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Destabilizing Giant Vesicles with Electric Fields: An Overview of Current Applications

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Abstract This review presents an overview of the effects of electric fields on giant unilamellar vesicles. The application of electrical fields leads to three basic phenomena: shape changes, membrane breakdown, and uptake of molecules. We describe how some of these observations can be used to measure a variety of physical properties of lipid membranes or to advance our understanding of the phenomena of electropermeabilization. We also present results on how electropermeabilization and other liposome responses to applied fields are affected by lipid composition and by the presence of molecules of therapeutic interest in the surrounding solution.

Keywords Deformation · Electric field · Fusion · Giant unilamellar vesicle · Poration

Introduction

Our conception of the role of lipid molecules in biological membranes has dramatically evolved over the past few decades. The lipid membrane was first considered as a purely passive barrier and a substrate to house membrane proteins that carried out the biological functions of the cell membrane. However, it is now widely accepted that lipids play an active role in a number of biological processes. Their physicochemical properties, conferred by their structure and their amphiphilic nature, have consequences well beyond their self-assembly and ability to form a barrier. However, the study of the behavior of lipid molecules in a complex environment, such as a living cell, turns out to be a very difficult task as a result of the presence of other cell constituents such as the cytoskeleton and organelles, and also because of the large number of different lipid species present. Another source of complexity emerges from the presence of various membrane proteins and the cell glycocalyx.

Fortunately, we are now able to form cell-sized artificial membranes with well controlled compositions. These objects are called giant unilamellar vesicles (GUVs), also referred to as giant vesicles or giant liposomes. Their study has become increasingly popular in chemistry, biology and physics laboratories. Their primary interest is probably their size which, ranging from a few to hundreds micrometers in diameter, allows their direct observation via optical microscopy techniques. During the 1980s, Angelova and Dimitrov developed an efficient protocol for GUV production known as electroformation (Angelova and Dimitrov 1986; Mathivet et al. 1996), which was later shown to indeed produce unilamellar vesicles (Rodriguez et al. 2005). Because of the ease of their fabrication and their rich phenomenology, artificial vesicles have received an ever growing interest from the scientific community, as attested by the increasing number of studies using GUVs as membrane model systems. We anticipate that this tendency could even accelerate in the future, as a number of obstacles in the preparation of giant liposomes under physiological conditions have recently been overcome (Estes and Mayer 2005; Horger et al. 2009; Montes et al. 2007; Pott et al. 2008).

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Here we aim to introduce the various phenomena that occur when GUVs are subjected to electric fields. Dimova and colleagues have written comprehensive reviews about the effects of electric fields on GUVs (Dimova et al. 2007, 2009) and about the physics of GUVs in general (Dimova et al. 2006). The present paper is a natural sequel to these articles. It reviews newer results published after 2009, with an emphasis on works from our group. As review paper, it naturally suffers from certain subjectivity and is obviously not exhaustive; however, we hope it will be of use to newcomers in the field or to people familiar with Dimova et al.'s reviews and who wish to know about the most recent developments. It is organized in the following way: we first describe results on the influence of electric fields on giant liposomes, along with their theoretical explanations when they exist. We then describe some applications resulting from these behaviors. In addition, we present some preliminary results on the effect of lipid composition and the presence of poloxamers on the electropermeabilization of GUVs. Some results on the electromediated uptake of DNA by GUVs are also described.

Some Effects of Electric Fields on Giant Vesicles

Both direct current (DC) square pulses and alternating current (AC) sinusoidal fields can strongly destabilize giant vesicles and influence their behavior. Depending on the parameters of the applied field (amplitude, frequency, and number and duration of pulses), giant vesicles can porate, fuse, deform, or even exhibit domain motion in the case of multicomponent liposomes. The purpose of this section is to present these phenomena.

Deformation

To our knowledge, the deformation of giant vesicles by electric fields (or electrodeformation) was first extensively studied by Kummrow and Helfrich (1991). The authors applied sinusoidal AC electric fields of ~ 1 kHz frequency and amplitudes ranging up to 100 V/cm, and found that spherical liposomes deformed into prolate ellipsoids oriented in the field direction, the degree of deformation increasing with the magnitude of the applied field. Ellipsoidal deformation of spherical vesicles in water by AC and DC electric fields had already been predicted earlier (Winterhalter and Helfrich 1988). Monitoring the relative area dilation of the membrane yielded an experimental estimate for the bending rigidity k_c of the lipid bilayer. A few years later, Mitov et al. (1993) explained theoretically and observed experimentally a prolate to oblate shape transition when increasing the field frequency above a few kilohertz. However, these experiments were limited to vesicles in pure water (internal–external conductivity ratio $x = \lambda_{in}/\lambda_{out} \approx 1$), and to AC field frequencies in the kHz range. An extensive study of the influence of the media's electrical conductivities ($x > 1$ or $x < 1$) in a wider range of frequencies f (from 10^2 to 10^8 Hz) was published 15 years later (Aranda et al. 2008). Aranda et al. observed that GUVs could attain oblate, prolate or spherical shapes upon varying x or f, and were able to construct a morphological phase diagram describing four different transitions whose characteristic frequencies depend on the conductivity ratio x. At high frequencies (several MHz), all vesicles were found to adopt spherical shapes. At low frequencies ($f <$ a few kHz), all vesicles deformed into prolate ellipsoids, as predicted in (Winterhalter and Helfrich 1988). The prolate to oblate transition happens at intermediate frequencies for $x < 1$, i.e., when there is more salt outside the liposomes than inside. Another transition of this type could also be triggered at a fixed frequency f by varying x, for example by adding pure water to the sample and thus increasing the conductivity ratio. These behaviors can be qualitatively understood if one considers the motion of ions and the distance they travel in presence of an AC electric field. If the field frequency is large they are virtually immobile, while if the frequency is lowered they can be pushed against the membrane and then modify the vesicle shape. Nevertheless, at that time there was no theory able to fully describe these four transitions as a function of f and x. Winterhalter and Helfrich's (1988) theory did not account for the recovery of the spherical shape at high frequencies, and Mitov's theory could not either (Mitov et al. 1993). A theory explaining the morphological diagram of (Aranda et al. 2008) was developed in (Vlahovska et al. 2009). In this approach, vesicle shape was determined by balancing electric, hydrodynamic, bending and tension stresses exerted on the membrane. This force balance approach also provides information about the kinetics of liposome deformation, unlike the former theories based on energy minimization (Mitov et al. 1993; Winterhalter and Helfrich 1988). A more recent theoretical study (Yamamoto et al. 2010), despite not considering the effect of the electric double layer, provides an explanation for all the features of the morphological diagram of (Aranda et al. 2008). Rigorous evaluation of the work done by the Maxwell stresses, for different frequency and salt conditions, finally enabled to clarify the molecular mechanisms underlying the morphological transitions.

Winterhalter and Helfrich's (1988) model also predicted that DC pulses should deform spherical vesicles into prolate ellipsoids too. However, DC pulses usually have durations of the order of several tens or hundreds of microseconds, or at most of some milliseconds. Imaging at such high rates is impossible with classical video cameras, so if one wants to get insight at what happens during an

electric pulse application, one has to work with a fast imaging setup allowing image acquisition at several thousand frames per second. This approach was first applied by Riske and Dimova (2005), and they indeed found that GUVs deformed into prolate ellipsoids upon the application of electric pulses of 50–300 μs duration and 1–3 kV/cm amplitude. They worked with salt-free solutions, but still at a conductivity ratio of $x \approx 1.3$, meaning that the solution inside the vesicle was more conductive that the outer one. Later, when the same authors wanted to work in conditions closer to physiological ones and to investigate the role of salts in the exterior medium, it was somewhat surprisingly found, that vesicles subjected to electric pulses in salt solutions always adopted cylindrical shapes, irrespective of their ionic content (and thus of the conductivity ratio) (Riske and Dimova 2006). This finding still lacks a quantitative theoretical explanation.

Electropermeabilization

Electric pulses may also have much more dramatic effects on lipid bilayers than simple deformation. They can sufficiently disrupt the membrane as to allow the uptake of otherwise nonpermeant molecules such as Propidium Iodide, plasmid DNA, poloxamer, bleomycin or cisplatin. Depending on the field strength used this can occur in a reversible manner and without affecting cell viability (Neumann et al. 1989). This process, called electropermeabilization, has led to biomedical applications in the fight against cancer, or in the field of gene therapy (Belehradek et al. 1993; Escoffre et al. 2009). Despite its increasing popularity among physicians, biologists and oncologists, no one knows for sure what really happens when subjecting a living cell to a permeabilizing electric pulse. Uptake mechanisms are triggered by the electric potential difference across the membrane $\Delta\Psi$ induced by the field and seem to be dependent on the size and charge of the transferred compounds. Electric potential difference across the membrane is among others influenced also by both membrane surface potentials (inner and outer) which depends on the physical properties of the electric double layer at both sides of the membrane (McLaughlin 1989).

No consensus exists on the way the membrane reorganizes at the molecular level and this question still remains a challenge. Here again, GUVs are convenient tools to investigate these questions. Some studies have been performed and have shown that indeed, giant vesicles can be permeabilized by electric pulses. By coupling electropermeabilization and the micropipette aspiration technique, it was shown that the critical membrane voltage $\Delta\Psi_c$ required for breakdown ranged from 1.1 to 1.8 V, depending on membrane composition (Needham and Hochmuth 1989). It was also observed that a macropore of several micrometers size could be induced (Zhelev and Needham 1993). It was later shown by Tekle et al. (2001) that the mechanism of pore formation is asymmetric. A macropore is formed on the cathode-facing side of the vesicle, whereas the presence of many smaller pores on the other hemisphere can be inferred from the size decrease of the liposome after pulse application. This phenomenon of GUVs shrinkage during electropulsation was then extensively studied by our team (Portet et al. 2009). By applying a sequence of long (5 ms) electric pulses, we found that vesicles shrank, down to a critical radius beyond which their size no longer changed. We identified three mechanisms for the lipid loss: formation of macropores on the cathode-facing side as already reported (Tekle et al. 2001), formation of tubular structures on the anode facing side, and formation of small vesicles at both poles. These three features should probably not be considered as distinct mechanisms. Macropore and tubules formation probably reflect the same phenomena, as they were found to occur together. Small vesicles expulsion can be understood as another way of expelling lipids, and may under certain conditions be more energetically favorable than tubule formation. While assuming that the area lost per pulse was proportional to the permeabilized area (the area on the vesicle where the induced transmembrane voltage $\Delta\Psi = (3/2)\mathrm{RE}\cos(\theta)$ exceeded the critical transmembrane voltage $\Delta\Psi_c$, R denoting the radius of the vesicle, E the pulse amplitude and θ the angle with respect to the field direction), we were able to analytically predict the decrease of the GUV radius as a function of the number of applied pulses. We fitted our experimental data with the appropriate formula (equation (10) in Portet et al. 2009), and finally obtained the values of $\Delta\Psi_c$ for DOPC and EggPC vesicles, 0.85 and 1 V, respectively. Later on, we conducted the same experiments with liposomes of another composition: DOPC/Sphingomyelin/Cholesterol (2/2/1, mol/mol/mol) [unpublished results]. We found $\Delta\Psi_c \approx 0.4$ V for this composition closer to the actual lipidic composition of a real cell membrane. This finding is interesting, as to our knowledge, such a low value for permeabilization threshold of GUVs has never previously been found. Typical values are about 1 V for giant liposomes containing a single phosphatidylcholine, or a simple two component mixture, while typical critical transmembrane voltages are about 0.2 V for many cell types (Teissie and Rols 1993). Our results thus indicate that the reason for this discrepancy with respect to the case of simple model membranes may be the result of the presence of several lipid phases. Macropore formation was also studied by Riske and Dimova (2005). The pulses they applied were shorter and more intense than ours, but could still cause macropore formation. It was shown that poration of the vesicles affected the membrane relaxation dynamics, and that the initial membrane tension had a strong influence on the critical potential difference for porating the GUV.

Another study reported that the presence of anionic lipid species in the membrane could cause vesicle bursting, whereas simple phosphatidylcholine membranes usually reseal after a permeabilizing electric pulse (Riske et al. 2009). More recently, the same group investigated the electropermeabilization of GUVs in the gel phase (Knorr et al. 2010). Wrinkling patterns of the membrane were reported, along with poration thresholds 10 times higher than those typically measured with fluid membranes. It was also observed that the macropores created by the electric pulse did not reseal in these gel phase vesicles; the pores were stable for several minutes.

Motion of Domains

Increasing the membrane complexity by addition of different phospholipids and cholesterol leads to phenomena that cannot be observed in single component GUVs. Staykova et al. (2008) managed to monitor the motion of liquid ordered domains in giant vesicles made of DOPC/DPPC/cholesterol (different molar ratios were explored) subjected to AC electric fields of $\approx$500 V/cm amplitude in the kHz frequency range. They report that this movement had characteristic features depending on the field parameters, and that it was caused by the inhomogeneous surface tension induced by the field because of the chamber geometry. It was the first time this phenomenon of induced charge electroosmosis was observed and studied on a lipid membrane.

Fusion

Membrane fusion is a key process of life. It is one of the most common ways for molecules to enter or exit cells. The process of fusion occurs in a variety of important biological processes such as nerve signal transmission provided by the fusion of synaptic vesicles to the outer membranes of nerve cells. Other processes where fusion occurs are intracellular trafficking (Eitzen 2003; Hay 2007) and viral infection by membrane-enclosed viruses (Kielian and Rey 2006; Weissenhorn et al. 2007).

However, membrane fusion does not occur spontaneously because of large energetic barriers in biological membranes. These energetic barriers are caused by Van der Waals, electrostatic and steric repulsions and by strong hydration (Helm et al. 1992; Kozlovsky and Kozlov 2002). To promote membrane fusion it is essential to overcome these barriers. In biological membrane systems this process occurs via fusion proteins (Carr and Kim 1993; Yu et al. 1994) in a multistep process. For most viruses, a fusion event is achieved in two steps. First, fusion proteins recognize a site for fusion on the membrane of the host cell. Then, the hydrophobic domain of the fusion protein inserts itself into the membrane of the host cell.

Studies of the fusion pathway have shown two important types of intermediate structures in the fusion mechanism: hemifusion structures and fusion pores (Lentz et al. 2000). Hemifusion structures form a connection between outer leaflets but not between inner leaflets which remain distinct. They are transient structures that either dissociate to give two independent membranes or induce fusion pores (Lentz et al. 2000). Fusion pores are connections between both outer and inner leaflets, they form an aqueous connection between the two aqueous compartments. The propensity to obtain these two intermediates depends on lipid composition, as fusion depends on the ability of the membrane to bend into these states and thus on the spontaneous curvature and bending rigidity of the associated monolayers and bilayers (Chernomordik and Kozlov 2003). It has been recently shown that the stability of the fusion pore may strongly depend on the anisotropic, intrinsic shape of lipids in the fusion pore (Jorgacevski et al. 2010). Obviously another condition to induce fusion is the establishment of a sufficiently close interbilayer proximity. As hydration forces contribute to the repulsion between membranes, the fusogenic state can be induced via bilayer dehydration (Lentz 1994). In biological systems dehydration can be induced by calcium ions that neutralize the negatively charged phospholipid head groups, reducing electrostatic repulsion between membranes, and/or help the formation of Ca^{2+}-phosphate bridges between opposing bilayers (Jeremic et al. 2004). Dehydration can also be induced by the application of an electric field on membrane systems, which can rearrange the interfacial water molecule network (Lopez et al. 1988).

Electrofusion is a very convenient way to control, both spatially and temporally, fusion events. It is thus possible to trigger and observe the whole fusion process to study the underlying mechanisms. To occur, electrofusion requires two conditions: (a) electropermeabilization and (b) contact between lipid membranes. When the two membranes are close enough and lipid perturbation is high enough, fusion occurs; for cells this was demonstrated by Zimmermann (1982). It has been shown that membrane fusion can be obtained, not only by pulsing cells already in close contact, but also by bringing them into close contact after their permeabilization (Rols and Teissie 1989; Sowers 1986; Teissie and Rols 1986). Electropermeabilized membranes are thus fusogenic. For a better understanding of the fundamental processes involved in membrane fusion, lipid vesicles are often used as model systems (Neumann et al. 1989; Tamm et al. 2003). An AC field can be used to align vesicles in the field direction and bring two vesicles into contact. A subsequent application of a DC pulse induces the permeabilization of the two vesicles and if permeabilization is induced in the contact area fusion is induced. Membrane fusion is a fast process. It has been shown that

the fusion process occurs within two stages (Haluska et al. 2006). The first stage is the opening of the fusion neck with an average expansion velocity of about 2 cm/s. In the second stage, the neck-expansion velocity slows down until complete opening of the fusion neck. In the absence of salt, the fusion typically occurs at several contact points of the vesicles. In the case of several contact points (more than two), the coalescence of these fusion necks can lead to small vesicles enclosed in the contact zone. In the presence of salt in the solution outside of the vesicles, the DC pulse induces vesicles deformation and the vesicles are pushed together and form a at contact zone. As no enclosed vesicles are observed, one may infer that only one fusion neck or a small number of such necks has been formed.

In summary, it is possible to control and observe vesicle fusion by the method of electrofusion. Vesicle fusion has applications that we discuss below.

Some Applications

In the last section, we have reviewed the various possible responses of GUVs subjected to different kinds of electric fields. These phenomena have led to applications, which we describe in this section. We first describe how shape analysis of giant liposomes in an AC field can allow the measurement of the lipid bilayer bending rigidity k_c. Then we show how electric pulses can be used to efficiently load GUVs with nonpermeant molecules. Below we highlight some applications of membrane fusion. Finally we explain how to measure the edge or line tension γ of lipid membranes, and how electric fields can be used to improve the current measurement methods.

Measuring Bending Rigidities

The bending rigidity k_c is a material property of lipid membranes introduced by Helfrich (1973) in his theory of the elasticity of lipid bilayers. It has the dimensions of energy and, roughly speaking, can be related to the energy price one has to pay in order to bend a membrane. It plays a role in the control of the membrane shape, in the determination of the amplitude of thermal fluctuations, or in the modulation of membrane proteins activity, and thus being able to measure it is of interest in the understanding of various biological events. Here we will just describe the k_c measurement method on the basis of electrodeformation. There exist other ways of measuring bending rigidities which are described in the review of Marsh (2006).

In the first bending rigidity measurements via electrodeformation, giant vesicles were exposed to AC fields of a few kHz frequency and $\approx$100 V/cm amplitude, and shapes were recorded while increasing the field amplitude (Kummrow and Helfrich 1991). The liposomes deformed gradually into a more and more elongated prolate ellipsoid, and also changed their apparent surface area as a result of the flattening of thermal fluctuations. The basic idea of the method is first to calculate the lateral tension σ induced by the field. To do so, one combines the Laplace relation at the pole and the equator for a vesicle with uniform internal pressure, which yields: $\sigma(c_1 + c_2)_e - (T_{rr})_e = \sigma(c_1 + c_2)_p - (T_{rr})_p$, where c_1 and c_2 are the principal curvatures of the ellipsoid (computed from the measured values of the axes of the ellipsoid), T_{rr} is the stress exerted on the outer surface of the vesicle by the field which gives rise to the deformation and the flattening of undulations, and the subscripts e and p indicate that the associated quantities are calculated at the equator and the poles of the vesicle, respectively. The difference between the equatorial and polar stresses is proportional to the square of the magnitude of the applied field. The tension σ can then be obtained on the basis of the known value of the applied field and the observed shape of the GUV. After that, the relative area change α should be computed using the measured values of the axes of the ellipsoid. This relative area change is given by $(k_BT/8\pi k_c)\ln(\sigma/\sigma_0)$, where k_BT is the thermal energy and σ_0 a positive parameter obtained by extrapolation to $\alpha = 0$. Note that this parameter is not necessarily the tension at zero field, but is not required to determine k_c by fitting a straight line to the logarithmic plot of α as a function of σ. This method for measuring k_c was later applied by Gracia et al. (2010) in order to study the influence of cholesterol on the rigidity of membranes of various compositions. The authors also used another measurement method for k_c on the basis of the analysis of membrane fluctuations (see, e.g., Faucon et al. 1989; Milner and Safran 1987; Schneider et al. 1984). It was found that the electrodeformation method leads to slightly lower k_c values than the fluctuation method (as noted in Kummrow and Helfrich 1991). Nevertheless, both methods give the same trends and reproducible results. The results suggest that the effect of cholesterol addition on the membrane bending rigidity is concentration dependent, but is also highly influenced by the lipid type to which cholesterol is added (Gracia et al. 2010). An important point to keep in mind is that the electrodeformation method using the theoretical analysis described above cannot be applied if one works in salt solutions or with charged lipid species.

Loading Vesicles

As previously stated, DC pulses are useful tools to enable the entry of nonpermeant molecules into cells. They can thus also enable one to load a vesicle with compounds of interest. This has applications in drug delivery, where lipid vesicles play the role of cargo carriers, but is also of interest in fundamental research, for example when trying

to understand the nature of the DNA/membrane interaction occurring during cell electropermeabilization (Golzio et al. 2002). As far as we know, very few papers focusing on the electromediated DNA uptake by liposomes have been published. Chernomordik et al. (1990) applied a single electric pulse of 12.5 kV/cm amplitude and 300 μs duration to large unilamellar vesicles (≈100 nm size, two orders of magnitude smaller than GUVs) composed of DPPC/cholesterol (7/3, mol/mol) in the presence of high molecular mass DNA of size similar to that of the plasmid DNA usually used in gene transfer protocols. They claim that the uptake mechanism took place via the electrostimulated formation of endosome-like vesicles rather than via electropores. These conclusions were questioned 10 years later by Lurquin and Athanasiou (2000), who found that DNA could enter DPPC GUVs under free form by a mechanism involving electro-induced membrane pores. They used longer pulses (12 ms) of smaller amplitude (1,500 V/cm), but which caused greater induced transmembrane voltages than those of Chernomordik et al. (1990).

In order to resolve these conflicting results, we carried out DNA loading experiments on DOPC GUVs. Our results clearly favor the second mechanism involving DNA entry via electropores. As can be seen on Fig. 1, showing a vesicle before (left) and a few seconds after (right) electric treatment (15 pulses of 5 ms duration, 370 V/cm amplitude and 0.33 Hz frequency), plasmid DNA stays trapped inside the vesicle, mostly under free form and not in endocytosis-like vesicles. Tubules and small vesicles associated with lipid loss and vesicle shrinkage, as described in 2.2, can also be observed. The small vesicles may contain some DNA, but DNA entry under free form is the predominant mechanism. The tubules appear to stay mostly attached to the vesicle and diffuse along the vesicle surface after the pulses application. The encapsulation is stable; DNA stays trapped in the GUVs several minutes after the electric treatment. The extent of lipid loss was comparable to the one reported in Portet et al. (2009); about 30 % of the permabilized area was lost, per pulse. No macropores were detected in these experiments, but this is not surprising because the temporal resolution of our setup was 3 s whereas the macropores typically reseal within a few hundred milliseconds. Using confocal microscopy, we were able to quantify the amount of DNA entering the liposome after each pulse, and have developed a theoretical model able to account for the increase of DNA concentration inside the GUV (Portet et al. 2011).

Applications of Fusion

Because membrane fusion induces a mixing of the lipids initially present in each membrane and a mixing of the aqueous compartments, electrofusion could be used to introduce molecules into a membrane or into an aqueous compartment. In the case of the fusion of two vesicles having different lipid compositions, it is possible to produce multicomponent vesicles of well-defined composition (Dimova et al. 2007; Riske et al. 2006). This provides the possibility of studying the dynamics of domain formation and stability. When giant vesicles are prepared directly with a multicomponent lipid mixture, the compositions of the different vesicles are dispersed about the composition of the initial lipid mixture. Electrofusion of two vesicles

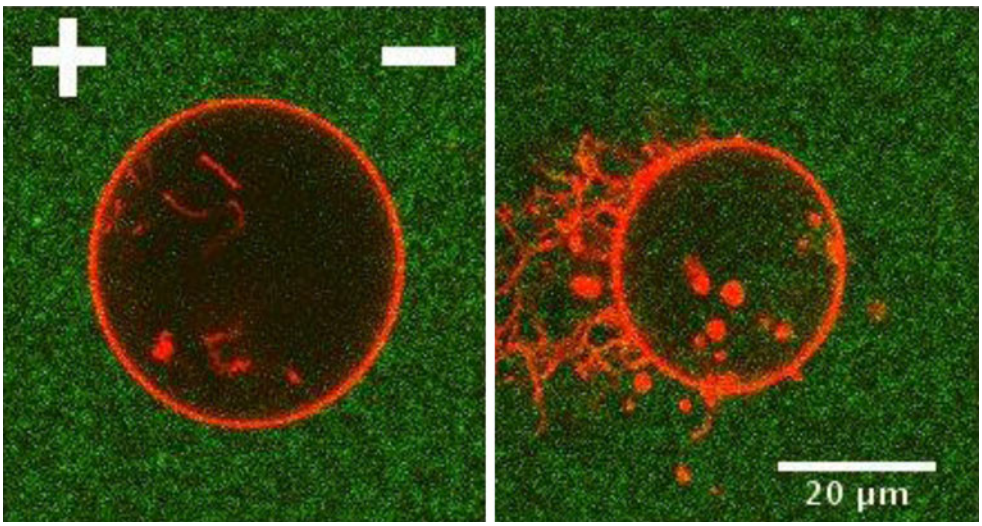

Fig. 1 Confocal microscopy image of a DOPC GUV fluorescently labeled with rhodamine-PE (*red*) in the presence of plasmid DNA labeled with TOTO-1 (*green*). *Left* There is no DNA present inside the liposome before electric treatment. *Right* A few seconds after electropermeabilization, the vast majority of electrotransferred DNA is under free form and not enclosed in endocytosis-like vesicles. Tubules and small vesicles associated with lipid loss can also be observed. Field polarity is indicated on the *left*

each made of different components thus allows one to obtain vesicles with a well specified composition. Fusing two vesicles of nonmiscible lipid composition produces microdomains in the resulting vesicle's membrane.

The fusion of two vesicles of different content is a realization of a microreactor. It has been shown that two vesicles can encapsulate one or more reagent molecules (Chiu et al. 1999). When electrofusion is induced between these two vesicles, the contents of the two vesicles are mixed and a chemical reaction between the two reagent molecules can occur. This method needs one condition to be efficient: the lipid membranes of the two compartments have to be impermeable to the reactants. The first use of vesicles as microreactors was demonstrated with a chemical reaction between the calcium sensitive fluorescent dye Fluo-3 and Ca^{2+} (Chiu et al. 1999). A batch of vesicles was loaded with Fluo-3 and another was loaded with Ca^{2+}. The two types of containers were mixed, electrofusion was induced, and fluorescence enhancement due to the formation of a complex between Fluo-3 and Ca^{2+} was observed. The method of electrofusion provides a promising tool for studying and following one chemical reaction at a time where spatial and temporal localization can be precisely controlled. Furthermore, the small volume used in this technique could also provide a general chemical or biochemical delivery system.

On the basis of this previous experiment, electrofusion has been used as a method for nanoparticle synthesis in vesicles (Yang et al. 2009). In this case, one vesicle is loaded with Na_2S and the other one with $CdCl_2$. The application of an AC field allows the bringing of vesicles into contact, DC pulse application then induces fusion and as a consequence mixing of the two compartments. Fluorescence in the interior of the resulting vesicles indicates the formation of CdS nanoparticles. Thanks to this approach it is possible to observe and monitor the whole reaction with an optical microscope. This experiment suggests that nanoparticles could be synthesized in biological compartments even without the intervention of macromolecules.

Measuring Edge/Line Tensions

The hydrocarbon chains of lipids are hydrophobic and the energy cost per unit length for leaving the hydrophobic core of a membrane in contact with water is called the line or edge tension γ. It is often hypothesized that for large edges the lipid heads can bend over the edge to make the edge hydrophilic; however, there is still an edge tension due to the bending energy associated with this lipid rearrangement. The edge tension is a lipid material property and depends on the external medium. It is the edge tension that makes a membrane patch embedded in an aqueous environment spontaneously bend to form a vesicle, and it also generates the force driving pore closure in lipid bilayers (Fromherz et al. 1986; Sandre et al. 1999). Roughly speaking, the higher the edge tension, the faster the pore closes and one can deduce γ from the evaluation of the corresponding pore closure times.

We put this idea into practice while studying the influence of a poloxamer molecule on EggPC GUVs. It was seen in Sharma et al. (1996) that poloxamers could modify lipid membrane properties under electric pulses. We explored the effect of the presence of poloxamer in the surrounding solution. Using poloxamer L64 at concentrations of 2.5 and 5 g/L, we first observed no effect on giant liposomes at rest. Then we applied porating electric pulses of 5 ms duration and $\approx$500 V/cm amplitude to these liposomes, and with a fast EMCCD camera we were able to measure the size of the pores and their closure time. We saw no difference of the pore size between liposomes with or without poloxamer, regardless of the concentration. A study of pore closure shows a strong effect: the average measured closure time without poloxamer is 0.2 s while it is 0.4 s with poloxamer, and we did not detect any effect of the concentration of poloxamer. Pore closure is thus twice as slow with poloxamer, and this seems to indicate that L64 reduces the edge tension of EggPC membranes, hence helping to maintain the membrane in a permeabilized state. Because the above results come from very few measurements (6 without poloxamer and 2 for each poloxamer concentration) they are only preliminary and still need to be corroborated.

It is possible to go beyond these qualitative observations and to accurately measure edge tension values. Harbich and Helfrich (1979) observed open cylindrical EggPC giant vesicles in AC fields and then deduced a value of 20 pN for γ. Zhelev and Needham (1993) obtained values in the same range for SOPC and SOPC/cholesterol (1/1, mol/mol) liposomes while coupling the electroporation technique and micropipette aspiration. A more recent edge tension measurement method emerged from the theoretical work by Brochard-Wyart et al. (2000). Considering a spherical vesicle of size R, it was shown that in the limit corresponding to experiments on giant vesicles, a pore of radius r spent the vast majority of its lifetime in a so-called slow closure stage during which the quantity $R^2 \ln(r)$ decreased linearly with time, the coefficient of proportionality being equal to $(-2\gamma/3\pi\eta_0)$, where η_0 denotes the external medium viscosity. The same group later applied this method to pure DOPC GUVs and also to systems containing cholesterol or a surfactant, opening pores via intense visible light illumination. Adding glycerol to the aqueous solutions caused slowing down of the dynamics, and they were thus able to measure the pore size during the slow closure stage using classical fluorescence microscopy. They obtained values of

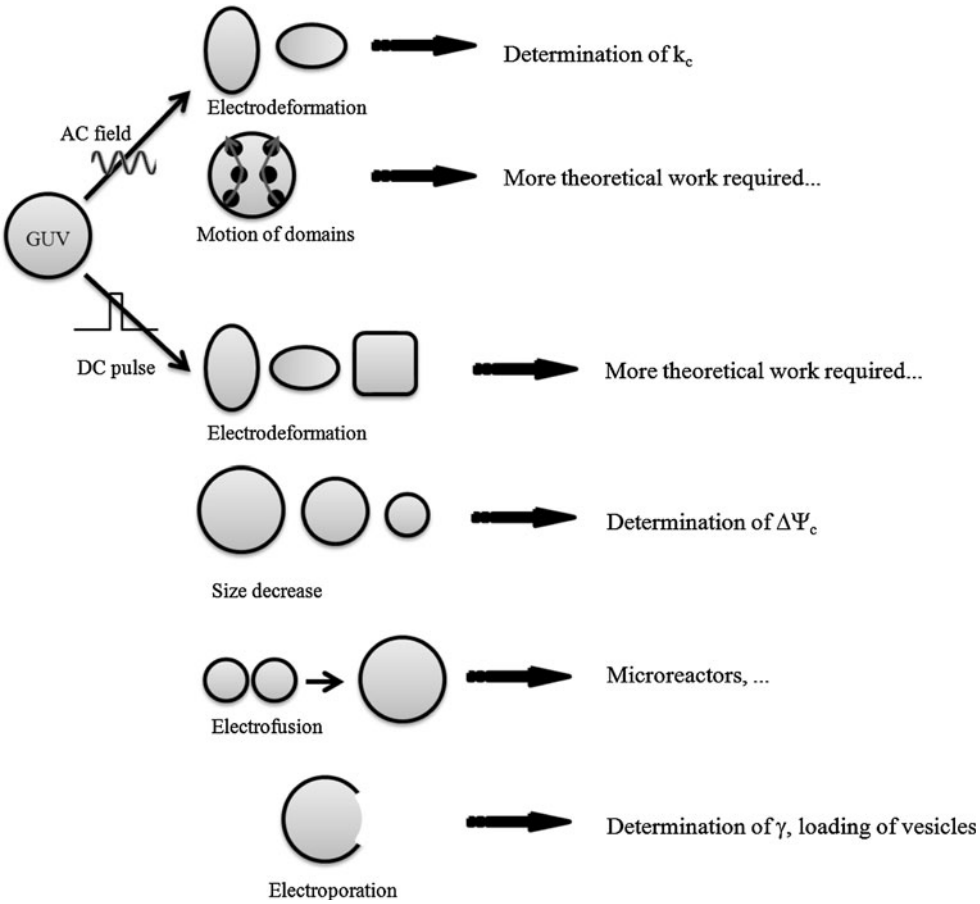

Fig. 2 Diagram summarizing the influence of electric fields on giant lipid vesicles and their practical uses. The mention "More theoretical work required…" means that the current understanding of the phenomenon does not allow us to design practical uses yet, and that deeper knowledge is still needed to fully exploit this effect of the electric field. Indeed, further identification of gaps and opportunities in the field should be exploited

γ for their different vesicle compositions, and found that cholesterol caused an increase of γ whereas the surfactant led to a decrease (Karatekin et al. 2003). The main problem of this approach is that glycerol influences the lipid bilayer organization, and that fluorescent dyes embedded in the membrane behave as impurities and may hence lead to inaccurate edge tension measurements. We thus proposed an improvement of Brochard-Wyart's method based on the application of an electric pulse to porate the vesicle and on ultrafast phase contrast imaging to monitor pore size evolution (Portet and Dimova 2010). This method is easy to implement, requires little equipment, and does not suffer from the drawbacks related to the presence of glycerol and fluorescent dye. We confirmed that the addition of cholesterol increased the edge tension of a bare DOPC membrane, and surprisingly, we found that DOPE addition to a DOPC bilayer caused a decrease of γ.

Conclusions

Giant vesicles provide a useful model system for measuring a variety of physical properties of lipid membranes and for improving our understanding of the electropermeabilization and electrofusion phenomena. In this review, we have summarized the presently known effects of electric fields on giant vesicles and some of their practical applications, and presented current research topics covered by our group. Subjecting GUVs to DC pulses or alternating AC fields can destabilize the lipid bilayer, inducing different behavior such as deformation of the vesicles, poration, motion of domains, or fusion. An outline of these various behaviors and their uses is presented in Fig. 2.

It is now known that the deformation of the vesicles depends not only on the frequency and the intensity of the applied field, but also on the solutions conductivities. Furthermore, the deformations are not exactly the same, depending on the presence or not of salt in the external medium and on the shape of the applied electric field (AC or DC). By monitoring the relative area dilatation of the membrane, it is possible to estimate the bending rigidity k_c of the lipid bilayer.

Electric pulses have more dramatic consequences than just deformation. With suitable parameters for the electric field, it is possible to electropermeabilize the membrane. This effect can lead to exchange of molecules between the

inside and the outside of the vesicle. If the electropermeabilization is strong enough, membrane macropores can be visualized via optical microscopy. When a sequence of pulses is used, such observations are in general accompanied by loss of lipid membrane material and a subsequent GUV size decrease allowing the determination of a critical transmembrane voltage. Moreover, by monitoring the evolution of the pore radius it is possible to measure another lipid material property, the edge or line tension γ.

When two vesicles are in close contact, electropermeabilizing them with suitably chosen electric field parameters can induce their fusion. This electrofusion method allows the control and observation of vesicle fusion and is thus useful for insertion of molecules into the membrane or for mixing different chemicals in the resulting vesicle, thus giving a microreactor.

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