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Content Delivery of Lipidic Nanovesicles in Electropermeabilized Cells

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Abstract Lipidic nanovesicles (the so-called liposomes) were among the one of the earliest forms of nanovectors. One of their limits was our lack of knowledge on the delivery pathway of their content to the target cell cytoplasm. In most models, it appears to be linked to endocytotic transfer. Their direct content delivery can be enhanced by electric field pulses applied to a cell liposomes mixture. The optimal form for liposomes was shown to be large unilamellar vesicles (LUV). The present communication describes an optimization to enhance the delivery. When lipidic nanovesicles (LUVs) are electrostatically brought in contact with electropermeabilized cells by a salt bridge, their content is delivered into the cytoplasm of electropermeabilized cells. The PEF parameters are selected to affect specifically the cells leaving the vesicles unaffected. Cell viability is positively affected by the treatment. High-field short pulses are more efficient than low-field long pulses. A homogeneous cytoplasm labeling is observed under digitized videomicroscopy. The process is a content mixing, not an endocytotic pathway. The lipidic composition of the LUV should contain charged lipids (phosphatidylserine), fusion promoting lipids (phosphatidylethanolamine), and cholesterol.

Keywords Drug delivery · Electropermeabilization · Electroporation · Lipidic nanovesicles · Liposomes · LUV

Introduction

Nanovesicles are now described as efficient tools to target drug delivery to specific cells and organs (Verderio et al. 2014). Most of them are internalized by active processes, generally called endocytotic pathways. In such cases, no direct delivery to the cytoplasm is obtained. There is a need to chemical or physical methods to affect the interactions at the nano–bio interface, which in turn mediate the nanoparticle internalization routes. Lipidic nanoparticles (the so-called liposomes) were the first nanovesicles brought to the market for drug delivery (Allen and Cullis 2013; Bozzuto and Molinari 2015). Again delivery is in most cases obtained by endocytosis. Modification of the lipid composition of liposomes by the addition of fusogenic lipids or membrane-active inserted peptides was proposed to disrupt the cell membrane to obtain a direct cytoplasmic delivery of the drug cargo (Parente et al. 1988; Bailey et al. 1997; Torchilin et al. 2001), but the use of this approach remained very limited. Fusion of liposomes can be obtained by delivering electric pulses to giant unilamellar vesicles brought into contact (Haluska et al. 2006; Stoicheva and Hui 1994). This is indeed a development of the intercellular electrofusion (Rems et al. 2013; Usaj et al. 2010). We proposed some years ago an adaptation of these methods to enhance the direct cytoplasmic delivery of the content of large unilamellar vesicles.

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Taking advantage of the size dependence of electropermeabilization (Sixou and Teissie 1990), cells in contact with smaller size liposomes were electropermeabilized under conditions preserving their viability (Ramos et al. 2002). Such a heterofusion was suggested previously (Chernomordik et al. 1991). Liposomes were too small to be directly affected by the field (Teissie and Tsong 1981). Fusion was assayed by content mixing. Fusion was detected only when cell electropermeabilization was induced. As a constant electric field intensity was used in all experiments and as the delay between the pulses was too short for the cell to rotate during the application of the train of pulses, the part of the cell surface that was brought to the permeabilized state was constant in all experiments (Teissie and Ramos 1998). An increase in the number of pulses is known to bring an increase in the number of defects in the permeabilized part of the cell (Teissie and Ramos 1998). Such an increase in defects may affect the cell viability by making the membrane permeabilization irreversible. Cell fusion was shown to be controlled by the number of pulses (Teissie and Ramos 1998). It was proposed that this was linked to the density of defects as long as the cell viability was not affected. The conclusion was that a high density of defects brings a high level of permeabilization and a membrane state highly competent for fusion. Vesicle spontaneous fusion with an electropermeabilized cell was facilitated when the membrane state was strongly competent for fusion for a given number of vesicles.

The present study is an approach to bring more details in the description of the transfer from LUVs. Optimization of the amount of lipid particles and in their lipid composition is investigated. Consequences on our understanding of the basic processes supporting the delivery as well as on the practical procedures are briefly reported.

Materials and Methods

Materials

8-Hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS), and propidium iodide (PI) were obtained from Molecular Probes (Eugene, OR, USA). Lipids were obtained from Avanti Biochemicals (Alabaster, AL, USA) (egg yolk phosphatidylcholine (PC), bovine brain phosphatidylserine (PS, 840032), 1,2-dioleoyl-sn-Glycero-3-Phosphocholine (DOPC, 850375), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE. 850757)). Cholesterol was purchased from Sigma (362794). Phosphate buffered saline (PBS) was purchased from Gibco (Grand Island, NY, USA). Salts were all analytical grade. Ultrapure water was obtained from a MilliQ system (Millipore, France).

Cells

The wild-type Toronto (WTT) is derived from Chinese hamster ovary cells (CHOs) and was first introduced in the 1960s. It can grow in suspension and in culture flasks (generation time = 18–20 h).

The possibility to grow in suspension prevents the necessity of trypsinisation.

MEM 0111 buffer (Eurobio France (ref: CM1 MEM40 K-BP)) with Fetal Calf Serum 8 %, (SVF EUROBIO, ref: CVFSVF00-01, lot no: S155839), D (+)—Glucose 45 %, (3.5 g/l) (Sigma, USA), Tryptose phosphate (2.95 g/l), Vitamins (GIBCO, ref: 043-01040), and Antibiotics (penicillin 100 units/ml, streptomycin 100 mg/ml, L-glutamin 0.58 mg/ml) is used to cultivate the CHO cells under slow agitation (70 to $100\times g$, 37 °C). Cells stay in the exponential growth phase by a control of the cell number (dilution from 0.55 up to 0.7×10^6 cells every day).

Liposome Preparations

The PC/PE/PS/Cholesterol lipid mixture (at different molar ratios) (10 mg total) was dissolved in chloroform. Lipids were dried under a nitrogen flow and then under vacuum (30 min). They were then resuspended in 5 mM Hepes (pH 7.2) containing HPTS (0.5 mM) and vortexed to form large multilamellar vesicles (MLV). The lipid dispersion was treated by thawing and freezing several times. Large unilamellar vesicles (LUVs) were obtained by extrusion by forcing the lipid suspension five times through two successive through 0.2 μm pore polycarbonate membrane (The Avanti Mini-Extruder (AVANTI)).). The free HPTS was separated from the liposomes by a Sephadex G 75 gel filtration equilibrated with a NaCl (0.145 M) HEPES 10 mM buffer (pH 7.4). The temperature of HEPES buffer was kept above gel–liquid crystal transition temperature of the lipid mixture (i.e., −20 °C for DOPC).

The final concentration in liposomes was obtained by an assay of the total final phosphorus concentration (Rouse et al. 1970). It was about 1 mg/ml after the final wash by the gel filtration.

The size homogeneity was checked by dynamic light scattering (Proteinsolution Dynapro) (Supplementary Fig. 1).

Electropulsation Protocol

Just before electropulsation, cells were washed in a pulsation medium with an iso-osmotic low ionic content (PBCa) (HEPES 10 mM, sucrose 250 mM, $CaCl_2$ 5 mM). Cells washed in this appropriate pulsing buffer are finally resuspended at 4×10^7 cells/ml. Mixtures with different ratio of cells and liposomes were evaluated (see results). In

most experiments, 50 µl of the cell suspension was mixed with 200 µl of the liposome solution. 5-min incubation at 4 °C was observed. The volume was placed between thin stainless steel parallel electrodes with an inter-electrode distance of 4 mm. The edges of the electrodes were in contact with the bottom of a plastic Petri dish (Nunc 153066 TC) to build an open pulsing chamber. The field was uniform in the sample. Voltage pulses were performed then by a voltage generator that gave a constant electric field (CNRS Cell Electropulser, Jouan, France). In this way, the pulse intensity (480 V, 1.2 kV/cm) and duration (100 µs) could be kept constant (HFSP, high-field short pulse). The voltage pulse applied to the cell suspension was monitored with an oscilloscope associated to the cell pulsator (Supplementary Fig. 2). 10 Repetitive pulses were applied with a 1-s delay. In another set of experiments, another protocol was applied where ten pulses lasting 5 ms at a strength of 800 V/cm were delivered (LFLP low-field long pulse). The suspension was then incubated for 10 min at room temperature, washed, and then resuspended in 1 ml PBS washing buffer (Dulbecco's phosphate buffered saline, Eurobio, France, pH 7.2,–Ca^{2+}–Mg^{2+}). $Ca^{2+‡}$, when present, was therefore chelated, and almost no free ions were left. This step removed the electrostatic bridges. The unfusioned nanovesicles were not bound anymore to the surface of the cells and were diluted in the volume of the washing buffer. They could be washed out by centrifugation (5 min, $100 \times g$). The cell pellet was then resuspended once again in PBS and analyzed. This procedure was chosen (i) to preserve cell viability (Ramos et al. 2002) and (ii) to avoid a long contact between cells and liposomes, where a significant endocytotic uptake may occur.

Detection of Fusion

Single-cell fluorescence emission was evaluated by flow cytofluorometry on a Facscan (Becton–Dickinson, Cockeysville, MD, USA) using the FL1 channel (to detect HPTS-positive cells) and the FL3 channel (for the viability assay with PI). The population of intact cells was obtained from the double-scattering SSC/FCS dot plot to remove the debris. Gating of positive cells was manual (Supplementary Fig. 3). This gives access to the relative population of cells of interest (either where HPTS delivery was present (HPTS-positive cells) or where the PI emission was weak (viable cells)) and to the mean fluorescence signal in the HPTS-positive population.

Cells were analyzed with the fluorescence-digitized microscope Leica DM IRB (Wetzlar, Germany) using the filter set for fluorescein. Video monitoring was possible with a cooled CCD camera (Princeton Instruments, NJ, USA). The pictures were taken with the Metavue software (Molecular Devices, USA). Images were analyzed with ImageJ (for more information http://rsb.info.nih.gov/ij/index.html). No filtering was operated on the raw data. A region of interest (ROI) was selected covering the cell and was analyzed to obtain the associated fluorescence emission (Supplementary Fig. 4).

Cell viability

A short-term assay of cell viability was obtained by observing unsealed cells 20 min after the pulse delivery. Permeabilized cells gave a fluorescent signal when 0.2 ml PI (0.1 mM, i.e., 50 µM final) was added. The observation was performed within less than 30 min after adding the dye to prevent an endogenous uptake. The relative population was estimated by flow cytofluorometry using the FL3 channel.

Cell viability on the long term was measured by quantifying the cellular growth over 48 h by crystal violet staining. Briefly, cells were stained with 1 mL crystal violet (Merck 1-01407-02580; 0.1 % in pulsing buffer) for 20 min, washed with PBS, and then lysed with 500 µL acetic acid (10 %) for 5 min. Cell density was evaluated by 595 nm OD measurement.

Viability evaluated by the short-term and the long-term methods gave comparable conclusions.

Fluctuations of viability after the fusion treatment on a week to week schedule were classically observed. To limit their consequences, we kept into account only experiments where the viability was preserved over about 40 %.

Results

Effect of the Electrical Parameters

Liposomes (LUVs) were prepared with PS as the charged partner (PC/Cholesterol/PS/PE 4/3/2/1), and Ca^{2+} was present in the pulsing buffer to mediate the electrostatic interaction between liposomes and cells (0.7 mM final concentration). The unpulsed cells displayed a faint peripheral labeling under the microscope as already observed (Ramos et al. 2002). The FACS data were just telling that a background signal was present due to the LUVs that were present in the solution around the cell. This was of course increased with the concentration of LUVs and was used to correct the signal associated with the uploaded HPTS from cells. The cell viability was not affected with the procedure where the PEF treatment was not delivered.

Under the HFSP condition, the uptake of HPTS from the LUVs by the pulsed cells showed under the microscope a uniform cytoplasm labeling in a significant number of cells as reported in Ramos et al. (2002) (data not shown and Supplementary Fig. 4). This was indicative of the LUVs to

cell content mixing and confirmative of previous conclusions that electropermeabilization was the support for HPTS delivery. As it will be reported later, the HFSP treatment did not significantly affect the cell viability.

A more quantitative analysis was obtained by the data from the cytofluorimeter (Supplementary Fig. 3).

Optimization of the Cell to LUVs Ratio

At a given number of cells, the volume of LUVs was increased, i.e., the LUVs per cell ratio was increased. The mixture was pulsed under the HFSP conditions. An increase in the percentage of cells that were HTPS positive was observed (Fig. 1). Again the protective effect of LUVs on the cell viability was observed but slightly increased with the LUVs amount. Loading of cells was directly related by the occurrence of fusion events and was monitored by the fluorescence of each single cell. This was evaluated from the mean fluorescence measured on the population by flow cytofluorometry (Fig. 2). This intensity was the sum of the background signal, the fluorescence of the dye entrapped in the cell as a consequence of the fusion, and the fluorescence of the LUVs that were in the buffer around the cell. The background was observed on pulsed cells with no added LUVs. The fluorescence of the non-trapped LUVs was directly related to the amount of added LUVs even if they were diluted after the addition of the PBS solution to the cell suspension post-pulse delivery. Its intensity was obtained by observing non-pulsed cells. We observed that the mean fluorescence entrapped in pulsed cells increased sharply when 50 and 100 µL LUVs were added and then leveled off (Fig. 2). This was indicative that a saturation in fusion was obtained when adding more than 200 µL under our pulsing protocol.

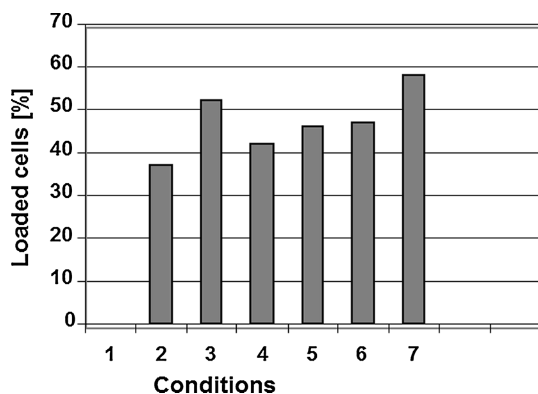

Fig. 1 Dependence of the percentage of loaded cells on the amount of LUVs. The volume of cells was kept constant at 0.1 ml (2×10^7 ml^{-1}). Increasing volumes of LUVs are added (*1* no LUVs, *2* 50 µl, *3* 100 µl, *4* 150 µl, *5* 200 µl, *6* 300 µl, and *7* 500 µl). The mixture was pulsed and washed

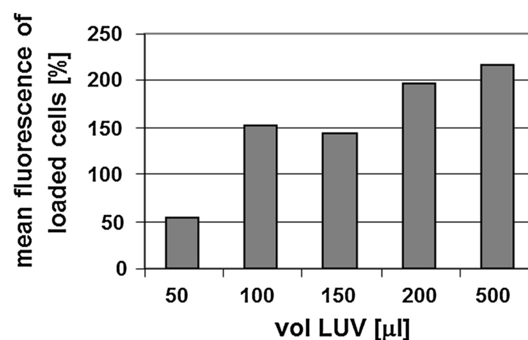

Fig. 2 Mean fluorescence per loaded cells with increasing amount of LUVs. The volume of cells was kept constant at 0.1 ml (2×10^7 ml^{-1}). Different volumes of LUVs were added. The mixture was pulsed and washed

At a given volume of LUVs (0.1 ml), increasing amounts of cells were added (from 10^6 to 2.3×10^6 by adding increasing amount of a 2 10^7/ml suspension) (Fig. 3). The relative percentage of HPTS-positive cells was observed to decrease with the number of cells, but their total number remains constant about 2×10^5. The mean fluorescence per loaded cell was observed to decrease with the increase in the number of cells in the pulsed sample (Fig. 4). This is in agreement with the previous experiment showing that with the associated dilution of the LUVs solution, the conditions were below the saturation in LUVs.

Further experiments were therefore performed on a mixture obtained by adding 200 µL LUV to 50 µL of cells (10^6). This condition appeared as the most efficient to load cells (high-percentage, high-mean fluorescence).

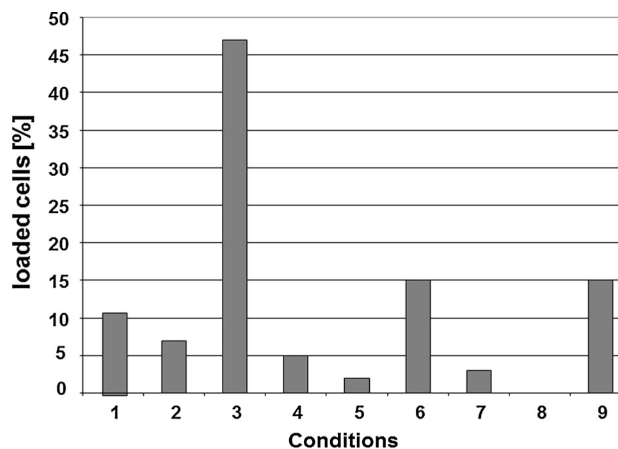

Fig. 3 Dependence on the percentage of loaded cells on the amount of added cells. The volume of LUVs was kept constant at 0.1 ml. Increasing volumes of cells (2×10^7 ml^{-1}) are added. Conditions were (*1*) LUVs, 50 µl cells, no pulse, (*2*) 50 µl cells, pulsed, (*3*) LUVs, 50 µl cells, pulsed, (*4*) LUVs, 100 µl cells, no pulse, (*5*) 100 µl cells, pulsed, (*6*) LUVs, 100 µl cells, pulsed, (*7*) LUVs, 150 µl cells, no pulse, (*8*) 150 µl cells, pulsed, and (*9*) LUVs, 150 µl cells, pulsed

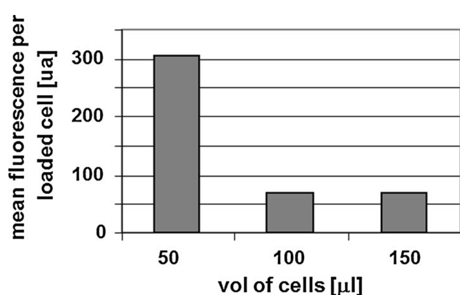

Fig. 4 Mean fluorescence per loaded cells with increasing amount of cells. The volume of LUVs was kept constant at 0.1 ml. Different volumes of cells (2×10^7 ml^{-1}) were added. The mixture was pulsed and washed

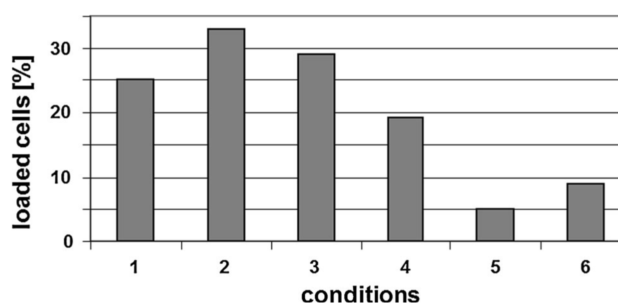

Fig. 5 Percentage of loaded cells as a function of the lipid mixture. The volume of cells was kept constant at 0.05 ml (2×10^7 ml^{-1}). 0.2 ml of LUVs with different compositions were added. The mixture was pulsed and washed. Conditions were (*1*) PC/PS/PE/Chol 4/1/2/3, (*2*) PC/PS/PE/Chol 3/2/2/3, (*3*) PC/PS/PE/Chol 3/1/3/3, (*4*) PC/PS/PE/Chol 5/1/1/3, (*5*) PC/PS/PE/Chol 6/1/0/3, and (*6*) PC/PS/PE/Chol 5/1/2/2

Control by the Lipid Composition of the LUVs

Fusion of lipid vesicles by the addition of calcium was observed to be dependent on the composition of the lipid mixture used to prepare the liposome. This was explained by the molecular forces present in the lipid bilayer (Nir 1991). The occurrence of a similar control in the LUVs fusion with cells under the electric field pulse effect was investigated.

The main host phospholipid was chosen to remain phosphatidylcholine (PC) with chain lengths such that the phase transition temperature was lower than the experimental temperature (room temperature). Its phase was fluid. PC is known to be the main component of the plasma membrane, and we tried to avoid to induce a large change in the membrane composition along the fusion with LUVs. Cholesterol (CL) was present to mimic the mean lipid content of the plasma membrane of mammalian cell membrane (33 %) and not to induce local unbalances in cholesterol when the fusion was induced. Phosphatidylserine (PS) was requested in our approach to allow the formation of the Ca^{2+} electrostatic bridge between the LUVs and the cell surface. Phosphatidylethanolamine (PE) was described to a critical constituent in the liposome homofusion due its non-bilayer behavior (Teissié and Rols 1992) and its effect was evaluated in the present approach. Due to the large size (diameter larger than 200 nm), no significant constraint on packing was present on LUVs, and one might assume that the same lipid distribution was present on each monolayer in the bilayer. It should be mentioned that no spontaneous fusion between the LUVs was expected under our experimental conditions (5 mM Ca^{2+}) (Verkleij 1991).

Different compositions between these 4 lipids were evaluated. HFSP electrical conditions were applied. Results are shown in Fig. 5. Fusion was clearly affected by the lipid composition. The most significant results were that high levels in PS (20 %) and PE (30 %) were promoting the most efficient fusion. Indeed PE was requested to obtain a significant fusion. Cholesterol was needed at a high (physiological) level (30 %) to obtain a high fusion.

Effect on Cell Viability

Viability of fused cells showed that a high level of fusion was associated to a low level of protection. Taken into account that high level in fusion was associated with a high loading in HPTS, the question of a toxicity of HPTS was open. Similar experiments were therefore performed with empty LUVs with a similar composition. Amazingly, a protective effect was observed. It was observed that the protective effect of LUVs was dose dependent as the fusion efficiency (Fig. 6). It was observed that the highest viability was observed with LUVs that provided the highest level in fusion, but under this HFSP protocol, the viability was always largely preserved (data not shown).

Two different pulsing conditions were investigated either the high-field short pulses (HFSP 1.2 kV/cm, 0.1 ms) or the low-field long pulses (LFLP 0.8 kV/cm, 5 ms) (Fig. 7). This second condition was not investigated in previous studies, but our present conclusion is that HFSP conditions were more effective for fusion (data not shown). LFLP are known to be highly effective for the transfer of macromolecules (pDNA) but always with a high loss in cell viability (see Fig. 7). When LUVs were present electrostatically bound to the cell surface, the cell viability was less affected. Under the two protocols, LUVs played a protective role against the damaging effect of the electric pulses.

Discussion

Content transfer from LUVs to the cell cytoplasm was easily obtained by our procedure as previously reported. More information were provided by the present study where up to 50 % of the pulsed cells were observed to be loaded with the dye initially trapped in the LUVs.

An increase in fusion was obtained when more vesicles were present. This suggested that the probability of contact

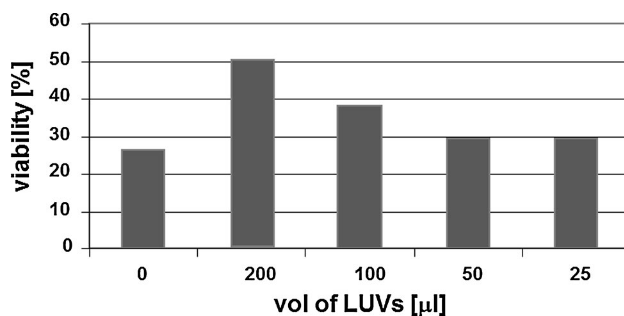

Fig. 6 Short-term viability as a function of added empty LUVs. The volume of cells was kept constant at 0.05 ml (2×10^7 ml^{-1}). Different volumes of empty LUVs were added. The mixture was pulsed and washed. LUVs were not loaded with the dye to avoid its toxicity

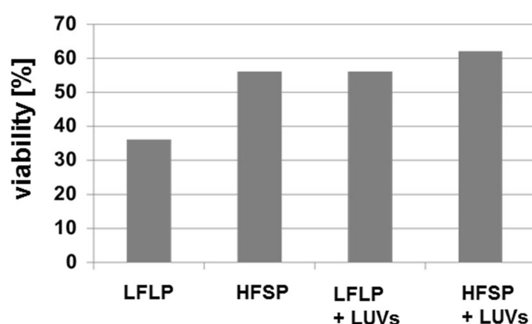

Fig. 7 Short-term viability as a function of the pulsing conditions. The Cell [0.05 ml (2×10^7 ml^{-1})] LUVs (0.2 ml) mixture was treated under HFSP or LFLP conditions with or without added LUVs

between vesicles and cells was increased at high vesicle concentration, but a saturation was observed. This may result from a steric hindrance between LUVs when they were bound on the cell surface. This would limit the number of LUVs in contact with the cell surface and therefore available for fusion with the cell. Increasing the amount of LUVs in the solution would not increase the number of LUVs in this critical layer. Another explanation took into account that due to fusion the cell surface was modified and turned to a non fusogenic state when this modification reached a critical level.

The effect of the nature of the lipidic composition of the LUVs might result from the specificity of the interaction at the level of the head groups. PS promoted binding of LUVs to the cell surface by the Ca^{2+} bridge. An increase in PS resulted in an increase in the contact as more Ca^{2+} bridges could be present for a given LUV. In a PS/Ca^{2+} system, a number of studies had emphasized the role of dehydration of the bilayer surface and local packing defects in bilayers in initiating fusion. X-ray diffraction studies of the interlamellar spacing between hydrated PS membranes in the presence of Ca^{2+} showed that in the presence of Ca^{2+}, much less water was left between the lipid layers (Nir 1991). In fusion experiments, one Ca^{2+} probably bound to two PS molecules from apposed bilayers forming a 'trans' complex between the bilayers, dehydrating the interface. This dehydration of the interface was assumed to occur on electropermeabilized cell surface to explain electrofusion and the occurrence of a long-lived fusogenic state after the pulse delivery (Teissié and Rols 1992).

PE is known to promote liposome fusion due to its molecular geometry (small head group) and the possibility that it forms transiently non-bilayer lipid organization (Verkleij 1991). It was suggested that such organizations are the pathways for lipid vesicles fusion (Rand and Parsegian 1986). Its presence plays a critical role in the present case. Cholesterol is known to increase the rigidity of fluid lipid assemblies (Wilschut 1991).

This dependence on the nature of the lipid mixture is confirmative that the LUVs fusion is not due to the field effect on the LUVs. This is clearly different from what was observed with the electrofusion of Giant vesicles (GUVs) where neutral lipids were used (contact being mediated by dielectrophoresis) and that the vesicle size made them sensitive of the field (Haluska et al. 2006; Stoicheva and Hui 1994). Fusion resulted from a direct destabilization of the lipid bilayer by the field pulse. In the present case, the fusion resulted from the bioelectrochemical alteration of the electropermeabilized cell surface. It resulted a change in the environment of the LUVs. Previous analysis of fusion of lipid assemblies showed that changes in local pH or hydrophobicies triggered the spontaneous fusion that was dependent on the lipid composition (Nir 1991; Teissié and Rols 1992). They were proposed to depend on associated dramatic fluctuations in the shape of lipid vesicles.

The toxicity that is associated to the fusion-mediated transfer of HPTS is a direct evidence that this approach is prone for an enhanced drug delivery by providing a direct access to the cytoplasm of the content of the lipidic nanovesicles with a very limited time of contact.

A peculiar information was that it was not possible to obtain loading by LUVs fusion in all pulsed cells, while under our HFSP protocols, we previously observed that all cells were "electropermeabilized." One should keep in mind that indeed the molecular processes supporting electropermeabilization remained poorly explained and therefore controlled (Teissie et al. 2005).

An interesting effect is the protecting effect against the loss of cell viability due to electropermeabilization. Our data report that pulsing cells with highly fusogenic empty LUVs always improve the viability of the cell population, suggesting a positive use of LUVs as long, of course, as the LUV content is not toxic as we observed with HPTS. This was obtained whatever the pulsing conditions (HFSP as well as LFLP, known to induce a high level in gene transfer and expression) and opens the way to useful applications as

the loss of viability in gene electrotransfer is a limit in most applications.

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