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Cationic liposomes formulated with DMPC and a gemini surfactant traverse the cell membrane without causing a significant bio-damage

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ABSTRACT

Cationic liposomes have been intensively studied both in basic and applied research because of their promising potential as non-viral molecular vehicles. This work was aimed to gain more information on the interactions between the plasmamembrane and liposomes formed by a natural phospholipid and a cationic surfactant of the gemini family. The present work was conducted with the synergistic use of diverse experimental approaches: electro-rotation measurements, atomic force microscopy, ζ-potential measurements, laser scanning confocal microscopy and biomolecular/cellular techniques. Electro-rotation measurements pointed out that the interaction of cationic liposomes with the cell membrane alters significantly its dielectric and geometric parameters. This alteration, being accompanied by significant changes of the membrane surface roughness as measured by atomic force microscopy, suggests that the interaction with the liposomes causes locally substantial modifications to the structure and morphology of the cell membrane. However, the results of electrophoretic mobility (ζ-potential) experiments show that upon the interaction the electric charge exposed on the cell surface does not vary significantly, pointing out that the simple adhesion on the cell surface of the cationic liposomes or their fusion with the membrane is to be ruled out. As a matter of fact, confocal microscopy images directly demonstrated the penetration of the liposomes inside the cell and their diffusion within the cytoplasm. Electro-rotation experiments performed in the presence of endocytosis inhibitors suggest that the internalization is mediated by, at least, one specific pathway. Noteworthy, the liposome uptake by the cell does not cause a significant biological damage.

1. Introduction

Due to their biocompatibility, partial tissue selectivity and relative simplicity of manufacturing, liposomes are among the most studied drug delivery systems (DDS). The validity of liposomes as vehicles for the transport of specific drugs depends on a number of physico-chemical parameters, that are determined by the type and amount of lipids used in their formulation and the physiology of the target system. In the last two decades a particular kind of liposomes, the cationic ones, has raised an increasing interest. Cationic liposomes are formulated with cationic lipids which do not occur naturally and, because of their positive charge, interact more easily with the negatively charged cell membrane and nucleic acids [1]. As a consequence, after the pioneering work of Felgner [2], most investigations have concerned their application as carriers of nucleic acids and, more recently, as vaccine carrier/adjuvants [3,4]. Further, abilities of cationic liposomes to deliver in a fairly specific manner their payload to specific tissues such as tumor endothelium, lungs and liver [5], and gastro-intestinal tract upon oral delivery [6], make them attractive DDS of therapeutic agents for a number of pathologies, among which are the neoplastic ones [7,8]. Due to their ability to increase bacterial cell wall and membrane permeability, thus causing a higher susceptibility to drugs [9,10], cationic liposomes have also been considered as DDS in antibacterial therapies against Gram-negative or antibiotic-resistant bacteria.

As mentioned above, the initial interaction of cationic liposomes with plasma membranes is of electrostatic nature. However, the mode

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of interaction of these, as well as of other nanoparticles, with the cell membrane, the routes of internalization and the intracellular trafficking, yet remain to be fully clarified. On the other hand, the assessment of the internalization pathway, that in turn controls the intracellular traffic and hence determines the target within the cell [11], is a main prerequisite for an optimized drug delivery. As a matter of fact, this information is of the utmost importance to address an adequate design of both new and improved formulations.

The aim of the present study is therefore to analyze the process of the interaction with the cell of a cationic liposome formulation that already demonstrated satisfactory characteristics for pharmacological and gene therapies in terms of delivery and transfection efficiency, stability, and the efficacy of interaction with the cell surface [7,12–15]. To this aim, murine fibroblasts (known as 3T6) in culture were used as a model system.

In particular, the main aim of this work is to discriminate between two possible mechanisms of interaction: the adsorption or adhesion of the liposomes onto the outer surface of the cell membrane, favored by electrostatic interactions and eventually followed by their fusion with the membrane, or their internalization into the cell. This investigation was conducted with the synergistic use of diverse experimental approaches, i.e. electro-rotation (ER) measurements, atomic force microscopy (AFM), ζ-potential measurements, and laser scanning confocal microscopy (LSCM) as well as biomolecular/cellular techniques. ER may reveal membrane alterations measuring the dielectric parameters: specific capacitance, C, and conductance, G, that are related to the membrane structure/functions [16–18]. The surface of cytoplasm membrane of untreated 3T6 cells and of cells of the same line after their exposure to the liposomes was studied using AFM, to gain qualitative and quantitative information about cell surface features [19]. Electrophoretic light scattering technique (ζ-potential measurement) can be informative of the overall charge of the cell surface [20]. LSCM allowed following the pathway of fluorescently labeled liposomes inside the cell [21]. Finally, the biological effects of the liposomes were assessed by means of standard biomolecular/cellular techniques that allow investigating the cell survival/proliferation. By collecting and combining all the results obtained by these different techniques, we were led to conclude that the cationic liposomes under investigation cross the cell membrane, are diffuse within the cytoplasm and do not cause a significant bio-damage.

2. Materials and methods

2.1. Liposome preparation

In all the experiments we used a liposome formulation formed by the zwitterionic phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), (Avanti Polar lipids, USA) with the addition of the cationic gemini surfactant (2S,3S)-2,3-dimethoxy-1,4-bis(N-hexadecyl-N,N-dimethylammonium)butane bromide (hereafter Ge1) (Fig. 1), at the molar ratio of 8:2.

The aqueous dispersions of liposomes were prepared by extrusion according to the usual procedure described in the literature [22,23]. Briefly, a film of lipid was prepared by evaporation of a $CHCl_3$ solution containing the appropriate amount of DMPC and Ge1 to obtain the desired molar percentage mixture. One milliliter of PBS buffer solution (Aldrich, 10^{-2} M, pH 7.4) was added to obtain a 12.5 mM lipid dispersion. The solutions were vortex-mixed and freeze-thawed 6× from liquid nitrogen to 307 K. Dispersions were then extruded (10×) at 307 K using a 2.5 mL extruder (Lipex Biomembranes, Vancouver, Canada). The size of liposomes was 110–120 nm, as determined by DLS (cumulant analysis), with a polydispersity index lower than 0.15. The value of ζ-potential was around 26 mV, as obtained from electrophoretic measurements. Liposome suspensions were stable up to 4–5 days.

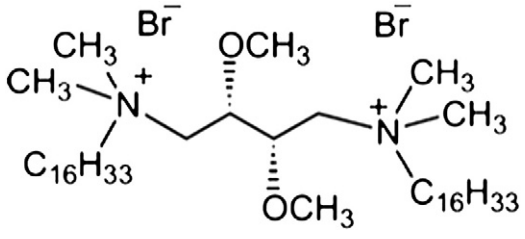

Fig. 1. The synthetic gemini surfactant used for liposome preparation.

Fluorescent liposomes were prepared for the LSCM experiments by adding the fluorescent lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-lissamine rhodamine B sulfonyl ammonium salt (DMPE-RHoB), (Avanti Polar Lipids) to the DMPC–Ge1 mixture in the film preparation (1% of total lipids).

2.2. Cell cultures

The mouse fibroblast cell line 3T6 was used and cells were routinely grown as previously reported [24]. Cultures were exposed to a vast excess of liposomes (in the order of magnitude of 10^6 per cell) for 1 h. These treatment times were selected on the basis of literature data [7,14,15].

In selected electro-rotation experiments bafilomycin A_1 [25] and chlorpromazine [26] were used as endocytosis inhibitors. A bafilomycin A_1 solution was added to the growth medium at 100 nM (f.c.). After 45 min, an aqueous solution of chlorpromazine was added to the growth medium, at 28 μM (f.c.). Total incubation time was 60 min.

The effect of both cationic liposomes and endocytosis inhibitors on cell survival was assessed by the MTT assay (a standard colorimetric assay that measures the reduction of 3-(4,5-diMethyThiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT, by mitochondrial succinate dehydrogenase) [27].

2.3. Electrorotation: theory and apparatus

A rotating electric field applied to a poly-dispersed cell suspension induces on each cell a dipole moment, due to the accumulation of charges at the interphase plasma-membrane/solvent [18]. This charge accumulation is a consequence of the high difference in the polarizability between the dispersing medium and the cell membrane. When this mechanism of interfacial polarization relaxes, a phase shift between the electric field and the induced dipole moment occurs. As a consequence, a torque is generated and the cells rotate in an anti-field fashion. The phenomenon is generated in the range of approximately 10^4–10^6 Hz; as the frequency is further increased (≳10 MHz), the electric field traverses the plasma membrane and the direction of cell rotation is inverted in a co-field fashion. In the kHz range, a further relaxation occurs, associated to the double electrical layer formed by the counterions and the mechanisms of surface conductivity. These three relaxations are known as β, γ and α dispersion, respectively. In this study we only considered the β dispersion, directly related to the dielectric properties of the plasma membrane [16,28].

The rotation period (T) of the cell depends on the frequency (f) of the applied field, according to the following equation, which describes a Debye-like relaxation:

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

where f^* is the relaxation frequency and T_{min} is the corresponding value of the period. Since the relaxation frequency changes with the conductivity of the dispersing medium σ_e, the measurements were carried out on an osmolar sucrose solution (300 mM) supplemented with NaCl at