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molecular pharmaceutics

Efficiency of Liposomes in the Delivery of a Photosensitizer Controlled by the Stereochemistry of a Gemini Surfactant Component

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Received July 16, 2009; Revised Manuscript Received October 19, 2009; Accepted November 24, 2009

Abstract: Liposomes formulated with dimyristoyl-*sn*-glycero-phosphatidylcholine, DMPC, and either one of the cationic gemini surfactants (*S*,*S*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonio)butane bromide, **1a**, and (*S*,*R*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonio)butane bromide, **1b**, were investigated as vehicles of the photosensitizer *m*-tetrahydroxyphenylchlorin, *m*-THPC, to cell models of malignant glioma. The delivery efficiency of DMPC/**1a** and DMPC/**1b** liposome formulations were evaluated on the murine glioblastoma cell line C6 and on the human glioblastoma cell line LN229 by flow cytometry and laser scanning confocal microscopy. The stereochemistry of the spacer of the gemini was found to strongly influence the delivery efficiency of *m*-THPC to cells, the mode of interaction with the cell membrane, and the intracellular distribution of *m*-THPC. The physicochemical features of liposomes were investigated with the aim of explaining the parameters that control their biological features. Differences that could account for the different biological activity of the formulations concern the values of surface potential and the environment of *m*-THPC at the water/liposome interface.

Keywords: Gemini surfactant; stereochemistry; cationic liposomes; photodynamic therapy; *m*-THPC; Foscan; malignant glioma

Introduction

Recently, there has been renewed interest in liposomes, mainly due to their potential as drug delivery systems (DDS). Advantages of liposomes compared to other DDS include the lack of immune and inflammatory response, and then the possibility of repeated administration of the drug, the ease of large scale preparation, the versatility at the molecular and at the formulation level, and their lower costs. Liposome formulations are investigated at both preclinical and clinical level in many therapeutic protocols. Among liposome formulations, the cationic ones have been shown to be highly promising. Actually, liposomes composed of a natural phospholipid and cationic lipids or surfactants were shown effective in delivering DNA or RNA to cells;[1] further, because of their enhanced ability to interact with cell membrane, they have been investigated, with good results, as drug delivery systems for many disease and cancer treatments.[2]

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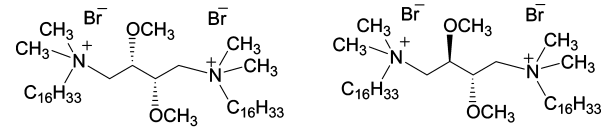
10.1021/mp900173v
Published on Web 11/24/2009

The use of liposomes may improve the efficacy and intrinsic safety of photodynamic therapy (PDT), a protocol that involves the administration of a photosensitizer (PS), and its irradiation with visible light to induce formation of cytotoxic species. In fact, liposomes can efficiently solubilize hydrophobic PSs,[3] thus increasing PS photoactive population,[4] improve the PS accumulation in tumor,[5] improve the PS pharmacokinetic, and reduce the side effect of skin photosensitization due to poor target specificity.[6]

We reported on the use of liposomes formulated with dimyristoyl-*sn*-glycero-phosphatidylcholine, DMPC, and the cationic gemini surfactant (*S*,*S*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonio)butane bromide **1a** (Chart 1), to deliver *m*-THPC to a human colon adenocarcinoma cell line[7] and to murine and human glioblastoma cell lines.[8] It was shown that the gemini surfactant increases both cell uptake of *m*-THPC[7,8] and its cytotoxic effect after laser irradiation with respect to the pharmaceutical formulation (Foscan), and the highest efficiency was found for the formulation at a DMPC/**1a** 60:40 molar ratio.[8]

Chart 1. The Two Diastereoisomers of the Gemini Surfactant **1** Used for Liposome Preparation



Besides, in the investigation on the condensation and transfection of DNA by mixed liposomes formulated with DMPC and either **1a** or its stereoisomer (*S*,*R*)-2,3-dimethoxy-1,4-bis(*N*-hexadecyl-*N*,*N*-dimethylammonio)butane bromide, **1b**, it was shown that the stereochemistry of the gemini strongly influences DNA condensation and transfection.[9] Actually, it is known that the stereochemistry of the monomer, besides affecting the morphology,[10] the stability,[11] and the physicochemical features of lipid aggregates,[12] might affect their interaction with the biological environment. In fact, many investigations account for implication of stereo-

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chemistry in drug delivery,[13] transdermal delivery[14] and sustained release of active agents.[15]

Here we report on a biological investigation on the influence of the stereochemistry of the gemini surfactants **1**, included in mixed DMPC/**1** liposomes, on the delivery efficiency of *m*-THPC to cell lines of malignant glioma (GBM), and on the physicochemical characterization of the formulations, aimed at explaining the parameters that might control the interaction with cells.

Experimental Section

Materials. Dimyristoyl-*sn*-glycero-phosphatidylcholine (DMPC, purity >99%) was purchased from Avanti Polar Lipids (Alabaster, AL); exclusion gel Bio-Gel A-15 m gel was purchased from Bio-Rad Laboratories (Hercules, CA); *m*-tetrahydroxyphenylchlorin (*m*-THPC) was a kind gift by Biolitec (Jena, Germany); 4-heptadecyl-7-hydroxycoumarin (HC) was purchased from Fluka; PBS buffer and Hanks' balanced salt solution (HBSS) were purchased from Sigma-Aldrich (St. Louis, MO); Dulbecco's modified Eagle medium (DMEM) and streptomycin were purchased from Gibco Life Technologies, (Paisley, U.K.); FBS was purchased from Hyclone, (Carmlington, U.K.); RPE and HPLC grade solvents (chloroform, ethanol, isopropanol, bidistilled water) were purchased from Carlo Erba Reagenti (Milano, Italy); polycarbonate membrane were purchased from Whatman Nuclepore (Toronto, ON, Canada).

Gemini surfactants **1a** and **1b** were prepared as previously described.[16]

Cell Culture. Human (LN229) and murine glioblastoma (C6) cell lines (kindly provided by Dr. S. Ciafrè, University of Rome "Tor Vergata", Italy) were grown as monolayer in DMEM supplemented with 5% (LN229) or 10% FBS, 1% penicillin (50 IU/mL) and streptomycin (50 IU/mL) in a humidified atmosphere of 5% CO_2 in a water-jacketed incubator at 310 K.

Preparation of Liposomes. The aqueous dispersions of DMPC/**1** liposomes were prepared according to a procedure described previously.[17] Briefly, a film of lipids (total 20.0 μmol) was prepared on the inside wall of a round-bottom flask by evaporation of a $CHCl_3$ solution containing the proper amounts of DMPC and **1** to obtain the 60/40 molar percentage mixture. The obtained films were stored in a desiccator overnight under reduced pressure, and 1.6 mL of PBS buffer solution (10^{-2} M pH 7.4) was added in order to obtain 12.5 mM lipid dispersions. The solutions were vortex-mixed and then freeze−thawed six times from liquid nitrogen to 313 K. Dispersions were then extruded (10 times) through a 100 nm polycarbonate membrane. The extrusions were carried out at 307 K, well above the transition temperature of DMPC (297.2 K), using a 2.5 mL extruder (Lipex Biomembranes, Vancouver, Canada).

m-THPC or HC containing liposomes was prepared by adding to the chloroform solution of the lipids the proper volume of a *m*-THPC (5×10^{-4} M, EtOH abs) or HC (6×10^{-4} M, THF) stock solution to obtain, after hydration, a final concentration of 50 μM *m*-THPC or HC.

Flow Cytometry. The time course analysis of liposome/*m*-THPC uptake was performed on human glioblastoma (LN229) and murine (C6) cells treated for 30 min, 1 h and 4 h with the following liposome formulations: (i) *m*-THPC in DMPC, (ii) *m*-THPC in DMPC/**1a** 60/40, (iii) *m*-THPC in DMPC/**1b** 60/40. *m*-THPC and total lipid concentrations were the same in each formulation ([*m*-THPC] = 5×10^{-7} M, [total lipids] = 1.25×10^{-5} M).

After each treatment, cells were washed with ice-cold HBSS, detached with EDTA and trypsin, resuspended in ice-cold PBS and immediately analyzed for the PS content. Fluorescence signals were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15 mW, 488 nm, and air-cooled argon ion laser. The fluorescence emission was collected through a 670 nm band-pass filter and acquired in log mode, taking into account that fluorescence emission of *m*-THPC features two peaks at 650 and 714 nm, the more intense occurring at 650 nm. At least 10000 events were analyzed. The content of PS was evaluated as fluorescence intensity and expressed in arbitrary units (AU), calculated as the ratio between the mean fluorescence channel (MFC) of treated samples and the MFC of untreated cells. The analysis was performed by the CellQuest software (Becton Dickinson).

Laser Scanning Confocal Microscopy. Living cells were analyzed by laser scanning confocal microscopy (LSCM) in order to investigate the intracellular distribution of the PS delivered by liposome formulations. Cells, grown on 12 mm glass coverslips, were inoculated with the different liposome formulations, under stirring at 300 rpm. After incubation with liposome formulations for 1 and 24 h at 310 K, cells were fixed in 3.7% paraformaldehyde in PBS, for 10 min at room temperature. Observations were performed by using a Leica

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TCS 4D laser scanning confocal microscopy (Leica, Microsystems, Mannheim, Germany) equipped with an Ar/Kr laser. After excitation at 568 nm, fluorescence emission was collected using a 590 nm long-pass filter.

Determination of Transition Temperature, T_m, of Mixed Liposomes. Thermal phase transition of mixed DMPC/**1** liposomes was determined from the temperature-dependent changes of specific turbidity,[18] using a Cary 300 UV−vis double beam spectrophotometer (Varian Australia PTY Ltd., Mulgrave, Vic., Australia) with a quartz cuvette of 1.0 cm path length. The scan was carried out at 260 nm over the temperature range 288−318 K at a rate of 1 K/min. Results were expressed in turbidity (OD) as a function of temperature. The transition temperature of the liposome formulations correspond to the inflection point in the OD versus temperature plots. All measurements were made at 2.5 mM total lipid concentration in PBS buffer.

Determination of the Entrapment Efficiency (EE). Non-entrapped drug was separated by filtration of 200 μL of liposome dispersion on a 2.5 mL Biorad AM-15 gel column, equilibrated in PBS buffer solution. Eluted fractions containing liposomes were identified by fluorescence emission of *m*-THPC, then combined and diluted to a known volume. *m*-THPC concentration in liposome preparations before and after filtration was determined by measuring absorbance maximum (at 417 nm) of the *m*-THPC/liposome suspensions after the addition of an equal volume of isopropanol for disruption of liposomes, the molar extinction coefficient of *m*-THPC in 50% isopropanol/water being 158600 M^{-1} cm^{-1}. Percentage of entrapped drug was calculated by eq 1,[19] where $M^a_{m\text{-THPC}}$ and $M^b_{m\text{-THPC}}$ are the chlorin concentrations in liposomes, after and before gel filtration, respectively, M^a_{lip} and M^b_{lip} are the total lipid concentration after and before the filtration, respectively.

$$\%\ m\text{-THPC} = 100 \times (M^a_{m\text{-THPC}} M^b_{lip})/(M^b_{m\text{-THPC}} M^a_{lip}) \quad (1)$$

Effect of Storage on the Entrapment Efficiency (Leakage). After the extrusion and gel filtration, cationic liposome dispersions were diluted with PBS buffer in order to have *m*-THPC 8×10^{-6} M and total lipid 2×10^{-3} M. The fluorescence measurements were performed at room temperature on a Shimadzu RF5001PC spectrofluorimeter every 24 h for two days. Fluorescence of *m*-THPC was measured at 652 nm using an excitation wavelength of 517 nm (bandwidths 5 nm). The obtained fluorescence data were referred to a solution of 10^{-5} M *m*-THPC in absolute ethanol.

Because *m*-THPC is insoluble in water and once escaped from liposomes it aggregates, only the fraction of PS embedded in the double layer gives a fluorescence signal. Thus, the decrease of the fluorescence intensity was a direct measurement of the PS leaked from liposomes.

Liposome Stability. The stability of mixed DMPC/**1** liposomes was determined according to a known procedure,[18] i.e. from the time-dependent changes of specific turbidity, using a Cary 300 UV−vis double beam spectrophotometer with a quartz cuvette of 1.0 cm path length. All measurements were made at 1 mM total lipid concentration in PBS buffer; turbidity was measured at 260 nm, versus PBS buffer as blank, over 48 h. Results were expressed in turbidity (OD) as a function of time.

Determination of Surface Potential (ψ°). Surface potential was determined by an indirect method described in literature that exploits the pH-sensitive fluorophore HC.[20] Briefly, 100 μL of HC containing extruded liposome was diluted to 2.5 mL with PBS buffer. Fluorescence measurements were performed by scanning the excitation wavelength between 300 and 400 nm, at an emission wavelength of 450 nm, varying the solution pH between 2 and 12 by addition of concentrated sodium hydroxide or hydrochloric acid. The dissociation degree of HC incorporated into liposomes was monitored by the ratio of the excitation fluorescence intensities at 380 nm and 330 nm (pH independent isosbestic point). pK_a of HC in the liposome bilayer and, consequently, the surface potential (ψ°) was obtained by the plot of F_{380}/F_{330} ratio as a function of pH.

Fluorescence Quenching Measurements. Steady state fluorescence quenching experiments on *m*-THPC entrapped in DMPC/**1** liposomes were carried out on a Shimadzu RF5001PC spectrofluorometer. Fluorescence experiments were carried out on solutions with optical densities lower than 0.05 to minimize inner filter effects. Fluorescence quenching experiments were performed at 300 K by adding small aliquots of 2 M sodium iodide (I^-) solution, prepared in presence of 0.1 M $Na_2S_2O_3$ to prevent oxidation of I^-,[21] to the liposome formulations, previously filtered on a gel column for eliminating the free *m*-THPC as described above and diluted to obtain [*m*-THPC] = 0.75 μM. The decrease of *m*-THPC fluorescence was monitored at 652 nm by exciting at 517 nm.

Results

Cell Uptake of *m*-THPC. The delivery efficiency of DMPC/**1** liposomes at 60/40 molar ratio was measured by the quantitative analysis of content of PS by flow cytometry. In particular, both human and murine glioblastoma cells were treated for 30 min, 1 h and 4 h with the different formulations ([*m*-THPC] = 1×10^{-7} M, [total lipids] = 2.5×10^{-5} M).

The flow cytometric analysis demonstrated a time- and formulation-dependent uptake of *m*-THPC. As previously

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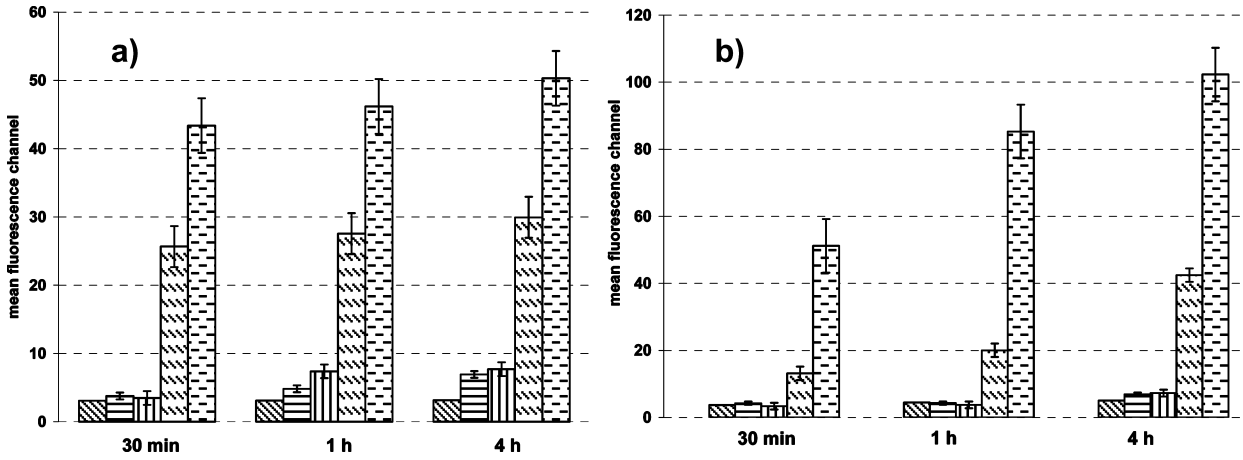

Figure 1. Time course analysis of the uptake of different liposome formulations was performed after 30 min, 1 h and 4 h with the following liposome formulations: (i) *m*-THPC in DMPC, (ii) *m*-THPC in DMPC/**1a** 60/40, (iii) *m*-THPC in DMPC/**1b** 60/40. *m*-THPC and total lipid concentrations were the same in each formulation ([*m*-THPC] = 5×10^{-7} M, [total lipids] = 1.25×10^{-5} M). After each treatment, cells were washed with ice-cold HBSS, detached with EDTA and trypsin, resuspended in ice-cold PBS and immediately analyzed for the PS content. Fluorescence signals were analyzed with a FACScan flow cytometer. Figure reports data from three experiments. Legend: for each time, from left to right, DMPC/**1a**, DMPC/**1b**, *m*-THPC/DMPC, *m*-THPC/DMPC/**1a**, *m*-THPC/DMPC/**1b**.

reported,[8] the incubation of all tested glioblastoma cell lines (LN229 and C6) with liposomes of neat DMPC slightly increased the value of mean fluorescence channel (MFC) indicating that *m*-THPC was scarcely delivered to the cells (Figures 1a and 1b). On the other hand, liposome formulations containing gemini **1** induced a strong increase of MFC value on both LN229 and C6 glioblastoma cultures. Actually, the formulation containing 40% of gemini **1a** was confirmed as very efficient, however, the *m*-THPC/DMPC/**1b** formulation were more efficient than *m*-THPC/DMPC/**1a** on both cell lines.

***m*-THPC Intracellular Distribution.** The qualitative analysis of the uptake and intracellular distribution of the PS was carried out by laser scanning confocal microscopy (LSCM), in both human and murine glioblastoma cells. Cultures were treated with the formulations tested in the quantitative analysis by flow cytometry, however, more concentrated liposome suspensions were used ([*m*-THPC] = 5×10^{-7} M, [total lipids] = 1.25×10^{-4} M) in order to improve the detection of fluorescent signal by the optical apparatus. The analysis was performed after 1 and 24 h of incubation.

As previously reported,[8] after both 1 and 24 h no fluorescent signal was detectable in both LN229 and C6 cell cultures treated with *m*-THPC/DMPC (data not shown). The low signal revealed in these samples by flow cytometry was not suitable for the LSCM imaging. In the cultures incubated for 1 h with *m*-THPC/DMPC/**1a**, the fluorescence of the PS was detectable at the membrane level of both LN229 (Figure 2a) and C6 (Figure 3a) cells. In addition, *m*-THPC was clustered and capped on the plasma membrane. A weak fluorescence was also observed in the cytoplasm. After 24 h of treatment with *m*-THPC/DMPC/**1a**, clustering was more evident on LN229 cells (Figure 2b), on the other hand cytoplasmic fluorescence was observable in C6 cells (Figure 3b). The treatment of cells with *m*-THPC/DMPC/**1b** gave significantly different results; in fact, after both 1 h and 24 h nuclear staining characterizes both LN229 (Figures 2c and 2d) and C6 cells (Figures 3c and 3d). In particular, after 1 h *m*-THPC appeared strongly clustered on specific regions of the plasma membrane of LN229 cells (Figure 2c). The fluorescence of nuclei, as well as fluorescent signal coming from the cell surface, increased after 24 h (Figure 2d). Also C6 cells displayed a significant amount of PS into the nuclei (Figures 3c and 3d). However, a minor amount of *m*-THPC was visible on the plasma membrane, when compared to LN229 cells. These differences in the uptake and intracellular transport could reflect a different composition of the plasma membrane between glioblastoma cell lines as well as the extent and the rate of receptor-mediated endocytosis.

Physicochemical Characterization of Mixed Liposomes. In Table 1 we summarize all the results relative to the physicochemical characterization of DMPC/**1** formulations at a 60/40 molar ratio. The T_m of the two investigated liposome formulations, measured by OD experiments, was very similar and also it was similar to the T_m of neat DMPC (T_m = 297.15 K). The two formulations also showed the same EE, i.e. ~65% of the preparation, whereas some relevant differences were found in their capacity of cargo retention monitored over 48 h. In fact, the DMPC/**1a** formulation lost ~15% of its cargo whereas the DMPC/**1b** formulation lost only 3%. The higher increase of OD at 260 nm showed by the DMPC/**1b** formulation with respect to DMPC/**1a** (20% versus 10%) demonstrated a minor stability of the formulation containing **1b**. Interestingly, the major retention was observed in correspondence of a minor stability of the liposomes.

Both formulations were characterized, as expected, by a positive value of surface potential, ψ°. Interestingly, the different configuration of one of the two stereogenic centers

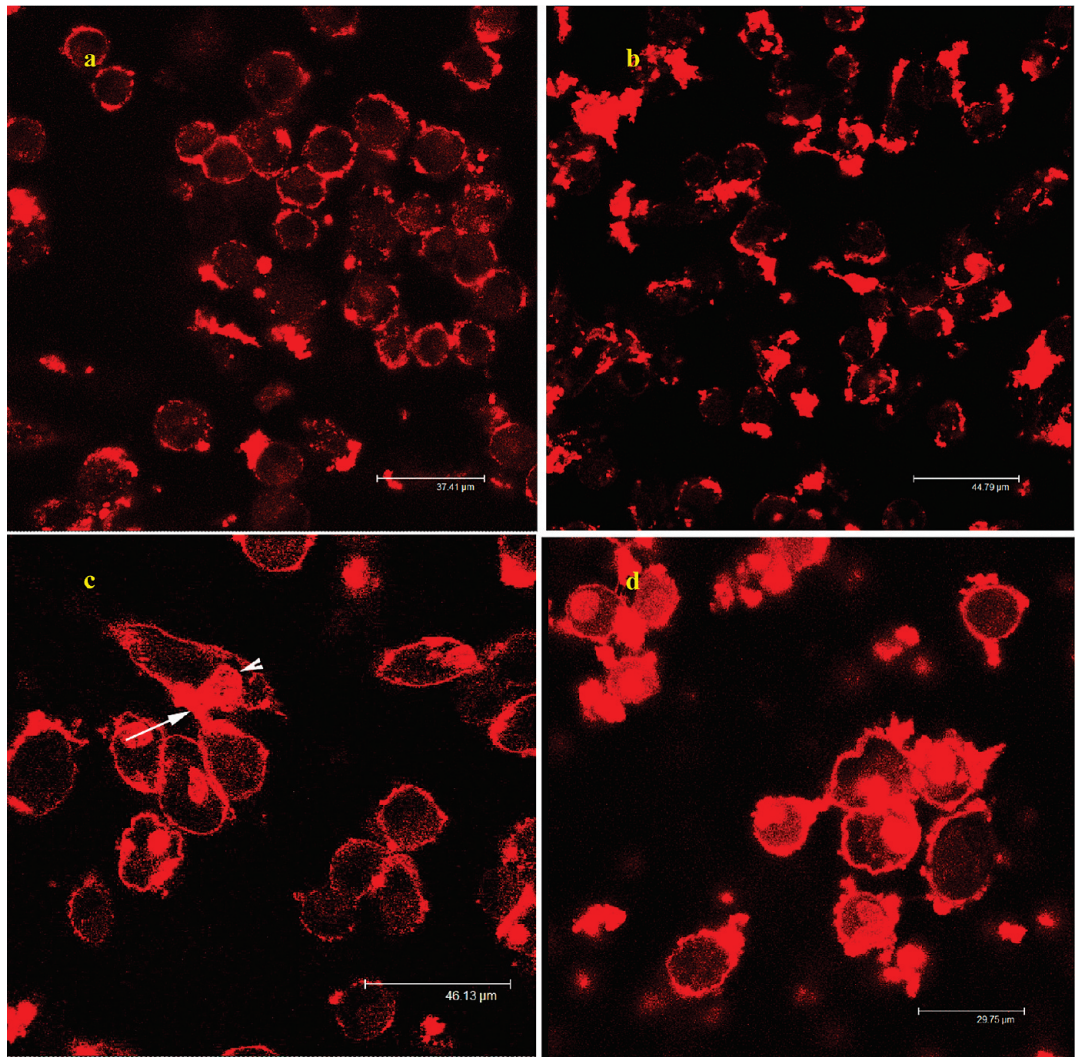

Figure 2. Analysis by LSCM of the intracellular distribution of *m*-THPC in LN229 human glioblastoma cells treated with *m*-THPC/DMPC/**1a** for 1 h (panel a) and 24 h (panel b), *m*-THPC/DMPC/**1b** for 1 h (panel c) and 24 h (panel d) ([*m*-THPC] = 5×10^{-7} M, [total lipids] = 1.25×10^{-4} M). The arrow indicates capping of *m*-THPC on plasma membrane whereas the arrowhead indicates the nucleus stained by *m*-THPC.

on the spacer of the gemini is responsible for a different exposure of the ammonium groups, thus determining a higher ψ° value for DMPC/**1a** compared to DMPC/**1b**.

Quenching of fluorescence with iodide (I^-) is one of the simplest ways to obtain information on the position of fluorophores within liposome membranes.[22] It should be pointed out that the fluorescence measurements were carried out above the T_m, this differently from the experiments on DMPC/**1a** liposomes reported previously.[7] In fact, below the T_m PS molecules may be located in more heterogeneous environments, thus making the interpretation of results more difficult.[23] For both formulations the fraction (f_a) of *m*-THPC readily accessible to I^-, and therefore localized in the interfacial region, was 0.5 of the loaded amount. However, the quenching constant, K_q, was different in the two formulations, being much higher in the case of DMPC/**1b** with respect to DMPC/**1a.**

Discussion

In a previous investigation we showed that the gemini surfactant **1a** made liposomes of DMPC efficient in the delivery of *m*-THPC to GBM cells, and that the DMPC/**1a** formulation was more efficient with respect to the pharmaceutical formulation of *m*-THPC, Foscan (a homogeneous solution formulation).[8b] This was an important result con-

(22) (a) Barenholz, Y.; Cohen, S.; Korenstein, R.; Ottolenghi, M. Organization and dynamic of pyrene and pyrene lipids in intact lipid bilayers. Photo-induced charge transfer processes. *Biophys. J.* **1991**, *60*, 110–124. (b) Lavi, A.; Weitman, H.; Holmes, R. T.; Smith, K. M.; Eherenberg, B. The depth of phorphyrin in a membrane and the membrane's physical properties affect the photosensitizing efficiency. *Biophys. J.* **2002**, *82*, 2101–2110.

(23) van Esch, J. H.; Feiters, M. C.; Peters, A. M.; Nolte, R. J. M. UV−Vis, Fluorescence, and EPR Studies of Phorphyrins in Bilayers of Dioctadecyldimethylammonium Surfactants. *J. Phys. Chem.* **1994**, *98*, 5541–5551.

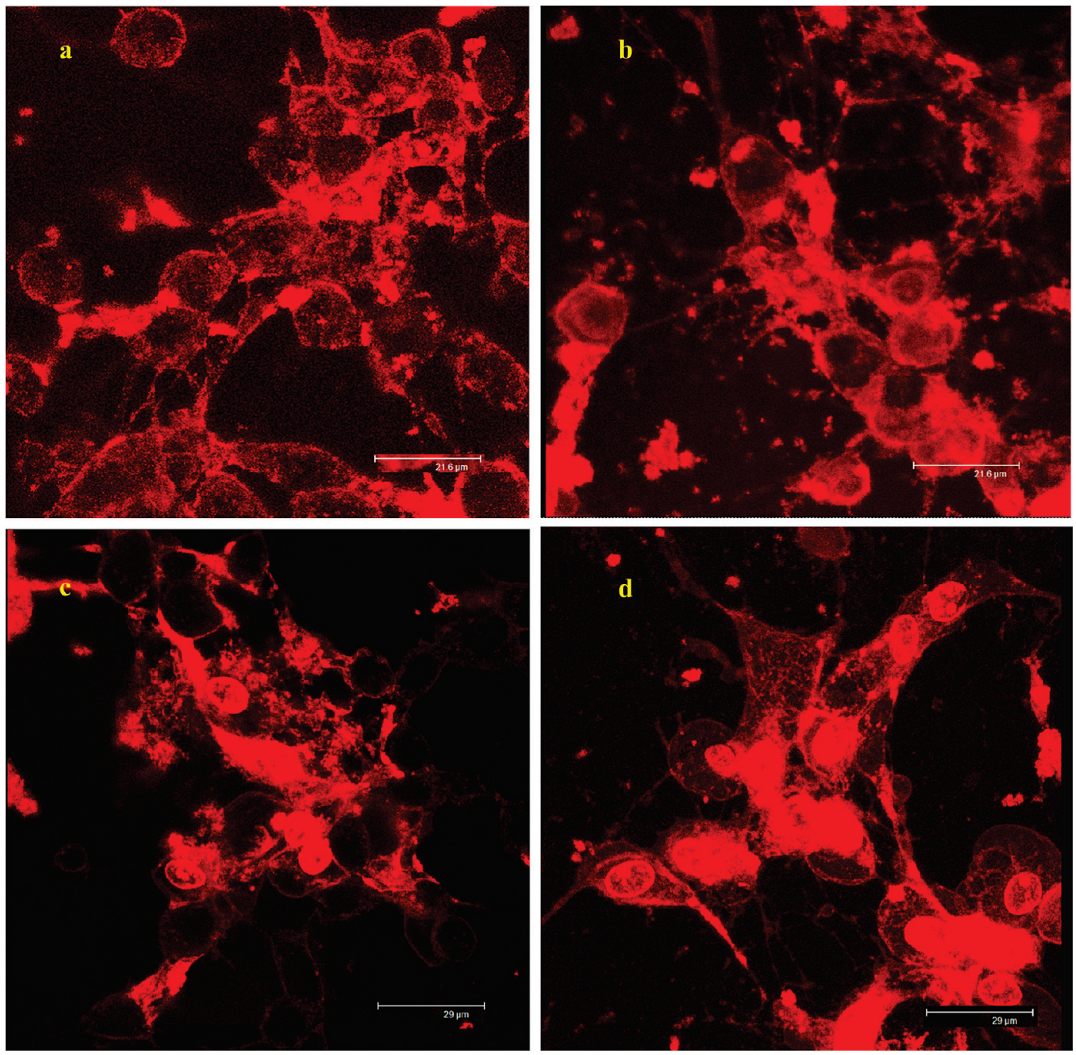

Figure 3. Analysis by LSCM of the intracellular distribution of *m*-THPC in C6 murine glioblastoma cells treated with *m*-THPC/DMPC/**1a** for 1 h (panel a) and 24 h (panel b), *m*-THPC/DMPC/**1b** for 1 h (panel c) and 24 h (panel d) ([*m*-THPC] = 5×10^{-7} M, [total lipids] = 1.25×10^{-4} M).

Table 1. Physicochemical Parameters of DMPC/**1** Liposomes at a 60/40 Molar Ratio

	DMPC/**1a**	DMPC/**1b**
T_m (K)	297 ± 0.5	298 ± 0.5
EE[a] (% *m*-THPC)	65 ± 5	66 ± 5
leakage (% of *m*-THPC lost after 48 h)	15 ± 2	3 ± 1
stability (% increase of OD_{260} after 48 h)	10 ± 3	20 ± 3
ψ° (mV)	124 ± 5	89 ± 5
f_a	0.53 ± 0.02	0.54 ± 0.01
K_q (M^{-1})	45 ± 8	180 ± 8

[a] Entrapment efficiency.

sidering that, in other investigations,[24] both the homogeneous formulation of *m*-THPC, Foscan, and the liposome formulation, Foslip, showed a similar efficiency of delivery with a similar intracellular distribution.

The main outcome of the biological evaluation reported here involved (i) the higher efficiency of *m*-THPC delivery of DMPC/**1b** liposomes with respect to DMPC/**1a** and (ii) the different mode of interaction with the cell membranes as a function of the stereochemistry of **1**. In fact, *m*-THPC delivered by DMPC/**1b** liposomes appeared strongly clustered on a region of the plasma membrane of LN229 cells in the proximity of the nucleus, and characteristic nuclear staining by *m*-THPC was observed both in human and in murine glioblastoma cells after the treatment with *m*-THPC/DMPC/**1b**. It could be argued that the nuclear staining might depend on the higher uptake of *m*-THPC in cells treated with *m*-THPC/DMPC/**1b**. However, LSCM images show a different intracellular distribution of *m*-THPC in cells treated with *m*-THPC/DMPC/**1a** and *m*-THPC/DMPC/**1b**, that suggest different pathways of internalization. The differences observed in the biological behavior of the two formulations

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should depend on some of the physicochemical parameters of liposomes. It is known, for example, that the size and the charge of liposomes control the interaction with cells and the pathway of internalization.[25] Other parameters could be of importance, such as the T_m and the localization of the cargo in the carrier. We investigated how, and to what extent, the molecular structure of the gemini component affects the physicochemical parameters that could control the biological behavior, for explaining the observed correlation between the stereochemistry of **1** and the biological behavior of the formulations.

We found that the stereochemistry of the spacer of **1** does not affect significantly either the double layer transition temperature or the *m*-THPC entrapment efficiency. It is worth noting that the favorable interaction of DMPC/**1** liposomes with cell membrane seemed to counterbalance the minor amount of PS entrapped in the liposomes as a consequence of the addition of **1** to DMPC liposomes.

A significant difference between the two formulations concerned the surface potential. In fact, the surface potential value of the DMPC/**1a** formulation is 30% higher than the surface potential of the DMPC/**1b** formulation. In a previous investigation on the aggregation features of surfactants **1**[16] we have shown that the different stereochemistry of the spacer influences the exposure of the charged ammonium groups, these being more exposed to water in surfactant **1a** with respect to **1b**. It is possible that also in the mixed liposomes, the ammonium groups of **1b** contribute to a minor extent to the bilayer surface potential. Because the reciprocal repulsion between liposomes decreases with the decrease of surface charge, the lower surface potential of liposomes formulated with **1b** can also account for their higher tendency to vesicle fusion, demonstrated by the higher increase of the percentage of OD value in 48 h (Table 1). Nevertheless, despite the higher tendency to vesicle fusion, DMPC/**1b** liposomes showed higher capability of retaining the entrapped chlorin compared to DMPC/**1a** liposomes, thus demonstrating that vesicle fusion did not affect leakage of the entrapped drug.

The results obtained in the fluorescence quenching experiments, that give information on the position of the loaded *m*-THPC in the lipid bilayer, have shown that the fraction (f_a) of PS accessible to the quencher is 0.5 in both cases. However, the stereochemistry of the gemini affects the quenching constant, K_q. In the DMPC/**1b** formulation K_q is four times higher than in the DMPC/**1a** one. The quenching constant measures the stability of the quencher−fluorophore complex, and it is related to the accessibility of the fluorophore to the quencher, in particular to the separation distance within the excited-state complex, affected by diffusion and steric shielding of the fluorophore. Therefore, despite the same values of f_a, *m*-THPC loaded in DMPC/**1b** liposomes is bound to a microenvironment more suitable for the interaction with the quencher compared to *m*-THPC loaded in DMPC/**1a** liposomes.

Thus, we found some relevant differences in the surface features of liposomes that might influence the interaction with the cell membrane and the internalization pathway either of liposomes or of PS. Unexpectedly, the formulation featuring a higher surface potential and a greater stability toward fusion (DMPC/**1a**), features that should favor the interaction with the cell membrane and the internalization process, was found the less efficient in the delivery of the PS. The mode of interaction with cells of the DMPC/**1b** formulation, characterized by clustering on a specific region of plasma membrane, suggests the mediation of specific rafts characterized by proper charge features. These specific rafts could be responsible for a defined internalization pathway of the carrier or for the release and internalization of the PS.

Conclusions

We have found that the stereochemistry of gemini surfactants **1** affects some of the physicochemical features of DMPC/**1** liposomes, the efficiency of delivery and the intracellular distribution of *m*-THPC. The correlation of physicochemical and biological features is not obvious. However the formulations containing the gemini with a different stereochemistry are characterized by different surface features that seem to control the interaction with cells, the mechanism of internalization and therefore the intracellular distribution of *m*-THPC. Investigations on the mechanism of internalization are under way.

Acknowledgment. Financial support from Dipartimento di Progettazione Molecolare of CNR and FUSINT project is gratefully acknowledged.

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