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Interactions of DMPC and DMPC/gemini liposomes with the cell membrane investigated by electrorotation

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ABSTRACT

The electrorotation technique was utilized to investigate the interactions between a mouse fibroblast cell line and zwitterionic liposomes formed by a natural phospholipid or cationic liposomes formulated with the same phospholipid and a cationic gemini surfactant. The application of this technique allowed an accurate characterization of the passive dielectric behavior of the plasma membrane by the determination of its specific capacitance and conductance. Changes of these parameters, upon interaction with the liposomes, are related to variations in the structure and or in the transport properties of the membrane. Cells were exposed to both types of liposomes for 1 or 4 h. Electrorotation data show a dramatic reduction of the dielectric parameters of the plasma membrane after one hour treatment. After 4 h of treatment the effects are still observed only in the case of the cationic liposomes. Surprisingly, these same treatments did not cause a relevant biological damage as assessed by standard viability tests. A detailed discussion to rationalize this phenomenon is presented.

1. Introduction

Liposomes have been extensively studied for the delivery of several drugs, proteins, peptides, and genetic materials [1] at both preclinical and clinical level in many therapeutic protocols. They present many advantages when compared to other drug delivery systems (DDS) such as biocompatibility, relative ease of preparation, and high drug payload. Among liposome formulations the cationic ones were shown to be very promising. The first formulation, proposed in 1987, was composed of a natural phospholipid (helper lipid) and a cationic synthetic lipid and was used to introduce genetic material into cells [2]. Since then, liposomes have been investigated as an alternative to viral vectors in gene therapy. More recently, they have been shown to feature an intrinsic selectivity for some tissues, such as the vascular endothelial cells of tumors thus supporting the idea that they might represent an efficient drug delivery system [3–5]. However, despite the high number of investigations on liposomes as DDS, only few formulations have been approved for therapy. This gap probably depends also on some specific drawbacks of many liposomes, such as stability problems, but it is mainly due to the still incomplete knowledge of the correlation between liposome structure and the composition as well as their influence on the cell biological activity. A crucial issue for the biological efficacy of a liposome formulation concerns the internalization pathway into cells. In fact, the intracellular trafficking of the liposome and the fate of its cargo are strictly dependent on the mode of internalization [6]. It is known that the internalization pathway of a liposome is dramatically influenced by its physicochemical properties, such as size, shape, rigidity, charge, hydrophobic/hydrophilic balance, and presence of specific ligands [7–9]. The uptake of liposomes at subcellular level is commonly investigated by the use of specific inhibitors, and by co-localization studies with definite intracellular organelles [10,11]. Dielectric spectroscopy proved to be very effective as a non-invasive technique for the study of the liposome–membrane interaction [see for instance 12–16]. Among the diverse approaches of dielectric spectroscopy, electrorotation is particularly informative of the cell membrane structure/function. In fact, this technique allows a very effective monitoring of the alterations of the plasma membrane due to external stimuli. This non-invasive tool clearly evidences also relatively small modifications of the dielectric parameters specific capacitance and conductance per unit area, on single cells. This strategy was successfully used to investigate the dielectric properties of prokaryotic and eukaryotic (animal as well as plant cells) exposed to different biological and chemical stresses [17–27].

Abbreviations: DDS, drug delivery systems; DMPC, 1,2-dimyristoyl-*sn*-phosphatidylcholine; Ge-1, DMPC–gemini (2S,3S)-2,3-dimethoxy-1,4-bis(N,N-dimethylamine)-butane; PBS, phosphate-buffered-saline solution

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Here we report the results of an investigation by electrorotation on the interaction of a mouse fibroblast cell line with two liposome formulations: the first one formed by the natural phospholipid DMPC, 1,2-dimyristoyl-*sn*-phosphatidylcholine, and the second one formulated with DMPC and the cationic gemini surfactant (2S,3S)-2,3-dimethoxy-1, 4-bis(N,N-dimethylamine)-butane in an 8:2 ratio. This formulation was chosen because liposomes made with this gemini surfactant were shown to deliver efficiently a photosensitizer, m-THPC, to human colon adenocarcinoma and glioblastoma cell lines [28–30]. Also, they were shown to transfect efficiently DNA into various cell lines [31]. In the light of these very vast literature data we aimed our study to the evaluation of the possible alteration of the dielectric membrane properties as a consequence of the interaction of the liposomes with the cell.

2. Materials and methods

2.1. Theoretical background of the electrorotation technique

When a cell suspension is subjected to an electric field, an effective dipole moment is induced on each cell because of the different polarizability of the solvent, as compared to the plasma membrane. In the case of the electrorotation the applied electric field is rotating. When the interfacial polarization relaxes, a phase shift appears between the induced dipole moment and the exciting electric field, thus causing a torque moment, and the cells rotate in an anti-field fashion. This process usually appears in aqueous cell suspensions in the range of approximately 10^4–10^6 Hz. At higher frequencies the electric field traverses the plasma membrane (i.e. the membrane capacitance is shorted out) and a co-field rotation is observed. At lower frequencies, in the kHz range, an additional relaxation may be observed due to the contribution of double layer polarization and surface conductivity [18]. These relaxations correspond to the so called α, β and γ dispersions, observed in the dielectric spectra of cell aqueous suspensions, in the same frequency ranges. In our study we focus only on the β dispersion, associated to the dielectric properties of the plasma membrane [32,33].

The rotation period of the β dispersion depends upon the frequency of the applied field, according to Eq. (1), which describes a Debye-like relaxation:

$$T(f) = T_{\min}\frac{1+\left(\frac{f}{f^*}\right)^2}{2\left(\frac{f}{f^*}\right)}. \quad (1)$$

Here, f is the frequency of the applied field, f^* is the relaxation frequency and $T_{\min}$ is the value of the rotation period at the relaxation frequency. The value of f^* depends on the solvent conductivity according to the expression (2):

$$f^* = \frac{1}{2\pi RC}\left[\frac{1}{\frac{1}{\sigma_e}+\frac{1}{\sigma_i}} + RG\right] \quad (2)$$

where C and G are, respectively, the specific capacitance and conductance of the cell plasma membrane with a radius R, σ_e is the solvent conductivity and σ_i is the conductivity of the cytoplasm considered as homogeneous. Since in general $\sigma_e \ll \sigma_i$, Eq. (2) becomes:

$$f^* = \left(\frac{1}{\pi RC}\right)\sigma_e + \left(\frac{1}{2\pi C}\right)G. \quad (3)$$

The technique is based on the determination of the relaxation frequency as a function of the external conductivity, from which C and G can be easily obtained.

Compared to the complexity of the real biological system, the Debye model, where the cell membrane is modeled as a sphere and is represented as a thin homogeneous layer characterized by a capacitance and a conductance per unit area, is obviously rather coarse. However this apparently rough model proved to be very effective to monitor even slight changes in the dielectric properties of the cytoplasmic membrane [16,17,19,21,22,24,25]. Clearly, the dielectric parameters C and G have the meaning of "effective quantities" that take into account, on the average, different effects and cannot be directly related to microscopic structures or specific transport mechanisms. For example, the capacitance C is a measure of the polarizabilty of the membrane which is influenced by the lipid composition, the presence and conformation of proteins, the permeability to water, but it is also influenced by the surface charge, by the structure and electrical properties of the membrane-medium interface, etc. However, the method proved to be very effective and very sensitive in monitoring changes, even very small alterations, of the structure/functionality of the cell membrane. Therefore, this simplified model is commonly accepted [see for instance 24,33]. A linear fit of the relaxation frequency, measured at different solvent conductivities, allows calculating the membrane parameters C and G. In our study, four different dispersion media were used, obtained from the same osmolar sucrose solution (300 mM) which was supplemented with three NaCl concentrations: (0.5; 1.0; 1.5 mM). The conductivities of the four solvents were accurately measured by an automatic impedance meter (HP 4194A). The electrorotation apparatus, previously described in detail [23,25], was implemented with a video-recording system that permitted a more accurate off-line image analysis. This analysis consists in the measurement of the rotation period at each frequency of the electric field, a minimum number of 15 cells were considered at each frequency. Measurements were done on untreated control or treated cells with natural or cationic liposomes for 1 and 4 h. Each experiment was repeated three times.

Cell culture details are reported in the Section 2.3.

2.2. Liposome preparation

Two liposome formulations were used, the first one formed by the zwitterionic phospholipid [1,2-dimyristoyl-*sn*-glycero-3-phosphocholine], DMPC and the second one formulated by adding to the DMPC and the cationic gemini surfactant [(2S,3S)-2,3-dimetoxy-1,4-bis(N,N-dimethylamine)-butane], (hereafter Ge-1), at a molar ratio of 8:2. The aqueous dispersions of liposomes were prepared by extrusion according to the procedure described [34,35]. Briefly, a film of lipid (total 12.5 μmol) was prepared on the inside wall of a round-bottom flask by evaporation of a $CHCl_3$ solution containing the appropriate amount of DMPC and Ge-1 to obtain the desired mixture. The film was stored overnight in a desiccator under reduced pressure then 1 mL of PBS buffer solution (Aldrich, 10^{-2} M pH 7.4) was added in to obtain a 12.5 mM lipid dispersion. The solutions were vortex-mixed and then freeze-thawed six times from liquid nitrogen to 307 K (well above the transition temperature of the bilayer). Dispersions were then extruded (10 times) through a 100 nm polycarbonate membrane (Whatman Nucleopore). The extrusions were carried out at 307 K, using a 2.5 mL extruder (Lipex Biomembranes, Vancouver, Canada).

2.3. Cell cultures

The mouse fibroblast cell line 3T6 was used in all electrorotation experiments. Cells were routinely grown as previously reported [23,25]. Cultures were exposed to a vast excess of liposomes (in the order of magnitude of 10^6 per cell) for 1 and 4 h. These treatment times were selected on the basis of literature data, indicating that a fluorescent drug, delivered by these supra-molecular aggregates, after these times was located at outer plasma membrane or at nuclear level [36].

The effect of both natural and cationic liposomes on cell survival was assessed by the standard MTT assay [37].

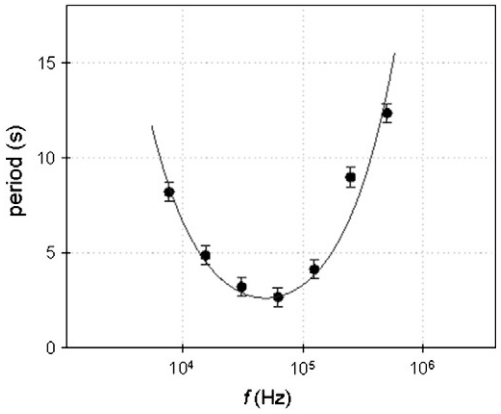

Fig. 1. Typical correlation between rotation period of the cell and applied electric frequency. Measurements were repeated at different conductivities of the solvent (0.5, 1.0 and 1.5 mM NaCl). As an example only the curve at 0 mM NaCl is shown. The curve results from the best fit according to Eq. (1) as reported in the Materials and methods section.

3. Results and discussion

3.1. Variation of the dielectric parameters of the plasma membrane after interaction with liposomes

Fig. 1 reports the typical dependence of the rotation period as a function of different field frequencies in the control, non-treated cell suspensions. The experimental data were fitted according to Eq. (1) as reported in the Materials and methods section. As expected from Eq. (3), the relaxation frequencies f^*, as a function of the solvent conductivity, form a straight line (Fig. 2). The figure reports the results obtained on control cells (empty circles) and after 1 hour treatment with cationic liposomes (full circles). The treatment clearly results in the variations of the angular coefficient and intercept, which reflect a decrease of the membrane parameters *C* and *G*. An analogous effect was observed also in the case of cells treated with DMPC. The percent variations of the membrane parameters, normalized to control cultures, are reported in Table 1 for the samples treated for 1 h or 4 h with both liposome species. Table 1 also reports the average cell radius. It is worth noting that the radius values change significantly as a consequence of the cell/liposome interaction. All data summarized in Table 1 are visualized in the Figs. 3, 4 and 5.

The reduction of the *C* and *G* values after 1 hour treatment, observed for both liposome species, is higher that 60%. On the contrary, at 4 h of treatment a dramatic difference occurs. As a matter of fact, for this longer treatment period, in the case of the cell interaction

Table 1
The percent variations of the membrane parameters and the average cell radius, for the samples treated for 1 or 4 h with both liposome species (A and C, natural liposomes; B and D cationic liposomes). The average radius estimated on untreated control cells was 8.5 ± 0.3 μm.

	ΔC/C (%)	ΔG/G (%)	r (μm)
A	−56 ± 7	−69 ± 5	9.5 ± 0.3
B	−5 ± 7	9 ± 9	8.6 ± 0.2
C	−70 ± 7	−71 ± 6	9.6 ± 0.4
D	−70 ± 8	−79 ± 7	9.6 ± 0.4

with DMPC the effect vanishes, while it persists for the Ge-1 cationic liposomes. Interestingly, a comparable phenomenon is observed on the cell radius: i.e. a significant increment occurs for both liposomes after 1 hour treatment, but at 4 h, such increase is observed for the cationic liposomes only.

The decrease of the specific capacitance *C* may be ascribed, in principle, to diverse and non-mutually exclusive reasons. One can hypothesize different alterations of the cell membrane morphology/structure which involve an increase of its thickness and/or a decrease of the membrane permittivity ε′. This decrease may reflect, for example, a slight overall misalignment of the membrane polypeptides due to the fusion of the liposomes with the cytoplasmic membrane. All these interpretations are also consistent with the observed reduction of the specific conductance *G*. It is reasonable, in fact, that this parameter is inversely proportional to the membrane thickness; in addition it is plausible to assume that the decrease of ε′, associated with the cited misalignment of the membrane polypeptides influences the ion transport mechanisms at cell membrane level.

Concerning the variation of the cell radius, more than one interpretation may be attempted. It is possible that, as a consequence of the fusion, or more in general of the interaction, of the liposome bilayer with the cell membrane, a variation of the lipid packing in the membrane occurs. An increase of the cell surface may be also due, for example, to local changes in the elastic modulus or may derive from a consequence of the resorption of microvilli causing the flattening of membrane ruffles. Another possibility is that the liposome bilayers are to some extent simply incorporated within the cell membrane, so that its surface increases proportionally. A rough estimate of the number of liposomes determining this increase can be obtained from a simple calculation. Assuming additivity, in fact, the area of the cell surface after the liposome interaction writes:

$$4\pi(R_C + \Delta R_C)^2 = 4\pi\left\lfloor (R_C)^2 + n(r_l)^2 \right\rfloor$$

where R_c is the radius of the cell, ΔR_c represents its increase subsequent to liposome interaction, r_l is the liposome radius and *n* is the

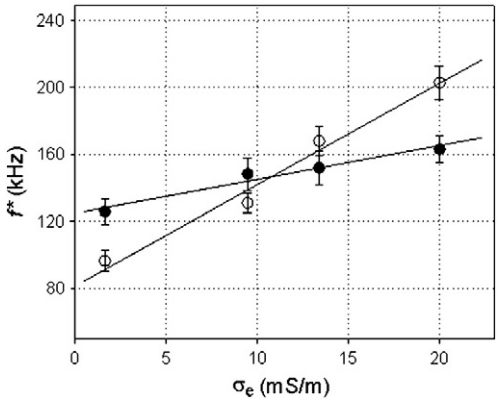

Fig. 2. Relaxation frequency (f^*) as a function of the solvent conductivity σ_e. The full circles refer to untreated control cell. Empty circles report the relaxation frequency of cells treated for 1 h with DMPC/1 liposomes. The straight lines were obtained from each relaxation frequency at the respective ion strength fitted according to Eq. (3).

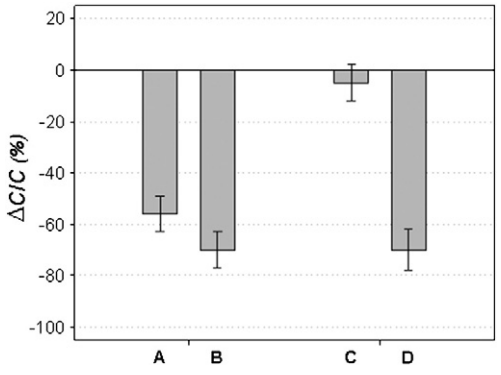

Fig. 3. Histograms reporting the effect on the cell specific capacitance. The y axis reports the percent variation Δ*C*/*C* normalized to non treated control cells, which is shown as the base line. The x axis reports the development of the individual treatments at 1and 4 h respectively (Bars A and C, natural liposomes; B and D, cationic liposomes).

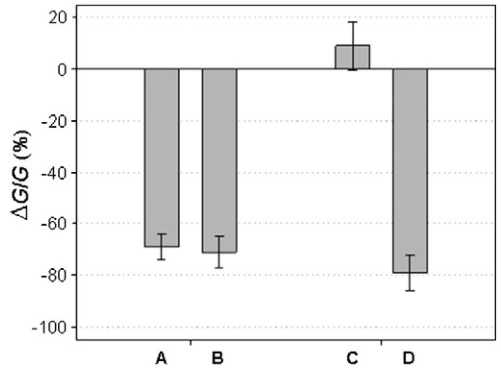

Fig. 4. Histograms reporting the effect on the cell specific conductance. The y axis reports the percent variation of ΔG/G normalized to non-treated control cells which is shown as the base line. The x axis reports the development of the individual treatments at 1and 4 h respectively (Bars A and C, natural liposomes; B and D, cationic liposomes).

number of fusing liposomes per cell. With the experimental values for R_c, ΔR_c and r_l as reported in [36] one may estimate that n is in the order of 10^4. Although this number may appear large, it must be remembered that in our experiments the liposomes were in large excess (approx. 10^6 liposomes per cell). On the other hand liposomes are very small in comparison to the cells and their surface ratio is approximately $R^2/r^2 \approx 10^4$. Although these numbers should not be interpreted literally, they suggest that the whole surface of the cell membrane is "saturated" by the interaction with the liposomes.

The biophysical schematic representation of a cell is usually a sphere surrounded by a thin homogeneous shell, mimicking the plasma membrane, according to the well known single shell model [38,39]. A more complex model, like the double shell one, is adopted if one wants to take into consideration also the nuclear membrane [38–40]. It is such an oversimplified scheme that has been employed to obtain the values of the dielectric parameters C and G. However, the uneven surface structures, however, render the cell very different from an ideal smooth sphere and increase the actual area exposed to the outer medium. To take into account the surface roughness, a form factor can be adopted in the specific capacitance expression of the spherical condenser. In this picture the cell is, in fact, assimilated to a spherical condenser where $C = K_m \varepsilon_0 \varepsilon'/d$, with d as the average membrane thickness. K_m is a dimensionless form factor, that accounts for the surface roughness and that is experimentally evaluated to be between 1.5 and 2.0, as extensively discussed in previous literature on electrorotation [see for instance the classical treatment of reference 41].

A point deserving discussion is the different behavior of the two liposome species as far as the variation of the membrane dielectric

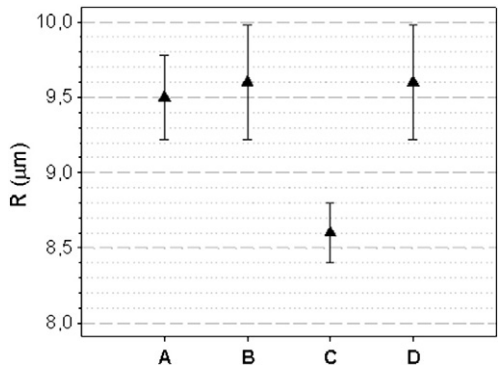

Fig. 5. Histograms reporting the effect on the cell radius. The letters A, B, C, and D refer to the same samples in Figs. 3 and 4. The highlighted area shows the statistical variation of the radius of non-treated cells with the average radius being 8.5 ± 0.3 μm.

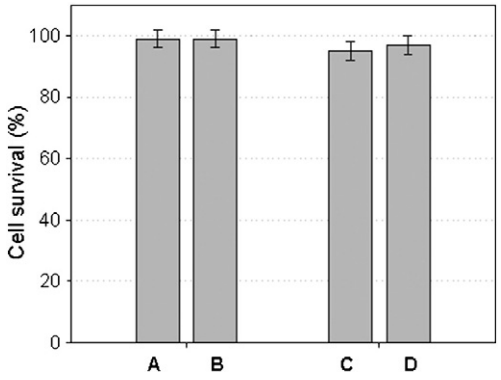

Fig. 6. Cell survival test after exposure to natural or cationic liposomes after 1 or 4 h of treatment. The letters A, B, C and D are as in the previous figures.

parameters and cell radius is concerned. Our results suggest that after the interaction with the natural liposomes the cells recover in a relatively short time so that already during the shorter time period treatment (1 h) a sort of "equilibrium" is reached. After 4 h of treatment, the plasma membrane returns to its homeostatic condition. On the contrary, the cationic liposomes show a longer lasting effect on the same cell parameters, possibly due to their electric charge, and even after several hours the cell membrane does not show a significant recover. As matter of fact, because of the weak negative character of the cell membrane, an electrostatic interaction with Ge-1 liposomes is most likely at the basis of the persisting effects.

It is worth to note that, in spite of the apparent alterations of the biophysical membrane parameters discussed above, no evident effect on the overall cell survival was monitored (Fig. 6). This is particularly surprising in the case of the Ge-1 liposomes, containing cationic surfactants. This class of surfactants may show, in fact, a moderate to relevant cytotoxicity [see for instance 42,43].

4. Conclusions

In this work we report on the interaction between two types of liposomes and cells in culture. The adopted strategy was electrorotation which is non-invasive and permits single cell analysis. This approach allows the evaluation of the membrane parameters: specific capacitance (C) and conductance (G). These are related to the structure/function of the plasma membrane. Results show that natural liposomes have a short term effect, while cationic liposomes produce an alteration of the membrane parameters lasting for a longer time period. This effect consists in a decrease of both C and G associated with a significant cell radius variation. This suggests that the neutral liposomes interact transiently with cell membrane, or better that the membrane recovers after a relatively short time, while the cationic liposomes establish a more stable perturbation of the cell membrane. It is noteworthy, however, that neither liposome species exerts a relevant effect on the cell survival after these treatment times.

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