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Increased binding of liposomes to cells by electric treatment

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The influence of electric field treatments on the interaction of large unilamellar vesicles (liposomes) with animal cells was monitored by the fluorescence assay based on the use of the liposomes loaded by a dye 1-hydroxypyrene-1,3,6-trisulfonic acid (HPTS). It was shown that application of a short electric pulse (100 μs of 3–4 kV/cm) to the suspension of cells in presence of vesicles resulted in significant (more than 2 times) increase of the fluorescence associated with cells. The pH-sensitivity of the excitation spectrum of the dye and its interaction with the quencher were used to determine the nature of the phenomenon as the increase of the liposome binding onto the cell surface but not the consequence of a promotion of liposome uptake into the cells by endocytosis. The higher affinity for the liposome caused by the electric field has a lifetime of several minutes. The possible relation of the effect described to the electroporation of cell membranes and to macroscopic changes in membrane structure is discussed.

Introduction

A variety of biotechnological approaches have evolved in the last few years based on the impulse electrical field treatment of the animal, plant, and bacterial cells. These include electroporation [1–3], electrofusion [1,3–5], and loading of DNA into cells [6]. This development has generated some general interest to the effects of electrical treatment of cells and especially cell membranes where effects of the applied field are greatly amplified. The most profound effect induced by the short application of electrical field is a significant and reversible increase of membrane permeability as a result of pore formation. Mechanisms of pore formation and resealing as well as the properties of the electropores have been extensively studied [1–3,7–11]. According to the results of these studies electroporation can be regarded as relatively local phenomenon: the sizes of pores were determined to be 1–20 nm. In addition to electroporation some other electro-induced changes of membrane properties were described including the appearance of a long-living fusogenic state [12,13] and bleb formation [14,15].

Here we report an additional effect of the electrical treatment indicating macroscopic changes of cell membrane properties. To monitor the interaction and to separate different types of it (binding, endocytosis, leakage of content of liposomes bound to cells [16,17]) a fluorescence assay based on the use of the liposomes loaded with a pH-sensitive water-soluble fluorescent dye 1-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) was employed [18,19]. The significant shifts of excitation spectrum of HPTS with changes in pH, namely increase of fluorescence peak at 450 nm and decrease of that at 403 nm, were described to occur at acidification of liposomes content during endocytosis [18]. In the present paper a significant increase of liposome binding onto the cell surface observed after the application of short duration pulse of electric field to the suspension of animal cells in the presence of large unilamellar vesicles was shown not to be accompanied by measurable changes of pH of liposomes content. The parameters of electrical treatment required to induce such effect were the same as those needed for the electroporation of cell membranes.

Materials and methods

Materials. Phosphatidylcholine from egg yolk (PC) and bovine brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids. Cholesterol was obtained from Sigma Chemical Co. 1-Hydroxypyrene-

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1,3,6-trisulfonic acid (trisodium salt, HPTS), *p*-xylene-bis-pyridinium bromide (DPX) and propidium iodide (PI) were obtained from Molecular Probes. All the chemicals were reagent grade.

Cell culture. The human T-cell leukemia line Molt-4 [19] was used. Cells were grown in RPMI medium supplemented with 2 mM glutamine, 10% fetal bovine serum in presence of 3% CO_2. The macrophage-like cell line J 774 [18] was maintained in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 3 g/l glucose and 10% calf serum (DMEM) and incubated under 7% humidified CO_2. J 774 cells were resuspended in 25 ml of medium ($4 \cdot 10^5$ cells/ml) and plated in Teflon vessel 24 h prior to use.

Liposome preparation. Lipid mixture (PC/PS/cholesterol, 1:1:1) was prepared in chloroform and the solvent was removed under reduced pressure. Solutions of HPTS (35 mM) in 10 mM Hepes (pH 7.4) or HPTS (35 mM) + DPX (50 mM) in 10 mM Hepes (pH 7.4) (adjusted to 290 mosM with 1 M NaCl) were added and liposomes were prepared by the reverse-phase evaporation procedure [20]. Liposomes were extruded four times through two 0.1-μm pore polycarbonate membranes under high pressure (Lipex Biomembranes Inc., Vancouver) and unencapsulated fluorescent probes was separated from liposomes by gel filtration on a Sephadex G-75 column (1 × 15 cm) equilibrated with 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4 (PBS). Phospholipid concentration was determined as described in Ref. 21. The liposomes averaged 112 nm in diameter as measured by laser light scattering [18].

Liposome-cell incubation. Cells were washed twice with PBS and resuspended in PBS to a final concentration $2 \cdot 10^7$ cells/ml. 200 μl of the suspension were incubated in semimicro disposable plastic optical cuvettes (VWR Scientific) with the liposomes (40 nmol lipid) at 37 °C for 30 min, unless otherwise stated, before electrical treatment. After incubation with liposomes at 37 °C for another 30 min cells were washed twice with 15 ml PBS using 10 min centrifugation to remove unbound liposomes. Then cells were resuspended in a 2 ml of PBS. In some experiments the isosmotic DPX containing buffer: 50 mM DPX, 2.7 mM KCl, 1.5 mM KH_2P04, 8.1 mM Na_2HPO_4 (pH 7.4) adjusted to 290 mosM with 1 M NaCl was used instead of PBS to resuspend the cells.

Then the fluorescence excitation spectra (λ_{ex} 390–465 nm, 1.8 nm bandwidth) of samples were measured at 510 nm emission with wide (4.5 nm) emission bandwidth using a SPEX Fluorolog 2 fluorimeter outfitted with a stirred temperature controlled cuvette (20 °C). In some experiments Triton X-100 (0.1%) was added and fluorescence was measured once again after 1 min.

Electrical treatment. To apply the rectangular pulses of electric field on cells and liposomes suspension the BTX T 800 Super System Electroporator as well as BTX Cuvette Electrodes 474 M (2 parallel gold plated plates with 0.68 mm gap) immersed into cuvette with cells and liposomes were used. The pulses were monitored with the use of BTX Optimizor. The resistance of samples averaged 20 Om.

Determination of percentage of electroporated cells. To determine the percentage of reversibly electroporated cells among the cells treated by electric field a DNA specific fluorescent stain, propidium iodide, was used. We compared under the microscope (Leitz Fluovert) the percentage of stained cells for the same electrical treatment of cells in two parallel experiments. The ratio of stained cells determined 10 min after electrical treatment of cell suspension in presence of $5 \cdot 10^{-5}$ M propidium iodide includes both dead and reversibly electroporated cells. It was compared with that obtained for the same electrical treatment of cells in the absence of dye. In this case propidium iodide was added after 30 min incubation of the treated cells at 37 °C. Then after another 10 min of incubation at 37 °C counting of stained cells was carried out. It gave the percentage of cells irreversibly damaged by the treatment. The difference between the ratios of stained cells for suspension treated in the presence and in the absence of PI was used as a rough estimation of the percentage of reversibly electroporated cells among all cells treated by electric field.

Results and Discussion

To determine the intensity of electric field for inducing electroporation of the particular cells used in this study we counted the ratios of dead (Fig. 1, curve 1) and reversibly electroporated (curve 2) cells after electrical treatment of the suspension of Molt 4 cells. Fig. 1 shows that the percentage of dead cells increased with the intensity and there was an optimum field intensity at approx. 3.75 kV/cm (length of pulse,

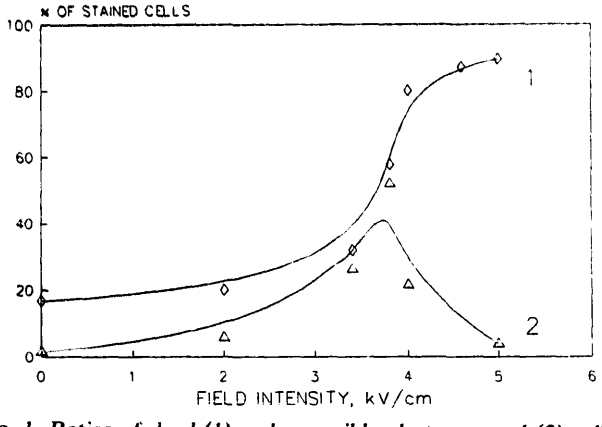

Fig. 1. Ratios of dead (1) and reversibly electroporated (2) cells in suspension after application of a 100 μs electric pulses of different intensity to Molt 4 cells.

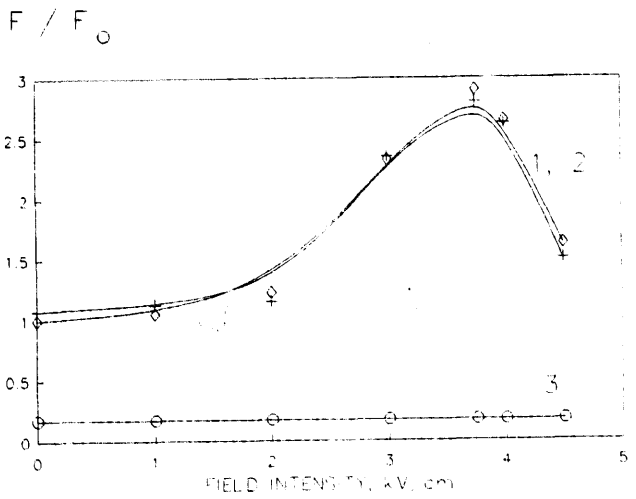

Fig. 2. Fluorescence associated with Molt 4 cells after their electrical treatment in the presence of HPTS containing liposomes as function of the field intensity. The fluorescence, F, (λ_{ex} = 413 nm, λ_{em} = 510 nm) was measured in PBS (curve 1); in isosmotic buffer containing 50 mM DPX (curve 2) and after disintegration of cells and liposomes in the presence of DPX by Triton X-100 (curve 3). F_0, the fluorescence associated with cells in PBS after incubation of cells with HPTS containing liposomes without electric treatment.

100 μs) for reversible electroporation of cells. For J 774 cells similar experiments gave this value at approx. 3.4 kV/cm (the same length of pulse).

In the same range of field intensities the electric pulses applied to the suspension of cells and liposomes resulted in a significant increase of the number of liposomes associated with the cells. The ratio of the fluorescence (λ_{ex} = 413 nm, λ_{em} = 510 nm) of Molt 4 cells treated by electric field in the presence of HPTS containing liposomes to that obtained for control untreated sample as a function of field intensity is presented in Fig. 2. Let us note that there was no influence of the buffer used for resuspending of cells before measurement (PBS or DPX containing buffer) on the fluorescence values obtained (Fig. 2, curves 1, 2). However the solubilization of liposomes and cells by Triton X-100 in the presence of DPX results in significant decrease of the fluorescence (Fig. 2, curve 3). It shows that the concentration of DPX used was sufficient to quench the HPTS fluorescence if dye and quencher were not separated by membranes. The absence of any HPTS fluorescence quenching at the high concentration of DPX before membrane solubilization allows one to exclude the possible role of the adsorption onto the cell membranes of the free HPTS released from the liposomes.

Additional control experiments showed that treatment of the liposomes by the same field intensities in the absence of cells did not result in any release of their contents or in liposome aggregation. The application of a 100 μs pulse of amplitude as high as 5 kV/cm on the suspension of liposomes loaded by HPTS and DPX did not give any measurable increase of HPTS fluorescence. The electrically induced release of the contents of the liposomes loaded simultaneously by fluorescent dye and quencher would have resulted in an increase of fluorescence due to the decrease of the quencher concentration. The laser light scattering measurements of the mean diameter of liposomes as well as the distribution of liposome size in suspension before and after electrical treatment did not reveal any differences indicating that the range of field intensities used did not induce any aggregation and/or fusion of liposomes. Since the transmembrane potential ($\Delta\phi$) generated on liposomes at the intensities (E) we used was relatively low (75 mV according the rough estimation [3,11]: $\Delta\phi = 1.5\ r \cdot E$, where r is the radius of vesicle), no effects on the liposomes were expected in these experiments [22–24].

The excitation spectra of the HPTS fluorescence associated with Molt 4 cells treated (Fig. 3a, curve 1) or untreated (curve 2) by electric field in the presence of

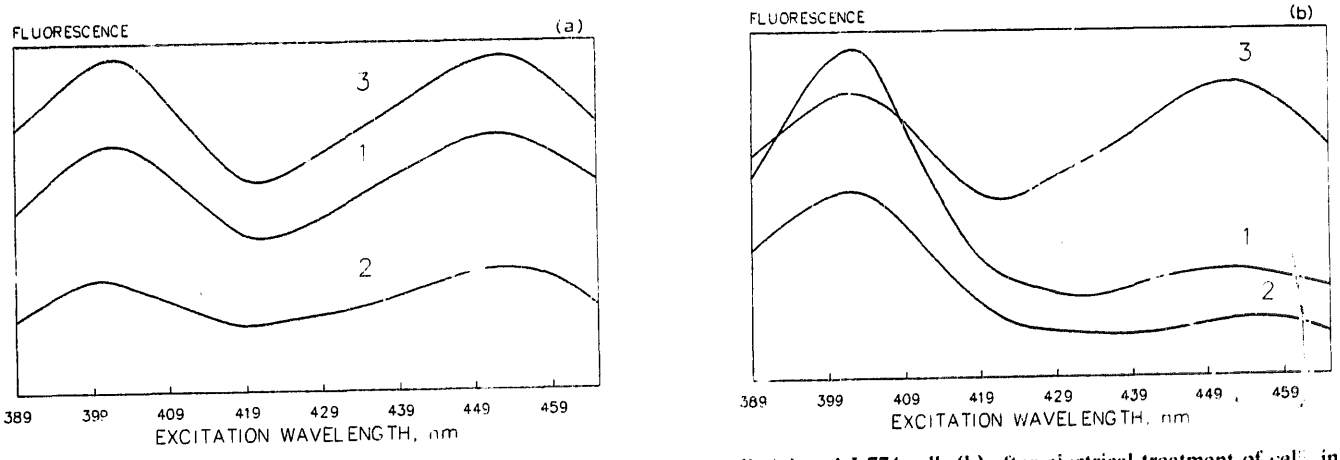

Fig. 3. (a, b) Excitation spectra of the fluorescence associated with Molt 4 cells (a) and J 774 cells (b) after electrical treatment of cells in the presence of HPTS containing liposomes. A 100 μs pulses of 3.75 kV/cm and 3.4 kV/cm were used for Molt 4 and J 774 cells, respectively (curves 1 in Figs. 3 a, b). Curves 2 were obtained for electrically untreated cells. Curves 3 on these figures show the spectrum of the HPTS containing liposomes in the absence of cells.

HPTS containing liposomes appeared qualitatively similar to each other and to that of the liposomes in the absence of cells (curve 3). The fluorescence spectrum of HPTS dramatically depends on the pH of a solution in pH range 6–9: lower pH, lower the ratio of the fluorescence at $\lambda_{ex} = 450$ nm to that at $\lambda_{ex} = 403$ nm; the fluorescence at $\lambda_{ex} = 413$ nm is invariant with pH [18]. For instance, decrease of buffer pH from 7.4 to 6.4 results in 3.8 times decrease of the ratio of the free HPTS fluorescence at $\lambda_{ex} = 450$ nm to that at $\lambda_{ex} = 403$ nm (data not shown, see also Ref. 18). Thus, the similarity of these spectra (the ratios of the fluorescence at 450 nm to one at 403 nm are 1.03; 1.05 and 1.01, respectively, for the curves 1, 2 and 3 in Fig. 3a) shows the lack of any acidification of the HPTS associated with the cells. Therefore there was no evidence for any uptake of liposomal contents into the acidified endosomes or secondary lysosomes of Molt 4 cells under our experimental conditions, with or without electrical treatment.

The situation was quite different in the case of the macrophage-like J 774 cells. There was a significant change in HPTS fluorescence spectrum after interaction of HPTS liposomes with these cells (Fig. 3b). The decrease of the ratio of the fluorescence at $\lambda_{ex} = 450$ nm to fluorescence at $\lambda_{ex} = 403$ nm in the presence of cells (curve 2) in comparison with that for only liposomes (curve 3) reflects the acidification of dye presumably following endocytosis of liposomes. The endocytosis of liposomes by macrophages was studied in detail by Daleke et al. [18]. The electrical treatment of J 774 cells increases the amount of fluorescence associated with cells at pH independent point (413 nm) (Fig. 3b, curves 1, 2). However the HPTS fluorescence spectra of these cells after application of electric field and without it (Fig. 3b, curves 1, 2) proved to be qualitatively similar: the ratios of the fluorescence at 450 nm to one at 403 nm are 0.32 and 0.29, respectively, for the curves 1 and 2 to compare with 1.03 obtained for curve 3. Therefore the electrical treatment of J 774 cells has no significant influence on the efficiency of endocytosis but rather it resulted in an increase of the number of liposomes (based on the amount of HPTS) associated with these cells as was also the case for Molt 4 cells.

Molt 4 cells were also incubated with liposomes containing HPTS and DPX. In this case there was no significant fluorescence associated with cells treated or untreated by the electric field. No fluorescence appeared during incubation of cells at 37 °C in RPMI medium without serum up to 4 h. However, disintegration of membranes by Triton X-100 resulted in appearance of fluorescence proving the presence of HPTS in these samples. Thus, before the disintegration of membranes, HPTS fluorescence was quenched by high concentration of DPX present inside the liposomes. These results indicate that the fluorescence associated with cells in the experiments described earlier was determined by the presence of intact liposomes.

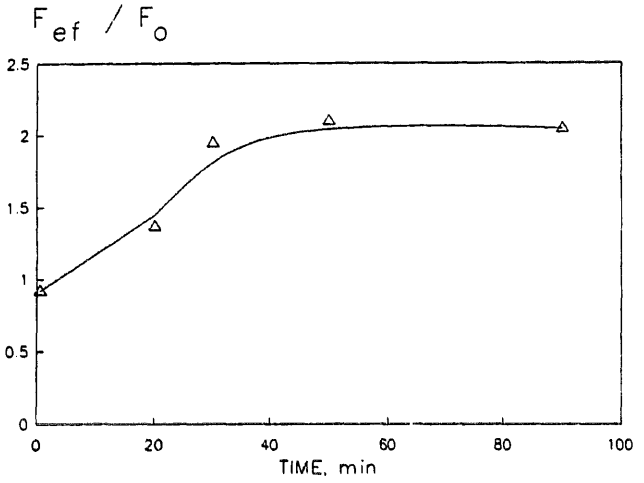

Fig. 4. Electric pulse induced increase of the fluorescence associated with Molt 4 cells as a function of time interval between the pulse and the moment of 100 times dilution of both liposomes and cells concentration. The ratios of the fluorescence at $\lambda_{ex} = 413$ nm for cells plus liposomes suspension treated, F, and untreated, F_0, by electric field (3.75 kV/cm, 100 μs) at $t = 0$ are plotted.

We have also carried out some experiments to study the kinetic properties of the electrostimulated increase of liposome binding to cells. In the experiment presented in Fig. 4, after 30 min of incubation of Molt 4 cells with HPTS-containing liposomes ($t = 0$ on time axis of the figure) the electric field was applied to the suspension. Then at time intervals of 0.5, 20, 30, 50 and 90 min after treatment, the suspension was diluted 100 times by PBS and the usual washing procedure was performed. The ratios of the cell-associated fluorescence of electrically treated cells to that of untreated cells incubated with the liposomes for the same time are plotted in Fig. 4. The data show no increase of liposome binding following electrostimulation for the case of fast dilution of the suspension of cells plus liposomes. This indicates that the processes responsible for liposome binding did not develop at the moment of electric field application. It took approx. 30 min to develop tight binding.

In Fig. 5, liposomes were added not only before but also after electrical treatment of cells ($t = 0$ on time axis of the figure). The increase of liposome binding to treated cells in comparison with the control untreated ones was observed even if the liposomes were added 10 min after pulse application. Thus, the modification of membrane properties under consideration has a lifetime in the scale of minutes.

In conclusion it appears that the electrical treatment of cells in the presence of liposomes loaded with a fluorescent dye results in significant increase of the fluorescence associated with the cells. The intensity of electric field required for this effect proved to be close

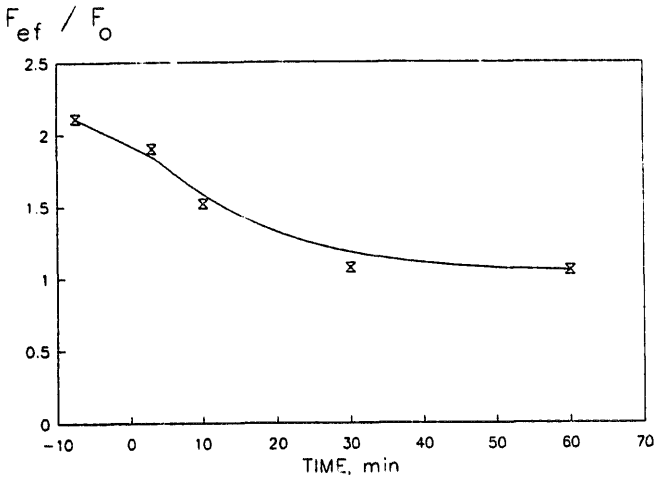

Fig. 5. Electric pulse induced increase of the fluorescence associated with Molt 4 cells, as a function of time interval between the pulse and the moment of the addition of liposomes into samples. The ratios of the fluorescence at $\lambda_{ex} = 413$ nm for samples treated, F_{ef}, and untreated, F_0, by electric field (3.75 kV/cm, 100 μs) at $t = 0$ are plotted.

to that of reversible electroporation of cell membranes. This result can not be explained by electrostimulated fusion of liposomes with cells, nor by the promotion of liposome uptake into the cells by endocytosis nor by the translocation of liposomes via electropores in cell membranes. The possibility of electrofusion of liposomes with cells discussed in Ref. 25 can be excluded here since the release of the internal content of HPTS + DPX liposomes into cells during fusion would have resulted in an increase of HPTS fluorescence due to DPX dilution. In addition we have not observed the characteristic changes of fluorescence spectra of HPTS normally observed during acidification of dye inside endosomes and secondary lysosomes, after electrical treatment of cells. This result testifies against the possible role of endocytosis (one of the most usual types of interaction between cells and liposomes [16,17]) as a driving force for the increase of cell association observed here. Finally, it is difficult to imagine the membrane of a viable cell developing pores with diameter more than 0.1 μm (the mean diameter of our liposomes) and lifetime of the order of minutes to explain the data presented on Fig. 5. Therefore, it seems that the electrostimulated increase of fluorescence associated with cells reflects the binding of intact liposomes onto the cell membranes.

The nature of changes of membrane structure underlying the observed effect is yet unclear. There are some indications in literature that electrical treatment of cells producing electroporation can also induce more macroscopic changes in the membranes related apparently to local disturbances of membrane organization. The changes of this type can be revealed for example as the electroinduced long living fusogenic state [12,13], bleb formation [14,15] and delay between the end of electrostimulation and the beginning of fusion [26]. We suppose that the significant increase of the binding of liposomes to cells after electrostimulation described in the present paper may be related to such changes in membrane organization. The binding of liposomes to cells can be also mediated by some molecules released from cytoplasm at electropermeabilization of cell membranes.

Acknowledgments

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References

1 Neumann, E., Sowers, A. and Jordan, C., eds. (1989) Electroporation and Electrofusion in Cell Biology, Plenum Press, New York.
2 Kinosita, K. and Tsong, T.Y. (1977) Nature 268, 438–441.
3 Zimmermann, U. (1982) Biochim. Biophys. Acta 694, 227–297.
4 Lo, M., Tsong, T.Y., Conrad, M.K., Strittmatter, S.M., Hester, L.D. and Snyder, S. (1984) Nature 310, 792–794.
5 Sowers, A. (1988) Biophys. J. 54, 619–626.
6 Neumann, E., Schaeber-Ridder, M., Wang, J. and Hobschneider, P.H. (1982) EMBO J. 1, 841–845.
7 Chernomordik, L.V., Sukharev, S.I., Popov, S.V., Sokirko, A.V., Pastushenko, V.F. and Chizmadzhev, Yu.A. (1987) Biochim. Biophys. Acta 902, 360–373.
8 Sowers, A. and Lieber, M.R. (1986) FEBS Lett. 205, 179–184.
9 Weaver, J. (1988) FEBS Lett. 299, 30–34.
10 Chang, D.C. and Reese, T.S. (1990) Biophys. J. 58, 1–12.
11 Kinosita, K. and Tsong, T.Y. (1977) Biochim. Biophys. Acta 471, 227–239.
12 Sowers, A.E. (1986) J. Cell Biol. 102, 1358–1362.
13 Teissié, J. and Rols, M.P. (1986) Biochem. Biophys. Res. Commun. 140, 258–267.
14 Escande-Geraud, H.L., Rols, H.P., Dupont, M.A., Gas, N. and Teissié, J. (1988) Biochim. Biophys. Acta 939, 247–259.
15 Gass, G.V. and Chernomordik, L.V. (1990) Biochim. Biophys. Acta 1023, 1–11.
16 Poste, G., Papahadjopoulos, D. and Vail, W.J. (1976) Methods Cell Biol. 14, 33–71.
17 Margolis, L.B. (1984) Biochim. Biophys. Acta 779, 161–189.
18 Daleke, D., Hong, K. and Papahadjopoulos, D. (1990) Biochim. Biophys. Acta 1024, 352–366.
19 Matthay, K.K., Abbai, A.M., Cobb, S., Hong, K., Papahadjopoulos, D. and Straubinger, R.M. (1989) Cancer Res. 49, 4879–4886.
20 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 145–149.
21 Barlett, G.R. (1959) J. Biol. Chem. 234I, 466–468.
22 Teissié, J. and Tsong, T.Y. (1981) Biochemistry 20, 1548–1554.
23 Needham, D. and Hochmuth, R.M. (1989) Biophys. J. 55, 1001–1009.
24 Chernomordik, L.V., Sokolov, A.V. and Budker, V.G. (1990) Biochim. Biophys. Acta 1024, 179–183.
25 Machy, P., Lewis, F, McMillan, L. and Jonak, Z.L. (1988) Proc. Natl. Acad. Sci. USA 85, 8027–8031.
26 Dimitrov, D. and Sowers, A. (1990) Biochemistry 29, 8337–8344.