained mechanically from the area conservation relationship upon increases in nanotube length using a second pipette

\[
R_{t} = \frac{\Delta L_{p}}{\Delta L_{e} \left(1 - \frac{R_{t}}{R_{c}}\right)}R_{c}
\]

(3)

in which ΔL_{p} and ΔL_{e} are the differences of the projection length and tether length, respectively [63,64]. The membrane-attached bead can also be pulled by using OT, as shown in Fig. 2A. In this set-up, both membrane tension, set by the aspiration pressure, and tube length can be controlled, and the force (F_{t}) applied on the tube is given by [60,65]

\[
F_{t} = 2\pi \sqrt{2\kappa \sigma}
\]

(4)

Fig. 2B shows the dependence of the square root of tension versus tube force for membranes in the liquid ordered phase (squares) and in the liquid disordered phase (circles), where κ is obtained from the fit [65]. From the data, it is clear that ordered membranes are stiffer than disordered membranes. In fact, since membranes with very low κ offer little resistance to bending, the formed nanotubes are very thin and typically below optical resolution. In contrast, nanotubes formed on stiffer membranes can be large enough to be resolved with optical microscopy [66].

From the mechanical data, R_{t} can also be measured according to

\[
R_{t} = \frac{\kappa}{\sqrt{2\sigma}}
\]

(5)

Alternatively, R_{t} can be obtained from fluorescence even if its diameter is below optical resolution. With fluorescently labelled membranes, tube fluorescence scales inversely with its diameter. The changes in fluorescence associated with tube thinning upon increases in tension can be used to measure tube radius using a fluorescence lipid reference (provided that this reference lipid shows no curvature preference) according to

\[
R_{t} = F \left(\frac{I_{t}^{0}}{I_{t}^{f}}\right)
\]

(6)

in which I_{t}^{0} and I_{t}^{f} are the lipid fluorescence in the tube and in the vesicle, respectively, and F is the calibration factor from the measurement of tube fluorescence versus tube radius as measured with optical tweezers [67]. More recent methods have been used to quantify the tube diameter in the absence of mechanical manipulation [68], which can be advantageous since they do not require any micropipettes or force apparatus.

From the relations above, it is clear that molecules that positively curve the membrane (concave shape) decrease the force necessary to pull a tube. This was shown for the curvature generator amphiphysin; at high enough concentrations (0.4 μM for DA-N-BAR domain of amphiphysin), the force to pull a tube decreased to zero as expected for tube-generating proteins [70]. Conversely, the force is increased if negative curvature is induced. Not only macromolecules but also ions can significantly induce membrane curvature and tubulation [71,72]. The possibilities to control membrane tension, force, tube diameter as well as to obtain physical-chemical information from fluorescence permit not only to measure but also to control many parameters from a single experiment. For example, Fig. 2C and D show the sorting of the lipid analogue DHPE and the glycolipid GM1 in nanotubes in vesicles of different compositions. In thick tubes (~70 nm), both fluorescence signals are similar (upper row). In thinner tubes (~20 nm), GM1 relative fluorescence decreases as it is excluded from more curved tubes as a result of a compositional sorting [69]. As shown in Fig. 2E, sorting depends on membrane composition. Lipids are equally distributed in the tubes and vesicles in membranes far from phase separation (open data points), but are positively sorted (enriched) in the tubes for membranes close to phase separation (filled data points). These findings were confirmed by other studies [64,73]. More complex lipids such as a cardiolipid, a cone-shaped lipid with four hydrophobic tails, do in fact sense membrane curvature in membranes far from phase separation, and are sorted in the inner leaflet of extruded nanotubes, as expected from its geometry [74].

Transmembrane proteins can also sense curvature and can be sorted in regions of curved membranes according to their molecular shape [75]. This curvature sensitivity can modulate underlying biophysical processes such as molecular mobility; the curvature-sensitive protein KvAP potassium channel increases its mobility under tension, whereas the curvature-insensitive Aquaporin 0 does not [76]. These experiments have revealed that curvature can be sensed or generated by curvature-sensitive proteins depending on its molecular geometry and concentration [67,77,78]. Additionally, it can influence the protein’s activity, such as polymerization [79]. The combination of micropipette aspiration and OT has also enabled measuring the force associated with tube scaffold generation and constriction by curvature-sensing proteins that ultimately lead to membrane fission [80–82]. As expected, fission occurs more easily on softer membranes and is promoted by an increase in membrane tension [83].

Combination of membrane aspiration and tube pulling with advanced fluorescence techniques can provide additional information on membrane mechanics. Membrane tension is a very important
mechanical parameter, but it can only be measured with complex techniques such as MPA or tube pulling with OT. Colon et al. developed a tension-sensitive fluorescent probe that changes its fluorescence lifetime under tension [84]. This probe was validated for specific membrane tensions set and measured on GUVs by micropipette aspiration, from which a calibration curve was built. It was then used to measure membrane tension in live cells [84] and intracellular organelles [85]. Although it requires the use of sophisticated FLIM (fluorescence-lifetime imaging microscopy) set-ups, the probe has the advantage that it can report tensions in membranes that are not accessible by mechanical manipulation in a high throughput manner.

Micropipettes can be used in applications that do not necessarily involve aspiration. For example, it can be used to locally inject controlled amounts of substances in the surroundings of single cells and vesicles to study effects of membrane-active substances [86], or to test the effects of curvature sensing proteins locally injected near the membrane tubes using a second micropipette. The changes in vesicle area and volume upon protein binding to membranes were shown to depend on the density of bound proteins [87]. Another study showed that the addition of lysolipids not only increases membrane area as expected, but also reduces bending rigidity, which was shown to facilitate membrane budding mediated by COPII proteins [88]. It also revealed that the location of membrane fission in tubes coated with the fission protein dynamin occurs at the interface between the membrane coated and the bare membrane part [83], although this view has recently been challenged using other membrane tube assays [89]. More unusual applications include using pipettes to simply hold two GUVs to induced controlled membrane fusion [90,91], or to apply shear flow on the cell membrane that triggered the activation of mechanically-sensitive intracellular signaling [42].

2.3. Electrodeformation and electroporation

Electric fields provide another way of manipulating micrometer-sized objects. Compared to other micromanipulation techniques, the use of electric fields has important advantages: it allows contactless manipulation, minimizing the risk of contaminating the samples, and is usually much cheaper and easier to apply in comparison with techniques such as MPA, OT or atomic force microscopy (AFM). The electric field can either be applied via an alternating current (AC) or a direct current (DC). DC fields are normally generated by electric pulses. Cells and vesicles can exhibit a variety of responses to the electric field.

Fig. 2. Mechanical properties of vesicles with extended lipid nanotubes. A) A portion of the GUV is aspirated with a micropipette (left white lines) and a lipid nanotube (right white box) is pulled with the help of a membrane-attached bead that is moved away from the membrane by using optical tweezers (right white circle). The membrane is fluorescently labelled. B) Plot of the square root of tension versus the force on the OT for ordered (square) and disordered (circle) membranes. The fit is used to calculate $k$ according to Eq. (4). C) Fluorescence of lipid nanotubes labelled with the lipid analogue DHPE (red) and the peripheral protein GM1 for thick and thin tubes. The fluorescence intensities are shown in D). E) Sorting of DHPE as a function of tube radius (curvature) for membrane far from (open data) and close to (closed data) phase separation. The ratio of BSM:chol:DOPC is shown in the legend. Scale bars 5 μm. Panels A, C-E reprinted from Proceedings of the National Academy of Sciences USA (ref. [69]) with permission; Panel B reprinted from EMBO Journal (ref. [65]) with permission.
depending on the way the electric field interacts with the membranes [92,93]. Possible responses include electrophoretic movement used for transporting vesicles and cells, as well as electrodeformation and electroporation, used to extract mechanical information and for the intracellular delivery of membrane-impermeable substances, respectively.

The application of an electric field to a membrane-bound object results in the accumulation of charges across the membrane and the creation of a transmembrane electric potential $V_m$ [94]. This potential builds an effective force that, for a spherical vesicle of radius $R_v$, is

$$V_m = 1.5R_v \cos \theta |E| \left(1 - \exp \left(-\frac{t}{\tau_{\text{charge}}} \right) \right)$$

where $\theta$ is the angle between the surface of the electrodes and the membrane normal, $E$ is the electric field, $t$ is the time and $\tau_{\text{charge}}$ is the membrane charging time [94,95], given by

$$\tau_{\text{charge}} = R_v C_m \left(\frac{1}{\lambda_{\text{in}}} + \frac{1}{2\lambda_{\text{out}}} \right)$$

in which $C_m$ is the membrane capacitance and $\lambda_{\text{in}}$ and $\lambda_{\text{out}}$ are the medium conductivities inside and outside the vesicles, respectively [94,95]. From the equations above, the electric potential drop across the membrane depends on operational parameters such as the applied field strength, on membrane properties such as its capacitance, and on medium properties. The medium conductivity can be tuned by the presence of ions, whereas membrane capacitance is a material property and depends on membrane composition. The membrane capacitance can be measured on GUVs using AC fields [96]. The tension applied on the membrane by the electric field ($\sigma_{el}$) is given by

$$\sigma_{el} = \epsilon_m \epsilon_0 \left(\frac{h}{2h_b}\right) V_m^2$$

where $\sigma_{el}$ and $\sigma_0$ are the permittivity of membrane and vacuum, respectively, and $h$ and $h_b$ are the bilayer total and dielectric thickness, respectively [97,98]. These relations are important to calculate the mechanical effects applied by the electric fields on the membrane.

Since electric fields can induce mechanical tension, it can be used to deform a vesicle in a similar fashion as with MPA or the optical stretcher method. The electric tension increases the membrane's apparent area $\Delta A$ according to

$$\Delta A = \frac{k_B T}{8\pi \epsilon_0} \log \left(\frac{C_0}{\sigma_{el}} \right)$$

where $k_B$ is Boltzmann's constant and $T$ is the absolute temperature, and $C_0$ is the positive parameter obtained by extrapolation to $\sigma_{el} = 0$ [98]. For low tension and for vesicles with excess area, electric fields of low magnitudes can be used to pull the membrane and the increase in membrane area in this low-tension regime is dominated by membrane's bending rigidity.

For small increments of the AC field, discrete increases in area can be pulled (Fig. 3A). The area is represented simply by the vesicle’s aspect ratio $a/b$ (see figure inset) [47,98]. Note that for a given tension, the projected area is stable since the system is in equilibrium. Plotting the relative area change as a function of log($\sigma$) gives a slope that is given by $\gamma$ (Fig. 3B). Using these approaches, Dimova's group has measured $\gamma$ for a number of different membrane compositions. They showed that the bending rigidity of DOPC membranes containing increasing fractions of cholesterol is nearly constant [47], despite the fact this was initially unexpected. A wealth of data on the effects of cholesterol on membranes indicated an increase in $\gamma$ for higher fractions of cholesterol [41,44]. However, it was shown that such effects are valid only for membranes with saturated or monounsaturated lipids [99], but not for lipids with unsaturation in both lipid tails such as DOPC. These findings were further supported by X-ray scattering [100] and micropipette aspiration [69].

The bending rigidity can also be measured with DC pulses. When an electric pulse is applied to a vesicle, the vesicle deforms, and the degree of deformation depends on the field strength and excess area. At the end of the pulse, the vesicle relaxes back to its initial shape. In the absence of excess area, relaxation depends on the properties of the membrane such as stretching, viscosity and bending rigidity [102]. Yu et al. have recently developed a theoretical analysis, supported by experiments, in which $\kappa$ and $\sigma$ can be obtained from the relaxation of vesicles upon DC electrodeformation [103]. In this case, although relaxation time depends on intrinsic and uncontrollable properties such as vesicle size and excess area, mathematical analysis of relaxation dynamics led the data to fall into a master curve from which $\kappa$ and $\sigma$ can be obtained. With this new method, they confirmed previous findings on the increase in $\kappa$ in POPC membranes as a function of cholesterol. This method has the advantage that it is experimentally much simpler than MPA and data can be obtained in much shorter time.

Another popular use of electrodeformation is on the quantification of membrane area changes. An example is the photooxidation of porphyrin-PE, a photosensitizer molecule bound to PE lipids, whose molecular area increases upon (photo)oxidation [101]. Illumination of porphyrin-PE GUVs leads to an increase in GUV area monitored in real time (Fig. 3C). As expected, the increase in vesicle apparent area scales with porphyrin-PE molar fraction (Fig. 3D and E). A similar approach has been used to measure the area increase associated with the insertion of detergents in membranes. Mattei et al. showed that addition of Triton X-100 to GUVs increases the vesicle area, and this increase is related to Triton X-100 partitioning in the membrane [104]. Knowing the molecular area of the components, it is possible to precisely calculate the number of molecules inserted in the membrane. Electrodeformation was also used to measure the growth of GUVs fused with multiple large liposomes. In the initial stages of fusion, when not much fusion had occurred, the incorporated area can be completely pulled by the electric field. On the other hand, when too many liposomes had fused, the GUV's tubulate due to an increase in membrane curvature [105].

If the applied electric field is too strong and exceeds the membrane's lysis tension $\sigma_{ly}$, the membrane ruptures and one or more pores are formed. The threshold is the so called the critical transmembrane potential, $V_c$, and it is given by

$$V_c = \frac{3}{2} E \sqrt{R_e^2 - r^2}$$

where $R_e$ and $r$ are the GUV and the pore radii [106]. $V_c$ is a material property that depends on membrane composition and can be measured by applying increasing pulses, starting from subthreshold pulses, until a pore of radius $r$ is detected in the membrane. When pores are formed, the membrane conductivity largely increases and the voltage across the membrane can no longer be held. This process is called electroporation, and it has been largely used for the insertion of foreign materials into cells [107]. In cells, pore lifetimes are in the order of seconds to minutes, and lifetime is dominated by cellular factors (i.e. membrane composition, anchorage to the cytoskeleton, etc.) [108]. In synthetic lipid vesicles, pore lifetime is in the order of a few hundreds of milliseconds [102,109].

Pore lifetime is controlled by the membrane's edge tension ($\gamma$), the energy per unit length of the pore that drives pore closure. It arises from the geometrical arrangement of lipids on the pore rim in order to avoid exposure of their hydrophobic tails to water, forming a hydrophilic pore in the membrane [110]. Edge tension can be measured by vesicle electroporation, and typical values for lipid bilayers lie around 10–50 pN [106]. Due to high $\gamma$, pores formed in pure lipid bilayers spontaneously close. According to the positively bent pore geometry, molecules that display an inverted-cone shape (i.e. micelle-like structures) reduce $\gamma$, whereas cone-shaped molecules increase $\gamma$ [106,111]. Because both lysis and edge tension determine how easy it is to open or close pores in the membrane, respectively, it is expected that their
balance will eventually determine membrane stability. In fact, the addition of cholesterol increases membrane stability by increasing both $V_c$ and $\gamma$ \cite{106,112}. The physical obstruction of pores by stiff materials (i.e. solid polymers) \cite{109,113}, and by physically supporting the membrane \cite{114} can artificially increase pore lifetime, and these examples help to explain why pores formed in cells are long-lived. Recently it has been shown that membrane charge density controls $\gamma$; highly charged membranes in the absence of salt display $\gamma$ values that are below the edge tension threshold necessary to keep membranes sealed. As a result, pores formed (by electric pulses) in these membranes open indefinitely and the whole vesicle collapses \cite{115}. The process can be reversed by the addition of salt, screening the charges and therefore increasing $\gamma$ to values high enough to maintain membrane stability.

2.4. Atomic force microscope and nanoindentation

Atomic force microscopy (AFM) has become one of the essential tools for the study of mechanobiology \cite{2}. One of its prime advantages is the possibility to study the biological sample of interest at the nanometer scale in liquid, and thus under close-to physiological conditions. On this point it outcompetes optical-based techniques such as MPA, electrodeformation and -poration. Additionally, and similar to OT, AFM provides subpiconewton force resolution. AFM allows temperature control and the characterization of the sample in terms of morphology and mechanics. To probe the mechanical properties of membranes, the tip is pushed onto the sample, applying a set force while recording a force-distance curve \cite{2}. Fig. 4A shows the operating principle of an AFM set-up.

There are multiple modes for imaging with AFM. The most commonly used modes are contact mode, oscillating mode and jumping mode. In contact mode, the scanning probe is continuously in contact...
with the surface of interest, and it is continuously applying a force to the sample. When imaging a relatively flat and hard surface, this mode provides an outstanding resolution and is fast and straightforward at the same time [116]. However, while working with soft, fragile biological samples, the continuous application of force leads to an increased probability of damaging the sample during imaging. In order to reduce the forces applied to the sample, the tip can be oscillated near or at its resonance frequencies. During a scan, most of the time the tip is not in contact with the sample and thus not applying a vertical or lateral force to the sample. Forces are only briefly applied, and only when the tip is touching the sample. An even more gentle operation mode is based on force spectroscopy AFM and is called “jumping mode” [117,118], “quantitative imaging (QI)” mode [119] or “PeakForce mode” [120]. In this imaging mode, force vs. distance curves are acquired at each pixel and the cantilever is moved laterally when it is not in contact with the sample and thus, minimizing shear forces. It allows imaging with forces below 100 pN [116,121], thereby minimizing force-induced damage.

In order to extract mechanical properties with AFM, nanoindentation experiments can be performed. During these experiments, the sample is indented with the cantilever tip until a pre-set force. In Fig. 4B and C a schematic representation of an indentation of a vesicle and an exemplary indentation curve acquired on a red blood cell (RBC) EV are shown. To analyze these force curves, theoretical models are used to extract the mechanical properties, e.g. Hertz, Sneddon and thin shell model, depending on the sample conditions and the geometry of the indenter. In reference [2] an overview of the most commonly applied models is provided, including their limitations. To perform nanoindentation experiments on vesicles and membranes, the substrate used should allow for a stable attachment, but preferably with little deformation of the sample due to adhesion. The use of a variety of substrates has been reported [123], e.g. glass coverslips coated with poly-L-lysine [121,122,124,125], BSA [126–128] and antibody-functionalized substrates [129–131].

AFM nanoindentation is the only technique that is able to measure the rigidity in submicrometer-sized vesicles. Experiments on artificial liposomes revealed a variety of responses [127,132–134]. For instance, by nanoindenting different liposome compositions, a decrease of the bending rigidity from solid ordered via liquid ordered to the liquid disordered phase state was reported (liquid-disordered (Ld) < liquid-ordered (Lo) < solid-ordered (So)) [127]. This agrees with literature showing the bending rigidities obtained for the different liposome compositions and a schematic representation of the different phase states. After calcine encapsulation inside these vesicles, as a model hydrophilic drug, the permeability coefficient of saturated lipid-based liposomes was studied with spectroscopic methods [126]. By measuring the leakage of calcine, it was demonstrated that the liposome bending rigidity is a quantitative parameter that relates to liposomal membrane permeability.

The link between mechanics and drug delivery efficacy of SUVs was studied using different lipid compositions and AFM nanoindentation [9]. It was shown that an increase in the bending rigidity goes hand in hand with increasing acyl chain length and saturation, in agreement with measurements performed on GUVs using different techniques, e.g. micropipette aspiration [136,137]. For differently composed liposomes, varying bending rigidities were found. Vesicles with long and fully saturated chains were stiffer than liposomes made from unsaturated chains. The softest liposomes were made from lipids with saturated or unsaturated but short chains (below 14 carbons) [9]. The accompanying cellular internalization experiments revealed that liposomes displaying intermediate stiffness are the most promising particles for pancreatic tumor therapy due to their increased deformability as compared to stiff vesicles, and higher diffusion velocities as compared to soft vesicles; in summary, a better penetration in the extracellular matrix [9,11].

While these experiments revealed valuable insights into vesicle dynamics, it turned out that it is not straightforward to extract consistent, quantitative mechanical properties from the AFM experiments. For instance, the shear modulus of fluid bilayers is often negligible and this is not always taken into account in the analysis. Taking membrane fluidity into consideration, a model based on Canham-Helfrich theory was developed to describe the deformation of small vesicles [125]. This model allows to extract the membrane bending rigidity κ using the Young-Laplace equation ΔΠ = 2κR−1 for the osmotic pressure and the expression for the so-called tether force \( F_t = 2\pi\sqrt{2\kappa} \) (see also Eq. (4)). Here, \( \sigma \) is the tension in the membrane, \( R \) is the radius of curvature of the vesicle, \( \Delta \Pi \) is the pressure difference across the membrane and \( F_t \) is the tether force measured in the retract curve of a nanoindentation experiment when a membrane tether is pulled out of the indented vesicle. This leads to the following relation

\[
\Delta \Pi = \frac{F_t^2}{4\pi^2 R_0 \kappa} \tag{12}
\]

Plotting the normalized stiffness \( (KR_0^2/\kappa) \) against the normalized pressure \( (\Delta \Pi/R_0^2) \), the bending rigidity \( \kappa \) can be extracted by fitting the data to the theory [125]. In this expression, \( K \) is the stiffness of the vesicle obtained by linearly fitting the force-indentation curve in the range 0.02–0.1 R0. It turns out that the stiffness of adhered vesicles is
dominated by the pressure built up due to adsorption. Furthermore, it was shown that the actual indentation response importantly depends on the tip size of the probe [125,138].

In addition to unilamellar vesicles, multilamellar vesicles have also been mechanically characterized [139]. Nanoinindentation experiments revealed that it was possible to distinguish uni- and multilamellar vesicles up to 5 bilayers, which was confirmed by cryo-EM. When more bilayers are present, the particles are more spherical and they exhibit an increase in their stiffness (~20% per added bilayer). In Fig. 5B and C, an exemplary indentation and the degree of lamellarity in relation to the stiffness are shown. The bilayers are identified by small breaks in the force-indentation curves. The inset in panel B of Fig. 5 shows a zoom on those events. In panel C, the stiffness measured as the slope of the initial indentation curve is plotted against the degree of lamellarity of the particle. A linear trend is observed, defining an additional stiffness of ~2.7 × 10⁻³ Nm⁻¹ per added bilayer. In fact, measurements of membrane stretching elasticity and bending rigidity have been classically used to characterize the lamellarity of GUVs [140].

In the previously mentioned OT experiments on Syt1 and Doc2b that revealed their influence on hemifusion probability (see Section 2.1), the bending rigidity of liposomes incubated with these proteins was determined by AFM [32]. It was found that both proteins reduce the membrane bending rigidity compared to liposomes without protein. Interestingly, the reduction of bending rigidity occurred for Doc2b at a lower concentration (~0.9 μM) than for Syt1 (~20 μM). Those measurements supported the findings with optical tweezers and suggest an active role of the protein (domain) in membrane remodeling during the fusion process.

AFM nanoindentation experiments were also performed on vesicles derived from cellular components. The mechanical properties of yeast cell plasma and inner membrane vesicles revealed a lipid composition-independent rigidity, pointing to the influence of membrane proteins on vesicle stiffness [141]. SUVs made from influenza lipids did not show a major change in elastic behavior upon temperature change, suggesting that the influenza virus has a fluid-like envelope [142]. Vorselen et al. [121] investigated EVs secreted by RBCs obtained from healthy donors and spherocytosis patients. Fig. 6A and B show a typical AFM image of RBC EVs and the difference in EV bending rigidity for healthy donors and spherocytosis patients. The bending rigidity of EVs from healthy RBCs was ~15 k_BT, while the bending rigidity of vesicles released from spherocytic RBCs was ~40% lower compared to their healthy counterparts. This major difference does not seem to be caused by differences in lipid composition, but rather by varying protein content between donor and patient EVs. The authors speculate that a decrease in membrane organization leads to local accumulation of specific membrane proteins lowering the bending rigidity. This in turn lowers the energy barrier for vesicle formation and can be linked to the reported increase in vesiculation in patients with spherocytosis.

The bending rigidities of EVs from malaria infected and non-infected RBCs were also studied. Unexpectedly, they displayed no significant difference in their bending rigidity [122]. In contrast, artificial liposomes were significantly stiffer compared to their naturally secreted
counterparts. However, it was found that mechanics from RBC EVs were affected by temperature conditions during incubation and different incubation times of the RBCs compared to freshly isolated EVs after blood drawing (see Fig. 6C). Not only the bending rigidity of EVs was lower the shorter the cells were stored at 4 °C, but also the size of the secreted EVs varied. Fig. 6D shows the bending rigidity determined for EVs generated at different temperatures as a function of the total protein fraction [122]. These findings were used to discuss potential vesiculation pathways at different temperatures, related to ATP levels [122].

While the AFM is typically operated in liquid for biological studies, it is also possible to operate the AFM in air. Several publications investigated EVs and membranes in air [130,143,144]. Hardij et al. [129] compared in-air tapping mode AFM with in-liquid PeakForce AFM, to identify the size and number of EVs under different conditions. As expected, they observed heights of 6-10 times higher in liquid compared to topographies acquired when operated in air. Noticeably, the operation in liquid was recommended for height determination and the same holds for mechanical measurements. Besides using AFM cantilevers for nanoindentation experiments, it is also possible to study mechanical properties by adsorbing membranous structures onto microcantilevers. In particular the adsorption free energy of lipid bilayers [145] as well as of EVs was determined upon adsorption onto a microcantilever [146].

In the context of imaging, one of the technique's limitations becomes apparent: the scan speed. Typically, it takes several minutes to acquire a topography image, depending on the parameters used. Efforts have been made to overcome this limitation. In 2001, an approach called high-speed AFM was reported [147]. In the following years the system was improved and in 2008 imaging rates of ~40 ms/frame over an area of ~250 nm × ~250 nm with 100 scan lines were reported [148,149]. However, the system still lacks a force spectroscopy tool for mechanical investigation of the samples. To address this, an alternative approach to probe the mechanical information of small membrane vesicles with increasing imaging force has been reported [150].

Efforts are made to combine different techniques for vesicle studies. Recently, a combination of AFM, scanning electron microscopy and Raman spectroscopy was used to characterize tumor-derived EVs [130]. In this approach, the EVs are captured on stainless steel substrates functionalized with anti-EpCAM (epithelial cell adhesion molecule). By means of position markers on the chip, the same locations were identified in the different instruments. Chemical, morphological and size information could be correlated at single EV level. The measurements were performed in air, but as no absolute size determination was required, this did not affect the outcome. In a complementary

Fig. 6. AFM nanoindentation experiments on EVs. Bending rigidities estimated for EVs from different origins and under different conditions. A) Typical AFM topography image showing RBC EVs bound on an APTES-coated glass slide. Color scale indicates height. Scale bar 1 μm. B) Bending rigidities determined of EVs from three healthy donors and the three spherocytosis patients. Histogram bars indicate means, and error bars indicate standard errors (s.e.m.) of the 3 samples in each condition. Values of individual measurements are marked by black crosses. C) Influence of temperature during incubation of RBCs on the bending rigidity. For comparison the bending rigidity estimated for liposomes mimicking RBC membrane is displayed, too. D) Values of bending rigidities obtained from EVs whose parental cells were incubated at different temperatures plotted against the estimation of total protein fraction. Errors in all bending rigidity estimations mark 68% confidence intervals determined by bootstrapping. Panels A,B reproduced from Nature Communications (ref. [121]) with permission. Panels C,D reproduced from Small (ref. [122]) with permission.
micropipette aspiration approach of GUVs and AFM experiments on small vesicles of identical composition, the mechanical properties of ESCRT (endosomal sorting complex required for transport) protein-covered vesicles were scrutinized [150]. By imaging SUVs with increasing force using a high-speed AFM, the mechanical response of differently coated liposomes was studied. In particular, vesicles coated with the ESCRT protein CHMP2B showed a higher rigidity than bare vesicles. This result fitted with the MPA experiments using GUVs and it is suggested that CHMP2B contributes to the rigidity of ESCRT-III spirals in the cytokinetic bridge. Although the experiments using AFM and micropipettes were performed on different vesicles, unlike the combined use of micropipettes and OT which are performed on the same vesicle, they show good agreement. This highlights the importance of using complementary techniques to study a given process.

2.5. Comparison of different methods

Vesicles and membranes in general are soft and elastic structures that react to mechanical disturbances according to their material properties. Four of the major techniques used to study the mechanical properties of vesicles and their membranes were described here. Notably, these methods share similarities, e.g. the investigation at a single vesicle level, and all techniques do this by actively probing the samples and recording their response. On the other hand, they also have large differences in their sample requirements. This dictates the type of measurement that can be performed and the specific type of vesicle that can be used. An important advantage of using large vesicles is the possibility to directly observe and manipulate them. With techniques, such as MPA, OT and electrodeformation, the mechanical properties of GUVs have been characterized. These techniques work well with free-standing or free-floating membranes, but measurements on adhered vesicles can also be performed. However, in the latter case the vesicles are usually more tense and both the experiment as well as the data analysis becomes more challenging. Typically, in these experiments the vesicles are de-territorialized so as to decrease membrane tension and to provide excess area for deformation. Depending on the degree of stress applied, regimes such as membrane bending, stretching and rupture can be assessed, and they can be used to infer mechanical information from these vesicles. Ideally, several material parameters are obtained from a single experiment performed on one and the same vesicle. This is in fact often the case with these experiments. Classically, MPA or electrodeformation (and -poration) can measure membrane bending rigidity, stretching elasticity, excess area, membrane curvature, permeability and lysis tension from a single measurement. The values obtained by these different techniques are comparable [99]. In experiments with OT, bending rigidity and spontaneous curvature can be assessed, but these measurements usually require the previous knowledge of membrane tension. For this reason, OT experiments are often combined with MPA.

While using optical microscopy is a straightforward and effective way to study large vesicles, most vesicles produced by cells are of submicrometer-size, and hence below optical resolution. AFM is the only method to reliably collect mechanical information from such submicroscopic vesicles. As it physically probes the sample, the vesicles need to be deposited on a support, and thus measurements are carried out on adhered, and consequently usually pre-stressed vesicles. Nowadays, correction methods for this adhesion are available and therefore do not limit this approach [125]. AFM can not only be used for studying synthetic vesicles, but also native vesicles produced directly by the cells. Additionally, AFM allowed successful mechanical characterization of structures combining membranes and for instance a surface protein shell, which were irreversibly damaged when investigated with MPA [151]. Finally, AFM can be applied simultaneously with optical microscopy, e.g. in order to combine the chemical information provided by fluorescence and the membrane mechanics assessed from nanopindentation.

3. Conclusions

Characterizing the mechanical properties of vesicles and membranes is not only critical for a better understanding of vesicle and membrane properties, but also for the understanding of cellular function. In addition it supports the rational design and development of new vesicular systems for biotechnological and medical applications (i.e. drug delivery system). Here, we discussed the major techniques used to actively probe the mechanical properties of natural and synthetic vesicles. With these techniques, a strain is applied onto the system of interest and their material properties are extracted by analyzing the response of the sample to this stimulus. Depending on the sample size, there are different techniques available to study the mechanics of the sample. For instance, micropipettes, optical tweezers and electrodeformation and -poration are applicable to GUVs, AFM nanopindentation is suitable for smaller particles such as SUVs and EVs. Combined approaches, in which different but complementary techniques are used to actively probe the membrane, are becoming more popular. The power of the combined approaches lies in the possibility of extracting several parameters from a single experiment by combining the capabilities of the different technique. In order to obtain a thorough insight, theoretical models are needed to interpret the increasingly complex data collected in these experiments. Using these models, relevant parameters such as the bending rigidity can be extracted. Despite some discrepancies, over the last years a much better understanding of the mechanical properties of vesicles and membranes has been obtained. This provides unique information about the system and deepens our views of biological processes such as cellular organization, particle uptake and vesicle release, under healthy as well as diseased conditions.

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Declaration of Competing Interest

There are no conflicts of interest.

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