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Fusion of gemini based cationic liposomes with cell membrane models: implications for their biological activity

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ABSTRACT

The interaction of neutral and anionic phospholipid liposomes, used as cell models, with cationic liposomes formulated with 1,2-dimyristoyl-*sn*-glicero-3-phosphocholine and stereomeric cationic gemini surfactants was investigated by differential scanning calorimetry, fluorescence experiments and dynamic laser light scattering. This study was aimed at rationalizing the different biological features shown by liposomes based on different gemini stereoisomers observed in previous investigations. In fact, to correlate the observed biological activity of liposomes with the molecular structure of their components is critical for a rational and systematic approach to the design of new carriers for drug delivery. The obtained results show that the different stereochemistry of the gemini surfactant controls the interaction and the extent of fusion with different cell models.

1. Introduction

Liposomes can be used as drug carriers to transport a variety of biologically active molecules into cells in vitro and in vivo [1], including those that would not ordinarily be taken up by the cells. There is now a large body of evidence, including pre-clinical and clinical studies, showing that many different drugs packaged in liposomes exhibit significantly reduced toxicity, while retaining or even gaining efficacy [2], so that liposomes are regarded as most promising in the panorama of drug delivery systems. However, in most studies the emphasis has been focused on demonstrating that the material encapsulated within the vesicles can affect the cell activity and functioning, and less attention has been paid to the mechanisms of liposome internalization. A deeper understanding of the mechanisms involved in the drug uptake mediated by lipid vesicles, together with a more complete information on the fate of the vesicles inside the cell and on the intracellular distribution of the vesicles content, is necessary to the best design of new lipid based drug delivery systems.

We previously reported detailed investigations on the efficacy of liposomes formulated with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and the cationic gemini surfactants (*S*,*S*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium) butane bromide **1** or its stereoisomer (*S*,*R*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonium)butane bromide, **2** (Chart 1) as drug delivery systems. It was shown that the stereochemistry of the gemini components significantly affects important features of the mixed liposomes as carriers: the efficiency of the delivery, the intracellular distribution of the drug, and the DNA condensation and transfection in gene delivery [3–8]. Moreover, the delivery efficacy was shown to depend on cell lines [6,7]. The DNA-cationic liposome aggregates (lipoplexes) employed for gene delivery are known to form complex structures [9–11]. While simple liposomes cannot be considered adequate models to study the interaction of these structures with cell membranes, nevertheless also in this case differences in the molecular structure of lipid molecules apparently play an important role [12]. On these premises, we investigated by differential scanning calorimetry (DSC), fluorescence and dynamic light scattering (DLS) experiments the influence of the stereochemistry on the interaction of the cationic formulations with different liposomes employed as cell membrane models,

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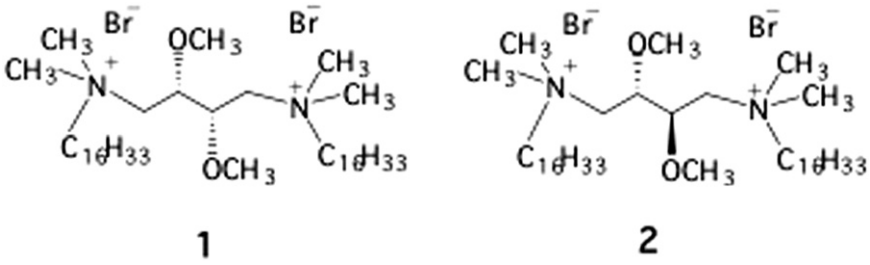

Chart 1. Structure of the stereomeric cationic gemini surfactants **1** and **2**.

namely DMPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcoline (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (DPPG) liposomes.

While liposomes, due to the complete absence of protein components, can be righty considered an excessively oversimplified model of cell membrane under several aspects, studies of the molecular mechanism of membrane fusion and/or lipid exchange in simple, protein free, model systems provide fundamental insights into the fusion process in biomembranes, where complex protein machines promote the lipid molecule rearrangements required for fusion [13–15]. Indeed, the study of the interaction between different bilayer structures such as liposomes, black lipid membranes and supported bilayers has been instrumental not only in defining the sequences of the intermediate structures formed in the course of bilayer merger, but also in determining the properties of the lipid bilayers and the characteristics of the individual lipid molecules that control the propensity of membranes to fuse [16].

The data presented here give clear evidence that the presence of the cationic gemini in the bilayer composition promotes an extensive exchange of lipids between the liposomes, probably as a consequence of "hemifusion". Hemifusion is the partial and reversible fusion of the external leaflet of two bilayers in close contact (see for example Ref. [17]). This process, which is sometimes colorfully described as "kiss-and-run", does not imply a "full fusion" of the liposomes, with the mixing of their internal aqueous cores. In what follows, since our experimental approach focuses on the lipid exchange between the gemini based cationic liposomes and the cell models, and we cannot discriminate between hemifusion and full fusion, we will use the term "fusion" in the general sense, meaning a significant degree of lipid-transfer and mixing of the bilayers.

The cationic liposomes employed in this study were formulated at 6/4 DMPC/gemini molar ratio, because these formulations had shown in previous studies the highest efficiency of delivery [4–8], leading to different intracellular distribution of the transported drug [8].

2. Materials

DMPC, DPPC, DPPG, (purity > 99%) 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (*N*-NBD-PE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(Lissamine Rhodamine B Sulfonyl) (*N*-Rh-PE) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Calcein (Bis[*N*,*N*-bis(carboxymethyl)aminomethyl] fluorescein), Sephadex® G-50 (20–80 μm, CAS 9048-71-9) and PBS buffer solution (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4 at 25 °C) were purchased from Sigma Aldrich. Laurdan (6-dodecanoyldimethylaminonaphthalene) and Triton® X-100 were purchased from Fluka. Gemini surfactants **1** and **2** were prepared and purified as reported previously [18].

3. Methods

As already specified in the introduction, in all the experiments, the gemini liposomes were formulated at 6/4 DMPC/gemini molar ratio. The interaction of the DMPC/gemini liposomes with the phospholipid membrane models was evaluated on multilamellar vesicles (MLVs) in DSC experiments, while large unilamellar vesicles (LUVs) were employed in fluorescence, DLS and Zeta potential experiments.

DSC allowed us to study the interaction of gemini based cationic liposomes with cell models (DMPC or DPPG liposomes) by analyzing the thermal behavior of the interacting bilayers.

The fluorescence investigation involved different experiments.

The extent of the interaction of the cationic liposomes with the DMPC cell model was evaluated by using a membrane associated fluorescent probe, 4-heptadecyl-7-hydroxycoumarin (HC), commonly used to study the electrostatic properties of liposomes at water–lipid interface. Here HC was exploited to monitor the change of the electrostatic surface potential of the neutral DMPC liposomes upon the interaction with the gemini cationic liposomes: a significant increase of the electrostatic surface potential would be a clear indication of lipid (gemini) transfer.

The influence of the gemini sterochemistry on the interaction with the cell models was also investigated by fluorescence resonance energy transfer (FRET) experiments, following a procedure described in the literature [19,20]. Briefly, the fusion of the cationic liposomes with the cell models, containing both a donor and an acceptor fluorescent phospholipid analogue, by diluting the fluorescent probes induces a decrease of the energy transfer efficiency.

Finally, because the fusion of liposomes is usually accompanied by leakage of the solution from the internal liposome pool into the environment [21], the leakage of the internal water pool during the interaction of the cationic liposomes with the cell models was also investigated. To this aim we evaluated the fluorescence of calcein, a fluorescent hydrophilic probe which is self-quenched when confined at high concentration in the liposomes internal water pool, and fluoresces when, as a consequence of leakages, is diluted in the bulk.

The size, size-distribution and electrophoretic mobility of the DMPC or DPPG cell models and of the cationic liposomes were analyzed by dynamic light-scattering (DLS) and zeta potential measurements before and after the mixing.

3.1. Sample preparation

3.1.1. Preparation of MLVs

A lipid film was prepared on the inside wall of a round-bottom flask by evaporation of $CHCl_3$ solutions containing the proper amount of lipids to obtain either the cationic liposomes or the cell models. The obtained films were stored overnight under reduced pressure (0.4 mbar), then a PBS buffer solution (Aldrich, 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4 at 25 °C) was added to obtain a lipid dispersion of the desired concentration for the preparation of the LUVs (see below), or a concentration of 1 mg total lipids/10 μL

for the DSC measurements. The solutions were then heated at 45 °C and vortex-mixed.

3.1.2. Preparation of LUVs

The MLV obtained as described above were freeze–thawed six times from liquid nitrogen to 45 °C. The dispersions were then extruded (10 times) through a 100 nm polycarbonate membrane (Whatman Nucleopore). The extrusions were carried out at 45 °C, i.e. above Tm [22] using a 2.5 mL extruder (Lipex Biomembranes, Vancouver, Canada).

3.2. DSC measurements

DSC measurements were carried out on mixtures, at a 1:1 ratio, of DMPC or DPPG MLVs and cationic MLVs. Each sample, kept under continuous stirring, was incubated at 45 °C, and 35 μL aliquots of the suspension were examined every 2 h. The calorimetric experiments were performed using an adiabatic differential scanning calorimeter Pyris1 (Perkin Elmer). The cells were pressurized with nitrogen to 2.7 mbar to prevent bubbling when heating, and the loss of solvent by evaporation. All heating scans were recorded at a 5 °C/min rate. A scan rate of 1 °C/min was occasionally used to verify if the shape of the heat capacity curves could depend on the scan rate, and based on these experiments, it was ascertained that the phase transitions under consideration were not influenced by the scan rate [23]. Each sample was heated several times, up to the achievement of reproducible thermograms. All the experiments were repeated several times (three at least) at the same incubation time and the reproducibility was excellent.

3.3. Fluorescence measurements

Steady state emission or excitation spectra were obtained using a FluoroMax-4 Horiba Jobin Yvon spectrofluorimeter. The excitation and emission slits were 2 nm. Suspensions of LUVs composed of phospholipids including the fluorescent probe and cationic liposome formulations in a 1:1 ratio were incubated at 45 °C and kept under continuous stirring. Samples were analyzed soon after mixing and then every 2 h. The extent of the lipid exchange between the bilayers was evaluated by monitoring the changes in the fluorescence intensity at the proper wavelength as a function of time.

3.3.1. Evaluation of the extent of bilayer fusion by changes in the surface potential

HC-containing DMPC liposomes were prepared as described above, and adding the proper amount of a HC stock solution (5×10^{-4} M in tetrahydrofuran) to the lipid chloroform solution, to obtain, after hydration, a 5 mM final lipid concentration. In cell model samples, the molar ratio lipid/HC was 375:1. The preparation of HC-containing liposomes and all the experiments dealing with this fluorophore were performed in the dark to avoid photodegradation. The HC fluorescence was measured by scanning the excitation wavelength between 300 and 400 nm and collecting at 450 nm. The extent and the rate of fusion between the cationic formulations and the DMPC cell model were estimated from the increase of the HC fluorescence intensity at 380 nm observed as a function of time after the mixing, and comparing this fluorescence to that of the same amount of HC included in a formulation mimicking 100% fusion. To this aim, liposomes were prepared at 16/4 DMPC/gemini molar ratio (10/0 in cell model + 6/4 in cationic liposomes) and 375:0.5 lipid/HC molar ratio (375/1 in cell model + 375/0 in gemini liposomes).

3.3.2. Evaluation of the extent of bilayer fusion by FRET

Fluorescence (or Förster) resonance energy transfer, FRET, is the non-radiative or resonant transfer of energy [24] where a donor chromophore (*N*-NBD-PE in our case), initially in its electronic excited state, may transfer energy to an acceptor chromophore (*N*-Rh-PE) through non-radiative dipole–dipole coupling. This transfer occurs over very short distances, typically a few nanometers, and since the FRET efficiency decays very rapidly with the distance *d* between the acceptor and the donor ($\approx d^{-6}$) a measure of this quantity allows a very effective evaluation of the dilution of a lipid bilayer where the donor and the acceptor are included at an appropriate surface density [25].

1% molar *N*-NBD-PE and of *N*-Rh-PE were incorporated in DMPC or DPPG liposomes 2.5 mM prepared as described above. The rate of the bilayer fusion between the cationic formulations and the phospholipid cell models was evaluated by monitoring the changes of the fluorescence intensity at 530 nm and 585 nm ($\lambda ex = 450$ nm) as a function of time. Finally, the vesicles were disrupted with Triton X-100 (10% final concentration) to completely eliminate the energy transfer, thus obtaining only the donor fluorescence (100% fluorescence of the donor, at 530 nm). Then, the extent of the bilayer fusion was estimated from the fluorescence emission intensity of *N*-NBD-PE at 530 nm in the absence (F) and in the presence (F_0) of Triton X-100, i.e. $(F/F_0)\cdot 100$. Values obtained in the presence of Triton X-100 were corrected for sample dilution and for the effect of Triton X-100 on the quantum yield of *N*-NBD-PE (1.39) [26].

3.3.3. Evaluation of liposome leakage

DPPC and DPPG liposomes were hydrated with 3 mL of calcein solution (80 mM in PBS buffer at pH 7.4) in order to obtain a 12.5 mM lipid dispersion. The non-entrapped calcein was separated from the liposomes on a Sephadex G-50® gel column (100 μL of liposome solution on 2.5 mL of gel), equilibrated in a PBS buffer solution enriched in NaCl (0.2 M). Calcein-loaded liposomes were added, after filtration, with equimolar (in total lipids) cationic liposomes and the obtained suspension was diluted with PBS buffer in order to achieve a final concentration of ≈1 μM calcein. Calcein fluorescence was measured at $\lambda_{ex} = 490$ and $\lambda_{em} = 515$ nm. To calibrate the assay, a 100% release was obtained by adding Triton X-100 (10% final concentration). The percentage of calcein release was calculated according to Eq. (1):

$$\%F_i = (I_t - I_0)/(I_f - I_0) \times 100 \quad (1)$$

where I_0 is the initial fluorescence, I_t is the fluorescence at the various times and I_f is the total fluorescence observed after the addition of Triton X-100, corrected for dilution and for the variation of the quantum-yield of calcein due to the presence of Triton X-100 [27,28]. In fact, the presence of Triton X-100 affects the calcein fluorescence in a way that is a function of their relative concentrations. Briefly, the variation of quantum yield was evaluated by measuring the fluorescence at 515 nm of a 1 μM solution of calcein in PBS before and after the addition of Triton (1.25 mM final concentration). The resulting correction factor was 1.22.

3.4. DLS measurements

The particle size and their size-distribution in the LUV co-suspensions of cell models and cationic liposome formulations at a ratio 1:1 (1.25 mM in total lipids, 15 mM PBS buffer) were analyzed by DLS measurements. The samples were incubated at 45 °C, kept under continuous stirring and examined soon after mixing and then every 2 h. For all light-scattering measurements, a MALVERN Zetasizer apparatus equipped with a 5 mW HeNe laser was employed. This instrument employs backscatter detection, i.e. the scattered light is collected at an angle of 173°. The main advantage of this detection geometry, when compared to the more conventional 90°, is that it is less sensitive to multiple scattering effects [29]. Intuitively, since nor the illuminating laser beam, nor the detected scattered light need to travel through the entire sample, the chance that incident and scattered photons will

encounter more than one particle is in fact reduced. Moreover, since large particles scatter mainly in the forward direction, the backscattering geometry helps to reduce the effects of dust or, as in this case, of large irregular aggregates (lumps or clots) on the size distribution. To obtain the size distribution, the measured autocorrelation functions were analyzed using the CONTIN algorithm [30,31].

3.5. Zeta potential measurements

The zeta potential of the particles in LUV co-suspensions of cell models and cationic formulations at a 1:1 ratio (1.25 mM in total lipids, 15 mM PBS buffer) was measured by electrophoresis. The samples were incubated at 45 °C, kept under continuous stirring and examined soon after mixing and then every 2 h. Low voltages were applied to avoid possible artifacts due to sample damage caused by Joule heating. The particles ζ-potential was obtained from the electrophoretic mobility u measured using the MALVERN NanoZetasizer apparatus described in the previous paragraph. By combining laser Doppler velocimetry (LDV) and phase analysis light scattering (PALS) [32], this instrument allows the accurate determination of the average mobility and of the mobility distribution. LDV measurements are performed using a so called "mixed mode measurement" (M3) procedure [33] to reduce the effect of electroosmosis [34]. The mobility u was converted into the ζ potential using the Smoluchowski relation $\zeta = u\eta/\varepsilon$, where η and ε are the viscosity and the permittivity of the solvent phase, respectively.

4. Results

4.1. DSC measurements

The interaction of the gemini based cationic liposomes with neutral (DMPC) and anionic (DPPG) cell membrane models was investigated by DSC. In these experiments, we analyzed the changes in the thermotropic behavior of both cationic liposomes and cell models after mixing and incubating them at 45 °C (i.e. at a temperature higher than the Tm of cationic formulations and cell models) for increasing periods of time. The observed thermotropic behavior gives a strong indication that a substantial lipid rearrangement occurs upon the interaction between the gemini cationic liposomes and our cell models the phospholipids liposomes. Particularly, the new peak (or new peaks, in the case of gemini **1**) that appears in the thermogram clearly suggests a fusion and rearrangement of the lipid bilayers.

The thermograms of the fusion experiments are reported in Fig. 1 and Fig. 2. These thermograms, obtained at the same scan rate and after incubation at controlled temperature, were highly reproducible. In each figure the thermograms are shown in the order of increasing incubation time (from bottom up). The traces at the bottom are the thermograms of the liposomes suspensions (cationic liposomes and cell models) before mixing (*bm*); the second trace is the thermogram obtained immediately after mixing (*am*), where often the transition peaks of the cell models and of the cationic formulation are still clearly distinguishable; the following thermograms are those measured after 2, 4, 6 and 8 h of incubation, respectively.

The thermograms clearly show that the interaction of the cationic formulations with the cell models strongly depends both on the stereochemistry of the gemini surfactant and on the composition of the cell model.

When the DMPC cell models were incubated with cationic liposomes formulated with **1**, a complex behavior was observed: after 2 h of incubation a new peak was observed, at a temperature slightly higher than the transition of the cell model, while at the same incubation time the transition of the cationic liposomes disappeared almost completely (Fig. 1, panel A). At increasing incubation time, a complex

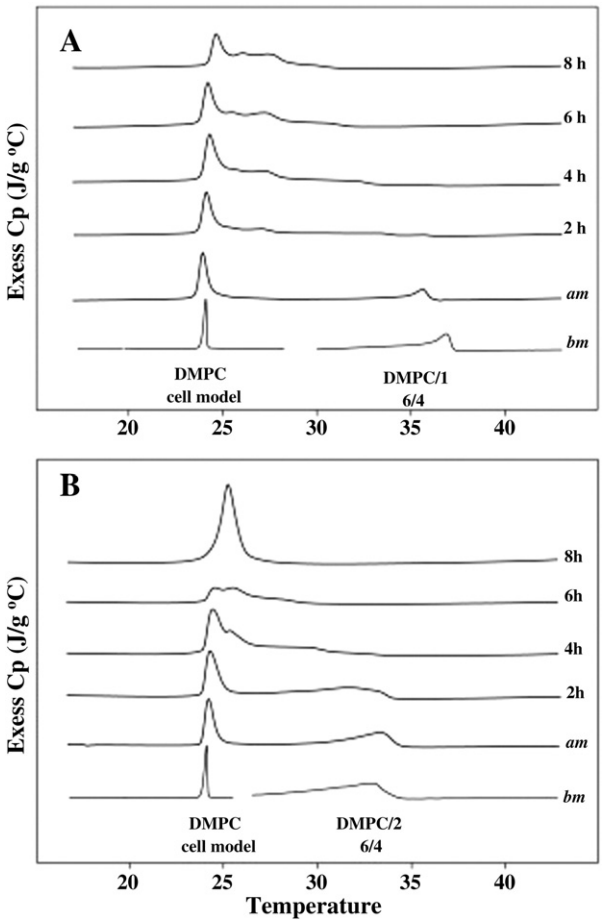

Fig. 1. Thermograms obtained in the experiment of interaction of DMPC cell model with DMPC/**1** and DMPC/**2** MLV (panel A and panel B, respectively) at 6/4 molar ratio. In each figure the thermograms are organized according to increasing time of incubation, the first thermogram from the bottom being relative to the start of the experiments (immediately before mixing). Scan rate is 5 °C/min.

endotherm was obtained, where two new peaks partially overlapping with that of the cell model were observed.

The thermogram relative to the experiments involving liposomes containing **2**, showed after 2 h of incubation a significant broadening of the peak of the cationic formulation (Fig. 1, panel B) partially overlapped to a new broad peak. After 4 h of incubation the peak corresponding to the cationic formulation almost disappeared and a new peak appeared as a shoulder of the cell model peak. At increasing incubation time the two peaks showed similar intensity and after 8 h of incubation only a single peak was present in the thermogram, probably due to the complete overlap of the two peaks.

The lipid rearrangement upon interaction of both the cationic liposomes with the anionic cell model (Fig. 2) was faster than that observed with the neutral cell model (Fig. 1). In fact, soon after mixing the peaks of the main transition of both the cationic liposomes were shifted to a lower temperature (at ~32 °C and at ~30 °C for DMPC/**1** and DMPC/**2**, respectively), indicating an almost immediate lipid rearrangement.

In the thermograms relative to the interaction of the **1** containing formulations with DPPG cell model (Fig. 2, panel A), after 2 h of incubation three new peaks were observed, with the most intense one partially overlapped to the peak of the cationic liposomes. At increasing incubation time, a significant broadening of the new peaks, was observed, especially for the most intense one, while the peak corresponding to the cationic formulation almost disappeared.

The thermograms obtained for the formulations containing **2** when interacting with the DPPG cell model (Fig. 2, panel B) showed

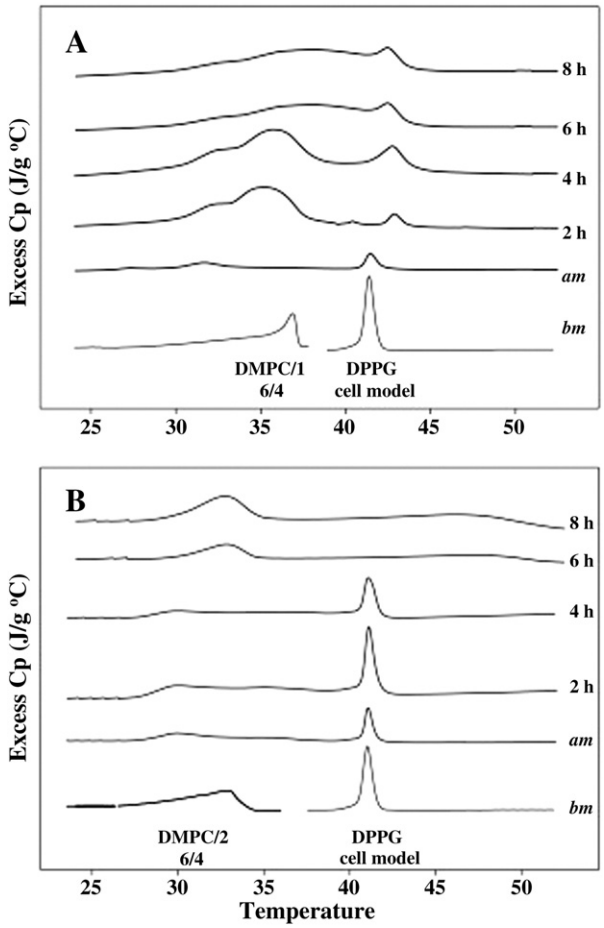

Fig. 2. Thermograms obtained in the experiment of interaction of DPPG cell model with DMPC/**1** and DMPC/**2** MLV (panel A and panel B, respectively) at 6/4 molar ratio. In each figure the thermograms are organized according to increasing time of incubation, the first thermogram from the bottom being relative to the start of the experiments (immediately before mixing). Scan rate is 5 °C/min.

a rather different behavior. In fact, after 4 h of incubation, the characteristic peaks of both the cationic liposomes and of the DPPG liposomes were still clearly observable, and only after 6 h of incubation a peak centered at ~33 °C, and a broad but pronounced peak, tailed on the high temperature side, clearly appeared, indicating the presence of a newly formed lipid mixture. Thus, although both the gemini liposome formulations show a strong interaction with the anionic cell model, the kinetics of the interaction and the characteristics of the newly formed aggregates appear to be rather different for the two gemini stereoisomers, with an apparently faster mixing kinetics observed for **1**.

4.2. Fluorescence measurements

4.2.1. Evaluation of the extent of bilayer fusion by changes in the surface potential (HC)

In these experiments, the variation of the surface potential upon the interaction of the cationic liposomes with the neutral cell models was evaluated by exploiting the presence in the cell model bilayer of HC, a fluorescent probe whose emission features depend on the value of the surface potential. This method demonstrated to be unsuitable to evaluate the effect of the interaction with the anionic cell model, because in this case the variation of the surface potential is too small to be detected by changes in the HC fluorescence at 380 nm.

In a preliminary experiment, we verified that leakages of HC during the incubation time were negligible, so that the eventually observed variations can be confidently ascribed to a fusion of the bilayers. To evaluate the extent of the fusion, the fluorescence intensity at 380 nm of HC embedded in a formulation mimicking 100% of fusion (DMPC/gemini liposomes at 16/4 molar ratio, see Section 3.3.1) was measured: the obtained value was then used to normalize the fluorescence intensity observed for the cationic liposome/cell model mixture observed at different incubation times. The results are reported in Table 1. Both the cationic formulations interact to a great extent with the DMPC cell model, however in the case of DMPC/**1** liposomes the percentage of observed fusion was higher; this difference being relevant yet after 2 h of incubation.

4.2.2. Evaluation of the extent of the bilayer fusion by FRET experiments

Changes in FRET efficiency are commonly used for a quantitative evaluation of the fusion process based on the F/F_0 ratio as described in the experimental section.

In our case, as a result of the interaction of the gemini liposome formulations with the cell models containing the two chromophores, the lipid mixing causes a significant decrease of their FRET efficiency. The results are reported in Table 1. The interaction of both the cationic formulations with the two different cell models was very fast (after 2 h of incubation all samples showed a percentage of fusion higher than 70%). After 8 h of incubation DMPC/**1** liposomes showed a higher tendency to fuse with DMPC cell model compared to DMPC/**2** liposomes, whereas in the case of DPPG cell model both cationic formulations fused almost completely (95%).

4.2.3. Evaluation of liposome leakage

Since the fusion of liposome bilayers can involve leakage from the internal aqueous pool of liposomes, we investigated on LUV the effect

Table 1
Results of fluorescence experiment after 2 h and 8 h of incubation. First row: percentage of bilayer fusion obtained in experiments using DMPC cell model containing HC. Second row: percentage of bilayer fusion observed in the FRET experiments using DMPC and DPPG cell models. Third row: percentage of calcein released obtained using DPPC and DPPG cell models. Errors in determination are within 5% for all methods.

	DMPC/**1**			DMPC/**2**		
	DMPC	DPPC	DPPG	DMPC	DPPC	DPPG
% bilayer fusion	51 (2 h)	–	–	39 (2 h)	–	–
evaluated by HC	83 (8 h)	–	–	72 (8 h)	–	–
fluorescence						
% bilayer fusion	77 (2 h)	–	77 (2 h)	72 (2 h)	–	73 (2 h)
evaluated by FRET	87 (8 h)	–	95 (8 h)	74 (8 h)	–	95 (8 h)
% calcein release[a]	–	29 (2 h)	28 (2 h)	–	12 (2 h)	31 (2 h)
	–	36 (8 h)	33 (8 h)	–	25 (8 h)	33 (8 h)

[a] Values corrected for the spontaneous leakage of the cell models (25% for DPPC, 35% for DPPG).

of the interaction of cationic formulations with cell models also by measuring the calcein release from the internal aqueous pools of cell models loaded with this fluorophore. In these experiments we used DPPC liposomes as neutral cell membrane models because, due to their higher main transition temperature (41 °C vs 24 °C of DMPC liposomes), they feature a reduced permeability compared to DMPC liposomes. The values obtained in these experiments (Table 1) were corrected for the spontaneous leakage of the cell model in the absence of cationic liposomes; this was found to be 25% and 35% for DPPC and DPPG liposomes, respectively. The interaction of DMPC/**1** liposomes with both cell models induced the same release of calcein (>28% after 2 h incubation and >34% after 8 h). Noteworthy, the interaction of DMPC/**2** liposomes with DPPC and DPPG cell models shows instead some differences. While **2** causes a similar release as **1** from the DPPG cell model, the calcein release from the DPPC cell model appears significantly reduced, and in any case slower (12% after 2 h incubation).

4.3. DLS measurements

The interaction of the cationic formulations with the cell models was evaluated by analyzing the evolution of the size of the vesicles upon mixing. Table 2 reports the hydrodynamic diameters as mean values of the intensity-weighted size distributions obtained from the CONTIN analysis of DLS correlation functions. In all the experiments a stable, although quite large, size distribution was reached in a few minutes after the mixing. This distribution did not change significantly even after 8 h incubation. In any case the average diameter measured after the mixing, is significantly larger than that of both the component vesicles. This systematic although small increase suggests that upon mixing some aggregation occurs. Since both the preparations are separately very stable, and since they are prepared at the same concentration and in the same buffer, so that upon mixing nor the overall concentration nor the pH changes, the aggregation most probably results from the interaction of different vesicles.

In Table 2, for each mixing experiment, in correspondence of the measured final size, we also indicate within brackets an expected diameter calculated assuming a one-to-one complete fusion between a cell model and a cationic liposome. In this calculation we assume that in the fusion process the vesicles' surface area is conserved, with two fusing vesicles that becomes one single larger vesicle. Clearly, with these assumptions the resulting diameter is simply the square root of the sum of the squared diameters of the initial vesicles. Although these assumptions might appear naïve or even simplistic, it is surprising that for all the formulations the ratio between the so calculated diameter and the average diameter of the vesicles effectively measured is 0.90. This might suggest that at least part of the bilayer fusion evidenced by the fluorescence and DSC measurements could result in a complete fusion.

Table 2
Size and half-width of size distribution (intensity average) of cell models, of cationic formulations and of the resulting aggregates after 8 h incubation. Data were obtained by CONTIN analysis. The expected size calculated assuming a complete one-to-one fusion of the vesicles (see text) is reported in brackets.

Diameter (nm)	DMPC/**1**	DMPC/**2**
	103 ± 20	131 ± 35
DMPC	134 ± 33	155 ± 42
110 ± 17	(150)	(171)
DPPG	132 ± 22	141 ± 29
115 ± 18	(154)	(174)

4.4. Electrophoretic mobility measurements

We investigated the interaction of the cationic formulations with the cell models also by electrophoretic mobility measurements. This technique is a valuable complement to the size determination by DLS in evaluating the occurrence of a fusion process, thanks to the possibility of distinguishing between particles with the same size but with different surface charge.

Table 3 reports the mean values of zeta-potential of the vesicles employed as cell models, of the cationic formulations, and those of the vesicles obtained upon mixing and 8 h incubation, Fig. 3 shows the relative distributions. As expected, the neutral (zwitterionic) DMPC cell model shows a zeta potential close to zero, while DPPG exhibits a strongly negative value. Noteworthy, DMPC/**1** liposomes show a slightly higher zeta potential compared with DMPC/**2** liposomes. This is probably due to a different exposure of the charged group to the bilayer surface due to the different stereochemistry [14].

After the incubation, the mixed vesicles formed by the DMPC/**1** liposomes with the DMPC cell model showed a zeta potential value that is only slightly smaller than that of the cationic formulation alone. A similar behavior is observed for the cationic formulation DMPC/**2**. The comparison of the zeta-potential distributions (Fig. 3) gives further evidence for the occurrence of a significant bilayer fusion. The zeta-potential distribution of the mixed vesicles appears slightly shifted to lower values compared to DMPC/1. More significant is the complete disappearance of the peak relative to DMPC, a finding that clearly suggests that after the 8 h incubation the fusion of the bilayers and the lipid exchange proceeded to such extent that in practice all the vesicles have now a similar composition of the bilayer, with the charged lipids distributed over all the particles. Upon the interaction with DPPG liposomes, the mixed vesicles show negative zeta potential values for both the DMPC/**1** and DMPC/**2** formulations, with final distributions of the mixed vesicles that are clearly different from the distributions of the original (unmixed) vesicles.

5. Discussion

By the synergistic use of several different techniques, DSC, fluorescence, FRET, DLS and ζ-potential, we investigated the interaction of gemini based cationic liposomes with neutral (DMPC, DPPC) and anionic (DPPG) cell membrane models, to gain a better understanding of the basic interaction of gemini based cationic liposomes with typical phospholipid bilayers employed as simplified models of cell

Table 3
Zeta potentials of cell models, of cationic formulations and of the aggregates present in the sample after 8 h of incubation (± the half-width of the distribution).

Zeta potential (mV)	DMPC/**1**	DMPC/**2**
	55 ± 6	45 ± 6
DMPC	48 ± 10	49 ± 8
4 ± 3		
DPPG	−28 ± 8	−30 ± 6
−50 ± 12		

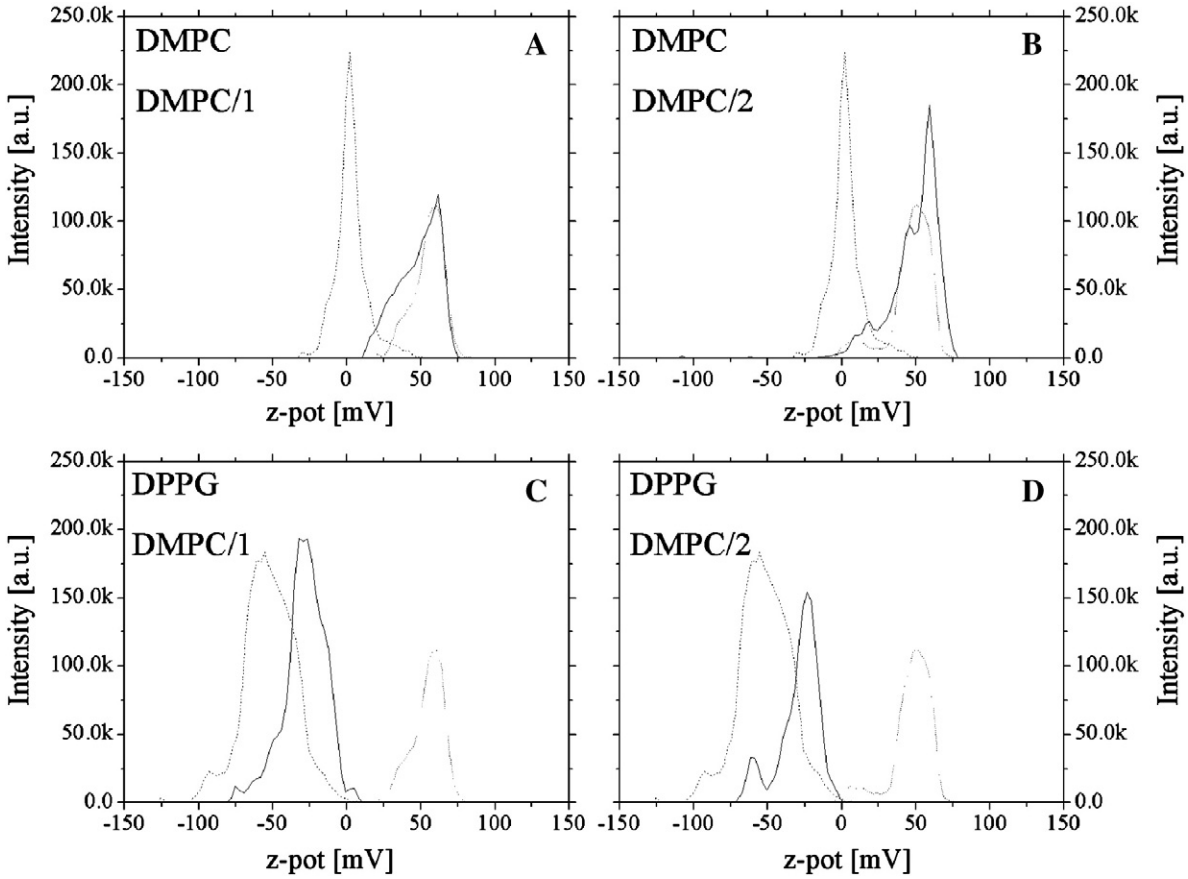

Fig. 3. Zeta potential distributions of the cell models (dashed line) and of the cationic formulations (dotted line) before the mixing, and of the single populations (continuous line) that appear shortly after mixing. Here the "mixed" distributions are those measured after 8 h incubation.

membranes. As we will show in details, this complex experimental approach allowed us to point out the key role of the stereochemistry of the gemini cationic component of the liposomes formulations in such interaction. In fact, while our findings clearly suggest that in any case as a result of the gemini-liposome/cell-model interaction there is a significant exchange of lipids between the bilayers, there is also significant evidence that such exchange is quantitatively different for the two gemini stereoisomers.

The finding that gemini-based cationic liposomes show fusogenic properties is interesting.

Even long-term contacts between protein-free liposomes mimicking the compositions of biological membranes usually do not result in fusion. Actually, the propensity of lipid bilayer to hemifuse usually depends on the presence of "fusogenic" lipids, i.e. lipids that show a packing parameter [35]

$$P_p = v/lA > 1$$

(here v is the volume of the hydrophobic tail, l its length and A is the area of the cross section of the hydrophilic headgroup). A value of $P_p > 1$, meaning that the hydrophobic part is somehow "larger" than the hydrophilic one, increases the propensity of these lipids to form structures with a "negative" spontaneous curvature (the curvature is considered negative when the surface bulges in the direction of the hydrophobic tails). The presence of such "negative curvature" lipids is considered a prerequisite for the formation of a "hemifusion stalk" where the proximal leaflets of two bilayer in close contact begin to fuse (while the distal ones remain unfused) [16]. In fact, in the stalk the curvature must be negative.

While in general charged liposomes are considered "non-fusogenic" due to the strong electrostatic repulsion between their head that favors a positive curvature, there are several evidences that cationic gemini, as the length of the spacer decreases and at relatively low degree of protonation tend to form inverted structures ($Pp > 1$) [36,37]. In different conditions, they form wormlike micelles, ribbons, inverted hexagonal phases [38] and more complex aggregates of elongated vesicles, where the clustering could be also due to the embedding of the two tails in bilayers of different vesicles [22]. This last tendency could also be reconnected to the fact that for an "activated collision" that result in the formation of a stalk, a local "defect" in the bilayer organization is needed, i.e. a lipid that protruding from the bilayer, increases the probability of fusion in the collision [15]. Such "pointlike protrusions" favor the bilayer fusion [17] both by decreasing the distance to a nearby bilayer and by decreasing the hydrophobic energy of the monolayer rupture.

Due to the different requirements of the experimental techniques, DSC measurements were carried out on highly concentrated MLVs, while fluorescence and DLS measurements were performed on more diluted LUV samples. Nevertheless, data from the different experiments gave complementary information and are perfectly consistent in pointing out an extensive bilayer fusion and lipid exchange. The extent of the fusion (without indications on the organization of the bilayer) between the cell models and the cationic formulations was evaluated i) by analyzing the thermotropic behavior of the mixtures by DSC; ii) by following the variation of the surface potential of the neutral cell model containing HC; iii) by monitoring (by FRET experiments) the lateral diffusion of the fluorescent probes included in the cell models; iv) by measuring the leakage of calcein; and v) by monitoring the changes of the vesicle size (hydrodynamic radius by DLS) and ζ-potential (electrophoretic mobility). Since the fusion is usually accompanied by leakage of the internal aqueous phase, the "calcein" experiment gives only an indirect estimation of the extent of fusion.

In any case, the results obtained by all these different methods and techniques pointed out consistently to a significant lipid-transfer between the gemini-based cationic liposomes and the cell models.

Particularly, DSC results show for both cationic formulations a high extent of lipid rearrangement upon interaction with both the cell models. The kinetics of lipid rearrangement is significantly faster for the liposomes formulated with the gemini **1** compared to **2**, independently of the composition of the cell models and of their surface charge (neutral or anionic). However, the thermograms at 8 h in the experiments with DMPC/**1** liposomes show a more complex behavior than that of DMPC/2 liposomes, in fact two new peaks, partially overlapping with that of the cell model, are observed. In other words after a faster exchange, gemini **1** seems to experiment greater difficulties than gemini **2** in diffusing and rearrange within the bilayer. This finding suggests a better miscibility of DMPC/**2** liposomes with the cell models.

This significantly different thermotropic behavior points out the important role of the stereochemistry of the gemini component in the mode of lipid rearrangement upon the interaction of the cationic liposomes with phospholipid bilayers.

The DSC results are well consistent with the fluorescence experiments on the neutral cell model.

In fact, both the HC and FRET fluorescence experiments show a slower and minor extent of lipid rearrangement upon the interaction of the neutral cell model containing the fluorescent probes with DMPC/**2** liposomes compared to DMPC/**1** liposomes. Also the experiments of calcein release confirm a different kinetics of interaction.

On the other hand, in all the fluorescence experiments the differences between the two gemini cationic formulations were negligible when they interact with the anionic (DPPG) cell model. The reason of such different behavior is unclear, but it is probably to be connected with the presence of the electrostatic interactions between gemini and DPPG. The presence of these interactions, being in general much stronger and long ranged than sterical interactions, tends in fact to attenuate the differences between stereoisomers. The size of the aggregates formed by the interaction of the cell models with the DMPC/**1** liposomes did not show any dependence on the composition of the cell model. Conversely, the size of the aggregates formed by the interaction with the DMPC/**2** liposomes depended on cell model, the larger size, and the larger polydispersity, being observed upon mixing with the neutral cell model.

As expected on the basis of the surface potential values reported previously [8], DMPC/**2** liposomes feature lower zeta potential compared with DMPC/**1** liposomes, probably because of a different exposure of the cationic head group of the gemini surfactant [18], and this feature could be, among others, responsible of the different kinetic, extent and mode of lipid rearrangement. However, the systems formed by the two gemini upon the interaction with both the cell models feature the same zeta potential ($\approx$50 mV and ≈ -30 mV upon interaction with neutral and anionic cell model, respectively), thus suggesting that after fusion the mode of exposure of the cationic head groups is similar for the two gemini components.

Summarizing, the extent, the kinetic and the mode of interaction of gemini cationic liposomes with cell models were shown to depend on the cell model and on the stereochemistry of the gemini. In particular, DMPC/2 liposomes showed a significantly slower lipid rearrangement, possibly due to a different exposure of the cationic gemini head group, though accompanied by a higher lipid miscibility with both cell models when compared to DMPC/**1** liposomes.

These evidences could explain the different biological behaviors of the cationic formulation i.e. different uptake and intracellular distributions of the delivered drug that are controlled by the interaction with specific compartments of the cell membrane.

6. Conclusions

DSC, fluorescence, DLS and electrophoresis experiments were carried out to evaluate how and to what extent the stereochemistry of the two gemini surfactants included in liposome formulations affect their ability of interacting and fusing with neutral and anionic membrane models.

DMPC/**2** liposomes, that feature the highest efficacy in drug delivery [8], showed a lower zeta potential and, upon the interaction with both the neutral and anionic cell model, a slower rate of lipid rearrangement and a higher miscibility compared to the corresponding formulation containing **1**; the stereochemistry of the gemini, by affecting the exposure to water of the cationic head group of the surfactant, controls some physicochemical properties of liposomes (zeta potential, bilayer organization) important for their interaction with lipid bilayers.

Obviously, cell membranes are very complex lipid bilayers, they are composed by hundreds of lipids that can interact differently with liposome bilayers and are organized in domains with specific composition (rafts); furthermore cell membrane contains many different receptors, that could play a fundamental role in the interaction with liposomes and in their internalization. On the other hand, the models used are very simple and can mimic a region of cell membrane featuring a specific composition and/or surface charge.

The results of this investigation clearly show that the surface charge of liposomes (in this case controlled by the different stereochemistry of gemini component), cell membrane composition and lipid miscibility might control the uptake and the biodistribution of the drug delivered by liposomes.

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