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Embedding calix[4]resorcinarenes in liposomes: experimental and computational investigation of the effect of resorcinarene inclusion on liposome properties and stability

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Two calix[4]resorcinarenes, which differ in the length of the alkyl chain on the methylene bridge between the aromatic rings, have been embedded in unilamellar liposomes prepared from 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine in three host/guest ratios, following two different procedures. The effect of the insertion of the guests has been evaluated through the measurements of the viscosity and the kinetic stability of the liposomal systems by means of the fluorescent probes pyrene and 5(6)-carboxyfluorescein. The presence of the guests reduces the viscosity of the liposomes, suggesting a modification of the bilayer structure. However, this does not affect liposome stability. A calix[4]resorcinarene cavitand with a more rigid conformation compared to the parent resorcinarene, has been also synthetized and embedded in liposomes . The free energy of the insertion of the substrates in the lipid bilayer has been evaluated through Molecular Dynamics simulations.

Keywords: liposomes; calix[4]resorcinarenes; free energy calculations

1. Introduction

Calix[n]resorcinarenes (RESs) were first reported in the late XIX century by Adolf Baeyer (1), but only later, in 1968, their structure was proven by Erdtman (2) by single crystal X-ray analysis. RESs are a class of macrocyclic compounds typically obtained by acid-catalysed condensation reaction between resorcinol (or its derivatives) and an aldehyde (3). They are characterized by a basket-shaped structure with a hydrophobic

interior and an upper- and a lower-rim which can be easily functionalized to modify their properties. Like their closely related and more extensively studied calix[n]arenes, RESs are particularly interesting as host systems in supramolecular chemistry. The hosting ability of RESs was first demonstrated by Schneider and co-workers (4). Since then, many efforts have been made in the elucidation of the supramolecular chemistry of these macrocycles. They can form stable complexes with non-ionic polar (5) and cationic (6) guests and the macrocycles can be easily modified by suitable substitution at their upper rim of methylene bridges and extra annular –OH as well as at their lower rim, for designing tailored systems with peculiar properties and increased binding abilities (7-12) useful in a variety of applications, from molecular recognition (13) to drug carriers (14). Based on this approach, a wide number of RES derivatives have been synthesized and their complexing properties have been examined under different conditions, from solutions (15, 16) to crystalline state (17, 18), to Langmuir-Blodgett films (19, 20).

The ability of RESs to complex guests is a consequence of the presence of the hydroxyl groups on the hydrophilic upper rim, which can form intermolecular hydrogen bonds, while the in−cavity molecular recognition results from cation···π, and C−H···π or π···π interactions between aliphatic or aromatic portions of the guest and the aromatic rings of RESs (21).

Amphiphilic RESs have been prepared from resorcinol condensation with long chain aliphatic aldehydes (22, 23). Due to the presence of the hydrophobic tail, these RESs can interact with lipid membranes and be potentially useful in several applications as biosensors or biomimetic and biochemical systems.

Tanaka and co-workers have demonstrated that calix[4]resorcinarene with C18 alkyl chains forms an ion channel with K^+ selectivity, in planar soybean lecithin

bilayers (24). Nikolelis and co-workers have incorporated a calix[4]resorcinarene with C11 alkyl chain in planar lipid bilayers, as a receptor for the selective electrochemical detection of dopamine and ephedrine (25).

In recent years, many studies have been reported on calix[n]arene interactions with liposomes. Some functionalized calixarenes have been successfully inserted into liposomes (26, 27). Sidorov and co-workers have demonstrated that calix[4]arene tetrabutylamide forms a synthetic, non peptidic ion channel which binds and transports HCl across the liposomal membrane (28). Tris-N-phenylureidocalix[6]arene inserted into liposome bilayer forms pores able to transport Cl^- across the membrane (29). Costa et al. (30) have investigated, by molecular dynamics, the ability of a calix[4]arene bearing four $-NH_3^+$ groups to interact with liposomes and promote transmembrane chloride transport.

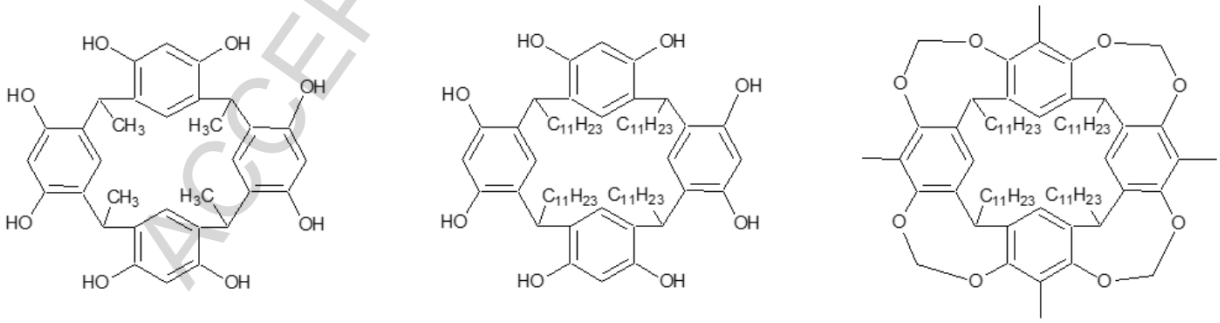
In spite of a vast available literature regarding calixarenes, very little has been done on the resorcinarenes inserted into liposomes. Resorcinarene cavitands, obtained by bridging the neighbouring hydroxyl groups on the upper rim of the macrocycles, have been incorporated in aqueous phosphocholine micelles, acting as simple biomimetic receptors for hydrophobic guests (31,32). More recently, a fluorescently labeled resorcinarene cavitand has been successfully included in 1,2-dilauroyl-sn-glycero-3-phosphocholine giant unilamellar vesicles (33). The embedded cavitand located in the lipid bilayer has been visualized by means of fluorescent confocal microscopy.

While it is well known that hydrophobic molecules such as calix[n]resorcinarenes are able to enter liposome membranes, there is a lack of detailed information about their positioning inside the liposome membrane, their effect on liposome stability and how these effects depend on the RES structure. Indeed, the

incorporation of hydrophobic molecules inside liposomes may alter the liposome physicochemical properties such as bilayer organization, liposome morphology, surface charge and liposome stability. Such kind of information will allow to design properly tailored macrocycles acting as artificial ion channels and/or artificial molecular receptors which don't interfere with the integrity of the membrane model.

The aim of this work was to determine the changes in lipid bilayer characteristics induced by the incorporation of two calix[4]resorcinarenes, which differ in their hydrophobicity, and a resorcinarene cavitand (see Scheme 1) into model POPC liposomes. In particular, we have investigated their localization in the lipid bilayer, through microviscosity measurements, and the subsequent effects on liposome dimension, surface charge and stability.

To confirm the hypotheses raised from the experimental observations, concerning the interaction between the liposome and RES, we have evaluated the free energy of the insertion of RES into the lipid bilayer using potential of mean force calculations through Molecular Dynamics simulations.



1 2 3

Scheme 1. Structures of the calix[4]resorcinarenes and of the resorcinarene cavitand used as guest in the POPC liposomes.

2. Experimental section

2.1 Materials

All the solvent used for both liposomal suspension preparation and synthesis of the 2-methyl-undecyl-calix[4]resorcinarene cavitand were reagent grade and were used as received. Methyl-calix[4]resorcinarene (**1** in Scheme 1), undecyl-calix[4]resorcinarene (**2** in Scheme 1), 2-methyl-resorcinol, dodecyl aldehyde, bromochloromethane, 5(6)-carboxyfluorescein (CF) and pyrene were purchased from Sigma Aldrich and used without further purification. 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL).

Reactions were monitored by thin layer chromatography on silica gel plates (60F-254, Sigma Aldrich) and the analysis of the plates was carried out using a UV lamp 254/365 nm. Flash chromatography was performed on silica gel 60 (Merk).

2.2 Instrument

UV-Vis absorption measurements have been performed on a Jasco V-550UV/Vis spectrophotometer. Fluorimetric measurements have been performed on Jasco FP-6200 or Jasco FP-6500 spectrofluorimeters.

NMR spectra were recorded on a Varian Mercury 300 spectrometer with ^{1}H at 300.060 MHz and ^{13}C at 75.475 MHz. Proton chemical shifts were referenced to the TMS internal standard. Chemical shifts are reported in parts per million (ppm, δ units). Coupling constants are reported in units of Hertz (Hz). Splitting patterns are designed as s, singlet; d, doublet; t, triplet; q, quartet; dd, double doublet; m, multiplet; b, broad.

2.3 Preparation of liposomal systems

Large unilamellar vesicles have been prepared by hydration of a thin film of POPC, following the procedure reported in the literature (34). The insertion of RES into the bilayer has been carried out by following two different protocols:

Protocol A: addition of RES before liposome formation. The appropriate amounts of a RES stock solution (1×10^{-4} M for **1** and 2×10^{-4} M for **2**) in $CHCl_3$ have been added to 250µL of a stock solution in $CHCl_3$ of POPC (10 mg mL^{-1}) to give the desired RES/lipid ratio. The mixture was evaporated at 40°C under reduced pressure and the obtained thin lipid film was vacuum dried and rehydrated with an aqueous buffer solution (pH = 7.4). After a hydration period of 30 minutes, the lipid suspension was extruded five times through polycarbonate membranes (pore size 100 nm, Osmotics, Relau, Penang) mounted on an extruder (Lipex Biomembranes, Vancouver). The extrusion was performed at room temperature.

Protocol B : insertion of RES into preformed liposomes. 250µL of a stock solution in $CHCl_3$ of POPC (10 mg mL^{-1}) were evaporated at 40°C under reduced pressure. The thin lipid film formed was vacuum dried and then rehydrated with an aqueous buffer solution (pH = 7.4). The resulting heterogeneous liposomal suspension was extruded five times through polycarbonate membranes (pore size 100 nm) mounted on an extruder, at room temperature. After the extrusion, the appropriate amount of a stock solution of RES in DMSO was added to the liposomal suspension to give a 0.5:100, 1:100, 2:100 RES/lipid ratio and a maximum of 0.2% DMSO (v/v).

The liposomal suspension obtained following both protocols were diluted with the appropriate isosmotic buffer to give a final concentration of POPC of $2.64x10^{-4}$ M for viscosity measurements and $1.32x10^{-5}$ M for stability measurements.

The buffer used for rehydration of the thin lipid film for viscosity measurements was made from NaCl (174 mM), Na_2HPO_4 (105 mM) and KH_2PO_4 (20 mM). The buffer used for stability measurements was made from NaCl (121.5 mM), Na_2HPO_4 (25.2 mM) and KH_2PO_4 (4.8 mM) and CF (50 mM). In this case the unentrapped dye was removed by passing the liposomal suspension through a Sephadex G-25 column, at room temperature.

2.4 Fluorimetric measurements

The membrane microviscosity change induced by RES incorporation into liposomes has been quantified fluorimetrically, by using pyrene as fluorescent probe. The fluorescent intensity ratio I_E/I_M, where I_E is the fluorescent intensity of the excimer at λ=480 nm and I_M is the fluorescent intensity of the monomer at λ=395 nm, was used to estimate the membrane viscosity. The final concentrations of RES were 5.28×10^{-6}M (RES/lipid ratio = 2:100), 2.64×10^{-6} M (RES/lipid ratio = 1:100) and 1.32×10^{-6} M (RES/lipid ratio = 0.5:100). Experiments were carried out following a previously reported procedure (35). In each cuvette 1µL of a stock solution 2×10^{-3} M of pyrene in ethanol was added every 3 minutes for a period of 30 min so that a final concentration of pyrene of 1×10^{-5} M was reached. Care was taken that the total amount of added ethanol did not exceed 0.5% as it is known that at high concentrations, ethanol can induce a change in the distance between the acyl chains of the phospholipids and/or their organization inside the membrane (36) and enhances the membrane permeability through the occurrence of

a lateral phase separation, i.e. the coexistence of non-interdigitated and interdigitated phases (37).

The excitation wavelength, λ_{ex}, was 335 nm.

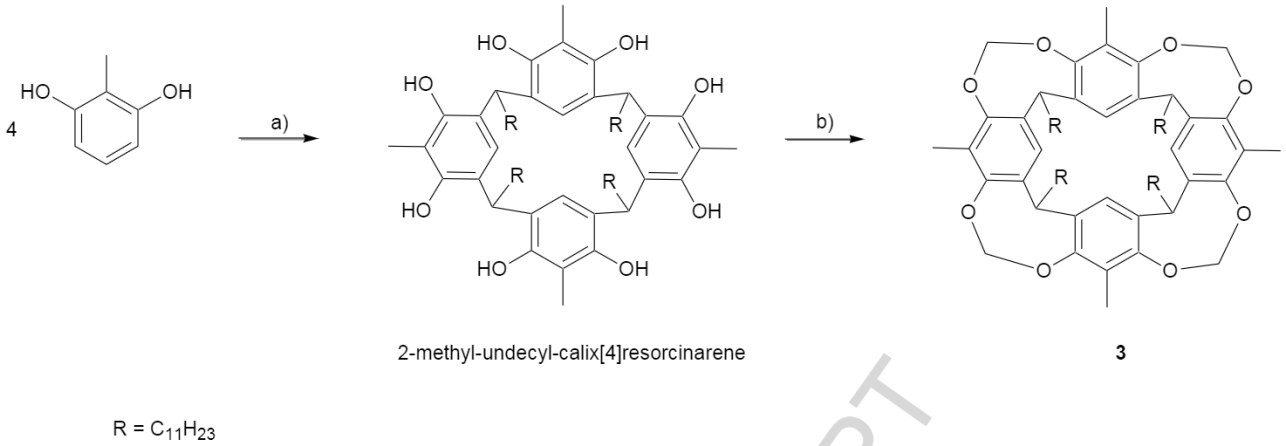
The stability of liposomes was investigated fluorimetrically by determining the time-dependent leakage of entrapped CF at 25.0±0.1 °C. The increase of fluorescence emission intensity at λ = 516 nm, using 490 nm as the excitation wavelength, due to the release of CF from the liposomes, was followed over a period of 24 h. This method is based on the principle of self-quenching of CF. At the concentration of 50 mM of CF used in the hydration of the lipid film, the fluorescence intensity is low, due to the self-quenching property of CF. When leakage of the entrapped CF from the liposomes starts, the emission intensity increases due to CF de-quenching upon dilution.

2.5 DLS analysis

The size and ζ potential values of the pure and loaded POPC liposomes were measured by using a 90Plus/BI-MAS ZetaPlus multiangle particle size analyzer (Brookhaven Instruments Corp., Holtsville, NY). For size measurements, the autocorrelation function of the scattered light was analyzed assuming a log Gaussian distribution of the vesicle size. The mean size and polydispersity index have been obtained. The ζ potential values were calculated from the electrophoretic mobility by means of the Helmholtz-Smoluchowski relationship.

2.6 Synthesis of the RES cavitand

The 2-methyl-undecyl-calix[4]resorcinarene cavitand **3** was synthetized as illustrated in Scheme 2.



Scheme 2. Synthesis of cavitand **3**. Reagents and conditions: a) dodecyl aldehyde, iodine, refluxing ethanol, 3h (61% yield); b) bromochloromethane, potassium carbonate, DMF, 70°C, N_2, 28h (93% yield).

The 2-methyl-undecyl-calix[4]resorcinarene was obtained by reaction of 2-methylresorcinol and dodecyl aldehyde, in the presence of molecular iodine as the catalyst, in refluxing ethanol for three hours (yield 61%) (38). It was then reacted with bromochloromethane, in the presence of potassium carbonate, in DMF at 70°C for 28 hours. Purification on silica gel of the crude product afforded **3** in good yield (93%).

2.6.1 Synthesis of 2-methyl-undecyl-calix[4]resorcinarene

Iodine (1 mmol) was added to a solution of resorcinol (5 mmol) and dodecyl aldehyde (5 mmol), in 10 mL of absolute ethanol. The mixture was heated at reflux for three hours, then the solution was cooled at room temperature and a saturated solution of sodium thiosulfate (10 mL) was added. The obtained precipitate was collected by filtration and washed with methanol. Pale orange solid, 61% yield; ^{1}H NMR (acetone-d_6) δ 0.89 (t, 12H, *J* 6.9 Hz, C*H*$_3$), 1.29 (s, 72H, C*H*$_2$), 2.03 (s, 12H, C*H*$_3$), 2.26-2.29 (m, 8H, C*H*$_2$), 4.37 (t, 4H, *J* 7.5 Hz, C*H*), 7.40 (s, 4H, C*H*$_{Ar}$), 7.96 (s, 8H, O*H*); ^{13}C NMR (acetone-d_6) δ 8.9, 13.5, 22.4, 28.1, 29.4, 29.7, 31.8, 34.5, 111.1, 121.2, 124.7, 149.5.

2.6.2 Synthesis of 2-methyl-undecyl-calix[4]resorcinarene cavitand 3

Potassium carbonate (18.3 mmol) and bromochloromethane (15 mmol) were added to a solution of 2-methyl-undecyl-calix[4]resorcinarene (1 mmol) in DMF (20 mL), at room temperature and under nitrogen. The pink solution was stirred at 70°C for 22 hours, then a new portion of bromochloromethane (2 mmol) was added. The mixture was heated for further 6 hours, then the mixture was cooled at room temperature and extracted with diethyl ether (2 x 20 mL). The organic phase was dried on sodium sulfate and evaporated under reduced pressure. The crude mixture was submitted to column chromatography (silica gel, chloroform) to afford a yellow oil, 93% yield. ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 12H, *J* 6.9 Hz, CH_3), 1.26 (s, 72H, CH_2), 1.96 (s, 12H, CH_3), 2.15-2.22 (m, 8H, CH_2), 4.25 (d, 4H, *J* 7.2 Hz, CH_2O), 4.74 (t, 4H, *J* 7.5 Hz, C*H*), 5.88 (d, 4H, *J* 7.2 Hz, CH_2O), 6.96 (s, 4H, CH_{Ar}); ^{13}C NMR ($CDCl_3$) δ 10.3, 14.1, 22.7, 27.9, 29.4, 29.7, 29.8, 31.9, 35.4, 98.4, 117.5, 123.5, 137.9, 153.2.

2.7 Free energy calculations

All the simulations were carried out using the Gromacs package, version 4.5.5 (39) with the Gromos force-field (gromos53a6) (40). The species **2** and **3** (hereafter concisely termed as RES) as reported in the Scheme 1 were simulated in the presence of water molecules and a lipid bilayer (POPC)

The charges of RESs were calculated using standard fitting procedures with the program Gaussian 09 (41) in the framework of the Density Functional Theory . For the lipid bilayer (POPC) we utilized the force field and the equilibrated box taken from the literature (42) and for water we utilized the Simple Point Charge (spc) model (43). The simulation was then performed in the NVT ensemble with a time step of 2.0 fs adopting a standard protocol: after an initial energy minimization, the system was gradually

heated from 50 to 250 K using short (20 ps) MD simulations. Finally, a further pre-equilibration of the system, arrived at 298K, was carried out by running 1.0 ns of MD simulation in all the systems. The temperature was kept constant using the velocity rescaling procedure (44). The LINCS algorithm was employed to constraint all bond lengths (45). Long-range electrostatic interactions were computed by the Particle Mesh Ewald method with 34 wave vectors in each dimension and a fourth-order cubic interpolation, and a cut-off of 1.1 nm was used (46).

Determination of the free energy of insertion of RES within the lipid bilayer was carried out using the following procedure.

First, we performed pulling simulations utilizing a constant velocity steered MD simulations. The center of mass of RES was pulled in the z-dimension (normal to the bilayer) forcing RES to move from the solvent (water) toward the middle of the membrane. A harmonic potential with a force constant of 1000 kJoule mol^{-1} nm^{-2} was adopted in conjunction with constant velocity of 0.001 nm ps^{-1}.

Secondly, we carried out umbrella sampling (47) in conjunction to the weighted histogram analysis method (WHAM) (48). At this purpose, harmonic potential of 3000 kJoule mol^{-1} nm^{-2} was applied in the z direction between the centre of mass of RES and the centre of mass of the POPC bilayer. 20 windows separated by 0.15 nm were produced and independently simulated for 12 ns. The error was evaluated through bootstrap analysis (49).

3. Results and discussion

3.1 Influence of the RES-insertion protocol and of the RES-lipid ratio on the membrane viscosity

Microviscosity is a parameter which can give useful information about the structural properties of membranes, such as packing, permeability and diffusivity (50). Several methods have been developed to determine membrane viscosity, from mechanical methods (51) to magnetic rheometry (52), to fluorescent probe-based techniques (53). Pyrene is used extensively as a fluorescent probe in model membrane studies (54), with the aim of obtaining lateral diffusion coefficient but also to obtain information on the inter-bilayer lipid transfer and lateral lipid organization, due to the sensitivity of its fluorescent spectra to the environment where it is solubilized (55). Pyrene has a characteristic emission spectrum with two emission bands: at 395 nm, relative to the pyrene monomer (I_M) and at 480 nm, relative to the pyrene excimer (I_E). The excimer is formed through the collision of a molecule of pyrene in the excited state and a molecule of pyrene in the ground state. Excimer formation clearly depends on the ability of pyrene to laterally diffuse through the membrane. The ratio of the fluorescent intensities, I_E/I_M, provides information on the lateral diffusivity of pyrene in the membrane (55). Indeed, a low value of I_E/I_M implies a high viscosity of the system because of the difficulty to form excimer, while a high value of the ratio implies a low viscosity because pyrene can move through the membrane and form the excimer.

The recorded spectra of pyrene in the liposomal system **2**/POPC (RES/lipid ratio = 2:100), obtained following the protocol A, are reported in Figure 1, as an example.

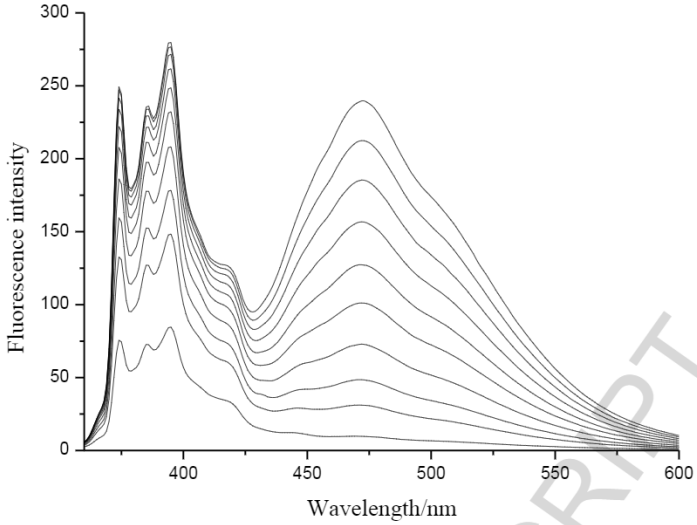


Figure 1. Fluorescent emission spectra of pyrene in the liposomal system **2**/POPC (guest/lipid ratio = 2:100 – protocol A) registered at $1x10^{-6}$ M ≤ [pyrene] ≤ $1x10^{-5}$ M

The results obtained, rationalized also in the light of previous studies present in the literature (see references throughout this section), have led us to formulate hypotheses on how resorcinarenes can interact with bilayer's phospholipids and where, probably, RESs are located in the liposome.

The data reported in Table 1 show that the sample preparation protocol and the RES/lipid ratio have a significant effect on the membrane microviscosity.

The insertion in the POPC liposomes of RES **1**, added to the phospholipid solution before the formation of liposomes (protocol A), leads to a concentration-dependent decrease in the membrane microviscosity. Indeed, the short alkyl chains of **1** interfere with the cooperative chain-chain interaction between the phospholipid molecules; the hydrophobic mismatch reduces the order of lipid packing and, consequently, the microviscosity.

The insertion of RES **1**, solubilized in DMSO according to protocol B, significantly increases the microviscosity of the liposomal membrane. This can be quite surprising

because it is reasonable to assume that the RES, added from the outside to preformed liposomes, should remain positioned at the interface of the bilayer, directly interacting with the lipid headgroups, and therefore it should not disturb the environment experienced by the probe, located at the inner of the membrane. However, it is known that organic solvents, such as DMSO, increase the motility of the alkyl chains in supramolecular aggregates (56). Based on this knowledge, we hypothesized that the presence of DMSO allows the internalization of **1** in the core of the bilayer, hindering the diffusion-dependent intermolecular formation of the pyrene excimer. The perturbation is greater the higher is the value of the RES-lipid ratio.

Liposomal system	**Viscosity (η)/cP**		**Polarity**	
pure POPC	110 (± 1)		0.950 (± 0.001)	
POPC + DMSO	94.6 (± 3.4)			
	Protocol A	**Protocol B**	**Protocol A**	**Protocol B**
1 + POPC 0.5:100	58.9(± 3.2)	141(± 7)	0.999 (± 0.001)	1.001 (± 0.001)
1 + POPC 1:100	44.8(± 0.7)	147(± 2)	1.004 (± 0.001)	1.004 (± 0.001)
1 + POPC 2:100	39.7(± 0.8)	171(± 2)	1.015 (± 0.002)	1.006 (± 0.001)
2 + POPC 0.5:100	79.2(± 2.6)	74.1(± 2.3)	1.007 (± 0.001)	1.007 (± 0.001)
2 + POPC 1:100	95.3(± 5.6)	82.0(± 1.4)	1.016 (± 0.002)	1.009 (± 0.002)
2 + POPC 2:100	112(± 2)	90.2(± 0.2)	1.029 (± 0.001)	1.016 (± 0.003)
3 + POPC 2:100	132(± 2)	-	-	-

Table 1. Membrane viscosity (η) and polarity in pure POPC liposomes and in liposomal systems containing resorcinarenes

The effect of the insertion, according to protocol A, of RES **2** on the membrane microviscosity is not linearly related to RES concentration. In fact, microviscosity decreases, albeit less markedly than with RES **1**, for a RES/host ratio = 0.5:100 but it increases again as the concentration of **2** increases, until, for RES/host ratio = 2:100 it becomes equal to that of pure POPC liposomes.

This result suggests that, at high concentration, the molecules of **2** arrange themselves in the liposome with their long hydrophobic tails immersed in the phospholipid chains below the phosphorus position. The good match between RES alkyl chains and POPC acyl chains, allows **2** to accommodate into the bilayer without interfere with the motion of the lipid chains.

The microviscosity shows the same trend also when **2**, solubilized in DMSO, is added to liposomes after their formation (protocol B).

For both RES **1** and **2** and for both protocol A and B, the insertion of the RES guest allows a deeper penetration of water molecules, probably retained as hydration water, as suggested by the increase in the local polarity experienced by the probe (see Table 1).

The cavitand **3**, which has a more rigid structure, when embedded into liposomes, leads to a significant increase in the membrane microviscosity. We hypothesized that the lipophilic **3** is able to diffuse within the highly packed media of the lipid tails, and to intercalate much deeply into the hydrophobic core of the POPC bilayer, reducing the mobility of the lipid acyl chains and preventing the pyrene from moving into the bilayer.

3.2 Particle size and zeta potential analysis

The effect of the insertion of the highest RES concentration (RES/lipid 2:100) on the liposome morphology has been investigated by DLS analysis and the liposome surface charge has been quantified by measuring the ζ potential. For pure POPC liposomes a ζ value of -26.37 has been measured. This suggests that the POPC zwitterionic liposomes behaved as negatively charged particles probably because the phosphate groups are more exposed than the choline on the surface of the liposomes (57). The obtained results are collected in Table 2.

Liposomal system	**Protocol A**		**Protocol B**	
	size (nm) **polydispersity index**	**ζ potential**	**size (nm)** **polydispersity index**	**ζ potential**
pure POPC	118.17 (± 0.42) 0.082 (± 0.012)	-26.37 (± 1.90)		
POPC + DMSO			116.60 (± 3.92) 0.105 (± 0.01)	-21.36 (± 1.23)
1 + POPC 2:100	112.63 (± 3.12) 0.126 (± 0.013)	-34.25 (± 1.48)	117.70 (± 1.76) 0.094 (± 0.01)	-27.33 (± 0.32)
2 + POPC 2:100	114.03 (± 0.94) 0.104 (± 0.013)	-38.45 (± 1.28)	124.50 (± 4.80) 0.100 (± 0.01)	-24.25 (± 1.73)
3 + POPC 2:100	107.65 (± 4.20) 0.090 (± 0.06)	-36.31 (± 0.81)	-	-

Table 3. Liposome size and zeta potential values measured for pure POPC liposomes and for the liposomal systems at RES/lipid ratio = 2:100 obtained following protocol A.

The size distribution and the surface charge of the investigated liposomal systems critically depend on the followed protocol of liposome preparation.

The insertion of RES **1** and RES **2**, according to protocol A, leads to a slight reduction of the hydrodynamic radius of the liposome but to a significant increase of the negative surface charge. This result would confirm the hypothesis that **1** and **2** are arranged into the liposome bilayer featuring the resorcinol rings right above the polar head of lipids and with the hydrocarbon chains pointing toward the liposomal hydrophobic core. RESs located immediately below the liposome surface interact with the phosphate head groups and affects their orientation, inducing an increase of their exposure at the interface. An analogous behaviour has been previously evidenced for some polyphenolic compounds inserted into DPPC liposomal bilayer for which the location close to the glycerol and C_1-C_{10} region of the DPPC acyl chains has been demonstrated by thermal analysis and ^{31}P-NMR (58). The same authors have suggested that molecules located closer to the center of the bilayer did not alter ζ potential, as they did not affect phosphate group exposure (58). Nevertheless, the insertion of **3** which we have considered to reside deep in the bilayer, leads to an increase in the negative ζ potential value, suggesting that the less flexible and more bulky and constrained structure of **3**, which reduces the liposome size more than **1** and **2** do (Table 2), increases liposome curvature and then the phosphate headgroups exposure at surface.

When RES **1** and **2** are solubilized in DMSO and added to preformed liposomes (protocol B), no evident change was observed in the ζ potential, suggesting that they reside closer to the center of the bilayer.

3.3 Stability of the liposomal systems

The mechanism of the spontaneous release of the entrapped 5(6)-carboxyfluorescein from liposomes is still not completely clarified, however it is supposed to take place through the formation of pores and defects in the phospholipidic bilayer of the liposomes (59). Indeed, the release of CF cannot occur by simple diffusion as the probe is practically insoluble in the hydrophobic membrane. The presence of RES into the bilayer can alter the physicochemical properties of the membrane and therefore can affect the rate of the release of CF . The kinetic stability of liposomes can then be regarded as a proper parameter to evaluate if a certain molecule is suitable to be inserted as a guest into a liposome bilayer without destabilizing it.

In the light of the obtained results on the system characterization in terms of RES effect on the liposome viscosity, and because it is known that DMSO disfavours the interactions between the membrane phospholipids and favours the formation of transient pores (60), the stability study was carried out only for the liposomal systems obtained following the protocol A. The spontaneous release of CF was monitored fluorimetrically as described in the Section 2.4 and the increase in fluorescence, due to the de-quenching of the anionic dye upon dilution, followed a first-order kinetic. The kinetic profiles of the spontaneous release of CF from pure POPC and from **1**/POPC and **2**/POPC liposomal systems, prepared following the protocol A, are reported in Figure 2 and 3, respectively.

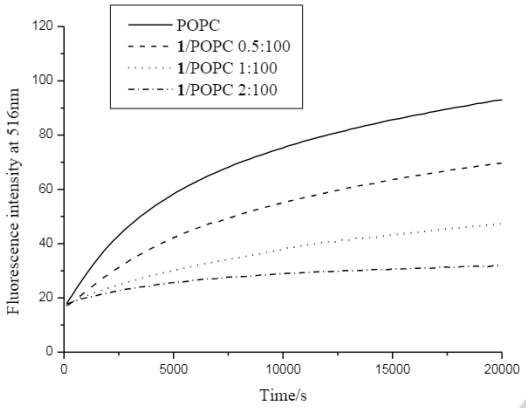


Figure 2. Kinetic profiles of the spontaneous CF release at 25 °C from pure POPC and from the RES **1**/POPC liposomal systems obtained following the protocol A.

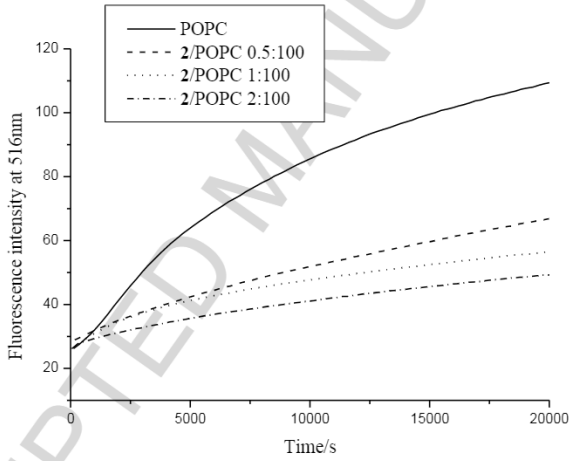


Figure 3. Kinetic profiles of the spontaneous CF release at 25 °C from pure POPC and from the RES **2**/POPC liposomal systems obtained following the protocol A.

The general rate expression for the process is (35):

$$d[CF]/dt = k_{obs}[CF]$$

The values of the first order rate constants, k_{obs}, for the investigated liposomal systems are reported in Table 3, together with that previously reported for pure POPC liposomes.

The data in Table 3 show that the rate of release of CF in the extra-liposomal environment is reduced by the insertion of calix[4]resorcinarenes **1** and **2** into the bilayer and that the stabilization of the corresponding liposomes increases with the concentration of RES. Such results suggest that calix[4]resorcinarenes can interact with the POPC molecules hindering the formation of transient pores or defects in the bilayer, thus lowering the efflux of CF. Both hydrogen bonding and hydrophobic interactions should be involved in this stabilization. Indeed, while for **2** the stabilizing effect could be ascribed to the favourable hydrophobic interaction between its alkyl chain and the phospholipid tail, the lack of such a long hydrophobic alkyl chain in **1** points to a stabilization through hydrogen bonding interaction between the -OH groups of the macrocycle and the phospholipid phosphate headgroups. A similar effect was previously observed in the presence of other hydrogen bonding forming guests (61).

Liposomal system	k_{obs} x 10^{-4} [s^{-1}]
pure POPC	1.31 (± 0.05)
1 + POPC 0.5:100	1.07 (± 0.07)
1 + POPC 1:100	0.83 (± 0.06)
1 + POPC 2:100	0.71 (± 0.05)
2 + POPC 0.5:100	0.75 (± 0.02)
2 + POPC 1:100	0.71 (± 0.05)
2 + POPC 2:100	0.60 (± 0.04)
3 + POPC 2:100	1.85 (± 0.09)

Table 3. First order rate constants, k_{obs}, for the spontaneous release of CF from the investigated liposomal systems.

Despite the effect on increasing the order of the acyl chains of POPC, the insertion of the methylene-bridged cavitand **3** into the liposomal bilayer adversely affects the stability of the liposome, as evidenced by the increase in the rate of the release of CF for the **3**/POPC liposomal system compared to pure POPC liposomes (see Table 3).

Since the main difference between **2** and **3** is that the methylene-bridged **3** cannot interact with the phospholipid phosphate headgroups, this suggests that the hydrogen bonding interactions between the -OH groups on the upper rim and the headgroups of phospholipids play a dominant role in the stabilizing effect of RES.

3.4 Free Energy calculations

To provide a more quantitative insight for interpreting the results shown in the sections 3.1 and 3.2, we modelled by means of atomistic simulations the insertion of RES into the liposome. We selected both RES **2** and **3** as prototypical examples.

This was accomplished through free-energy calculations based on MD simulations. In Figure 4 we report the free energy as a function of the z-direction (normal to lipid bilayer). Note that the zero value of the z-axis corresponds to the bilayer center of the mass.

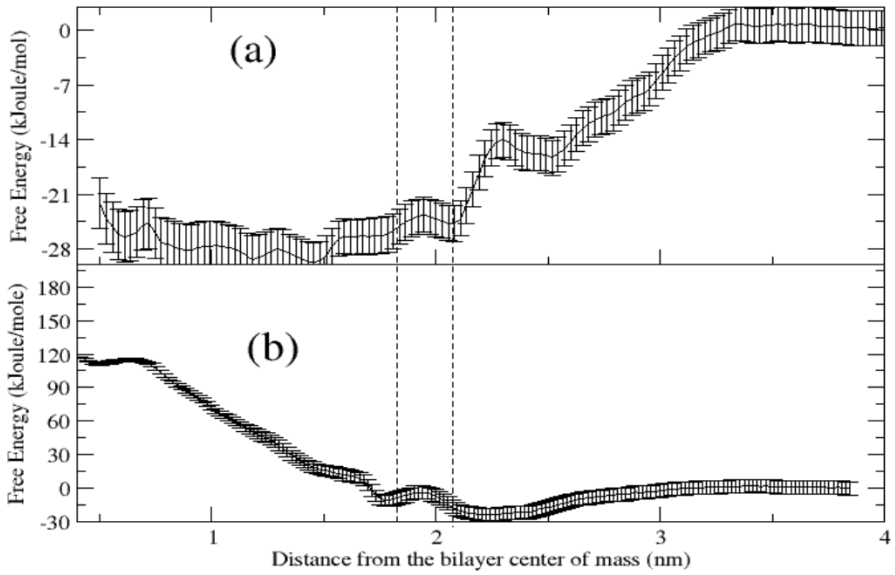


Figure 4. Free energy profile as a function of the distance from the bilayer center of mass for (a) **3** (panel 'a') and **1** (panel 'b'). Vertical dotted lines indicate the bilayer surface.

Our results show that the two free energy profiles are very different. In fact, in the case of **3**, RES shows a relatively good affinity toward the bilayer (the free energy of the system is lowered when the substrate interacts with the bilayer). Once RES has interacted with the bilayer surface (indicated with two vertical dotted lines) at about 2.0 nm, it is able of moving within the membrane following a quasi-diffusive regime. It follows that in the case of **3** we can expect a homogeneous distribution of RES within the membrane at different values of z.

Where **2** is concerned, similarly to the previous case we observe a good affinity toward the membrane (also in this case the free energy of the system is lowered when the substrate interacts with the bilayer) but, differently from **3**, in this case the substrate is practically blocked a few Angstroms below the surface. This is probably due to the

presence of polar groups which enhance the hydrophilicity of RES favoring the affinity to the higher polar region of the bilayer.

4. Conclusions

In this work, two calix[4]resorcinarenes, which differ in the length of the alkyl chains on the methylene bridges between the aromatic rings, and a resorcinarene cavitand, in which the hydroxyl groups at the upper rim of the macrocycle are linked covalently, have been embedded in POPC liposomes. Due to their different molecular structure, conformational flexibility, steric hindrance and hydrophobicity, these molecules can be located at various level into the bilayer and, differently interacting with the membrane phospholipids, they can affect liposome properties such as size, surface charge, viscosity and kinetic stability in different ways. Two preparation protocols were followed, that is premixing RES with lipids before liposome preparation and adding RES in DMSO to preformed liposomes. Differences were found in the physicochemical properties of the same liposomal formulation prepared according to the two different methods. In particular, viscosity and ζ potential values change as a function of the employed preparation protocol, probably due to a different location of the RES in the bilayer, induced by the presence of DMSO that, increasing the alkyl chain motility of the phospholipids in the liposomes and favoring the formation of transient pores, allows RES internalization.

The presence of a long alkyl chain on the lower rim of the RES macrocycle allows a better match with the phospholipid molecules, which is concentration-dependent. Indeed, at the highest concentration, RES **2** do not alter the orderliness of the acyl chains of POPC, as the less hydrophobic RES **1** does; nevertheless, both RESs can enhance the kinetic stability of the liposomes. In this respect, hydrophobicity of RES as

well as hydrogen bonding were revealed to play a fundamental role. The absence of OH groups, and therefore of interaction with the polar head groups of the phospholipids, and the non-flexibility of the bulky structure are responsible for the alterations observed in the presence of the cavitand **3**, which increases the viscosity of the liposome but decreases its stability.

Resorcinarenes are a fascinating class of macrocycles which can form complexes with both cations and anions and with neutral molecules, so we believe that understanding their effects on liposome morphology and stability and how these effects depend on their structure and on the liposome preparation protocol, will allow to design the appropriate macrocycle and the most suitable liposome loading method to be used in studies aimed to mimic ion channels and/or artificial membrane receptors.

Notes

There are no conflicts of interest to declare.

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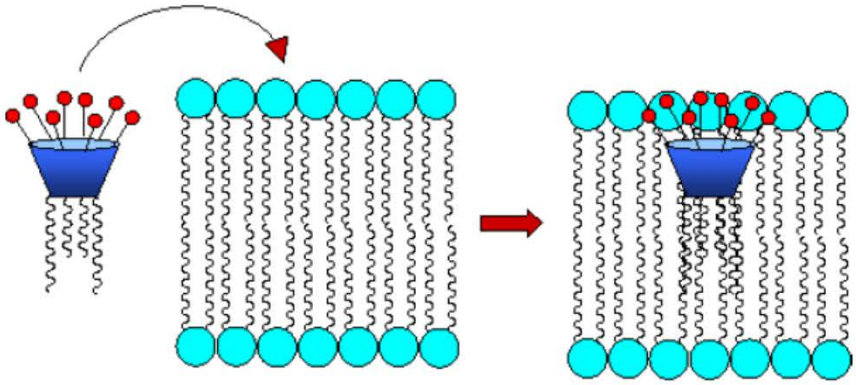
Conflict of interest

No conflict of interest to declare



Graphical abstract

Embedding calix[4]resorcinarenes in liposomes: experimental and computational investigation of the effect of resorcinarene inclusion on liposome properties and stability

Highlights

- Calix[4]resorcinarenes can be embedded in POPC liposomes.
- They interact with bilayer phospholipids at various level, depending of their molecular structure and the preparation protocol.
- Calix[4]resorcinarenes enhance the kinetic stability of the liposomes.
- The resorcinarene cavitand, in which the OH groups are missing, increases the viscosity and decreases the stability of liposomes.