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SWELLING OF CHARGED LIPIDS AND FORMATION OF LIPOSOMES ON ELECTRODE SURFACES

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Absract External electric fields can induce liposome formation on electrode surfaces if the swelling lipid is in liquid crystalline state. The lipid swelling and liposome formation depend on the type of the lipid, the dried lipid layer thickness, the medium parameters (temperature, osmolarity, ionic strength), the type and the parameters of the electric field (direct current (DC) or alternating current (AC), amplitude, frequency) and the time of exposure. This paper reviews some of our previous experimental results and theoretical estimates for those effects and presents new data and estimates for the effects of lipid charge and lipid layer thickness. The basic conclusion is that the electric fields can affect lipid swelling and liposome formation by at least six mechanisms: (1) electrostatic interactions between the field and the bilayers, (2) electroosmotically induced mechanical stresses, (3) redistribution of double layer counter-ions between the bilayers, (4) decreased surface, membrane and line tensions, (5) electrochemical reactions and (6) injection of charges from the electrodes. Speculation increases from 1 to 6.

INTRODUCTION

Bangham et al.[1] were the first who showed that swelling of negatively charged lipid mixtures on solid surfaces can lead to formation of closed membrane systems, called liposomes. This pioneering work induced a great number of different methods for liposome preparation (see, e.g., [2–4]). Cell–size liposomes have attracted much attention because of several basic reasons: (1) historically they were the first obtained liposomes, (2) the procedure for their preparation is rather simple – they form spontaneously by swelling of lipids on solid surfaces, if the temperature is above that corresponding to the main phase transition from gel to liquid crystalline state. (3) they serve as a simple model of cells and membranes.

Understanding the mechanisms of liposome formation can help in improving the methods for their preparation and is important in consideration of fundamental scientific problems, e.g., origin of life, etc. Surprisingly, in spite of the voluminous literature on lipids and liposomes, mechanisms of liposome formation, in particular those for cell–size liposomes, have received relatively little attention. Israelachvili et al.[5–8], and Mitchell and Ninham[9] suggested that lipid aggregates can spontaneously form small unilamellar vesicles (SUV), which are in a stable equilibrium state. Helfrich[10, 11], Fromherz[12, 13] and Lasic[14] supposed that the distortion of a planar membrane to form a SUV requires energy and therefore the SUV are inherently unstable. Fromherz founded experimental evidence for existence of transient discs, after sonication in detergent solutions, and showed that edge energy drives the disc–to–vesicle transformation. The role of the detergent is to decrease the edge energy and in this way to facilitate the disc formation[12]. Cornell et al.[15] proposed that the hydration energy tends to eliminate the exposed hydrocarbon edges of the bilayer by inducing curvature, while the configuration entropy and energy of the lipid opposes curvature. On the basis of their theoretical calculations they concluded that the formation of SUV from zwiterionic lipids represents a metastable state induced by a disruption of the

bilayer. Spontaneous formation of SUV occurs only for a limited class of lipid molecules, e.g. the dodecyldimethylammonium ion [16, 17]. Cell-size liposomes can, however, form spontaneously by swelling of egg lecithin in water solutions[18, 19]. Harbich and Helfrich[18] used an original procedure to make the swelling reproducible. They showed that there is no equilibrium spacing and that undulation forces can be dominant in lipid swelling. They observed tube formation and tube-to-vesicle transformation. The swelling and liposome formation was suppressed in NaCl solutions. Other investigators have also observed suppression of liposome formation in ionic solutions[20]. Mutz et al.[28] showed, however, that cephaline can form giant unilamellar vesicles in high salt concentration (up to 300 mM NaCl).

Presently, the exact mechanisms of cell-size liposome formation are not known. Liposome formation requires membrane separation and bending. Electric fields can affect both. They can change intermembrane forces and induce bending. They can also change the phase transition temperature. In addition, they can be precisely controlled. Therefore, electric fields can help to elucidate mechanisms of liposome formation. Knowing mechanisms will help in preparation of liposomes of predetermined properties. Electric field effects in liposome formation may be also important in physicochemical and biological processes.

This paper reviews some of our previous experimental results and theoretical estimates[21-26] for those effects and presents new data and estimates for the effects of lipid charge and lipid layer thickness.

MATERIALS AND METHODS

We used L-α phosphatidylcholine (EggL-) (Sigma P5394) which contains 71% phosphatidylcholine, 21% phosphatidylethanolamine, and 8% phosphatidylserine, i.e., it is negatively charged; dodecylamine (Merck 803527) / L-α phosphatidylcholine 99% (Sigma P5763) mixture (DA+/PC) of molar ratio 1:20 which is positively charged; The lipids were dissolved in

chloroform/methanol 9:1 solvent. Two drops of this solution (1μl each) were deposited on two parallel platinum electrodes (diameter 0.48 mm, separation distance 0.5 mm). The solvent was evaporated under nitrogen. The average number of bilayers was calculated from the amount of the lipid and the surface area on which that lipid was deposited using the data from [27] for the area per lipid molecule. The mean error was about 20%. Electric fields were applied and distilled water or water solutions added. The observations were performed under phase contrast microscope. In some cases Ficoll was added to improve visualization of the thin-walled liposomes.

EXPERIMENTAL RESULTS

NEGATIVELY CHARGED LIPID MIXTURES (EggL-)

Table 1 shows schematically the dependence of the swelling rate and the yield of thin-walled liposomes on the dried lipid layer thickness on the negative and positive electrodes and without field[24-26]. When the number of bilayers in the dried lipid layer is higher than approximately 500 there is no difference in the swelling rate on both electrodes and without field. In this case the swelling rate is very high and it is difficult to detect possible differences. The yield of vesicles is very low. Predominantly other structures as clumps, tubes, etc., are formed. By decreasing the thickness to about 90 bilayers, the differences in the swelling rate can be measured. On the negative electrode the swelling is faster than on the positive one. Without field the rate is smaller than on the negative electrode, but faster than on the positive one. The yield of thin-walled vesicles is relatively higher than that for the cases of larger numbers of bilayers. For thicknesses smaller than approximately 90 bilayers there is no sharp boundary between the swelling lipid and the bulk liquid. Therefore, the swelling rate can not be defined and measured. Commonly separate regions of vesicles and other structures are observed. There are no vesicles on the positive electrode and without field. On the negative electrode, however, very thin cell-size liposomes (diameters in the range of 25 μm) are

formed. Their yield relatively increases by decreasing the thickness of the dried lipid layer. The applied voltage to induce liposome formation increases with decreasing the thickness and is proportional to the logarithm of the lipid layer thickness. For lipid layer thickness below 2–3 layers there is no observable liposome formation.

TABLE 1 Effects of DC fields on Egg lecithin (71% PC, 21% PE, 8% PS – negatively charged mixture). Swelling medium – distilled water.

Number of bilayers	Relative swelling rate			Yield of thin-walled vesicles		
	No field	positive electrode	negative electrode	No field	positive electrode	negative electrode
> 500	the same			very low		
90 to 500	1	< 1	> 1	low		
10 to 90	No definite swelling front			No		high, U_c-Inl
3 to 10						highest, U_c-Inl
< 3						

ZWITTERIONIC LIPIDS

Figure 1 shows swelling of PC in distilled water without external electric field. The average number of bilayers of the dried lipid layer N = 50. Ficoll was added after 30 min to increase the phase contrast. Different non-vesicle structures can be seen. The non-homogeneity of the dried lipid layer led to rather large variations in the average thickness of the swelling lipid.

Figure 2 shows the effect of DC field (1 V amplitude) on PC swelling for the same conditions as those for the process shown in **Fig. 1**. Few vesicles, and a lot of clumps, tubes, and other non-vesicle structures can be seen on the positive electrode, unlike the picture on the negative electrode, where the vesicles dominate.

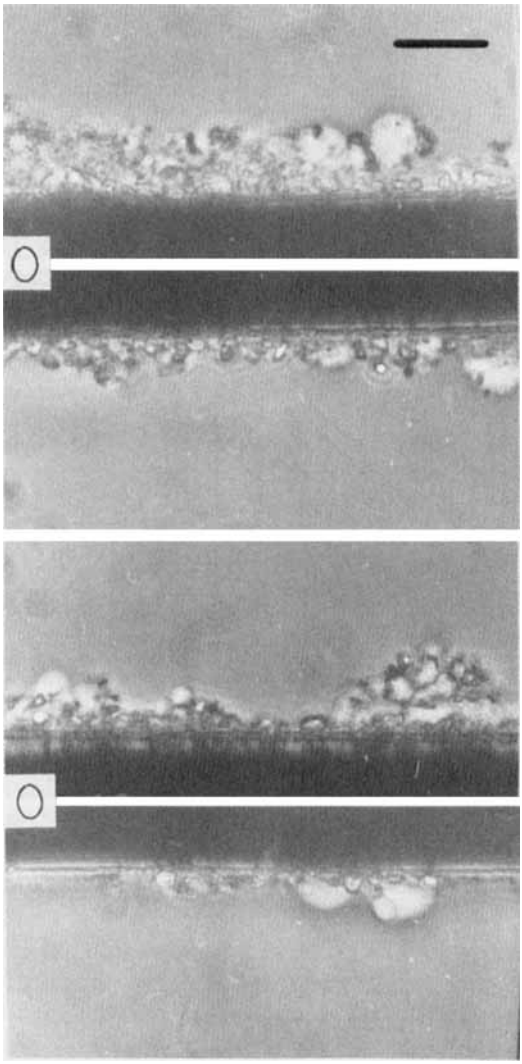

FIGURE 1. Swelling of PC in distilled water without external electric field, N = 50, 30 min, Ficoll added, bar = 50 μm.

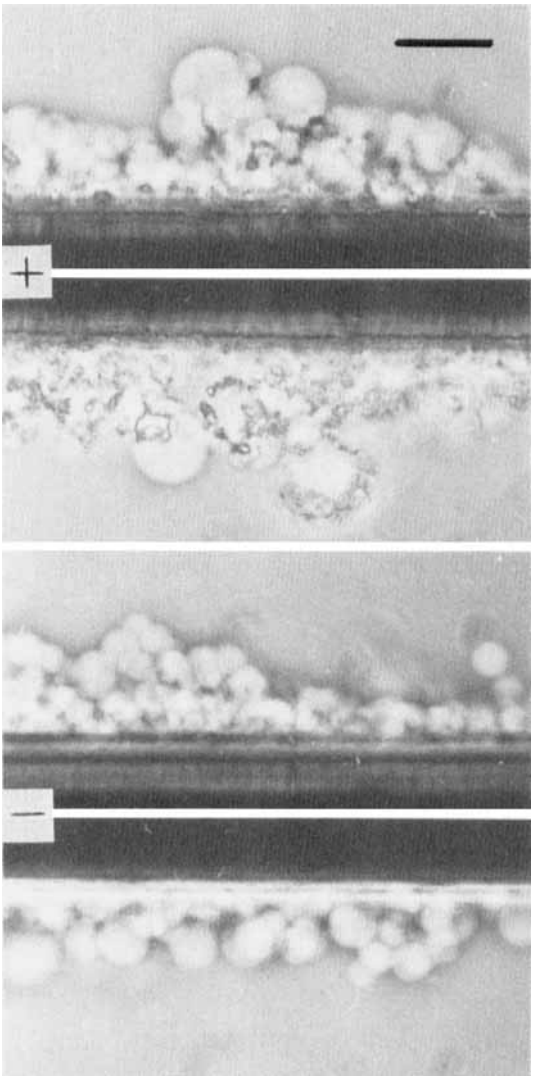

FIGURE 2. Swelling of PC in distilled water, N = 50, DC 1 V, 30 min, Ficoll added, bar = 50 μm.

Figure 3 shows effects of AC field (amplitude 1.4 V, frequency 10 Hz) on PC swelling (other conditions as in **Fig. 1**). Macromembranes not permeable to Ficoll can be seen. The extent of spherization (vesicle formation) is less than that in DC fields of the same effective amplitude (**Fig. 2**). The extent of swelling is larger on the internal side of the electrodes, which is probably due to the higher field intensity.

POSITIVELY CHARGED LIPID MIXTURES

Figure 4 shows spontaneous swelling of DA+/PC (molar ratio 1:20) at the same conditions as that shown in **Fig. 1**. It is seen that the addition of a positively charged component (DA+) leads to larger extent of swelling and formation of large number of vesicles (see [26b] as well). The picture is similar to that shown in **Fig. 2**. It means that DC fields may affect swelling of PC in a similar way as charged components.

LIPOSOME YIELD

Figure 5 shows formation of liposomes from very small amounts of PC (30 ng) in distilled water under DC field (amplitude 2 V) on the negative electrode. Similar picture was observed on the positive electrode. The average thickness of the dried lipid layer corresponds to 4 – 5 bilayers. Vesicles did not form for amounts of PC smaller than 20 ng, which correspond to average thickness of 2 to 3 bilayers. Therefore, probably, only the amount of 10 ng lipid is transformed into vesicles. This amount corresponds to 7.65×10^{12} molecules PC. We counted at average 10000 vesicles of diameter 6.0±2 μm. The area per one maximally hydrated PC molecule is 0.756 nm^2, [27]. Therefore, there are 2.99×10^{12} (from 1.33×10^{12} to 5.32×10^{12}) molecules in the vesicles if they are unilamellar. The yield is between 20% and 70%. This value is just a rough estimate. It gives, however, the feeling that much of the liposomes are unilamellar and the yield is high when the thickness of the dried lipid layer is very small.

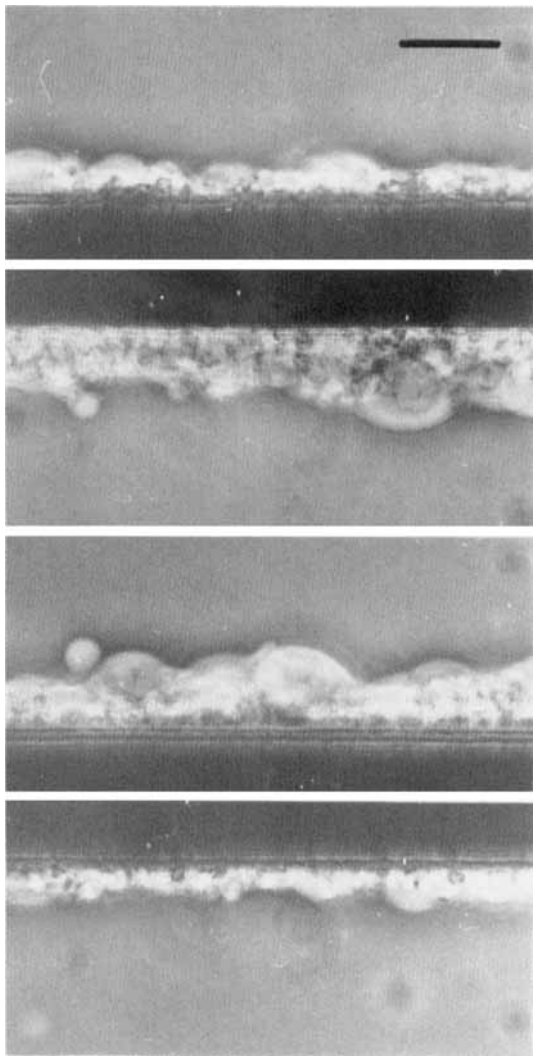

FIGURE 3. Swelling of PC in distilled water, N = 50, AC 1.4 V, 10 Hz, 30 min, Ficoll added, bar = 50 μm.

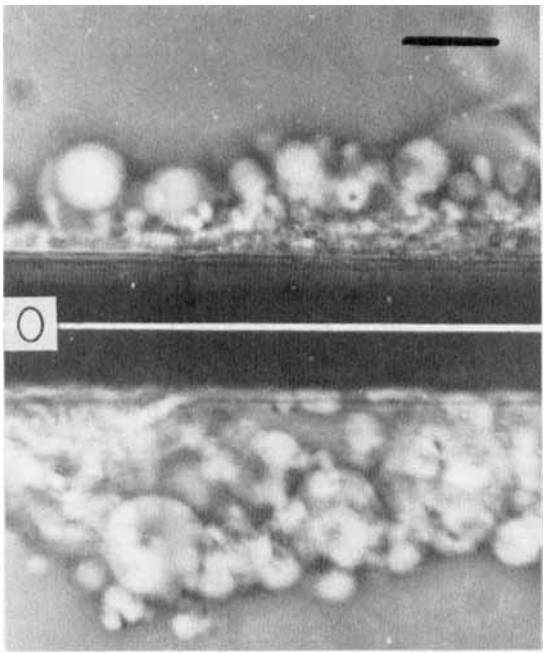

FIGURE 4. Swelling of DA+/PC 1:20 in distilled water, N = 50, 30 min, Ficoll added, bar = 50 μm.

DETACHMENT OF LIPOSOMES FROM THE ELECTRODE SURFACE

Liposomes are commonly adhered to the solid surfaces where they are formed by swelling. Brownian motion can be not effective especially for larger liposomes. Low-frequency AC fields can serve as an useful tool to detach them from the electrode surface. **Figure 6** shows the effect of AC field (amplitude 4 V, frequency 1 Hz, duration 1 min) on vesicles formed from PC (average lipid layer thickness 50 bilayers) in AC fields (amplitude 2 V, frequency 10 Hz, duration 30 min). It is seen that the vesicles have detached the electrode surface and dispersed into the bulk liquid.

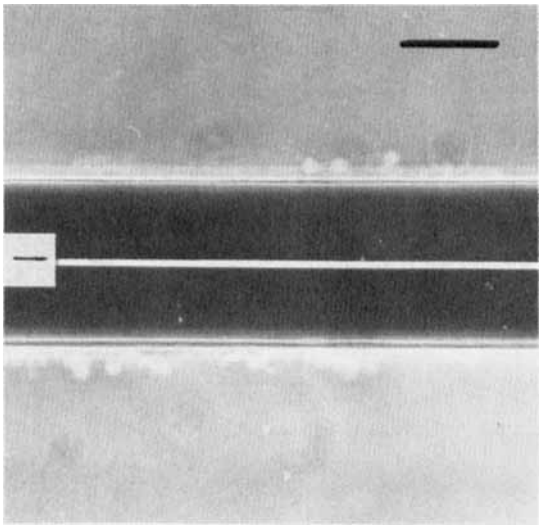

FIGURE 5. Swelling of PC in distilled water, N = 4 to 5, DC 2 V, 30 min, Ficoll added, bar = 50 μm.

CONCLUSIONS

Bilayer separation and bending are prerequisites for liposome formation from hydrating lipids. Therefore, a possible mechanism is that bilayers should be separated by increasing the intermembrane repulsive forces, and destabilized to bend, and form liposomes. This requires the right proportion between structured regions (in the form of bilayers), and defects and/or non-bilayer structures.

Two basic steps are involved in preparation of cell-size liposomes by swelling: (i) Drying of the lipids, dissolved in a mixture of polar and nonpolar solvent. During evaporation of the solvent, the lipids should adsorb onto the substrate surface and form a multilayer structure. The type of this structure will depend on the surface/lipid and lipid/lipid interactions. It may be expected that near the surface the lipid layer will have different properties than those far from the surface. (ii) Swelling of the lipid in water. When water or a solution is

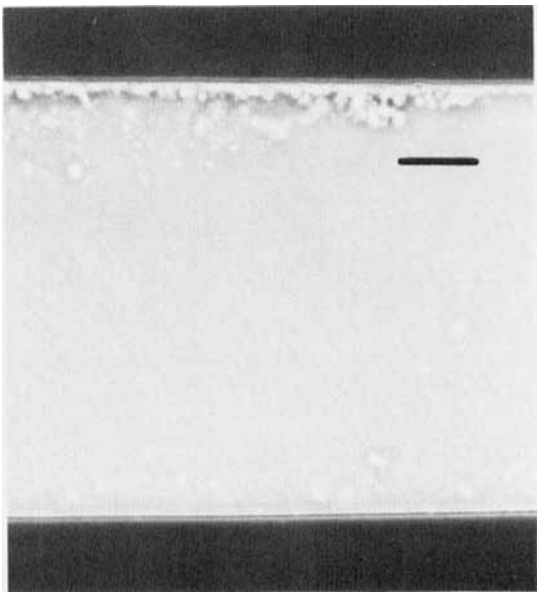

FIGURE 6. Swelling of PC in distilled water, N = 50, AC 2 V, 10 Hz, 30 min, Ficoll added; detachment and dispersion of the vesicles into the bulk after action of AC 4 V, 1 Hz for 1 min. Bar = 100μm.

added, it goes through the bilayers and/or through the defects, driven by the hydration forces and in less extent by undulation forces. The time and swelling distance of the hydration stage are very short: it takes $\sim 10^{-4}$ s to increase the interbilayer separation from 0 to 1 nm. For larger separations, electrostatic membrane–membrane, and van der Waals membrane–membrane and membranes–substrate surface interactions as well as undulation forces can be important up to membrane separations $\sim$ 0.1 μm. Lipid swelling to macroscopic distances (of the order of μm–s per lipid bilayer) can be driven by osmotic forces or external constraints – in particular, electric fields.

The electric fields can affect these processes by at least

six mechanisms: (1) electrostatic interactions between the field and the bilayers, (2) electroosmotically induced mechanical stresses, (3) redistribution of double layer counter-ions between the bilayers, (4) decreased surface, membrane and line tensions, (5) electrochemical reactions and (6) injection of charges from the electrodes. Speculation increases from 1 to 6.

(1) The direct electrostatic interaction leads to electrophoretic motion of the lipids. This effect is, however, relatively small and cannot explain the observed dependence of the potential to induce liposome formation on the lipid layer thickness. It is important for the last stages of lipid swelling for relatively thick lipid layers. It can be also important for the growth of the liposomes; the observation that the vesicles are a little elongated in the direction of the field can be explained on the basis of electrostatic interaction of the field with the lipid charge and the induced potential.

(2) Electroosmotic motion for AC electric fields has more pronounced effects than for DC ones. Unlike the case of DC fields, where the electroosmotic effect lasts very short time, AC fields induce significant periodical motion during the time of action of the field. There is a certain similarity between the effects in sonication and in AC fields. In both cases mechanical stresses are induced and they cause rupture of bilayers and formation of pieces of different size, which can bend to form liposomes. The electroosmotic vibrations are, however, in some aspects more gentle and lead to formation of larger thin-walled liposomes.

(3) Redistribution of the double layer ions between the lipid bilayers due to DC field can be the reason for the observed dependence of the potential to induce liposome formation and the lipid layer thickness. It is interesting to point out that the double layer thickness of distilled water for pH between 5 and 7 ranges from 100 nm to 1000 nm. This is just the range of thicknesses (10 to 100 bilayers) for which vesicles do not form from EggL- without fields. The increased interbilayer repulsion should overcome van der Waals attraction between membranes and between the membranes and the semi-infinite electrode material. The membrane/electrode interaction decreases with increasing the distance from the electrode surface. Probably, at lipid layer thickness corresponding to 90 bilayers, this interaction becomes equal to the sum of the other forces. One possibility is that the

applied electric field sucks out the counterions from between the membranes, thus increasing the interbilayer repulsion (on the negative electrode for negatively charged lipid mixtures). Increasing the potential will increase the intermembrane repulsion and therefore decrease the lipid layer thickness for vesicle formation. Similar effects can be produced by increasing the concentration of charged lipids. Unfortunately, the theoretical problem for ion distribution in multilayered systems in external electric fields is rather complicated and still unsolved. It is presently under consideration.

(4) The electric field can decrease the membrane surface and line tensions. The decrease of the surface tension leads to decrease of the membrane tension. The initial membrane tension (before applying the field) is small because there are no applied external forces to stretch or compress the bilayers. Our theoretical estimates showed that the external field leads to decrease of the membrane tension which can become negative. This can lead to instability of bending which can be driven by the line tension or other constraints. Electric field can also decrease the edge energy (respectively, the line tension)[29]. This can facilitate the initial stage of formation of lines of defects and holes and therefore the liposome formation (see also the discussion [13]).

(5) Electrochemical reactions can lead to chemical transformations of the lipids and dissociation of the water and chemical impurities. This can lead to a number of effects, including changes of the physical parameters of the lipid/water system.

(6) Injections of charges from the electrodes can induce electroviscous effects (see, e.g., Honda and Sasada[30,31]). It is interesting to note that the voltage to induce motion of the liquid due to interaction of injected space charges and the field is in the range of that used in our experiments. In addition, the injected charge carriers may change the charge of the lipid molecules, and therefore the interbilayer repulsion.

It must be pointed out that most of the above concepts are just hypotheses which need further experimental and theoretical work[25].

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