Liposome Destabilization by a 2,7-Diazapyrenium Derivative Through Formation of Transient Pores in the Lipid Bilayer

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The effect of the luminescent heteroaromatic electron acceptor N,N′-dimethyl-2,7-diazapyrenium dichloride (DM-DAP^{2+}) on the stability of 1-palmitoyl-2-oleoylphosphatydilcholine (POPC) liposomes is determined on the basis of the rate of release of different fluorescent probes entrapped within the liposome. The experiments show that DM-DAP^{2+} exerts a substantial destabilizing action on the liposomal bilayer, particularly at low concentrations. Molecular dynamics simulations suggest that the activity of DM-DAP^{2+} is related to its tendency to surround itself with water molecules, conceivably favoring the formation of transient pores across the bilayer.

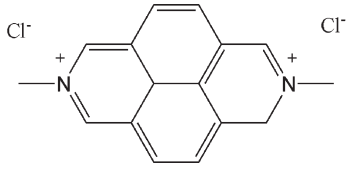
Keywords:
- fluorescent probes
- lipid bilayers
- liposomes
- molecular dynamics simulations

1. Introduction

Diazapyrenium compounds represent a potentially numerous class of heteroaromatic molecules characterized by a flat, hydrophobic, pyrene-type backbone containing two quaternized nitrogen atoms that render them dicationic. The 2,7-diazapyrenium (DAP) motif is structurally and functionally similar to the well-known and widely used[1] 4,4′-bipyridinium; however, DAP compounds have been much less investigated, most likely because of their difficult preparation.

DAP derivatives have been found to exhibit strong electron-accepting character and, in contrast with 4,4′-bipyridinium, show intense fluorescence and phosphorescence emissions.[2] The electron-accepting character of the DAP unit has been exploited in supramolecular chemistry to obtain self-assembling systems and interlocked compounds with characteristic spectroscopic and electrochemical properties.[3,4] Owing to their luminescence properties, diazapyrenium derivatives have been investigated as fluorescence probes for the detection of neurotransmitters.[5] Recently, the supramolecular charge-transfer complex between *N*,*N*′-dimethyl-2,7-diazapyrenium chloride (DM-DAP^{2+}, Scheme 1) and catechol (or dopamine) has been proposed for sensitive fluorescence detection of catecholamine neurotransmitters in solution, at macroscopic surfaces or on nanoparticles.[6]

The first studies on DAP cations were, however, directed to investigate the insertion of such molecules into the double helix of DNA for photocleavage purposes.[7,8] A series of



Scheme 1. *N*,*N*′-dimethyl-2,7-diazapyrenium chloride (DM-DAP^{2+}).

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DAP-tagged oligodeoxynucleotides with selective DNA-cleavage properties has also been synthesized.[9]

The dicationic nature of DM-DAP^{2+} and its lipophilic backbone let us speculate on a possible resemblance to positively charged antimicrobial peptides, which have recently been extensively studied for their tendency to induce membrane permeation and their critical affinity for the microbial membrane.[10,11] Therefore, we decided to investigate whether DM-DAP^{2+} exhibits a destabilizing activity towards 1-palmitoyl-2-oleoylphosphatydilcholine (POPC) liposomes, which are widely used as a model for cell membranes.

Over the last decade, several studies have been performed on the capability of some molecules — in most cases nonionic surfactants — to induce vesicle destabilization by taking the system from a vesicle-stable to a micelle-stable region.[12–15] The aim of the present investigation is to determine if and how DM-DAP^{2+} affects the stability of POPC liposomes, and to shed light on the mechanism associated with such effects. In fact, DM-DAP^{2+} is highly interesting in this context because it conjugates hydrophobic and hydrophilic features although it is not a surfactant, and it bears a positive charge.

Finally, it should be noted that the hydrophobic environment characterizing the phospholipidic bilayer is also typical of DNA coils, and that water molecules participate in the processes of both pore formation and drug intercalation.[16] Hence, we believe that a clarification of the interaction of DM-DAP^{2+} with the liposomal bilayer could be of help for a deeper understanding of the mechanism of intercalation[17] and photocleavage[7–9,18] of diazapyrenium derivatives towards DNA.

2. Results and Discussion

2.1. Spectroscopic Characterization

The UV–visible spectroscopic behavior of DM-DAP^{2+} was investigated in aqueous phosphate or Tris buffers, in both the presence and absence of liposomes, and in water. The absorption and fluorescence data are summarized in Table 1. In all cases the absorption spectra show three intense and structured bands, which can be attributed to transitions to the first, second, and third singlet ππ excited states, respectively.[2] It is also known[2,8,19] that DM-DAP^{2+} exhibits strong and structured fluorescence in solution at room temperature, and phosphorescence in rigid matrix at 77 K.

Table 1. Photophysical properties of DM-DAP^{2+} under the investigated conditions.

Medium	Absorption[a]		Fluorescence[a]		
	λ_{max} [nm]	ε [M^{-1} cm^{-1}]	λ_{max} [nm]	Φ	τ [ns]
H_2O	334	29 000	425	0.65	10
aqueous PB[b]	334	28 000	424	0.20	4.4
aqueous TB[c]	334	27 000	423	0.20	4.5
liposomes in PB	334	26 000	423	0.20	4.4
liposomes in TB	334	27 000	425	0.20	4.5

[a] Absorption and fluorescence spectra were obtained at 25.0 ± 0.1 °C. [b] PB = phosphate buffer. [c] TB = Tris buffer.

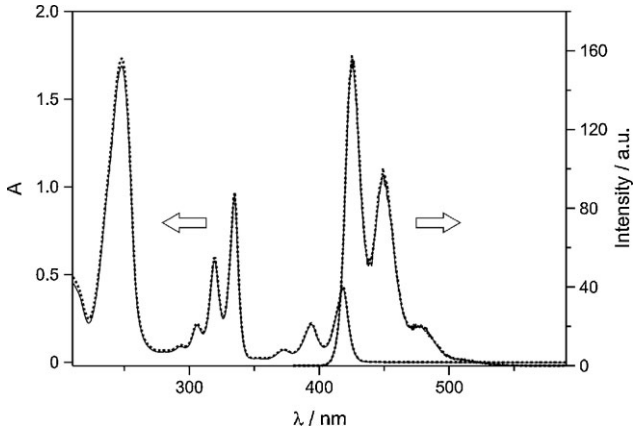

Figure 1. Absorption (left scale) and fluorescence (right scale; λ_{exc} = 300 nm) spectra of 30 μM DM-DAP^{2+} in phosphate buffer in the absence (solid lines) and presence (dotted lines) of liposomes.

The absorption and fluorescence spectra of DM-DAP^{2+} in phosphate buffer with and without POPC liposomes are reported in Figure 1. The absorption and fluorescence spectra, and the fluorescence quantum yields and lifetimes of DM-DAP^{2+} in the presence of liposomes are very similar to those in their absence (Table 1). Moreover, the DM-DAP^{2+} fluorescence shows no anisotropy in either buffer solutions or buffered liposome suspensions. All these observations suggest that DM-DAP^{2+} experiences an environment very similar to that provided by the bulk aqueous buffer, even in the presence of the liposomes.

Although it is likely that most of the DM-DAP^{2+} is dissolved in the buffer solution, it can be reasonably envisaged that a fraction of DM-DAP^{2+} molecules reside in a water-rich region of the membrane, such as the surface of the bilayer close to the phospholipid head groups. As a matter of fact, an electrostatic interaction between the positively charged DM-DAP^{2+} and the anionic phosphate groups of the POPC molecules may be expected. We cannot exclude, however, that a small amount of DM-DAP^{2+}, owing to its dual nature, may be able to drag along water molecules in the interior of the bilayer (see below).

2.2. Gel Permeation Experiments

To ascertain the preferred localization of DM-DAP^{2+} when liposomes are present in the buffer solution, we recorded fluorescence spectra after filtration of the liposomal suspension through Sephadex G-25 columns. Gel permeation should allow one to get rid of the DM-DAP^{2+} molecules located in the bulk aqueous phase. It is worth recalling that in all the experiments DM-DAP^{2+} was added to preformed liposomal suspensions, and hence it should not be present in the inner aqueous phase of the liposomes.

We found that repeated elution through Sephadex G-25 columns depleted the solution of both DM-DAP^{2+} and liposomes. After seven filtrations a nonfluorescent liposomal suspension was obtained, thus suggesting that all DM-DAP^{2+}

dissolved in the bulk buffer solution had been removed. However, subsequent rupture of the surviving liposomes by addition of isopropanol or a liposomal destabilizing agent (Triton X-100) caused the appearance of the typical DM-DAP^{2+} fluorescence. The presence of residual DM-DAP^{2+} in the gel-filtered suspension was confirmed by high-performance liquid chromatography (HPLC; see Figure S1 of the Supporting Information). These observations indicate that DM-DAP^{2+} is located both in the bulk buffer solution and at the liposome surface and that, although most molecules can be extracted by repeated gel permeations, a certain amount of DM-DAP^{2+} is tightly associated with the liposomal membrane. The lack of fluorescence before the rupture of the liposomes can be ascribed to self-quenching due to a high local concentration of DM-DAP^{2+} at the membrane surface. The affinity of this compound for the liposomal membrane is in agreement with the partition free energy determination (see below).

2.3. Liposome Stability Measurements

The kinetic stability of the POPC liposomes was evaluated at 25.0 ± 0.1°C by investigating the time-dependent leakage of the 5(6)-carboxyfluorescein (CF^{3-})[20,21] or of the calcein (CL^{4-})[22,23] anionic dyes from the liposomes.[24] The initial fluorescence intensity was very low because of self-quenching at the relatively high concentration of fluorescent dyes used in the hydration of the film. When liposomes started to release the dye, the emission intensity increased owing to the dilution-dependent dequenching, with time profiles (Figure 2) that are consistent with a first-order kinetic mechanism. Therefore, for each breakdown experiment, in the absence or in the presence of a constant DM-DAP^{2+} concentration, an apparent first-order rate constant k_{obs} can be determined according to the following equation:

$$\frac{d[\text{fluorescent dye}]}{dt} = k_{obs}[\text{fluorescent dye}] \quad (1)$$

Since strong binding of DM-DAP^{2+} cations with anionic substrates was indeed observed in water,[19] we investigated whether the fluorescence signals of CF^{3-} and CL^{4-} are affected by the presence of DM-DAP^{2+} under our experimental conditions. As a matter of fact, in phosphate buffer in either the presence or absence of [POPC] = 13.2 μM, only a small decrease (<5%) of the CF^{3-} fluorescence intensity was observed after the addition of DM-DAP^{2+} at the concentration associated with the maximum rate of release of the probe from the liposome, whereas a decrease of only 9% of the CL^{4-} fluorescence was observed upon an eightfold increase of the concentration of DM-DAP^{2+} (from 2.5 to 20 times higher than that of CL^{4-}; see Figure S2 of the Supporting Information).

The different rates of CF^{3-} release for a given concentration of DM-DAP^{2+} in different buffers is not surprising, because the presence of different ions in the aqueous subphase may disturb[25–27] the molecular interactions and packing of the phospholipids in the bilayer, and strongly influence the liposomal ζ-potential (see ζ-potential measurements below).[28] As a matter of fact, the mean diameter and the polydispersity of the liposomes in Tris buffer are respectively 33% lower and much higher than those observed in phosphate buffer.

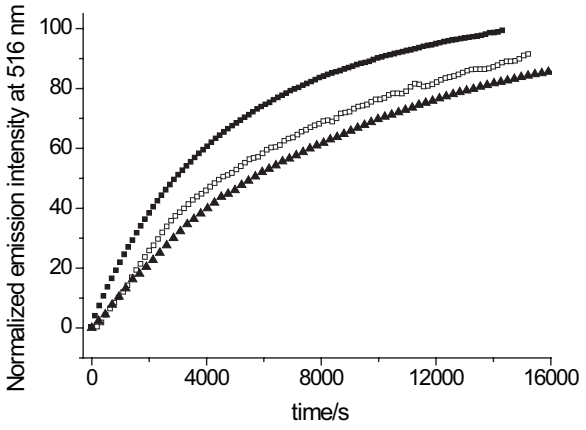

Figure 2. Kinetic profiles of the spontaneous (filled triangles) and DM-DAP^{2+}-induced (filled squares: [DM-DAP^{2+}] = 8 μM and empty squares: [DM-DAP^{2+}] = 56 μM) release of CF^{3-} from POPC liposomes in phosphate buffer.

The k_{obs} values reported in Table 2 show that DM-DAP^{2+} favors the fluorescent probe release from liposomes both in phosphate and in Tris buffer, at least at the lowest investigated concentrations. At 44 μM DM-DAP^{2+} concentration, the half-life of the CF^{3-} release halves, both in the phosphate- and Tris-buffered liposome suspensions, while the half-life of the CL^{4-} release reduces to one third.

Although the detailed mechanism of leakage of fluorescent dyes from POPC liposomes in the absence of destabilizing agents is not known, the most credited hypothesis, initially proposed by Kashchiev and Exerova,[29] is that the leakage is caused by the spontaneous formation of transient pores and defects in the phospholipidic bilayer of the liposome.

Concerning the release induced by destabilizing agents, such as nonionic surfactants, the most convincing mechanistic hypothesis involves a three-step model.[12b,30–32] At relatively low surfactant concentration, surfactant molecules are partitioned between the continuous aqueous phase and the phospholipid bilayer phase. Upon increasing the surfactant concentration, the number of surfactant molecules into the phospholipidic bilayer increases until a critical concentration of surfactant in the bilayer is reached. Eventually, upon further increase of the surfactant concentration, vesicles break down and transform into mixed phospholipid/surfactant micelles.

At first sight the destabilizing activity of DM-DAP^{2+} on liposomes could resemble that of a nonionic surfactant such as Triton X-100.[33,34] However, while the destabilizing effect of Triton X-100 on the liposomal membrane increases on increasing the concentration of the surfactant,[33] the destabilizing effect of DM-DAP^{2+} is stronger for the lower range of investigated concentrations, and reaches a maximum value at about 40 and 64 μM in phosphate and Tris buffers, respectively (Table 2). On increasing the concentration of DM-DAP^{2+}, the liposomal membrane appears less susceptible to the dication, until at the highest investigated concentrations the effect of

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Table 2. First-order rate constant for the spontaneous and DM-DAP^{2+}-induced leakage of carboxyfluorescein (CF^{3-}) or calcein (CL^{4-}) from POPC liposomes.

Concentration of DM-DAP^{2+} [μM]	CF^{3-}		CL^{4-}
	Phosphate buffer	Tris buffer	Phosphate buffer
	10^{-4} k_{obs} [s^{-1}][a]	10^{-5} k_{obs} [s^{-1}][a]	10^{-4} k_{obs} [s^{-1}][a]
0	1.3 ± 0.4	2.3 ± 0.5	1.4 ± 0.2
8	2.0 ± 0.1	1.3 ± 0.5	1.6 ± 0.2
16	2.1 ± 0.5		
24	2.5 ± 0.1		
32	2.4 ± 0.1	7.0 ± 0.1	3.3 ± 0.3
44	2.5 ± 0.3	5.3 ± 0.5	4.2 ± 0.6
56	1.6 ± 0.6		
60	1.5 ± 0.4	10.8 ± 0.4	3.1 ± 0.3
64	1.2 ± 0.1	11.5 ± 0.5	2.9 ± 0.3
80	1.3 ± 0.2	6.5 ± 0.7	2.4 ± 0.1
100	1.1 ± 0.1		
160	1.2 ± 0.2	3.3 ± 0.5	1.0 ± 0.1

[a] Each rate constant is the average of at least five measurements and the corresponding error is the standard error.

DM-DAP^{2+} is almost negligible. It is worth noting that the change of the fluorescent probe from CF^{3-} to CL^{4-} does not alter the above-mentioned trend.

It is difficult to deduce a mechanism for the destabilizing activity of DM-DAP^{2+} on the basis of our data. Several papers have been published[35,36] on the capacity of monomolecules (ligand-assembled π slides,[37,38] membrane-active rigid-rod molecules,[39] bioinspired peptide,[40,41] calix[4]arene derivatives),[42] self-assembled molecules,[43] or mobile carriers[44] to orient transmembrane and form stable pores in the phospholipidic bilayer for passive ion or active electron transport, but DM-DAP^{2+} cannot cross right through the bilayer and the formation of a stable channel in the present case is not feasible. We hypothesize that this species could favor the formation of transient pores in the membrane owing to its affinity for the liposomal bilayer (see Section 2.2) combined with a considerable hydrophilic character. The decrease in the destabilizing activity observed upon increasing the DM-DAP^{2+} concentration may be ascribed to electrostatic interactions between the fluorescent anions and the diazapyrenium dications either positioned inside the membrane defects or accumulated on the membrane surface at the pore rim, where the encounter between DM-DAP^{2+} and the fluorescent probes could be favored with respect to the bulk solution. A similar explanation was suggested[45] for the reduced peptide-induced leakage of CL^{4-} from 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA, monosodium salt) liposomes as compared to that from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes. In the former system the higher adsorption density of kininogen-derived peptides resulted in stronger electrostatic interactions between the cationic peptides positioned around the membrane pores and the negatively charged CL^{4-}, thereby hampering its release from the liposome.

Dynamic light scattering measurements confirmed the destabilization exerted by DM-DAP^{2+} on POPC liposomes. The addition of 40 μM DM-DAP^{2+} induced a slight decrease of the size and a doubling of the polydispersity index (PI)[46] of the liposomes, which passed from an average diameter of 187 ± 4 nm (PI = 0.07) to 166 ± 57 nm (PI = 0.16) in phosphate buffer. The size reduction was more pronounced (ca. 30%) for the lower range of investigated DM-DAP^{2+} concentrations (≤60 μM). Such a decrease in the size of the objects in the suspension cannot be ascribed to the transformation of the liposomes into mixed micelles, as is the case when a surfactant is added to a liposomal suspension. In this instance it is reasonable to assume that the original liposomes are remodeled into smaller ones, due to the leaving of fragments of planar bilayers from the original membrane and the subsequent closing of the remaining bilayer residues. The formation of planar bilayer fragments from liposomes has already been observed upon addition of positively charged peptides to DOPC liposomal bilayers deposited on a planar surface.[47] In fact, the increase of the PI, which indicates the transformation of the original nearly monodispersed liposomes into aggregates with a wider size distribution, is in agreement with such an interpretation. The formation of smaller liposomes could also be fostered by an increase in the curvature of the outer surface induced by the presence of DM-DAP^{2+} in the proximity of the membrane. Such an effect would be reminiscent of that exerted by membrane-associated proteins, which are able to induce an increase in the membrane curvature of cells.[48]

The liposomal ζ-potential did not change upon addition of DM-DAP^{2+} in the concentration range 8–160 μM. An average value of (−6.7 ± 2.6) and (−13.3 ± 2.7) mV was obtained for liposomes in Tris and phosphate buffers at varying DM-DAP^{2+} concentrations, respectively. The higher ζ-potential value observed in Tris buffer is in agreement with a variation of the composition of the electronic double layer of the liposomes in response to the different buffer used.

2.4. Molecular Dynamics Simulations

To gain more insight into the mechanism at the basis of the destabilizing activity of DM-DAP^{2+} on POPC liposomes, we performed molecular dynamics (MD) simulations. Such a theoretical/computational tool has been extensively applied in

recent years, to better understand both liposomal drug transport[49] and induced formation of pores in lipid membranes.[50] In all of the previous theoretical studies, the need always emerged for producing long-timescale sampling (from submicrosecond to microsecond) to obtain reliable information. The aim of the present study was to provide a semiquantitative theoretical support to the experimental observations. Therefore, we limited our simulations up to 20.0 ns, which might be considered a sufficiently wide time interval for our purposes. However, to achieve an internal consistency of the results and to minimize systematic errors induced by the approximations of the model, we also applied the same MD protocol to the POPC/water/pyrene system, which has already been the subject of recent theoretical studies.[51] First of all, we addressed the basic question as to whether DM-DAP^{2+}, according to our model, spontaneously interacts with the POPC bilayer. For this purpose we first evaluated the most stable "configuration" of the POPC/water/DM-DAP^{2+} system by calculating the reversible work (i.e., the Helmholtz free energy) associated with 1) the shifting of a DM-DAP^{2+} molecule along the z axis, that is, normal to the bilayer plane, and 2) the rotation of its symmetry plane with respect to the same axis, which is approximately parallel to the POPC lipid chain, as shown schematically in Figure 3a.

This calculation was carried out by extracting, from the MD trajectory, the probability density (p) of finding the geometrical center of a DM-DAP^{2+} molecule at generic z and z_{ref} quotas and θ and θ_{ref} angles, with reference values (z_{ref} and θ_{ref}) being the most sampled values. Within the simulated isothermal–isochoric conditions at equilibrium, the Helmholtz free energy values could then be calculated using the standard formula:

$$\Delta A(T)_{z_{ref},\theta_{ref}/z,\theta} = -RT \ln\left[\frac{p(z,\theta)}{p(z_{ref},\theta_{ref})}\right] \quad (2)$$

Note that Equation (2) is strictly valid only at the equilibrium, which may be typically reached after a long (order of hundreds of nanoseconds) simulation time. However, in the present case solutes only undergo rototranslational motions, which, according to our simulation conditions, do occur within the hundreds of picoseconds timescale. Therefore, an assessment of the relative stability of solute within the membrane, as revealed by our accord with previous MD data in the case of pyrene, may be obtained within our simulation time. To provide an estimation of the stability of our conditions, we also evaluated the free energy semidispersion using three subportions of the trajectory. The obtained value of ± 2 kJ mol^{-1} indicates that our system, during the 20.0 ns of simulation, has been rather homogeneously spanning its configurational space. Although more quantitative conclusions would require longer samplings as already remarked, we estimated the partition free energy between bulk water and the most stable configuration of DM-DAP^{2+} (Figure 3c) in the membrane, by using the standard thermodynamic integration (TI) procedure (see Experimental Section). The approximate estimation of -40 ± 10 kJ mol^{-1}, found with the TI procedure, qualitatively accounts for the affinity of DM-DAP^{2+} for the liposomal bilayer as revealed by HPLC and gel permeation experiments. An identical protocol was adopted for pyrene. The results, reported on the z/θ plane in Figure 3c and d, suggest that both DM-DAP^{2+} and pyrene experience a stabilizing interaction with the liposomal bilayer with some remarkable — although not unexpected — differences. In very good agreement with previous MD studies,[51] pyrene turns out to predominantly reside in the high-density alkyl chain region with an orientation essentially parallel to the lipid chains. On the other hand, DM-DAP^{2+} closely approaches the headgroup region, that is, the POPC/water interface, with a slightly tilted orientation. Interestingly, a small but significant fraction of DM-DAP^{2+} is able to reach the internal region of the bilayer, about 12 ± 2 kJ mol^{-1} higher in free energy than the absolute minimum, and adopts an almost-parallel pyrene-like configuration with respect to the lipid chains (see the dotted black circle in Figure 3c).

The second aspect investigated in our simulations concerns the potential role of DM-DAP^{2+} as destabilizing agent. A rather interesting feature has emerged by careful inspection of the water probability density, $p_{H_2O}(z)$, on the bilayer (xy) plane at different z quotas.

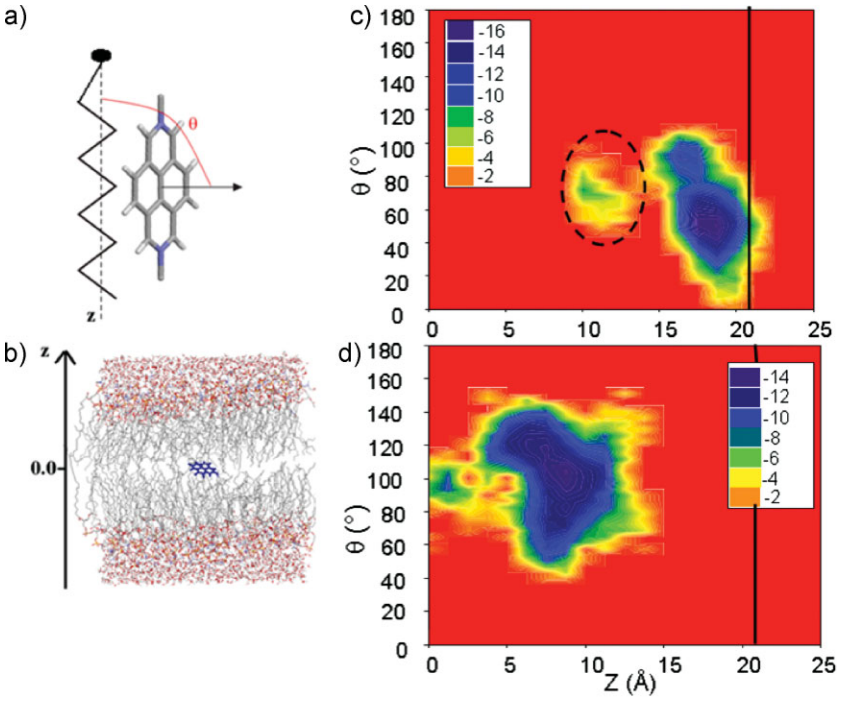

Figure 3. a) Schematic representation of the rotation angle between DM-DAP^{2+} (or pyrene) and the axis normal to the bilayer plane. b) Schematic view of the initial position of DM-DAP^{2+} (or pyrene). c,d) Relative Helmholtz (kJ mol^{-1}) free energy at 300 K (see Equation (2)) as a function of z position and angle θ for c) DM-DAP^{2+} and d) pyrene. The black line marks the approximate position of the POPC polar head groups. The energy intervals are indicated every 2.0 kJ mol^{-1}.

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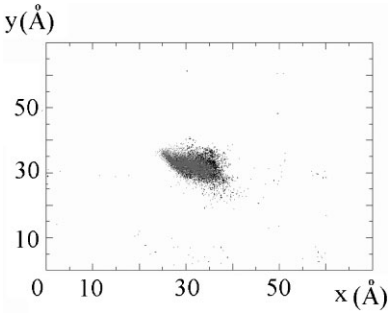

Figure 4. Probability density of water (black) and DM-DAP^{2+} (gray) as a function of the coordinate of bilayer plane *xy* at a *z* quota equal to 12.0 nm. See the text for more details.

In Figure 4 we report $p_{H_2O}(z)$ values for $z = 12.0$ nm, which is the quota corresponding to the deepest insertion in the bilayer observed for DM-DAP^{2+} (dotted black circle in Figure 3c). It is interesting to observe the perfect superposition of $p_{H_2O}(z)$ and $p_{DAP}(z)$. The same analysis carried out for pyrene did reveal the total absence of water molecules in the same region. Such a result nicely suggests that the ''internal'' diazapyrenium molecules tend to become surrounded by water molecules. Therefore, the presence of DM-DAP^{2+} in the membrane interior, roughly accounting for a molar fraction of 0.8% from the previously reported MD results, may favor the formation of internal ''water fingers'' or ''water wires'',[49,52] which could be plausibly, although partially, responsible for the creation of pores. The high affinity for water of the diazapyrenium unit could be the reason for the large negative entropy change associated with the intercalation of DM-DAP^{2+} in calf-thymus DNA.[17a] Such an observation was indeed unexpected and is not consistent with the dehydration of the diazapyrenium dication upon intercalation.

The similarity of the spectroscopic properties of DM-DAP^{2+} in phosphate buffer in the absence and in the presence of liposomes (Figure 1) and the invariance of the ζ-potential of the liposomes upon addition of DM-DAP^{2+} are in perfect agreement with these hypotheses.

3. Conclusions

We have investigated the effect of DM-DAP^{2+}, a hetero-aromatic cationic electron acceptor integrating hydrophobic and hydrophilic features, on the stability of POPC liposomes. Our results indicate that DM-DAP^{2+} accelerates the rupture of the liposomes, most likely by forming aqueous transient pores in the bilayer because of its tendency to attract water molecules and/or to accumulate on the liposomal surface.

The mechanism of action of DM-DAP^{2+} appears to be different from that of classical destabilizing agents such as nonionic surfactants. Actually, the tendency of diazapyrenium dications to surround themselves with water molecules even in strongly hydrophobic environments, such as the liposomal bilayer or the intercalation pockets of nucleic acids, could be one of the reasons — besides specific binding and photoactivity properties — why these compounds exhibit good DNA photocleavage activity.

4. Experimental Section

Materials and methods: POPC was purchased from Avanti Polar Lipids (Alabaster, AL). CF^{3-} (95% purity), CL^{4-} (indicator grade), acetonitrile, dimethyl sulfoxide, CH_2Cl_2, Triton X-100, and Sephadex G-25 were purchased from Fluka and Sigma.

DM-DAP^{2+} was synthesized according to a literature procedure.[53] DM-DAP^{2+} is soluble in water and in aqueous buffer solution at pH 7.4. Its aqueous solutions are stable for several months at room temperature. However, absorption spectral changes and a decrease in the fluorescence intensity are observed upon sonication, which indicates that DM-DAP^{2+} decomposes as a consequence of ultrasound treatment in water.

UV/Vis absorption measurements were performed on Jasco V-550 UV/Vis or Perkin Elmer λ40 spectrophotometers. Luminescence intensity measurements were performed with Jasco FP-6200, Jasco FP-6500, or Perkin Elmer LS-50 spectrofluorimeters. Luminescence lifetimes were determined by the time-correlated single-photon counting technique with an Edinburgh Instruments FLS-900 system. Luminescence anisotropy measurements were performed with the same FLS-900 fluorimetric apparatus. Fluorescence quantum yields were determined by the optically dilute method using quinine sulfate as a reference standard.[54] Experimental errors were estimated as follows: wavelength values, ± 1 nm; absorption coefficients, fluorescence quantum yield and lifetimes, $\pm 10\%$.

Measurements of vesicle size and ζ-potential values were performed by using a 90Plus/BI-MAS ZetaPlus multiangle particle size analyzer (Brookhaven Instruments Corp.) on dilute samples (final osmolarity of 58 mOsm). The osmolarity of the solutions was checked using a micro-osmometer (Advanced Instruments Model 3300) while the pH of the solutions was checked on a pHM93 pH meter from Radiometer Copenhagen.

Reversed-phase HPLC measurements were performed with a Spectra System P-200 pump equipped with a Spectra System UV6000LP photodiode-array detector and a computer-integrating apparatus. The column was a Vydac C18, and the mobile phase consisted of a filtered and degassed water/acetonitrile (95:5) mixture with added trifluoroacetic acid. The flow rate was kept constant at 1 mL min^{-1}.

Preparation and characterization of POPC liposomes: POPC liposomes were prepared by rehydration of a homogeneous thin film, obtained by evaporation of a chloroform solution (100 μL) of POPC (25 mg mL^{-1}). The phospholipidic film was kept at 4 °C overnight before rehydration with a phosphate buffer solution [2.5 mL; made of 174 mM NaCl, 105 mM Na_2HPO_4, and 20 mM KH_2PO_4 (pH 7.4, 578 mOsm) in the case of CF^{3-} release or of 533 mM NaCl, 105 mM Na_2HPO_4, and 20 mM KH_2PO_4 (pH 7.4, 1296 mOsm) in the case of CL^{4-} release] or a Tris buffer solution [made of 50 mM Tris, 42 mM HCl, and 0.12 M NaCl (pH 7.4, 578 mOsm)]. The obtained liposomal suspension was stirred and extruded five times through polycarbonate filters with 100-nm pores on a nitrogen-driven Lipex Biomembranes (Vancouver, BC, Canada) apparatus. Alternatively, the liposomal suspension was extruded 20 times through a polycarbonate membrane with 100-nm pores on an Avanti Polar Lipids (Alabaster, AL, USA) mini-extruder. Mean diameters d of 187 ± 4 nm (PI = 0.07)[46] and

125 ± 19 nm (PI = 0.3) were measured for the resulting unilamellar vesicles in phosphate buffer and in Tris buffer, respectively.

For stability measurements the liposomes were analogously prepared, the only difference being the rehydration with a 50 mM solution of CF^{3-} [21] or a 80 mM solution of CL^{4-} in aqueous buffer [made of 121.5 mM NaCl, 25.2 mM Na_2HPO_4, 4.8 mM KH_2PO_4 (pH 7.40), and 50 mM CF^{3-} or of 121 mM NaCl, 25.2 mM Na_2HPO_4, 4.8 mM KH_2PO_4 (pH 7.40), and 80 mM CL^{4-}, respectively]. Unentrapped CF^{3-} and CL^{4-} were removed by filtration of the liposomal suspension through a Sephadex G-25 column. Extrusion and column chromatography were performed at room temperature. The final liposome suspension was diluted with an isosmotic (578 mOsm in the case of CF^{3-} release and 1296 mOsm in the case of CL^{4-} release) buffer in order to achieve a final POPC concentration of 13.2 μM. CF^{3-}-loaded liposomes were analogously prepared in Tris buffer [made of 50 mM Tris, 42 mM HCl (pH 7.4), and 50 mM CF^{3-}].

The preparation of DM-DAP^{2+}-loaded liposomes by rehydration of the POPC film with an aqueous DM-DAP^{2+} solution failed. Therefore, DM-DAP^{2+} was added to already prepared liposomal suspensions in small aliquots from the proper aqueous stock solution, to obtain a final concentration between 8 and 160 μM. Stock solutions of DM-DAP^{2+} of concentration 1.6 and 10.0 mM were prepared for samples with a final DM-DAP^{2+} concentration in the ranges 8–64 and 80–160 μM, respectively. The osmolarity of the liposomal suspension was kept constant at 578 mOsm in the case of CF^{3-} release and at 1296 mOsm in the case of CL^{4-} release. In the liposome stability experiments, the fluorescence intensity of CF^{3-} was measured at 516 nm and that of CL^{4-} at 520 nm upon excitation at 490 nm.

In the gel permeation experiments a liposomal suspension containing 10 μM DM-DAP^{2+} was filtered seven times through a Sephadex G-25 column. The liposomes were finally disrupted with isopropanol (liposome dispersion/isopropanol 1:1 v/v) and the mixture separated by reversed-phase HPLC.

MD simulations: MD simulations were carried out in a canonical (NVT) ensemble using as starting configuration one solute molecule (DM-DAP^{2+} or pyrene) at the center (see Figure 3b) of a fully hydrated and equilibrated POPC membrane[55] consisting of 128 POPC molecules and 4480 water molecules, for which the single point charge (SPC) model was adopted.[56] Lipid force-field parameters were used as described by Berger et al.[57] For the solute molecules (DM-DAP^{2+} or pyrene), Gromos96[58] Lennard-Jones atomic point charges were recalculated by using a standard fitting procedure on density functional theory calculations (see Table S1 in the Supporting Information) with the B3LYP functional in conjunction with the 6-311g(d,p) basis set within the standard fit procedure by Merz and Kollman,[59] as implemented in the Gaussian 03 package.[60] By keeping the solute molecule frozen, we initially relaxed the system first mechanically then dynamically by using 50.0 ps of short MD simulations from 50 to 300 K. Then we allowed the solute to move. After an equilibration of 4.0 ns we performed two simulations of 20.0 ns, one using DM-$DAP^{2+} \cdot 2Cl^-$ ions, the other using pyrene. For all the simulations we adopted the following technical details: 1.0 fs integration step; nonbonded interactions evaluated using a twin-range cutoff of 1.0 and 1.4 nm; and Berendsen temperature coupling with a time constant equal to the integration step.[61] The Gromacs 3.0.5 package[62] was used for the production of the trajectories subsequently analyzed through homemade routines.

The standard TI procedure, used for evaluation of the partition free energy between bulk water and the most stable configuration of DM-DAP^{2+}, was adopted as implemented in the Gromacs software with soft core potential with $\alpha = 1.51$, $\sigma = 0.3$ nm, and $\Delta\lambda = 0.001$.[63]

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