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ORIGINAL ARTICLES

Optimization of the Electroformation of Giant Unilamellar Vesicles (GUVs) with Unsaturated Phospholipids

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Abstract Giant unilamellar vesicles (GUV) are widely used cell membrane models. GUVs have a cell-like diameter and contain the same phospholipids that constitute cell membranes. The most frequently used protocol to obtain these vesicles is termed electroformation, since key steps of this protocol consist in the application of an electric field to a phospholipid deposit. The potential oxidation of unsaturated phospholipids due to the application of an electric field has not yet been considered even though the presence of oxidized lipids in the membrane of GUVs could impact their permeability and their mechanical properties. Thanks to mass spectrometry analyses, we demonstrated that the electroformation technique can cause the oxidation of polyunsaturated phospholipids constituting the vesicles. Then, using flow cytometry, we showed that the amplitude and the duration of the electric field impact the number and the size of the vesicles. According to our results, the oxidation level of the phospholipids increases with their level of unsaturation as well as with the amplitude and the duration of the electric field. However, when the level of lipid oxidation exceeds 25 %, the diameter of the vesicles is decreased and when the level of lipid oxidation reaches 40 %, the vesicles burst or reorganize and their rate of production is reduced. In conclusion, the classical electroformation method should always be optimized, as a function of the phospholipid used, especially for producing giant liposomes of polyunsaturated phospholipids to be used as a cell membrane model.

Keywords Electroformation · Giant unilamellar vesicles · Phospholipid · Oxidation · Electric field · Membrane model

Introduction

Giant unilamellar vesicles (or GUV) are liposomal cell membrane models that have been widely used to study the properties of biological membranes (Dimova et al. 2006; Yamamoto and Ando 2013; Dimova 2014; Bagatolli and Needham 2014). In particular, GUVs have proven to be a simple yet effective model to analyze the mechanisms of membrane electroporation (Breton et al. 2012). These liposomes have a diameter close to that of a biological cell (around tens of micrometers) and contain the same phospholipids that constitute cell membranes. These phospholipids are able to form supramolecular assemblies such as lipid monolayers, micelles, or liposomes thanks to their amphiphilic character. Indeed, they present a hydrophilic phosphate head group, along with two hydrophobic fatty acid tails. GUVs are an advantageous cell membrane model compared to small unilamellar vesicles since GUVs are easily detectable by classical methods of optical microscopy (Walde et al. 2010) and since the curvature of GUVs, which can greatly impact experimental results is similar to

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that of biological cells (Dimova et al. 2006; Angelova 2007; Fenz and Sengupta 2012).

Several methods have been proposed for the synthesis of GUVs (Pott et al. 2008; Méléard et al. 2009; Walde et al. 2010). Initially, they were obtained thanks to a spontaneous swelling method. This protocol was time consuming (and therefore not applicable to rapidly degrading lipids) and rather inefficient concerning the rate of formation of the vesicles (Reeves and Dowben 1969). Nowadays, the most frequently used protocol is called electroformation. It was developed in 1986 by Angelova and Dimitrov (Angelova and Dimitrov 1986). Rapidly, the first part of the electroformation protocol consists in the deposit of a phospholipid layer on electrodes. An electroformation chamber is then made with these electrodes and filled with an appropriate buffer. The second part of the electroformation protocol is the application of an AC electric field to these electrodes in three steps. The first step is a progressive increase of the applied sinusoidal field up to a maximal amplitude. The second step is the application of this sinusoidal maximal field amplitude for a few hours. The third step is the application of a square-wave AC field of lower frequency. Depending on the lipid mixture, the formation buffer and the electric parameters chosen for the first step, the second and third step can sometimes be omitted. The vesicles produced thanks to this technique are unilamellar and their diameter ranges from 1 to 100 μm. This method allows for the production of a high number of vesicles in a short time (typically a few hours) and is therefore suitable for rapidly degrading lipids such as unsaturated phospholipids. Moreover, throughout the years, this method has proven to be applicable to a wide variety of lipid mixtures in various formation buffers (Méléard et al. 2009).

However, the potential chemical damages induced by the application of an electric field on unsaturated lipids have not been considered. The presence of oxidized lipids in the membrane of GUVs could greatly impact their permeability (Wong-Ekkabut et al. 2007) and their mechanical properties (Soto-Arriaza et al. 2008), hampering the use of these vesicles as cell membrane models. The presence of oxidized lipids in the cell membrane has also been evaluated to permeabilize the cell membrane (Volinsky and Kinnunen 2013). A single preliminary study has been previously published on the peroxidation of lipids during electroformation (Zhou et al. 2007). However, this study was based on indirect measurements of the peroxidation such as the appearance of malondialdehyde (MDA) or conjugated carbon double bounds. It has since been proven that these methods greatly lack in specificity for the measurement of the level of peroxidation of the lipids (Halliwell and Chirico 1993).

The goal of this work was to examine the chemical consequences of the electroformation protocol on phospholipids by mass spectrometry. This method was chosen since it has appeared as the most versatile, specific and quantitative method for the detection of peroxidized lipids (Reis and Spickett 2012). Therefore, we have decided to study the effect of various parameters, including the electric field strength and the duration of the preparation on the level of oxidation of the phospholipids. The extent of the lipid peroxidation will, of course depend on the nature of the phospholipids. Indeed, fully saturated phospholipids are not prone to peroxidation, whereas unsaturated phospholipids which contain hydrogens in a bis-allylic position have been demonstrated to be very sensitive to peroxidation (Reis and Spickett 2012). We have therefore worked with three phospholipids with various sensitivities towards oxidation (Fig. 1): DOPC (one double bound on each acyl chain, poorly oxidizable), DLPC (two double bounds on each acyl chain, mildly oxidizable) and DHAPC (six double bounds on each acyl chain, highly oxidizable). The consequences of the modification of the electroformation protocol on the size and the number of the GUVs was also studied by flow cytometry.

This article presents the optimization of the electroformation parameters (field amplitude and duration of the application of the field) to increase the efficiency of the method and decrease the level of phospholipid oxidation.

Materials and Methods

Materials

DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DLPC (1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine), DHAPC (1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine) and the fluorescent dye DOPE-Rhodamine (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt) were purchased from Avanti Polar Lipids (Alabaster, AL). All other chemicals and solvents were purchased from Sigma Aldrich (Saint Louis, MO). All reagents were used without further purification. The purity of DOPC, DLPC and DHAPC was checked by mass spectrometry before use to avoid any oxidation of the source material. The lipid solution were used only if their mass spectra presented a single peak corresponding to the lipid molecular weight (as presented in Fig. 4 and in supplementary Fig. S1).

Electroformation Protocol

The vesicles were prepared using a classical electroformation protocol (Angelova and Dimitrov 1986; Mauroy et al. 2012) presented in Fig. 2. The formation was conducted at 6 °C so as to avoid any lipid degradation due to

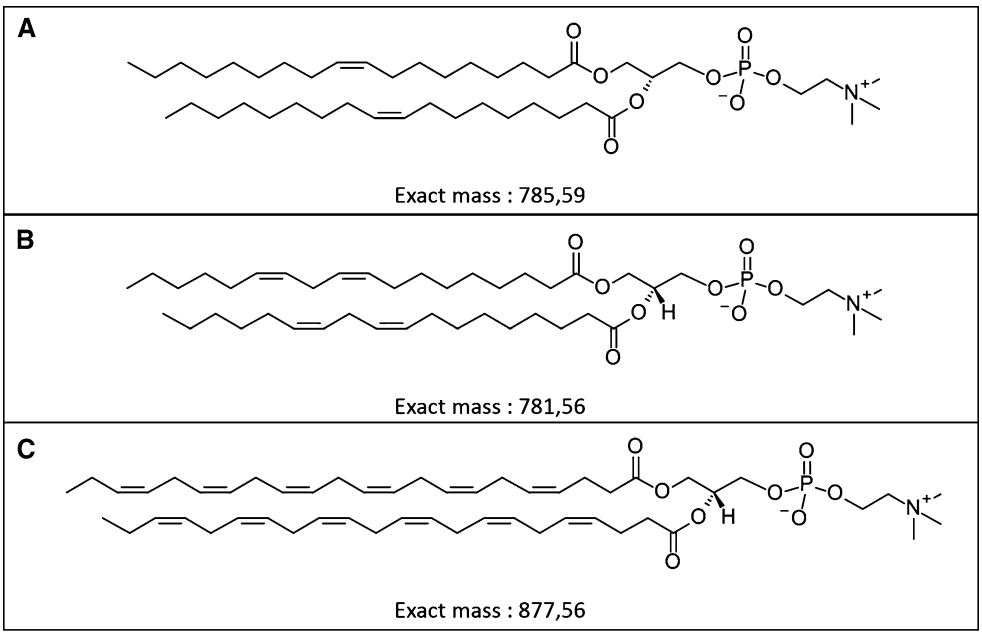

Fig. 1 Representation and exact masses (in g/mol) of the three phospholipids used in this study: DOPC (**a**), DLPC (**b**) and DHAPC (**c**)

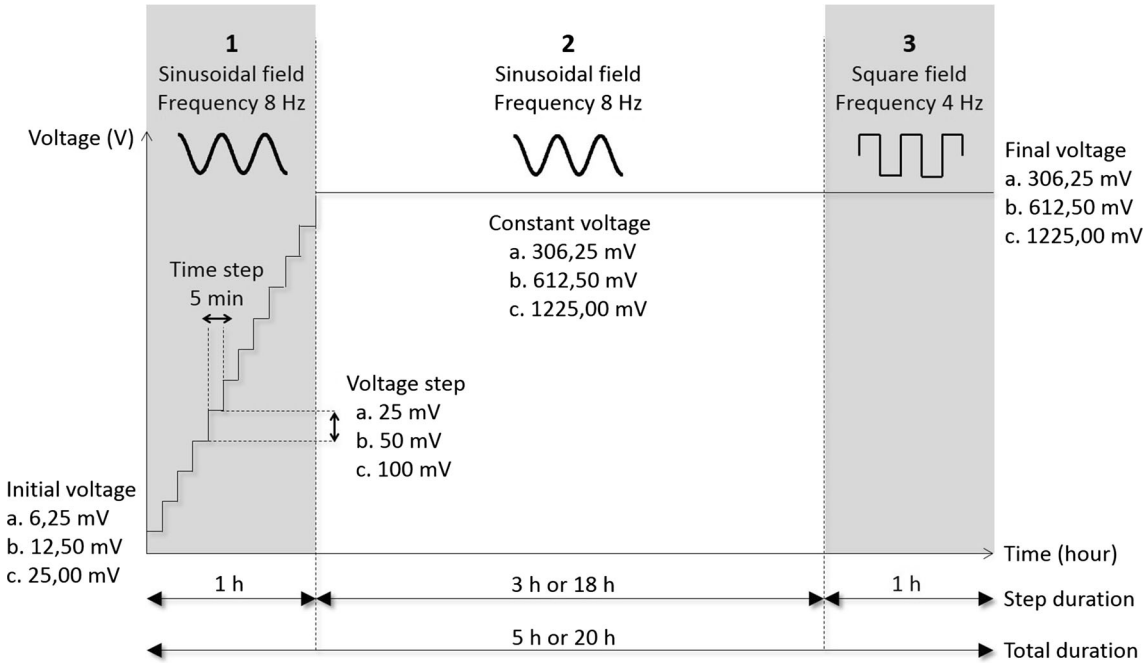

Fig. 2 Scheme presenting the electroformation protocol. First, application of a sinusoidal field of 8 Hz frequency and an initial voltage a (625 mV), b (1250 mV) or c (2500 mV). During 1 h, the voltage is increased every 5 min by steps of 25 mV for a, 50 mV for b and 100 mV for c. Second, during 3 or 18 h, application of a sinusoidal field of 8 Hz frequency at a voltage of 30,625 mV for a, 61,250 mV for b and 122,500 mV for c. Third, during 1 h, application of a square field of 4 Hz frequency at a voltage of 30,625 mV for a, 61,250 mV for b and 122,500 mV for c

the temperature, to ensure a consistency between the various experiments, as well as taking into account the phase transition temperature of the lipids to ensure that the vesicles were all in the fluid state. The desired lipid was dissolved in chloroform at a concentration of 0.5 µg/µl. A solution of the fluorescent dye DOPE-Rhodamine in chloroform was added to the lipid solution to obtain a dye concentration of 0.1 % w/w (0.6 % mol). 15 µl of the lipid solution were deposited on the conducting side of two glass slides coated with indium tin oxide (Sigma Aldrich, Saint Louis, MO, the slides were thoroughly washed with ethanol then distilled water then ethanol and dried before use). The

slides were then kept under vacuum for 2 h in a desiccator to remove all traces of organic solvent. A chamber was assembled with the slides spaced by a 1.5 mm silicone isolator (Sigma Aldrich, Saint Louis, MO). The chamber was filled with a buffer solution (240 mM sucrose, 1 mM NaCl, 1 mM KH_2PO_4/K_2HPO_4, pH 7.4). This preparation was conducted in a low conductive medium containing a minimal amount of salts so as to avoid any interference in the mass spectrometry analysis due to the salt adducts (for example, lipids complexed with sodium). The slides were connected to a function/arbitrary waveform generator (HP Agilent 33120A) and a sinusoidal AC field with a voltage of respectively 6.25/12.5/25 mV peak to peak and 8 Hz was applied. The voltage was increased by respectively 25/50/100 mV steps every 5 min, up to a value of respectively 306.25/612.5/1225 mV and maintained under these conditions for 3 or 18 h. Finally, a square-wave AC field of the same voltage amplitude was applied at 4 Hz for 1 h in order to detach the GUVs from the slides. The total formation time was therefore 5 or 20 h.

The minimal voltage required to obtain vesicles by electroformation and not by spontaneous swelling is 0.3 V in our set-up. A voltage of 1.2 V was the maximal voltage used in the literature for this protocol (Mauroy et al. 2012). The voltages studied were therefore chosen in the range 0.3–1.2 V. The presence of GUVs in the solutions were firstly checked by fluorescence microscopy. Then, the unilamellar vesicles formed were immediately analyzed by flow cytometry or mass spectrometry.

Fluorescence-Activated Cell Sorting (FACS) Cytometry of GUVs

Solutions of GUVs obtained by electroformation were diluted in a sucrose buffer (240 mM sucrose, 1 mM NaCl, 1 mM KH_2PO_4/K_2HPO_4, pH 7.4) to an appropriate concentration and were analyzed by flow cytometry using the C6 flow cytometer (BD Accuri, San Jose, CA). For each sample, 10000 data points were recorded. The flow cytometer simultaneously recorded the forward laser light scattering (FSC), the side laser light scattering (SSC), and the level of fluorescence of each event as well as the number of events. The fluorescence excitation wavelength was 488 nm, and the emission was detected through a 585 ± 40 nm band pass filter in order to detect the rhodamine-labeled vesicles. The graph of the SSC as a function of the fluorescence on all events was drawn (Fig. 3a). The vesicle population was selected to obtain the number of events per microliter and the mean fluorescence. Then, the graph of the FSC as a function of the fluorescence for the vesicle population only was drawn to obtain the mean FSC value (Fig. 3b). For each sample, the graph of the FSC height as a function of the FSC area was drawn to check for the absence of aggregates that could modify the results (Fig. 3c). In all cases, only a single population was detected which confirmed the absence of aggregates.

Mass Spectrometry Analysis of Phospholipid Oxidation

50 µl of the vesicle solution were added to 250 µl of a mixture of chloroform/methanol (2/1, v/v). Solutions were then vortexed and centrifuged. The inorganic phase was removed and the organic phase was evaporated under a stream of nitrogen. 50 µl of methanol were added and the solutions were vortexed. The Quattro LC mass spectrometer (Micromass, Manchester, UK) controlled by the MassLynx software system (version 3.4) and equipped with an electrospray ionization and a quadrupole analyzer was operated in the positive ion mode with a capillary voltage of 3 kV, a cone voltage of 30 V, the source block temperature set to 90 °C and the desolvation temperature set to 200 °C. The elution solvent was acetonitrile: 5 mM ammonium acetate (90:10, v/v). MS/MS (i.e. tandem mass spectrometry) spectra of the parent scan of the ion $m/z = 184$ were obtained over a mass range of m/z 500–1000. These spectra present peaks corresponding to the compounds that contain the 184 molecular fragment which is characteristic of the phosphatidylcholine head group. We could therefore specifically detect peaks corresponding to products containing a phosphatidylcholine head and an increase in weight compared to the non-oxidized lipids. We considered additions of up to six oxygen (corresponding to increases in mass of 16, 32, 48, 64, 80 and 96).

An example of the spectra obtained for DLPC are presented in Fig. 4. The spectrum of standard DLPC only displays the peak at $m/z = 782$ which is the molecular ion of DLPC. The mass spectrometry analysis therefore has no impact on the lipids. The MS/MS spectra of standard DOPC and DHAPC are presented in supplementary Fig. 1. The spectra of the organic extract of DLPC vesicles displays new peaks at $m/z = 798$, 814, 830 and 846 corresponding respectively to an addition of 1, 2, 3 and 4 oxygen atoms. The ion current as a function of the elution time was drawn for each ion peak present in the spectrum. For example, in Fig. 4, the ion currents corresponding to $m/z = 782$, 798, 814, 830 and 846 have been drawn. The spectra of the ion currents as a function of the elution time have been integrated. Finally, the percentage of oxidation of the samples were calculated by dividing the value of the integration of the ion current corresponding to the oxidized lipids in the MS/MS spectra (in Fig. 4, $m/z = 798$, 814, 830 and 846) over the integration of the ion current corresponding to all the masses of interest in the MS/MS spectra, both intact and oxidized lipids (in Fig. 4, $m/z = 782$, 798, 814, 830 and 846).

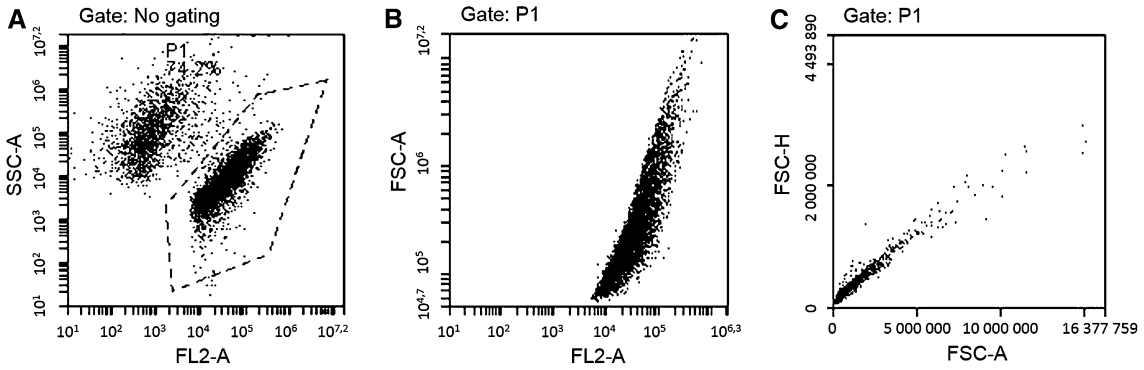

Fig. 3 Flow cytometry analyses of a solution of DLPC vesicles obtained after an electroformation of 20 h with a maximal voltage of 0.6 V. **a** Graph of SSC area (SSC-A) as a function of the fluorescence (FL2-A) that allows to gate the vesicle population (P1, *dotted line*). **b** Graph of FSC area (FSC-A) as a function of the fluorescence (FL2-A) gated for the vesicle population P1. **c** Graph of FSC height (FSC-H) as a function of FSC area (FSC-A) gated for the vesicle population P1

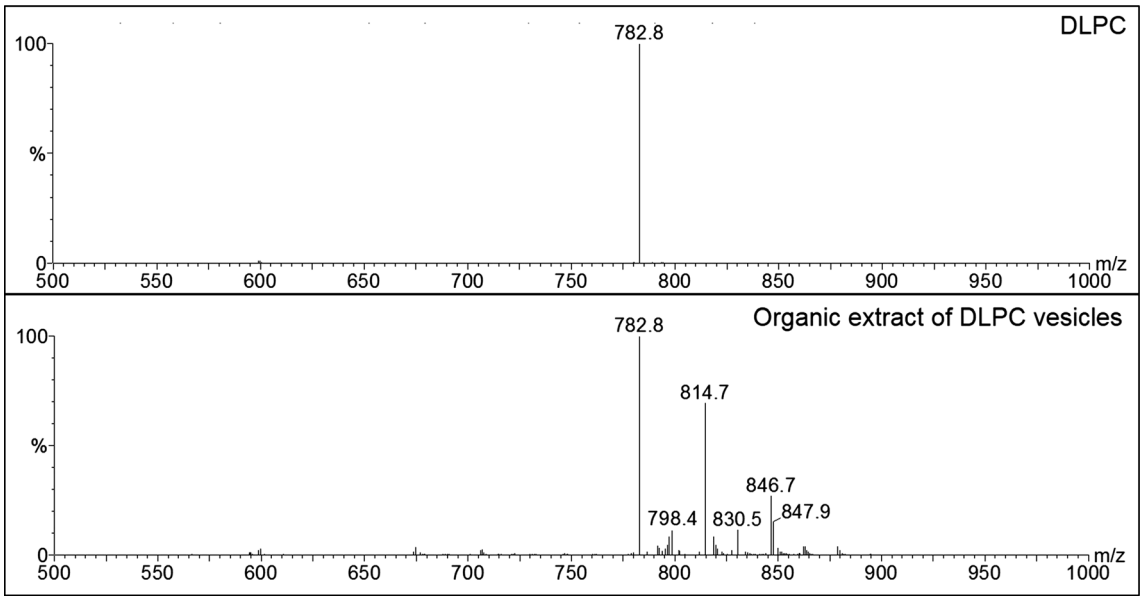

Fig. 4 Positive ion electrospray MS/MS spectra (parent scan of the ion $m/z = 184$) of DLPC and of organic extracts of solutions of DLPC vesicles obtained by a 20 h electroformation with a maximum applied voltage of 1.2 V

In some cases, the oxidation of phospholipids can be followed by a fragmentation which gives rise to low masses secondary products. Since the MS signals of these products were negligible, they have not been considered in this study.

Results and Discussion

Effect of the Maximum Applied Voltage and the Electroformation Duration on the Level of Lipid Oxidation

Our data reveal that GUV electroformation provokes an oxidation of the lipids constituting these GUVs. The effect of the maximum applied voltage on the lipid oxidation level in the vesicles is shown in Fig. 5.

For a 5 h electroformation, whatever the maximal voltage applied, DOPC lipid vesicles have the lowest level of lipid oxidation whereas DHAPC lipid vesicles exhibit the highest level of lipid oxidation. Whatever the duration of the electroformation the DOPC lipids still have a low level of oxidation, which shows that, in this voltage range, the electric field does not induce a significant oxidation of DOPC lipid. For DLPC vesicles, the oxidation level increases with the maximum applied voltage and the duration of electroformation as expected if the application of the electric field is responsible for the oxidation of the lipids. For the vesicles made of the very oxidizable

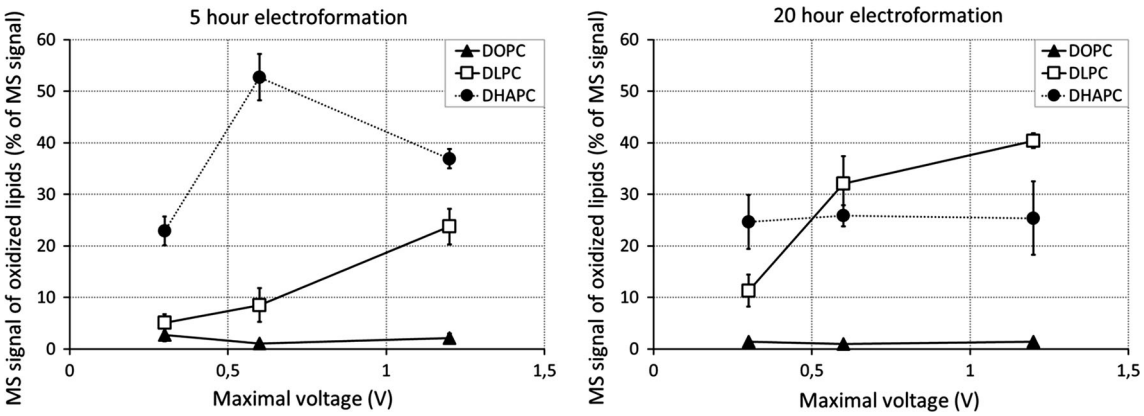

Fig. 5 Percentage of lipid oxidation in vesicles obtained by a 5 or a 20 h electroformation as a function of the maximum applied voltage, measured by mass spectrometry analysis. The displayed results are the mean of four independent experiments ± standard deviation (SD)

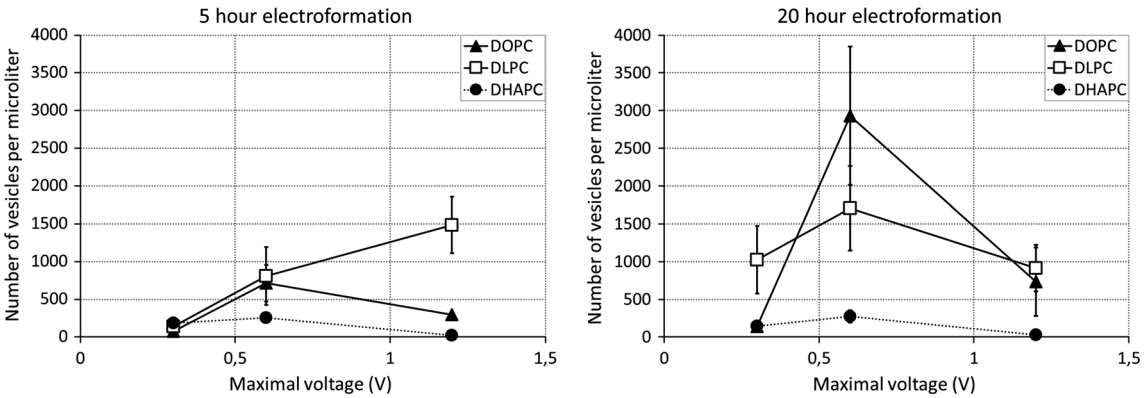

Fig. 6 Number of vesicles per microliter measured by flow cytometry in solutions of vesicles obtained by a 5 or a 20 h electroformation as a function of the maximum applied voltage. The displayed results are the mean of four independent experiments ± SD

DHAPC lipid, when the electroformation lasts for 5 h, an increase in the maximal applied voltage of 0.3–0.6 V induces an increase in the level of oxidation of the lipids of the vesicles. A further increase of the voltage should induce a higher oxidation level. However, a decrease is observed. This can be explained by the fact that the vesicles analyzed by mass spectrometry are those that can be recovered with a pipette, that is to say the vesicles remaining in the solution. It is possible to consider that the increase in the voltage from 0.6 to 1.2 V induces a level of lipid oxidation too high (which must be over 50 % since this is the maximal oxidation rate that has been measured), which then would cause the most oxidized vesicles to burst. Then, the only vesicles remaining in the analyzed solution would be the GUVs presenting a content of oxidized lipids compatible with the formation of stable vesicles. Indeed, it has been previously demonstrated that when vesicles burst, their lipid content does not remain in the solution but aggregates at the bottom of the vesicle solution container (Salomone et al. 2014). Concerning the 20 h electroformation, the level of DHAPC oxidation of the vesicles is constant whatever the maximal applied voltage. Increasing the duration of the electroformation should increase the level of lipid oxidation, but the opposite is observed. Again, it can be assumed that the very high level of oxidation would impair the stability of vesicles with a high percentage of oxidized lipids. Therefore, only moderately oxidized vesicles were recovered for analysis.

Generally, for oxidizable lipids, the rate of oxidation in the vesicles increases when the duration of the electroformation increases or when the applied voltage increases up to a threshold value above which the vesicles would explode.

Effect of the Maximum Applied Voltage and the Electroformation Duration on the Number of Vesicles

The effect of the maximum applied voltage on the number of vesicles obtained per microliter is shown in Fig. 6.

For vesicles made of DOPC, which are therefore nearly non-oxidizable, the number of produced vesicles reaches a

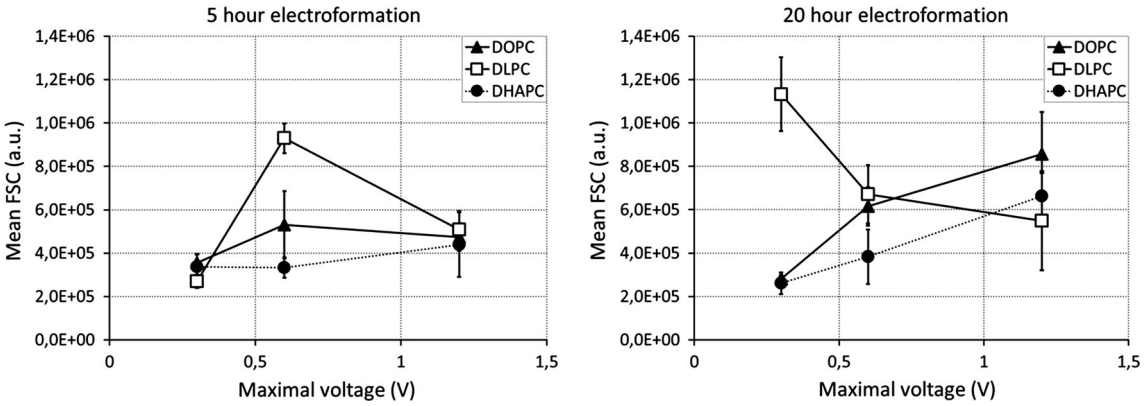

Fig. 7 Mean FSC (forward scatter) measured by flow cytometry for solutions of vesicles obtained by a 5 or a 20 h electroformation as a function of the maximum applied voltage. The displayed results are the mean of four independent experiments ± SD

maximum at an electroformation voltage of 0.6 V irrespective of the duration of the electroformation. The application of an electric field produces a force on the lipid layers called electromechanical stress that is responsible for a movement of the lipid layers. This movement allows the entry of water between the layers of lipids and the formation of vesicles (Angelova and Dimitrov 1988). For DOPC lipids, a voltage of 1.2 V appears to cause a decrease in the number of vesicles produced which could be due to an excessive mechanical stress. On the contrary, increasing the duration of the electroformation from 5 to 20 h appears to increase the efficiency of the vesicle production. For DLPC vesicles, for a 5 h electroformation, the number of vesicles increases when the voltage increases without reaching a maximal value. For the 20 h electroformation of DLPC vesicles, the number of produced vesicles reaches a maximum at 0.6 V. The decrease in the number of GUVs at 1.2 V is in agreement with the hypotheses made in the previous section: the presence of a very large proportion of oxidized lipids does not allow for the generation of stable vesicles. Concerning the vesicles made of DHAPC lipids, the number of vesicles produced is the lowest whatever the conditions. This appears to be correlated with the high level of oxidation of the lipids in the DHAPC vesicles. Indeed, it is possible to compare DHAPC vesicles and DLPC vesicles obtained after an electroformation of 5 h at 0.6 and 1.2 V and after an electroformation of 20 h at 0.3 and 0.6 V. For these voltages, the oxidation rate of DHAPC is always above 40 % and the oxidation rate of DLPC is always below 40 %. For these conditions, the concentration of DHAPC vesicles is always low (below 300 vesicles/µl), whereas the concentration of DLPC vesicles is always high (above 800 vesicles/µl). Moreover, when the oxidation rate of DLPC reaches 40 % (for an electroformation of 20 h at 1.2 V), the concentration of vesicles decreases, while it should increase as seen for the 5 h electroformation. This is in agreement with the hypothesis that a high level of oxidation impairs the formation of vesicles.

In conclusion, the number of vesicles produced increases as the duration of the second step of the electroformation increases or when the maximum applied voltage increases. However, when the rate of oxidation of the lipids in the vesicles is too high, the number of vesicles is decreased.

Effect of the Maximum Applied Voltage and Electroformation Duration on Size of Vesicles

The flow cytometry parameter called FSC (Forward Scatter) is related to the diameter of the detected objects that go through the laser light of the cytometer. An increase in the FSC thus reflects an increase in the diameter of the analyzed objects (in our case, vesicles). The effect of the maximum voltage applied during electroformation on the mean FSC value of the vesicle solutions is shown in Fig. 7.

For DOPC vesicles, increasing the voltage and the duration of the electroformation seems to induce an increase of the diameter of the vesicles. For DLPC vesicles electroformed during 5 h, the mean FSC reaches a maximum for an applied voltage of 0.6 V then decreases. The level of lipid oxidation, which is higher than 25 % for a voltage of 1.2 V, appears to hamper the production of large vesicles. Increasing the duration of electroformation to 20 h induces an increase in the FSC for an applied voltage of 0.3 V. However, once again, the FSC decreases for higher voltages, which can be explained by the corresponding levels of lipid oxidation which are superior to 25 % (Fig. 5). Finally, DHAPC is very sensitive to oxidation. Thus for a 5 h electroformation at a voltage equal to or greater than 0.6 V or for a 20 h electroformation whatever the voltage, the oxidation level of the lipid vesicles is always above 20 %, which could explain why the FSC remains low and follows the same trend as for the

DOPC-based vesicles. Indeed, the very few vesicles produced which remain in solution have a low lipid oxidation level and behave like the non-oxidized DOPC-based vesicles.

The diameter of the vesicles seem to increase as the duration of the electroformation increases or when the applied voltage increases. However, when the rate of oxidation of the lipids in the vesicles exceeds 25 %, a decrease in the diameter of the vesicles is observed.

Given the parameters studied, the conditions that allow for the most favorable balance between a high number of vesicles, a low level of oxidation, and a high size of the vesicles, are:

- For DOPC vesicles, a maximum voltage of 0.6 V applied during 20 h,
- For DLPC vesicles, a maximum voltage of 0.3 V during 20 h or a maximal voltage of 0.6 V applied during 5 h,
- For DHAPC vesicles, a maximum voltage of 0.3 V applied during 5 h.

Conclusion

Thanks to mass spectrometry and flow cytometry analyses, we could demonstrate that the electroformation technique used to form these vesicles causes the oxidation of the polyunsaturated phospholipids constituting the vesicles. This oxidation is probably the consequence of the application of the electric field. One of the most striking conclusions of this work is that the classical parameters of the electroformation method have proven to be inadequate for the three different phospholipids considered in this study. The oxidation level of the phospholipids increased with their level of unsaturation as well as with the duration and the maximum applied voltage of the electroformation. Oxidation is a very important parameter to consider. Indeed, according to our results, when the level of lipid oxidation exceeds 25 %, the diameter of the vesicles is decreased and when the level of lipid oxidation reaches 40 %, the vesicles burst or reorganize and their rate of production is reduced. Therefore, for each phospholipid (especially for oxidizable unsaturated lipids), a study must be conducted to determine which parameters allow for the best balance between the number of vesicles obtained and the lipid oxidation level. This will allow for an optimized preparation of the GUV. Noteworthy, it would be interesting to perform similar studies with mixture of lipids.

Finally, since an oxidation of the phospholipids can induce the modification of the physico-chemical properties and the structural ordering of the vesicle membrane, this study clearly points out that the classical parameters of the electroformation method should be used with caution especially for using giant liposomes of polyunsaturated phospholipids as a cell membrane model.

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