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New Cationic Liposomes as Vehicles of *m*-Tetrahydroxyphenylchlorin in Photodynamic Therapy of Infectious Diseases

Cecilia Bombelli,[†] Federico Bordi,[‡,§] Stefania Ferro,[||] Luisa Giansanti,[⊥] Giulio Jori,[||] Giovanna Mancini,[*,†] Claudia Mazzuca,[#] Donato Monti,[#] Fernanda Ricchelli,[||] Simona Sennato,[‡,§] and Mariano Venanzi[#]

CNR, Istituto di Metodologie Chimiche and Università degli Studi di Roma "La Sapienza", Piazzale A. Moro 5, 00185 Roma, Italy, Dipartimento di Fisica, Università degli Studi di Roma "La Sapienza", Piazzale A. Moro 5, 00185 Roma, Italy, INFM-CRS SOFT, Università di Roma "La Sapienza", Piazzale A. Moro 5, 00185 Roma, Italy, CNR Istituto di Tecnologie Biomediche, Sezione "Metalloproteine", Dipartimento di Biologia, Università di Padova, Viale G. Colombo 3, 35121 Padova, Italy, Dipartimento di Chimica, Università degli Studi di Roma "La Sapienza", Piazzale A. Moro 5, 00185 Roma, Italy, and Dipartimento di Scienze e Tecnologie Chimiche, Università degli Studi di Roma "Tor Vergata", Via della Ricerca Scientifica 1, 00133 Roma, Italy

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Abstract: Antimicrobial photodynamic therapy is emerging as a promising therapeutic modality for bacterial infections. For optimizing the antibacterial activity of the photosensitizer *m*-tetrahydroxyphenylchlorin, it has been encapsulated in mixed cationic liposomes composed of different ratios of dimyristoyl-*sn*-glycero-phosphatidylcholine and any of four cationic surfactants derived from L-prolinol. The delivery efficiency of the different liposomes formulations has been evaluated on a methicillin-resistant *Staphylococcus aureus* bacterial strain (MRSA), and one of the tested formulations shows a biological activity comparable to that of the free chlorin. In order to rationalize the physicochemical parameters of the carriers that control the biological activity, the new liposome formulations have been characterized by measuring (a) the zeta potential, (b) their capability of chlorin entrapping efficiency, i.e. entrapment efficacy, (c) the effect of storage on chlorin entrapment and (d) the localization of chlorin in the bilayer. The correlation of the physicochemical and biological features of formulations has allowed us to rationalize, to some extent, some of the parameters that may control the interactions with the biological environment.

Keywords: Methicillin-resistant bacteria; cationic liposomes; photodynamic therapy; bactericidal activity

Introduction

Photodynamic therapy (PDT) is a treatment for a variety of tumors and some other diseases.[1] This therapeutic modality involves the administration of a photosensitizer (PS), usually a porphyrin-based compound, which, upon photoactivation with visible light of appropriate wavelength, generates cytotoxic species, such as free radicals and singlet oxygen, that lead to irreversible destruction of the irradiated tissue.

* Author to whom correspondence should be addressed. Mailing address: CNR, Istituto di Metodologie Chimiche and Università degli Studi di Roma "La Sapienza", Piazzale A. Moro 5, 00185 Roma, Italy. E-mail: giovanna.mancini@uniroma1.it. Fax: +3906490421. Tel: +390649913078.

[†] CNR, Istituto di Metodologie Chimiche and Università degli Studi di Roma "La Sapienza".

[‡] Dipartimento di Fisica, Università degli Studi di Roma "La Sapienza".

[§] INFM-CRS SOFT, Università di Roma "La Sapienza".

[||] CNR Istituto di Tecnologie Biomediche, Sezione "Metalloproteine", Dipartimento di Biologia, Università di Padova.

[⊥] Dipartimento di Chimica, Università degli Studi di Roma "La Sapienza".

[#] Dipartimento di Scienze e Tecnologie Chimiche, Università degli Studi di Roma "Tor Vergata".

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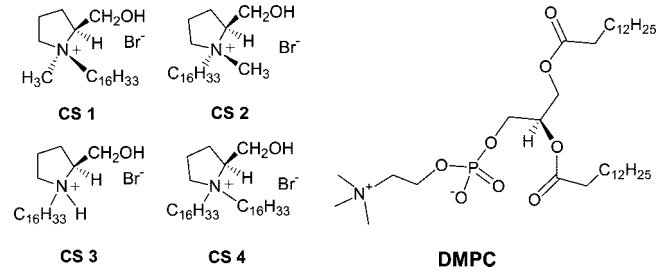
The multitarget nature of PDT, as well as the lack of involvement of the genetic material, makes it unlikely for the cell to develop resistance or repair mechanisms[2,3] and makes this protocol highly suitable as antibacterial therapy.

In fact, recent findings[3,4] suggest that PDT could have considerable potential for the treatment of localized infections of microbial origin. The use of cationic photosensitizers promotes their fast and selective association with the negatively charged moieties (e.g., teichoic acids, phospholipids), which are normally present in large amounts at the surface of the outer wall surrounding the bacterial or yeast cells. As a consequence, the survival of such cells can be notably reduced even by irradiation under mild experimental conditions. The use of PDT as a novel antimicrobial therapeutic modality is of special interest owing to the widespread emergence of antibiotic-resistant microbial strains, such as methicillin- or vancomycin-resistant *Staphylococcus aureus*, even in nosocomial environments, for which the presently available treatment options are often poorly effective.[5] At present, PSs under investigation at either a preclinical or clinical level are systemically administered after incorporation into lipophilic delivery systems, such as liposomes, oil emulsions or cyclodextrin inclusion complexes,[6] in order to minimize precipitation in the bloodstream or aggregation in a polar milieu, which produce a drop of PDT therapeutic efficiency.[7] Preliminary reports[8,9] indicate that the association of photosensitizing agents with liposomal vesicles can potentiate the cytocidal effects on bacteria and

Chart 1. Liposome Components



extend the type of effective photosensitizers to noncationic derivatives. Liposome physicochemical properties that are related both to the types and the amount of lipids used for their formulation and to the degree of drug encapsulation in the lipid bilayer affect their behavior in the biological milieu. The possibility of correlating the molecular structure of the components to the physicochemical features of the carriers and to their biological behavior is essential for a rational and systematic approach to the design of new drug delivery systems (DDS) engineered to promote a fast and specific interaction with bacterial cells.

Here we report the investigation on liposomes formulated with different percentages of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) and any of the cationic surfactants (CS) reported in Chart 1: (1*S*,2*S*)-*N*-hexadecyl-*N*-methylprolinolinium bromide, **1**, (1*R*,2*S*)-*N*-hexadecyl-*N*-methylprolinolinium bromide, **2**, *N*-hexadecyl-L-prolinolinium hydrobromide, **3**, *N*,*N*-dihexadecyl-L-prolinolinium bromide, **4**, in the presence and in the absence of *m*-tetrahydroxyphenylchlorin (*m*-THPC), a second generation PS. This study aims at correlating the physicochemical properties and the delivering efficacy of the formulations to their composition and to the degree of drug inclusion.

Liposomes formulated with cationic surfactants based on the pyrrolidinium skeleton with substitution on positions 3 and 4 were reported as more efficient carriers of DNA in gene therapy with respect to their corresponding open-head analogues.[10] This study is aimed at investigating if efficiency in delivery is a feature of amphiphiles featuring a pyrrolidinium structure. As a photosensitizer, we used *m*-tetrahydroxyphenylchlorin (*m*-THPC), which is electrically neutral at physiological pH values and is known to be a very active antitumoral PDT agent.[11]

Diasteromeric compounds **1** and **2** were previously prepared and fully characterized,[12] and it was shown that their different stereochemistry affects their aggregation behavior,

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so we decided to investigate how and to what extent the opposite nitrogen configuration might affect liposome properties. The stereochemistry of liposome components may play an important role in the interaction with the other chiral components in mixed liposomes, with the chiral components of cell membrane or in the entrapment of a chiral active principle thus influencing the physicochemical features of the aggregates. CS **3** and **4** have been chosen to investigate the effect of modifications in the hydrophobic portion of the surfactants on the liposome features. Moreover the protonation on nitrogen of CS **3** depends on pH conditions and it is well-known that pH-sensitive liposomes easily fuse with biomembranes releasing their content in the cytoplasm following endocytosis.[13]

Because CS **1**, **2** and **3** form micelles, we prepared mixed CS/phospholipid liposomes using DMPC, a natural saturated phospholipid stable to oxidative degradation commonly used in liposome preparation; its transition temperature (T_m) is 24.1 °C, and the T_m of mixed systems is not much higher, thus allowing to carry out all experiments at a relatively low temperature. Moreover, the use of a neutral phospholipid reduces cationic liposomes toxicity and adverse effects.

The various formulations at different DMPC/CS (8/2, 7/3, 6/4, 5/5) ratios were investigated to measure (a) the zeta potential by Doppler velocimetry measurements, (b) the percentage of entrapped *m*-THPC, i.e. entrapment efficacy (EE) by UV spectroscopy, (c) the effect of storage on liposome EE by fluorescence measurements using the lipophilic, pH sensitive fluorophore 4-heptadecyl-7-hydroxycoumarin (HC) and (d) the localization of *m*-THPC in the bilayer by fluorescence quenching experiments with collisional quenchers, namely iodide (I^-) and methyl viologen (MV^{2+}). All the measurements were performed on small unilamellar vesicles. The efficiency of photoinduced bacterial killing of a well-known antibiotic-resistant Gram(+) bacterium, namely methicillin-resistant *Staphylococcus aureus* (MRSA), by using DMPC/CS liposomes loaded with *m*-THPC was also investigated.

Materials and Methods

Liposome Preparation. A film of lipid was prepared on the inside wall of a round-bottom flask by evaporation of $CHCl_3$ solutions containing the proper amount of DMPC and CS **1**−**4** to obtain a defined molar percentage mixture. The obtained films were stored overnight under reduced pressure (0.4 mbar), 2.5 mL of PBS buffer solution (Aldrich, 0.15 M, pH 7.4) was added to the lipid film in order to obtain a 12.5 mM lipid dispersion, and 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 (Whatman Nucleopore). The extrusions were carried out well above the transition temperature of mixed liposomes, using a 2.5 mL extruder (Lipex Biomembranes, Vancouver, CA).

Table 1. Zeta Potential (mV) in DMPC/CS Liposome Formulations

	DMPC/**1**	DMPC/**2**	DMPC/**3**	DMPC/**4**
8/2	41 ± 1	44 ± 4	35 ± 1	60 ± 3
7/3	48 ± 2	40 ± 3	43 ± 2	61 ± 2
6/4	63 ± 4	57 ± 4	49 ± 1	73 ± 3
5/5	86 ± 3	60 ± 5	53 ± 2	77 ± 2

Table 2. Entrapment Percentage of *m*-THPC in DMPC/CS Liposome Formulations[a]

	DMPC/**1**	DMPC/**2**	DMPC/**3**	DMPC/**4**
8/2	92	89	85	82
7/3	77	69	63	54
6/4	60	46	46	42
5/5	32	33	39	19

[a] In all liposome suspensions the ratio between initial concentration *m*-THPC/total lipids is 0.004. Error in determination is 5%.

m-THPC containing liposomes were prepared by adding to the lipid chloroform solution the proper volume of a *m*-THPC stock solution (5×10^{-4} M, EtOH abs) to obtain, after hydration, a final concentration of 50 μmol of *m*-THPC.

Zeta Potential Measurements. Mixed DMPC/CS cationic liposome dispersions used in these experiments were 1.25 mM in 15 mM PBS buffer. The measurements of the electrophoretic mobility to determine zeta potential were carried out by means of the laser Doppler electrophoresis technique using a MALVERN Zetasizer apparatus equipped with a 5 mW HeNe laser. Analysis of the Doppler shift in the Zetasizer Nano series was done by using phase analysis light scattering (PALS) implemented with M3 (mixed mode measurement). Low applied voltages were used to avoid the risk of effects due to Joule heating.

Evaluation of EE. Gel filtration by size-exclusion chromatography (15 million Da) on Bio-Rad AM-15 gel column equilibrated in PBS buffer solution of extruded DMPC/CS liposomes containing *m*-THPC allowed us to separate the nonentrapped chlorin (i.e., chlorin in the buffer) from that entrapped in the lipid bilayer according to a reported procedure.[14] The amount of chlorin in the solution before and after filtration was measured by absorbance spectroscopy on a Cary 300 UV−vis double beam spectrophotometer (Varian PTY Ltd., Mulgrave, AU). The determination of the percentage of entrapped chlorin requires as an internal check the measurement of the total lipid amount before and after gel filtration. The percentage of entrapped drug was thus calculated by eq 1:

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

where $M^{a}_{m\text{-THPC}}$ and $M^{b}_{m\text{-THPC}}$ are the chlorin concentration in liposomes, respectively, after and before gel filtration and

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Table 3. Effect of Storage on the Entrapment Efficiency (Given in Percentage) of *m*-THPC in DMPC/CS Liposome Formulations

		DMPC/**1**				DMPC/**2**				DMPC/**3**				DMPC/**4**			
t (days)	DMPC	8/2	7/3	6/4	5/5	8/2	7/3	6/4	5/5	8/2	7/3	6/4	5/5	8/2	7/3	6/4	5/5
1	91	97	95	92	97	91	91	94	90	85	84	88	83	95	91	93	93
2	86	85	76	85	72	80	85	79	78	72	79	75	76	84	68	79	87
5	85	76	70	76	60	73	79	70	69	62	75	68	70	77	58	71	76
8	85	65	60	70	43	62	57	63	61	70	66	57	56	71	73	78	81

M^{a}_{lip} and M^{b}_{lip} are the total lipid concentration after and before gel filtration.

Effect of Storage on EE. HC-containing liposomes were prepared by 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 final concentration of 50 μmol of HC. In all samples, the molar ratio lipid/HC was 250 to 1. Preparation of HC-containing liposomes, as well as all the experiments dealing with HC, were performed in the dark to avoid photodegradation of the probe. After the extrusion, 100 μL of cationic liposome dispersion was diluted in 2.5 mL of PBS buffer. The fluorescence measurements were performed at ambient temperature on a Shimadzu RF5001PC spectrofluorimeter. Fluorescence of HC was measured by scanning at the excitation wavelength between 300 and 400 nm using an emission wavelength of 450 nm (bandwidths 5 nm). The obtained fluorescence data were referred to a solution of 10^{-4} M HC in ethanol.

Fluorescence Quenching Measurements. Fluorescent quenching experiments on *m*-THPC entrapped in DMPC and in DMPC/CS **1**−**4** were carried out on a Shimadzu RF5001PC spectrofluorimeter. All 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 288 K by adding small aliquots of 2 M sodium iodide (I^-) or 1 M methyl viologen dichloride (MV^{2+}) solutions 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.

MRSA Photoinactivation Experiments. The liposomal formulations obtained were dialyzed for 3 h against 10 mM PBS, pH 7.4. The concentration of the PS in the final liposomal suspension (7 mM in phospholipids) was l0 μM as determined by spectrophotometric analysis.

The methicillin-resistant strain *S. aureus* MRSA 110 was grown aerobically at 310 K in brain heart agar (BHA, Difco, Detroit, MI). The cells in the stationary phase of growth were harvested by centrifugation of broth culture (200 g for 10 min), washed twice with PBS and diluted in the same buffer to an absorbance of 0.7 at 650 nm, corresponding to 10^8-10^9 bacterial cells/mL.

Samples containing 10^8 MRSA cells/mL prepared by dilution from a previously washed bacterial suspension were incubated in the dark for 30 min or 2 h with 10 μM *m*-THPC. All the irradiation studies were performed by using white light emitted from a Teclas fluorescent lamp (Lugano, SWTZ), equipped with an ultraviolet and an infrared reflecting filter. The light beam was driven to the target by a bundle of optical fibers (external diameter = 8 mm). The rate of *m*-THPC photobleaching was followed spectrophotometrically by measuring the variations of the absorption spectrum in the 350−700 nm wavelength interval upon exposure of the chlorin in both homogeneous solutions and aqueous dispersions of liposomal vesicles to the Teclas lamp which was operated at a fluence rate of 100 mW/cm^2. The solutions/dispersions were placed in a quartz cuvette of 1 cm optical path and were gently stirred during irradiation. At the end, unirradiated and irradiated bacterial cells were serially 10-fold diluted with PBS and the number of colonies found after 24 h incubation at 37 °C was counted.

Cytotoxicity experiments with identical amounts of mixed liposome formulations were performed in order to study the cytotoxicity of the delivery system in the absence and in the presence of *m*-THPC.

Results

Zeta Potential Measurements. Measurements of zeta potential were carried out on samples in more diluted conditions with respect to those used in the other investigations reported here (1.25 mM in total lipid concentration and 15 mM in PBS buffer). In fact, the application of high voltage to more concentrated samples causes heating and decomposition of samples because of their high conductivity.

Zeta potential values obtained from electrophoretic mobility measurements for all DMPC/CS liposome formulations investigated are reported in Table 1.

All cationic liposome formulations investigated have a large positive zeta potential. In general, for DMPC/CS liposome formulations there is an increase of zeta potential with increasing percentages of CS in the formulation and the increase depends on the CS nature. The largest variation of zeta potential value is observed in DMPC/**1** liposomes between 20% and 50% of **1**.

DMPC/**1** and DMPC/**2** for molar percentages of CS ≤ 40 have comparable zeta potential values, while, though the difference in their molecular structure consists only in the configuration of nitrogen, the values observed at 5/5 ratio show a relevant difference.

DMPC/**3** liposomes show the lowest zeta potential values, whereas DMPC/**4** liposome preparations feature higher values of zeta potential at low percentages of **4** (20−30%), but the increase of zeta potential value at higher percentages of **4** is less relevant than in the case of **1** and **2**.

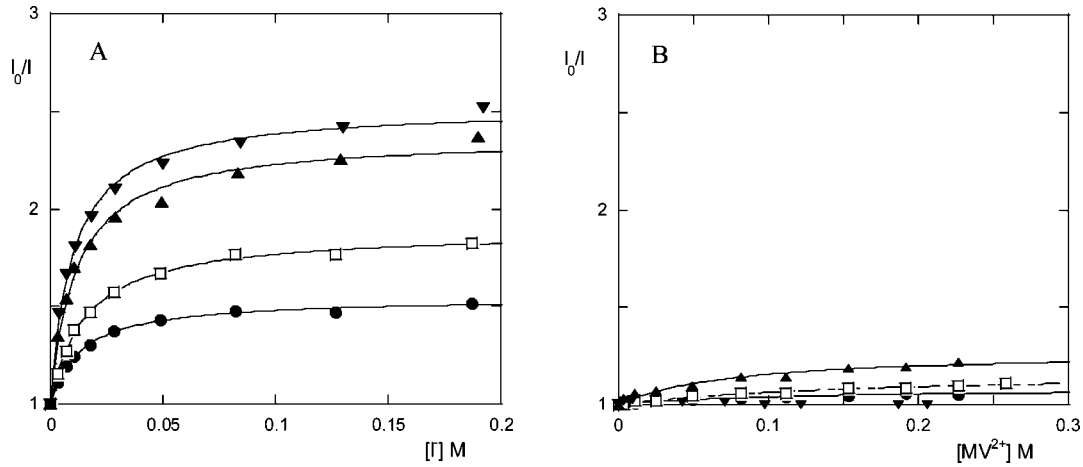

Figure 1. Fluorescence quenching experiments by (A) I^- and (B) MV^{2+} of *m*-THPC in mixed liposome formulations composed of DMPC/**1**: (●) DMPC/CS 8/2; (□) DMPC/CS 7/3; (▲) DMPC/CS 6/4; (▼) DMPC/CS 5/5.

The high values of zeta potential observed for all DMPC/CS liposomes investigated indicate relatively stable liposome suspensions.

Evaluation of EE. The percentage of entrapped chlorin in mixed DMPC/CS liposomes was determined by the ratio between the amount of *m*-THPC entrapped in the lipid bilayer and that of the whole chlorin contained in the solution (i.e., chlorin in the solution after and before gel filtration). In Table 2 we report the results of drug EE experiments in the DMPC/CS liposomes studied. For all the investigated liposome formulations we observed a decrease of entrapped chlorin with respect to the amount of chlorin entrapped in liposomes formulated only with DMPC (95%). In the mixed liposome formulation the percentage of entrapped chlorin decreases by increasing the percentage of CS.

In general, liposome formulations **1**, **2** and **3** show comparable EEs, whereas liposomes formulated with **4** show the lowest capability of *m*-THPC retention. This difference could be ascribed to the fact that **4** molecular structure is characterized by a larger hydrophobic portion with respect to DMPC and to the other CSs thus causing a less tight packing of the lipid bilayer and consequently an increased permeability.

Effect of Storage on EE. The effect of liposome storage on their capability of drug retaining was evaluated indirectly following the decrease in fluorescence intensity at 330 nm of a fluorescent probe, HC, entrapped in lipid bilayer. Fluorescent intensity at the wavelength of 330 nm is relative only to the actual level of the probe associated with the lipid bilayer; in fact, HC is nonfluorescent in an aqueous medium, where its aggregation quenches its fluorescence, so only HC associated with lipid assembly was observed.[15] This indirect method was used because it is known that HC fluorescence is unaffected by the temperature and by the physical state of lipids.[16] Results relative to the fluorescence measurements carried out for the evaluation of EE upon storage are reported in Table 3.

The decrease in fluorescence intensity of the sample referred to a standard is the evidence of leakage of the fluorophore from the liposomes. All liposomes formulated with CS show a higher leakage of the fluorophore with respect to liposomes formulated only with DMPC. The amount of HC initially entrapped in the lipid bilayer decreases of 20 and 30% within 5 days of storage for all the formulations studied (Table 3). After about one week liposomes formulated with either **1** or **2** lose a further amount of fluorescent probe whereas some of those formulated with **3** or **4** seem to bind part of the released probe. This finding is probably due to a phase transition of the aggregates, the new ones being more capable of binding the probe.

In general, the presence of the CS in the DMPC formulation decreases their capability of solute retention although liposomes formulated with different CS show a different behavior.

Fluorescence Quenching Measurements. The site of binding of *m*-THPC in the lipid bilayer of the mixed formulations was studied by their accessibility to the quenchers, i.e. by measuring the quenching of the chlorin fluorescence emission by both I^- and MV^{2+}. Both quenchers do not penetrate in depth into the bilayer, but their opposite charge allows the investigation of different regions of the bilayer.

In the experiments carried out with I^- and MV^{2+}, respectively, all investigated formulations gave similar results, i.e. smaller quenching constants and lower fractions of fluorescent molecules accessible to MV^{2+} with respect to I^-. A comparison of the results obtained with I^- and MV^{2+} is shown in Figure 1 where we reported as an example

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Table 4. Fraction *f* of *m*-THPC Readily Accessible to the Quencher (I^-) and Quenching Constant, *K*, Obtained by Fluorescence Quenching Experiments of *m*-THPC Included in DMPC/CS Liposome Formulations[a]

	DMPC/**1**		DMPC/**2**		DMPC/**3**		DMPC/**4**	
DMPC/CS	*f*	*K*, M^{-1}	*f*	*K*, M^{-1}	*f*	*K*, M^{-1}	*f*	*K*, M^{-1}
8/2	0.35	116 ± 5	0.48	33 ± 5	0.49	52 ± 6	0.47	51 ± 10
7/3	0.47	122 ± 6	0.57	219 ± 35	0.43	67 ± 6	0.45	110 ± 12
6/4	0.58	206 ± 17	0.60	226 ± 16	0.51	138 ± 5	0.37	99 ± 14
5/5	0.60	253 ± 17	0.51	116 ± 8	0.57	110 ± 11	0.46	146 ± 21

[a] Error in determination of *f* is 5×10^{-3}.

experimental results relative to the quenching of *m*-THPC included in DMPC/**1** liposomes. All other results are reported as Supporting Information.

In a previous paper[17] we reported on the fluorescence quenching experiments of *m*-THPC included in DMPC liposomes. A linear dependence on the I^- concentration was found; this indicates that the chlorin molecule can be approached by the quencher at very short distances, suggesting a location of the fluorescent probe proximal to the liposome surface. On the other hand, as found for other formulations of DMPC with cationic surfactants,[17] the inclusion of one of the CSs in the DMPC liposomes induces a different mode of binding of the chlorin with respect to DMPC liposomes. The quenching of *m*-THPC in DMPC/CS liposomes reaches constant values after the addition of a certain amount of I^-. These results can be described in terms of two different populations of the fluorophore: the first corresponds to the fraction (*f*) of molecules readily accessible to the quencher molecule, showing a characteristic quenching constant *K*, and the second to the fraction $(1 - f)$ that cannot be approached by the quencher, i.e.:

$$I_0/I = (1 - f) + f/(1 + K[Q]) \qquad (2)$$

The quenching constant *K* measures the stability of the quencher−probe complex; 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.

The results obtained by applying eq 2 to the steady-state fluorescence quenching experiments are reported in Table 4 for I^- titration.

In general, in liposomes formulated with CS **1**, **2** and **3** the population of *m*-THPC accessible to I^- increases at increasing amounts of CS, whereas small differences in fluorescence quenching values are observed for all samples formulated with **4** (Table 4).

Interestingly liposomes formulated with **2** include *m*-THPC differently with respect to those formulated with **1**. In fact, in liposomes formulated with low percentages of **2** (8/2, 7/3 formulations) the fraction of *m*-THPC accessible to I^- is larger with respect to the corresponding samples formulated with **1**. On the other hand, in samples formulated with 50% of **2** *m*-THPC is less accessible with respect to the corresponding sample formulated with **1**.

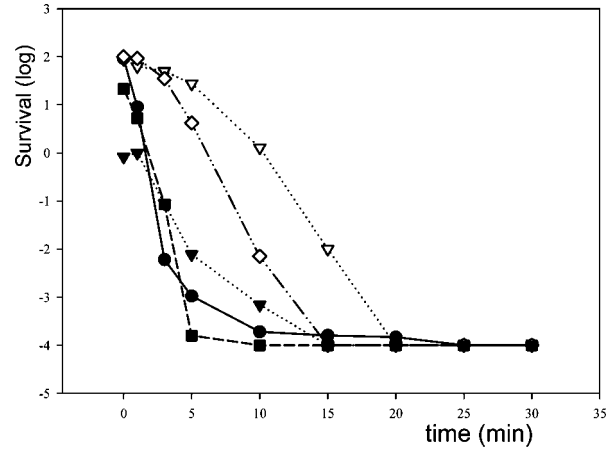

Figure 2. Survival of MRSA cells irradiated for different periods of time with white light (100 mW/cm^2) after 30 min incubation with 10 μM *m*-THPC bound DMPC/1 liposomes. The incubation of MRSA with free PS was performed for 30 min: (●) free *m*-THPC; (▽) DMPC/**1** 8/2; (◇) DMPC/**1** 7.5/2.5; (■) DMPC/**1** 7/3; (▼) DMPC/**2** 7/3.

In summary, the fluorescence quenching experiments reported above show that part of the *m*-THPC included in DMPC/CS liposomes is not accessible to surface collisional quenchers and is therefore embedded in the hydrophobic region of the liposomes. In most cases the accessible fraction increases at increasing amounts of CS, with small but significant differences in correspondence of different CSs. The use of two differently charged collisional quenchers allows us to suggest some hypotheses on the location of *m*-THPC in the lipid bilayer. On one hand, the chlorin may be in a location, driven by cation$-\pi$ interactions, close to the cationic headgroups and hence more accessible to the anionic quencher; on the other hand, the chlorine may be in a deep location, more accessible to I^-, a quencher more specific for the lipid bilayer with respect to MV^{2+}.[18]

MRSA Photoinactivation Experiments. The incubation of the bacterial cells with DMPC/CS liposomes formulated with **1** or **2** at a molar percentage ≤ 30 and with either **3** or

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Table 5. Biological and Physicochemical Features of the Formulations Evaluated in Vitro

	DMPC/**1**			DMPC/**2**		DMPC/**3**		DMPC/**4**
	8/2	7/3	6/4	7/3	6/4	6/4	5/5	6/4
biological activity	low	high	toxic	medium	toxic	absent	absent	absent
zeta potential (mV)	41	48	63	40	57	49	53	73
EE (%)	92	77	60	69	46	46	39	42
f m-THPC accessibile (I^-)	0.35	0.47	0.58	0.57	0.60	0.51	0.57	0.37

4 at a molar percentage ≤40, all devoid of *m*-THPC, had no detectable effect on survival. Analogously, no cytotoxicity was induced by visible light irradiation of MRSA in the presence of the above-mentioned empty liposome formulations. On the other hand, DMPC/**1** and DMPC/**2** at a molar percentage ≥40 showed high cytotoxic effect also in the dark. It is noteworthy that, at corresponding percentages, liposomes formulated with **2** are more toxic than liposomes formulated with **1**, though the two CSs differ only for the stereochemistry of the pyrrolidinic nitrogen.

Experimental results on inactivation of MRSA by *m*-THPC delivered by DMPC/**1** liposome formulations are reported in Figure 2 where they are compared with the inactivation yielded by free *m*-THPC (black circles). *m*-THPC delivered in liposomes formulated with DMPC/**1** at the 8/2 ratio (white down triangles) showed a good bactericidal activity after 20 min of irradiation, however this formulation is less efficient with respect to free *m*-THPC (black circles), which shows an analogous effect after 10 min of irradiation. *m*-THPC delivered in liposomes formulated with DMPC/**1** at 7/3 ratio (black squares) killed almost all bacterial cells after 5 min of irradiation, showing a bactericidal activity comparable to that of free chlorin. As expected, *m*-THPC delivered in liposomes formulated with DMPC/**1** at 7.5/2.5 ratio (white diamonds) had an intermediate activity between the two other formulations investigated.

m-THPC delivered in liposomes formulated with DMPC/**2** at 7/3 ratio (black down triangles) killed almost all bacterial cells after 15 min of irradiation, thus resulting less efficient with respect to free *m*-THPC and *m*-THPC delivered in the corresponding DMPC/**1** formulation (black squares) at the same ratio.

m-THPC delivered by DMPC/CS liposomes formulated with either **3** or **4** at a 6/4 ratio showed no appreciable bactericidal activity after 2 h of incubation and 30 min of irradiation.

Therefore the highest bactericidal activity was shown by the DMPC/**1** formulation at a 7/3 ratio.

These findings demonstrate that the composition of the formulation and the molecular structure of the components can significantly affect their physicochemical features and the efficiency of the photoinactivation process.

Discussion

Liposome behavior in the biological milieu is based on chemical interactions and the design of specific and efficient liposomes as DDS can be achieved by modulating their chemical composition and structure. It is known that cationic liposomes interact better than other liposomes with the negatively charged cell membranes.[19] It is clear and it has been shown[20] that subtle modifications of the composition may have dramatic effects on the biochemical interactions of these systems in vivo. On these premises our investigation on mixed cationic liposomes has given some indications on how and to what extent the chemical structure of the CS influences the physicochemical features of the lipid vesicles, and hence the interaction with the biological environment.

The comparison of the physicochemical and biological features of the formulations evaluated in vitro, reported in Table 5, can be of help to rationalize the physicochemical parameters that control the biological behavior.

In formulations containing **1** or **2**, in correspondence of high biological activity (high and toxic) we have a value of zeta potential in the range 48−63 (the highest value in both series, 63 mV and 57 mV, respectively, correspond to toxicity, i.e. in the dark in the absence of PS). Lower values of zeta potential correspond to a low or medium biological activity. Indeed zeta potential is a crucial parameter for the interaction with cell membranes; the correlation with biological activity suggests that a value of ∼40 mV is necessary for the interaction with cell membranes whereas higher values induce toxicity; however, Table 5 shows that formulations containing **3** or **4** feature high values of zeta potential in correspondence to the absence of biological activity. Actually, the different molecular structure and the relative specific interactions of components control the organization of the lipid bilayer and other parameters responsible for the biological behavior. Actually, liposomes formulated with CS **4** feature a transition temperature higher than the other formulations, ∼40 °C versus ∼27 °C (unpublished results); this means that in the experimental conditions formulations containing **1**, **2** or **3** are in a melted liquid crystalline phase whereas formulations containing **4** are in the rigid gel phase. Therefore the absence of biological activity in liposomes formulated with **4** could be ascribed to the state of the phase.

In liposomes formulated with **3** we observed T_m and zeta potential values that, according to what is discussed above,

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should be proper for observing biological activity; however the presence of **3** in the lipid bilayer induces a lower drug entrapment (EE 39−46%) with respect to the other formulations (EE 69−77%). Therefore the absence of biological activity in **3** containing formulations might be ascribed to a modest drug encapsulation. The comparison of the data do not allow one to evaluate properly the influence of the exposure of PS on the liposome surface.

Conclusions

The antimicrobial activity of the *m*-THPC encapsulated in liposomes formulated with DMPC and any of the CSs **1**−**4** was investigated in order to rationalize the physicochemical parameters of the carriers responsible for the biological activity. The comparison of the physicochemical and biological behavior of the different liposome formulations pointed out that (a) a zeta potential value of ∼40 mV is a necessary condition for the interaction with cell membranes; lower values correspond to low activity whereas higher values correspond to toxicity; (b) the melted liquid crystalline state of the lipid double layer is another crucial parameter; (c) high entrapment efficiency also is crucial for carriers featuring other necessary physical parameters.

Further, the comparison of the physicochemical and biological behavior of formulations containing the stereoisomers **1** or **2** demonstrates that also subtle variations of the molecular structure control the organization of components in lipid bilayer, and affect the physicochemical and biological behavior of the formulation. In fact, liposomes formulated with DMPC and 30% of **1** have a zeta potential higher than those formulated with **2** at the same molar percentage and, likewise, liposomes formulated with **1** display a higher biological activity than those formulated with **2**. These findings validate the approach of correlating the molecular structure to the physicochemical and biological behavior of the aggregates.

The finding that one of the formulations we have developed features the same bactericidal activity as free *m*-THPC enlarges the prospectives of exploitation of *m*-THPC and other PSs in PDT through the development of target specificity in these formulations.

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Supporting Information Available: Graphics of fluorescence quenching experiments relative to DMPC/**2**−**4** formulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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