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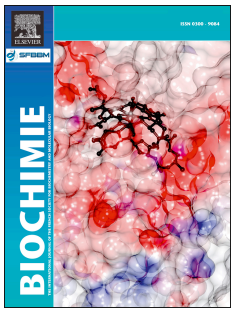

Interactions of sugar-based bolaamphiphiles with biomimetic systems of plasma membranes

Dr. Mehmet Nail Nasir, Jean-Marc Crowet, Laurence Lins, Firmin Obounou-Akong, Arnaud Haudrechy, Sandrine Bouquillon, Dr. Magali Deleu

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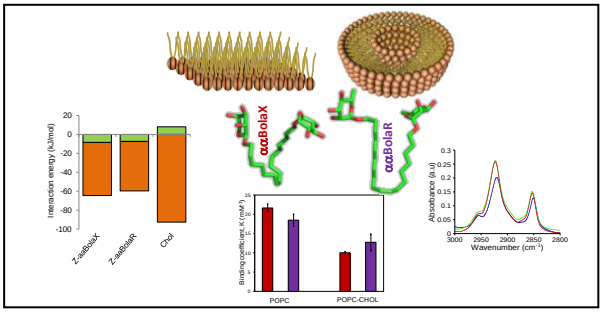
Graphical abstract



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Interactions of sugar-based bolaamphiphiles with biomimetic systems of plasma membranes.

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Keywords : glycolipid, membrane interaction, cholesterol influence, Langmuir monolayer, isothermal titration calorimetry, IR spectroscopy, *in silico* analysis

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Abstract

Glycolipids constitute a class of molecules with various biological activities. Among them, sugar-based bolaamphiphiles characterized by their biocompatibility, biodegradability and lower toxicity, became interesting for the development of efficient and low cost lipid-based drug delivery systems. Their activity seems to be closely related to their interactions with the lipid components of the plasma membrane of target cells. Despite many works devoted to the chemical synthesis and characterization of sugar-based bolaamphiphiles, their interactions with plasma membrane have not been completely elucidated. In this work, two sugar-based bolaamphiphiles differing only at the level of their sugar residues were chemically synthetized. Their interactions with membranes have been investigated using model membranes containing or not sterol and with *in silico* approaches. Our findings indicate that the nature of sugar residues has no significant influence for their membrane interacting properties, while the presence of sterol attenuates the interactions of both bolaamphiphiles with the membrane systems. The understanding of this distinct behavior of bolaamphiphiles towards sterol-containing membrane systems could be useful for their applications as drug delivery systems.

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1. Introduction

Glycolipid surfactants are surface-active molecules produced by several microorganisms such as bacteria, fungi and yeasts. They have interesting biological properties such as antifungal, antiviral and plant-eliciting, and are compounds of interest for pharmaceutical as well as for food and cosmetic formulations [1–7]. Their biological properties may be modulated by their action on the plasma membranes of target cells and more particularly by their interactions with membrane lipids[8,9]. It has been shown that dirhamnolipids, a class of glycolipid surfactant, were able to interact with phospholipid bilayers, to affect their transition temperature[9] and to have a fluidifying effect[10]. Moreover, the interactions between dirhamnolipids and phospholipids have been suggested to involve the carbonyl ester groups of phospholipids with a dehydrating effect[11,12].

Among glycolipid surfactants, bolaamphiphiles constitute an important class. They are composed of two hydrophilic heads connected by a hydrophobic carbon segment[13,14]. Their interest lies mainly in the development of efficient and low cost lipid-based drug delivery systems[15–17] due to their ability to form spontaneously stable vesicles[18]. They could also be useful to develop functionalized nanotubes[19] and novel self-assembling materials [20].

In this context, new sugar-based bolaamphiphiles have been synthetized from primary biological resources such as wheat and sugar beet[13,16–18,21–30]. Given their natural origin, sugar-based bolaamphiphiles are drawing increasing attention because of their biocompatibility, biodegradability and low toxicity[28]. They have been reported to form monolayers and insert into lipid membranes[13,26,31]. The interactions of sugar-based bolaamphiphiles with lipids could play a crucial role for their activity. Despite these several works carried out on the synthesis of sugar-based bolaamphiphiles and on the analysis of their macroscopic effect on the lipid membrane, to our best knowledge, there are only few limited

works devoted to the molecular analysis of the interactions of sugar-based bolaamphiphiles with membranes of different lipid compositions. The present work hence aims to analyze the interactions of two sugar-based bolaamphiphiles differing by their polar heads with lipid membranes composed of phospholipids and/or sterol in order to explore the effects of this structural trait and of the membrane lipid composition on the activity of bolaamphiphiles. For that purpose, a xylose-based and a rhamnose-based symmetric bolaamphiphiles were chemically synthesized. These sugars represent molecules of interest in the context of biomass valorization. Xylose is the main pentose in hemicellulosic biomass and, rhamnose is the methyl form of the xylose and is present in pectin of sugar-beet cell walls [32]. The synthesized bolaamphiphiles are composed by two identical hydrophilic heads constituted by xylose or rhamnose, with α anomeric carbon, connected by an ether link to a hydrocarbon segment of 18 carbon atoms with an unsaturation at the centre (Figure 1). They are called ααBolaX and ααBolaR for xylose- and rhamnose-based bolaamphiphiles, respectively. Previous works have described their synthesis and the characterization of their surface-active properties, showing their ability to form monomolecular films at the air-water interface [13,29].

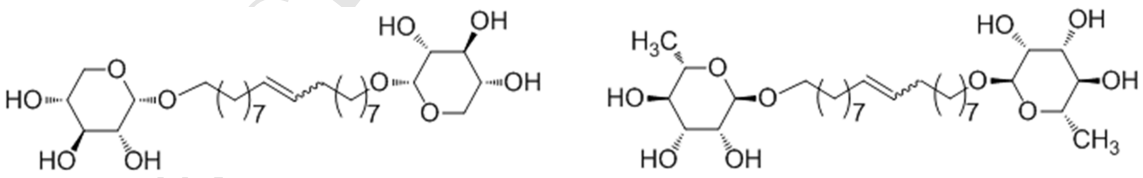

Figure 1: The structure of ααBolaX (left panel) and ααBolaR (right panel).

Three biomimetic membrane systems were used in our study, (*i*) Langmuir monolayers which mimic the external leaflet of the membrane in order to investigate the first steps of the interactions between bolaamphiphiles and membranes, (*ii*) small unilamellar vesicles (SUVs) and (*iii*) multilamellar vesicles (MLVs) to mimic the insertion of the bolaamphiphiles into a whole membrane. The adsorption kinetics of both bolaamphiphiles to Langmuir monolayers was evaluated by tensiometry measurements. Their extent of binding onto lipid monolayers constituted by phospholipid or phospholipid/sterol was evaluated by determining their maximum insertion pressure and the synergy factor α. The affinity of both bolaamphiphiles for membrane bilayers was thermodynamically confirmed using the isothermal titration calorimetry technique. In order to have information on the chemical groups involved in these interactions, FTIR spectroscopy in parallel with an *in silico* approach was used.

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2. Materials& Methods

2.1.Materials

1-Palmitoyl-2-oleoyl-*sn*-phosphatidylcholine- (POPC), 1,2-Dimyristoyl-*sn*-phosphatidylcholine (DMPC) and cholesterol were purchased from Avanti Polar Lipids and Sigma Chemical Co, respectively and used without further purification. Dimethylsulfoxide (DMSO), trifluoroethanol (TFE), Tris base and deuterium oxide (D_2O) at 99.9% isotopic purity were provided from Sigma Chemical Co. The ultrapure water was produced by a Millipore system and had a resistivity of 18.2 Ω.cm. Xylose and rhamnose-based bolaamphiphiles were chemically synthetized as described previously [29,31]. Their purity is superior to 95 % and was checked using different analytical methods [29,31].

2.2.Adsorption experiments at constant surface area by Langmuir monolayer

Xylose or rhamnose-based bolaamphiphiles adsorption experiments to air-water interface in the absence or in the presence of lipids were performed in a KSV Minitrough (9.5 cm x 21 cm) equipped with a Wilhelmy plate. The subphase was ultrapure water and its temperature was maintained at 25 ± 1°C during all experiments. The subphase was continuously stirred. Lipids (DMPC or DMPC/Chol (7/3, mole/mole)) were spread at the air-water interface to reach desired initial surface pressure. 30 minutes-waiting were required for solvent evaporation and film stabilization. Bolaamphiphiles, dissolved in DMSO at 1 mM , were then injected underneath the pre-formed lipid monolayer at a final concentration of 2.5 µM and their adsorption was followed by tensiometry as an increase of surface pressure as described previously[33–35]. Although POPC better mimics the mammalian plasma membrane, the saturated phospholipid DMPC was preferred to the unsaturated POPC in order to limit the risk

of oxidation at the interface. In terms of physical state, both of these phospholipids form a liquid-crystalline state in the experimental conditions.

The concentration of 2.5 µM in the subphase was chosen based on previous works. It is below the critical micellar concentration and large enough to observe an increase of surface pressure. The same volume of pure DMSO was injected underneath the lipid monolayer and no change of the surface pressure was observed. The uncertainty of the maximum insertion pressures and the synergy factor were calculated as described previously[34,36–38] using IgoPro Software.

2.3.Binding interactions to lipid bilayer by Isothermal Titration Calorimetry

2.3.1. Preparation of small unilamellar vesicles (SUV)

Small unilamellar vesicles (SUV) were prepared thanks to the lipid hydration technique. Pure POPC or POPC/cholesterol (7/3, mole/mole) was first dissolved in a chloroform/methanol (2/1, vol/vol) mixture in a 10-ml flask. In order to obtain a thin and regular lipidic film, the solvents were removed under vacuum using a rotatory evaporator. The last traces of solvent were removed by replacing the flask in a desiccator under vacuum during one night. The lipidic film was then hydrated above the transition temperature of lipids during 1h at 40°C and shaken every 10 min. 5 cycles of freeze-thawing were applied to spontaneously form multilamellar vesicles. To obtain SUVs, this suspension was sonicated to clarity (5 cycles x 2min) using a probe with 400 W amplitude keeping the suspension in an ice bath. At the end, the SUV solution was centrifuged during 10 min at 2000 g in order to remove titanium particles.

2.3.2. ITC experiments

ITC experiments were performed as described previously[35]. All measurements were performed at 25°C using a VP-ITC (MicroCal, Northampton, MA) with a sample cell volume

of 1.4565 mL. Before starting the experiments, solutions were degassed by stirring under vacuum or by ultrasonication. The 300 µL-syringe was filled out with lipid SUV suspension at 2 mM in Tris HCl buffer at pH 7.4. The sample cell was filled out with Tris HCl buffer at pH 7.4 (blank) or with a bolaamphiphile solution at 0.01 mM in Tris HCl buffer at pH 7.4 and the reference cell was loaded with milli-Q water. The sample cell was stirred continuously at 305 rpm during experiments. A titration experiment was performed by consecutive injections of 10 µL of SUV into the bolaamphiphile solution. Each injection took 14.5 sec and a delay of 200 sec between injections was applied in order to reach a steady state before each new injection. The effective heats were determined by subtracting the values obtained for the blanks from the observed heats. Raw data were processed using the software provided by the manufacturer (ORIGIN 7 – Originlab, Northampton, USA).

2.3.3. Calculation of thermodynamic parameters

The ITC data were treated according to the cumulative model described previously[39] and applied for other types of surfactants[35,40].

The binding coefficient (K) is calculated according to the equation (1) [41]

$$K = \frac{R}{C_{[bol,f]}} \qquad (1)$$

where R is the ratio of bolaamphiphile-to-lipid and $C_{[bol,\ f]}$ is the concentration of free bolaamphiphile (not bounded) in the solution.

R can also be defined as the ratio of the molar amounts of bounded bolaamphiphiles ($n_{[bol,b]}$) to total lipid in the sample cell ($n_{[lip]}$) or as the ratio of the concentrations of bounded bolaamphiphile ($C_{[bol,b]}$) to lipids ($C_{[lip]}$) in the sample cell (equation (2)).

$$R = \frac{n_{[bol,b]}}{n_{[lip]}} = \frac{C_{[bol,b]}}{C_{[lip]}} \quad (2)$$

The total amount of bolaamphiphile in the cell ($C_{[bol,\ tot]}$) is the sum of bounded and free bolaamphiphiles (equation (3)).

$$C_{[bol,tot]} = C_{[bol,f]} + C_{[bol,b]} \quad (3)$$

By combining equation (2) and (3), $C_{[bol,\ b]}$ can be expressed in terms of $C_{[lip]}$ (equation (4))

$$C_{[bol,b]} = C_{[bol,tot]} \times \frac{K \times C_{[lip]}}{1 + K \times C_{[lip]}} \quad (4)$$

After i^{th} injections of lipid vesicles to the sample cell containing bolaamphiphiles, the molar amount (then the concentration) of bolaamphiphile bounded to lipid vesicles is $n^{i}_{[bol,b]}$ and the cumulative heat produced by this phenomenon corresponds to the equation (5):

$$\sum_{k=1}^{i} \delta h_k = n^{i}_{[bol,b]} \times \Delta H_{bol}^{sol \to lip} = \Delta H_{bol}^{sol \to lip} \times C_{[bol,b]} \times Vcell$$

$$= \Delta H_{bol}^{sol \to lip} \times Vcell \times C_{[bol,tot]} \times \frac{K \times C_{[lip]}}{1 + K \times C_{[lip]}} \quad (5)$$

where $\Delta H_{bol}^{sol \to lip}$ represents the molar enthalpy change when the bolaamphiphile is transferred from the solution (sol) to the lipid vesicles (lip).

Then, from a curve representing the $\sum_{k=1}^{i} \delta h_k$ in function of $C_{[lip]}$, the parameters K and $\Delta H_{bol}^{sol \to lip}$ could be obtained by fitting.

From $\Delta H_{bol}^{sol \to lip}$, we can calculated the corresponding free energy $\Delta G_{bol}^{sol \to lip}$ and the reaction entropy $\Delta S_{bol}^{sol \to lip}$ as follows:

$$\Delta G_{bol}^{sol \to lip} = \Delta H_{bol}^{sol \to lip} - T \times \Delta S_{bol}^{sol \to lip} \quad (6)$$

$$\Delta G_{bol}^{sol \to lip} = RT \ln KCw \quad (7)$$

with R = 8.31 J mol^{-1} K^{-1} and C_w = 55.5 M.

2.4.Molecular interactions by FTIR spectroscopy

2.4.1. Preparation of multilamellar vesicles' samples

Multilamellar vesicles containing POPC or POPC and cholesterol (7/3, molar ratio) were prepared in the absence or in the presence of bolaamphiphiles as described previously by using deuterium oxide [34]. The lipid/bolaamphiphile molar ratio was 9/1.

2.4.2. IR measurement

Infrared spectra were recorded by means of a Bruker Equinox 55 spectrometer (Karlsruhe, Germany) equipped with a liquid nitrogen-cooled DTGS detector. The number of scans was 128 and the resolution was 4 cm^{-1}. All the experiments were performed with a demountable cell (Bruker) equipped with CaF_2 windows [34,42]. 20µL of the MLV solution containing or not bolaamphiphiles was deposited into the CaF_2 window-equipped cell and the FTIR spectra were representative of at least two independent measurements. During all experiments, the spectrophotometer was continuously purged with filtered dry air and the temperature was maintained at above the phase transition temperature of POPC.

2.5. Study of the interactions by *in silico* analysis

The conformational properties of the bolaamphiphiles as well as their interactions with model membranes were analyzed by molecular modeling. In a first time, the structure of xylose- and rhamnose-based bolaamphiphiles were constructed using HyperChem software, version 5.0

(Hypercube, Inc). A preliminary optimization of the molecule geometry was performed using the steepest descent method with the MM+ force field. A systematic analysis of the structure was then performed, as described previously [43] .

The most probable structure provided form this analysis was used for the following methods. The energy of interactions was calculated using the Hypermatrix procedure as described in [44,45]. Briefly, the bolaamphiphile molecule is oriented at the interface according to its hydrophilic and hydrophobic centers and is set constant. A lipid molecule is then moved along and around the bolaamphiphile and for each position (more than 10^6 positions are tested), the energy of interaction (van der Waals, electrostatic, torsional, and hydrophobic interactions) was calculated and the energies of all the positions were stored in a hypermatrix. The position of the first lipid was the lowest-energy complex; a second molecule was inserted as the next energetically favorable position in the hypermatrix, taking into account the presence of the first lipid. For the next lipids, the same process was repeated until the bolaamphiphile was completely surrounded with lipids.

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3. Results

3.1. Adsorption of ααBolaX and ααBolaR to lipid monolayers

The interactions of ααBolaX and ααBolaR with membranes were examined using DMPC Langmuir monolayers as simplified model for mammalian cells. For that purpose, the adsorption of both bolaamphiphile molecules to the preformed DMPC monolayers at different initial surface pressures was followed by tensiometry measurements as the increase of the surface pressure ($\Delta\Pi$) over time (data not shown) as it has usually been done for other molecules [46].

The plot of the maximal surface pressure variation ($\Delta\Pi_{max}$) due to the bolaamphiphile adsorption *versus* the initial surface pressure values of the monolayer (Π_i) (Figure 2) leads to the determination of the maximal insertion pressure (MIP), at the intersection of the linear regression with x-axis, and the synergy factor α as described previously [36,47].

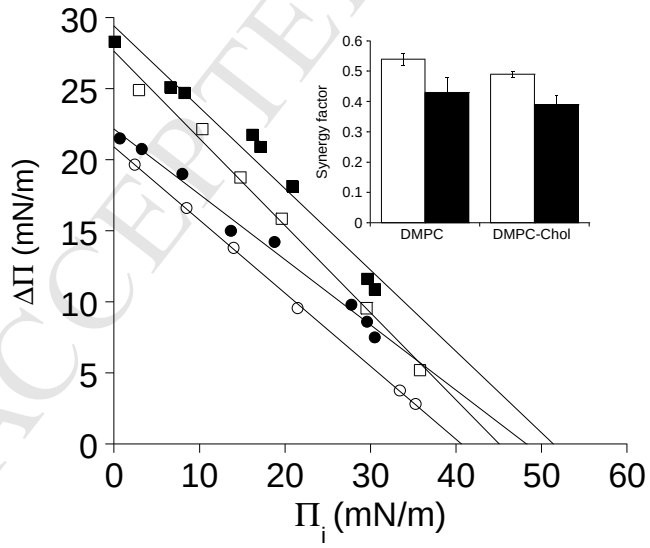

Figure 2: Determination of the maximal insertion pressure (MIP) of ααBolaX (●, ○) and ααBolaR (■, □) into pure DMPC (●,■) and mixed DMPC-cholesterol (Chol)

monolayers (○,□). Inset: Synergy factor of ααBolaX (white bars) and ααBolaR (black bars) towards DMPC or DMPC-cholesterol monolayers.

ΔΠ decreases linearly with increasing Πi values for both molecules. The increase of lipid packing associated with a greater initial surface pressure i.e. the reduction of the free space for bolaamphiphile adsorption as generally observed for amphiphile molecules interacting with membranes [34,48], could explain this observation. The MIP corresponds to the surface pressure beyond which no insertion can occur [36]. The MIP of ααBolaX in DMPC monolayer (48.2 ± 2.7 mN/m) is not significantly different from that of ααBolaR (51.4 ± 5.0 mN/m). The lateral pressure prevailing within biological membranes ranges from 30 to 35 mN/m [49] and even though the composition of biological membrane is more complex, a MIP value greater than 30-35mN/m is interpreted as favorable for the insertion of the molecule within biological membranes [34]. For both bolaamphiphiles, the MIP values were much higher than the estimated lateral pressure of biological membranes. It can be thus considered that these molecules could insert easily within biological membranes.

In order to determine the attractivity of ααBolaX or ααBolaR by DMPC monolayer, the synergy factor (α) was calculated as described by Calvez *et al* [36]. If α is greater than 0, there is a positive interaction (*i.e.* attraction) between the lipid monolayer and the bolaamphiphile; if it is equal to 0, it means that the bolaamphiphile adsorption to the monolayer is not influenced by the presence of lipids and finally if α is lower than 0, there is a negative interaction (*i.e.* repulsion) between the bolaamphiphile and the monolayer.

The calculated α values of ααBolaX and ααBolaR for DMPC monolayer were 0.54 ± 0.02 and 0.43 ± 0.05, respectively (Figure 3). Hence, calculated α values for both bolaamphiphiles showed positive interactions.

To analyze the influence of cholesterol (the sterol found in mammalian cells) on the behavior of both bolaamphiphiles towards monolayers, the same experiments with DMPC and cholesterol at a 7/3 molar ratio were performed. This proportion is currently used in the literature in the case of mixed systems composed of cholesterol and phopsholipids [50–52]. As for DMPC monolayers, kinetic profiles of ααBolaX and ααBolaR adsorption to DMPC/Chol monolayer were measured (data not shown) and MIP and α values were determined (Figure 2). Only a limited difference is observed between the MIP of ααBolaX (40.6 ± 0.5 mN/m) and that of ααBolaR (45.0 ± 2.3 mN/m). These values were sligthly lower than that obtained with pure DMPC monolayer for both bolaamphiphiles. Although decreasing, the MIP remains still higher than the estimated lateral pressure of biological membranes. Both values of synergy factor (0.49 ± 0.01 for ααBolaX and 0.39 ± 0.03 for ααBolaR) are somewhat lower compared to the pure DMPC monolayer, indicating that the presence of cholesterol tends to decrease the attractive interaction between bolaamphiphiles and lipid monolayers. Previous works have already demonstrated that the presence of cholesterol could attenuate the interactions of a membrane-active molecule with monolayer models[53,54].

3.2. Interactions of ααBolaX and ααBolaR with lipid bilayers

The interactions of ααBolaX and ααBolaR with lipid bilayers were thermodynamically characterized using ITC measurements. Both bolaamphiphiles have an affinity for POPC or POPC-Chol vesicles, for which the binding reactions are spontaneous ($\Delta G < 0$), exothermic ($\Delta H < 0$) and generate a positive change of the system entropy ($\Delta S > 0$) (Fig. 3 (A)).

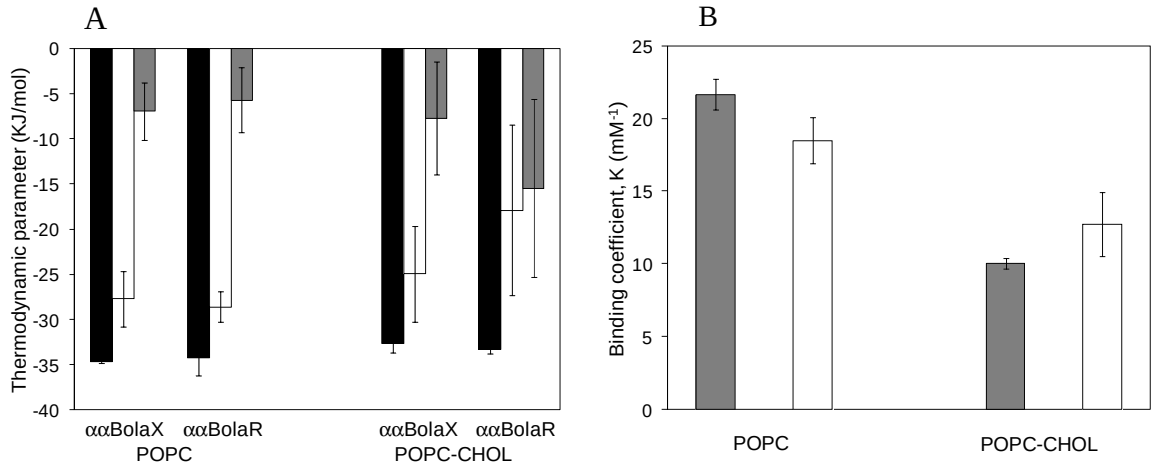

Figure 3 : (A) and (B) thermodynamic parameters (black bars: $\Delta G_{bol}^{sol \rightarrow lip}$, white bars: $-T\Delta S_{bol}^{sol \rightarrow lip}$, grey bars: $\Delta H_{bol}^{sol \rightarrow lip}$) and binding coefficient (grey bars: ααBolaX and white bars: ααBolaR) for the binding of ααBolaX or ααBolaR to POPC or POPC-Chol vesicles at 25°C.

The absolute values of T ΔS are higher than the absolute values of ΔH, indicating that the binding is dominated by hydrophobic interactions [55]. For a given lipid composition, the values of ΔG, ΔH and ΔS are similar for both bolaamphiphiles while limited differences in K values (Figure 3B) are observed. The nature of the sugar head has thus only a very slight influence on the binding interaction in terms of energy and affinity.

For both bolaamphiphiles, the composition of the lipid bilayer has a significant influence on the binding coefficient but not on the energies. The presence of Chol decreases the bolaamphiphile affinity for the lipid bilayer (Figure 3B) as also demonstrated for other membrane-active molecules as gramicidin S[56] and dermaseptin DDK [57].

3.3.Molecular analysis of the interaction between bolaamphiphiles and lipids

3.3.1. FTIR spectroscopy

Information at the molecular level about the interaction of both bolaamphiphiles with lipids was obtained using multilamellar vesicles, containing POPC alone or POPC and cholesterol prepared in the absence or in the presence of ααBolaX or ααBolaR. The FTIR spectra of multilamellar vesicles were recorded (Figure 4).

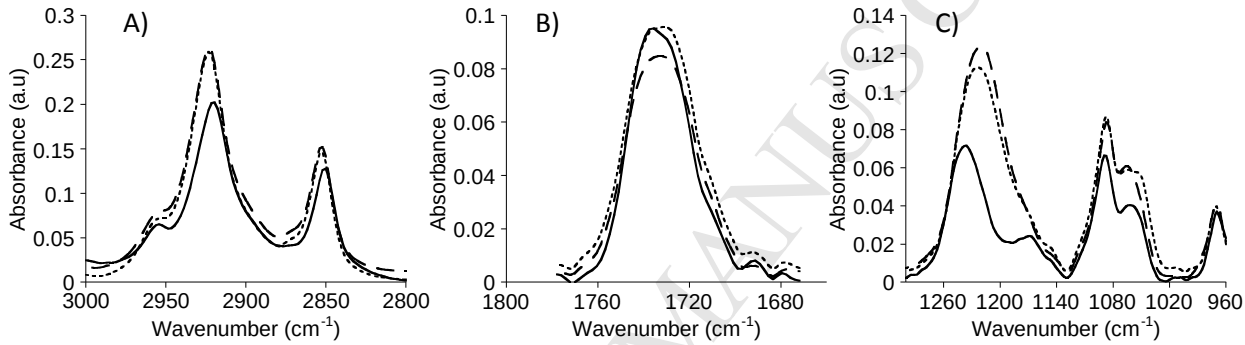

Figure 4: FTIR spectra of pure POPC MLVs (continuous line) and POPC MLVs containing ααBolaX (small discontinued line) or ααBolaR (large discontinued line). A) 3000-2800 cm^{-1} region, B) 1800-1650 cm^{-1} region, C) 1280-960 cm^{-1} region.

Three regions of interest were considered in the spectra, namely 3000-2800 cm^{-1}, 1800-1650 cm^{-1} and 1280-960 cm^{-1} regions for. In the 3000-2800 cm^{-1} region, the spectrum of pure POPC showed three bands centered at 2956, 2920 and 2851 cm^{-1}.The band at 2956 cm^{-1} corresponds to the asymmetric stretching of terminal $–CH_3$, the band at 2920 cm^{-1} corresponds to the asymmetric $-CH_2$ stretching and the band at 2851 cm^{-1} to the symmetric $-CH_2$ stretching [58]. When the MLVs contained ααBolaX or ααBolaR, the bands at 2920 and 2851 cm^{-1} were shifted to 2923 and 2853 cm^{-1} respectively. These shifts to higher

wavenumber values indicated an increasing mobility of the lipid alkyl chains and it can be interpreted as a lipid bilayer fluidizing effect of both bolaamphiphiles. It is worth to note that there were 60 alkyl chains from lipids for one alkyl chain from bolaamphiphiles; the contribution of the bolaamphiphile alkyl chain can then be neglected. In the 1800-1650 cm^{-1} region, the spectrum of POPC MLVs showed a band centered at 1737 cm^{-1} with a shoulder at 1728 cm^{-1}. This bands corresponds to the stretching vibrations of phospholipid C=O ester groups [58]. The absorption at 1737 cm^{-1} is due to the free C=O ester groups while the absorption at 1728 cm^{-1} is assigned to the C=O ester groups involved in hydrogen bonds [59]. When bolaamphiphiles were inserted within POPC MLVs, the relative intensity of the shoulder at 1728 cm^{-1} was increased, suggesting the involvement of the C=O ester groups in the interaction with both bolaamphiphiles. The increase of the "bonded" C=O population could be due to more hydrogen bonding between phospholipid molecules, between phospholipids and bolaamphiphiles or between phospholipids and the solvent. In the 1280-1150 cm^{-1} region of the POPC spectrum, a main band located at 1234 cm^{-1} corresponding to the anti-symmetric PO_2^- stretching band, was observed [58]. This band was shifted to 1224 cm^{-1} and to 1219 cm^{-1} in the presence of ααBolaX and of ααBolaR, respectively. These important shifts to lower wavenumbers indicated that the phosphate groups were involved in more hydrogen bonds and suggest an important contribution of phosphate groups of POPC in the interactions with bolaamphiphiles.

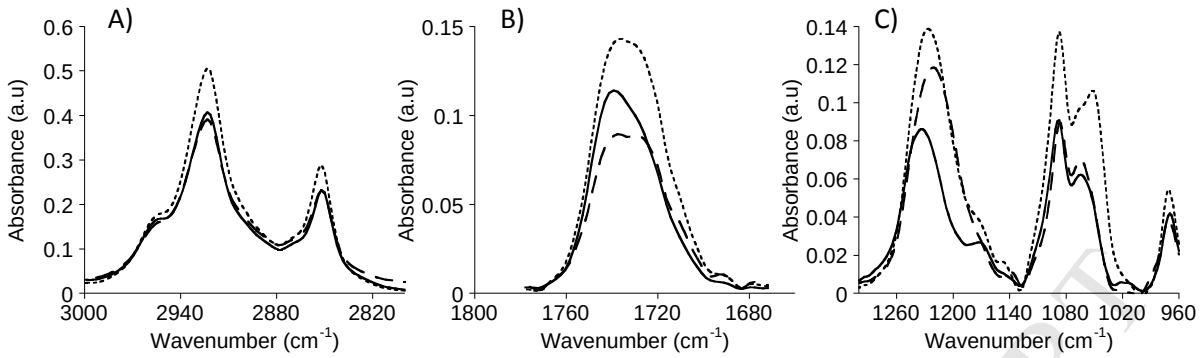

Figure 5: FTIR spectrum of pure POPC/cholesterol MLVs (continuous line) and POPC/chol MLVs with ααBolaX (small discontinued line) or ααBolaR (large discontinued line). A) 3000-2800 cm^{-1} region, B) 1800-1650 cm^{-1} region, C) 1280-960 cm^{-1} region.

The influence of cholesterol on the interactions at a molecular level was also investigated by FTIR spectroscopy (Figure 5). In the 3000-2800 cm^{-1} region, the spectrum of POPC/cholesterol MLVs showed three bands centered at 2954, 2924 and 2852 cm^{-1}. The presence of both bolaamphiphiles in the MLVs did not induce any significant shift of these bands, indicating that the alkyl chains were not involved in the interactions between lipids and bolaamphiphiles.It suggests that the presence of cholesterol within the bilayer changed the interactions between bolaamphiphiles and phospholipids, inducing probably a less deeper insertion of the bolaamphiphiles within the bilayer. This could be due to changes of the physical state of the bilayer in the presence of cholesterol or to a modification of the arrangement of lipid molecules preventing hydrophobic interactions between the alkyl chains of POPC and bolaamphiphiles. Concerning the C=O ester groups, a band centered at 1740 cm^{-1} with a shoulder at 1725 cm^{-1} was observed for POPC/cholesterol MLVs. When ααBolaX or ααBolaR was present, the band was shifted to 1737 cm^{-1} and the shoulder became more significant. These results suggest that the presence of both bolaamphiphiles within the

POPC/cholesterol MLVs increased the ratio of C=O ester groups involved in hydrogen bonds, as for POPC MLVs. In the 1280-1150 cm^{-1} region, the band corresponding to the anti-symmetric PO_2^- stretching vibrations was observed, as for POPC MLVs. It was shifted from 1231 cm^{-1} to lower wavenumbers when bolaamphiphiles were inserted into the lipids. The shift was more important for ααBolaR (to 1220 cm^{-1}) than for ααBolaX (1227 cm^{-1}), as observed for pure POPC MLVs.

3.3.2. Molecular modeling

In a first step, the 3D structure of both bolaamphiphiles was calculated (Figure 6). The structure of both bolaamphiphiles molecules was modeled taking into account the Z (Z-αα BolaX and Z-αα BolaR) or E (E-αα BolaX and E-αα BolaR) configuration of the double bond which could influence the conformation.

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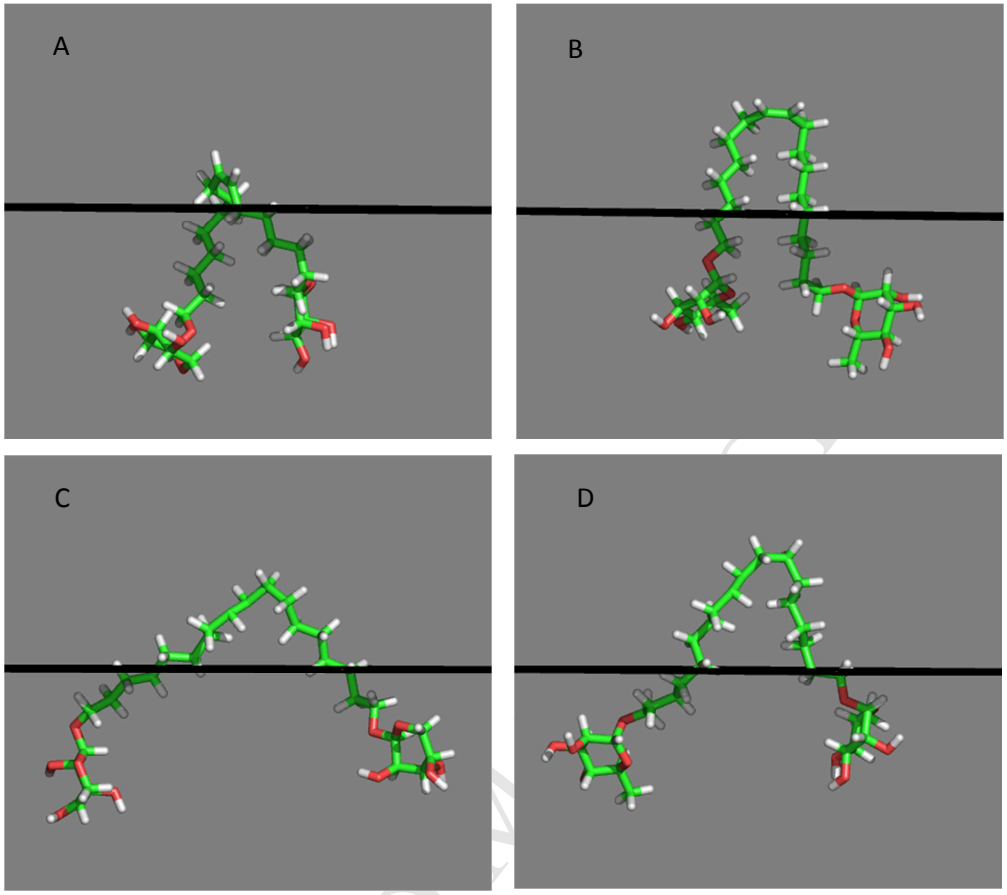

Figure 6: 3D-model of the Z-ααBolaX (A), Z-ααBolaR (B), E-ααBolaX (C), and E-ααBolaR (D). The figure was drawn using Pymol software. The black line corresponds to the interface.

Each model (*ie* Z and E-αα BolaX and BolaR) showed that the molecule has an amphiphilic configuration, with the hydrophilic sugar moeities located on the same side of the molecule and the hydrophobic alkyl chain on the opposite side. For both molecules, there was a hinge around the axis of the double bond leading to a U-shape conformation. The sugar moeities were oriented in opposite directions relative to each other. The Z configurations were more

folded, decreasing the interfacial area occupied by the bolaamphiphiles (Z-αα BolaX and BolaR occupy a molecular interfacial area of 85 and 70 Å² respectively). The E configurations were more extended with higher interfacial areas (E-αα BolaX and BolaR occupy a molecular interfacial area of 124 and 154 Å² respectively) (Figure 6 and Table 1).

Our previous work [31] has estimated by tensiometry measurements the area occupied by ααBolaX at an air-water interface to be 81 Å²/molecule and suggested a U-shape conformation of the bolaamphiphile. The interfacial area calculated from our modeling for ααBolaX Z and E configurations of (Table 1) is 85 and 124 Å²/molecule, respectively. The experimental value (81 Å²/molecule) was then in accordance with a Z configuration according to our calculations. It can further be suggested that the Z-αα BolaX is the most probable interfacial configuration compared to E-αα BolaX. For this the reason, only the Z configuration of each bolaamphiphile is taken into account for the following *in silico* analysis.

Table 1. Calculated interfacial area of bolaamphiphile models.

Bolaamphiphile Models	Calculated interfacial area (Å²/molecule)
Z-ααBolaX	85
E-ααBolaX	124
Z-ααBolaR	70
E-ααBolaR	154

The interaction energies of each bolaamphiphile and POPC molecules are calculated from Hypermatrix procedure and presented in figure 7A.

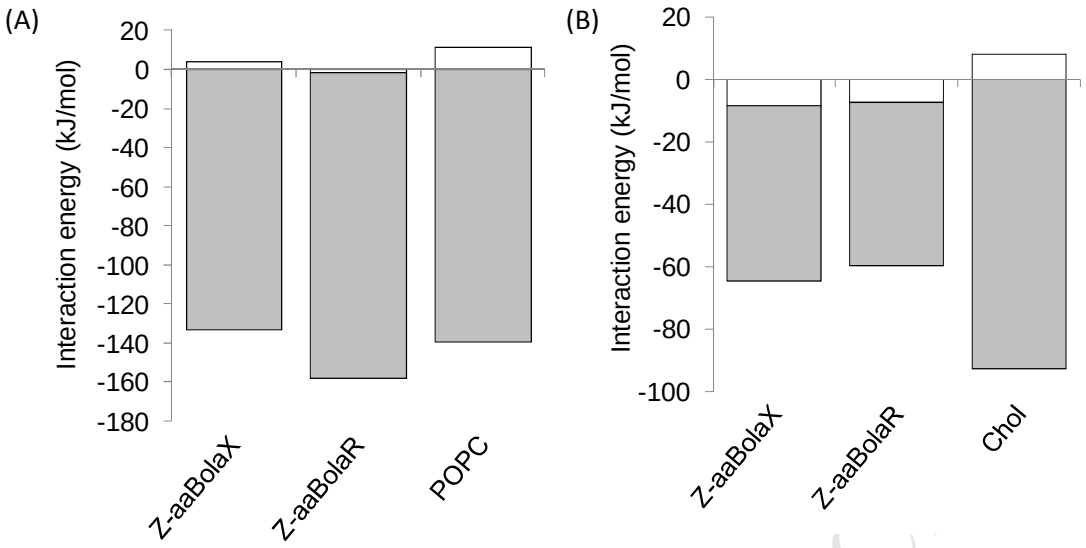

Figure 7: Total energy of interaction between POPC (A) or cholesterol (Chol) (B) and Z-ααBolaR, Z-ααBolaX or themselves in a multimolecular assembly. White and gray bars correspond to the polar and the hydrophobic contributions, respectively.

For all assemblies between the bolaamphiphile molecule and POPC, the total energy is in the same order as the one of pure POPC associations, suggesting a favorable interaction between each bolaamphiphile and the phospholipid. The energy is mainly hydrophobic and, although similar, is slightly higher (in absolute value) for αα BolaR than for ααBolaX.

The same procedure was carried out to calculate the interaction of bolaamphiphiles with cholesterol (Figure 7B). For both bolaamphiphiles, the interaction energies with cholesterol were lower compared to those of POPC and no difference was observed between ααBolaR and ααBolaX. In both cases, the total energy of the cholesterol-cholesterol interactions was higher than those of bolaamphiphiles with cholesterol, suggesting that the interaction of bolaamphiphile molecules with cholesterol is less favorable.

4. Discussion

4.1. Effect of bolaamphiphile conformation on their surface-active properties

In previous studies, both bolaamphiphiles were described as surface-active molecules with low critical aggregational concentration (CAC) and low corresponding surface tension (γ_{CAC}) compared to other conventional surfactants [29,31]. It was also shown that their aggregation behavior was mainly driven by an entropic process [29,31]. 3D models of ααBolaX and ααBolaR were constructed taking into the account Z- or E- configuration of the double bond. The structure calculations showed that each model of bolaamphiphiles (*ie* Z and E-αα BolaX and BolaR) adopt a conformation with distinct hydrophilic and hydrophobic as could be observed for other amphiphilic bolaamphiphile molecules [60,61]. Our results show an important role of the Z or E configuration on the surface organization of the bolaamphiphiles. The Z configuration occupying a reduced interfacial area is suggested to be more stable at the air-water interface and to mainly contribute to the surface-active feature of the bolaamphiphile.

4.2. What are the driven forces for the bolaamphiphile-phospholipid interactions?

A previous study showed that ααBolaX was able to interact with phospholipid monolayers constituted by DPPC [13,31]. Our results are in agreement with this study. They showed that ααBolaX and ααBolaR can insert within phospholipid monolayers (Π increase with time), can bind spontaneously to phospholipid bilayers ($\Delta G < 0$) and that they form energetically-stable assemblies with phospholipids (energy calculation). This is in accordance with the mixing behavior of other bolaamphiphiles with phospholipid models already demonstrated [62].

The synergy factor with PC monolayer was positive for both bolaamphiphiles suggesting an attractive interaction between bolaamphiphiles and phospholipids, as demonstrated for other molecules [36]. It means that in addition to the own surface activity of the bolaamphiphiles, other forces governed the bolaamphiphile binding to lipids. With the lipid bilayer models, it was shown that the bolaamphiphile-lipid associations are energetically driven by the hydrophobic interactions. At the molecular level, as shown by FTIR, both bolaamphiphiles interacted with the alkyl chains of PC. Both bolaamphiphiles increased the mobility of lipid alkyl chains and hence increased fluidity [63,64]. All together, our results indicate that the hydrophobic interactions involving the alkyl chains of lipids are the main driven forces for the interactions of bolaamphiphiles with phospholipids.

However, the interactions do not involve only the phospholipid alkyl chains but also their polar part, such as phosphate and carbonyl groups, as shown by FTIR spectroscopy. We suggest that during the interactions of bolaforms with phospholipids, a strong hydrophobic interaction between the alkyl chains of both molecules could occur, and could go along with interactions between sugar residues of bolaamphiphiles and polar head groups of phosphopholipids.

4.3. The effect of sterol on the interactions of bolaamphiphiles with biomimetic systems

The binding affinity of the bolaamphiphiles for lipid bilayers is markedly reduced in presence of cholesterol (smaller K). The unfavorable effect of cholesterol on bolaamphiphile interaction with phospholipids is also observed to a smaller extent for lipid monolayer models (lower MIP and α), even though the attractive nature of the interaction is still present ($\alpha > 0$).

At the molecular level, the presence of sterol within the MLVs system changed the interactions of bolaamphiphiles with lipids. Indeed, the interactions with the alkyl chains of

phospholipids seem to be less important in the presence of sterol while an involvement of the choline group of phospholipids is present. A different arrangement of the lipid molecules in the presence of sterol can hinder an accurate stabilization between phospholipids and bolaamphiphiles, as observed for other molecules [56,57]. Several studies of cholesterol/phospholipid monolayer or bilayer models have shown that the presence of cholesterol has a condensing effect on liquid-crystalline monolayers and increases the orientational order of the hydrocarbon chains of liquid-crystalline bilayers. The reduced mobility of the phospholipid hydrocarbon chains can explain the less favorable insertion of bolaamphiphiles into the lipid models containing cholesterol. This is in agreement with the interaction energy between bolaamphiphiles and sterol molecules, indicating that the association between bolaamphiphile and sterol is less favorable compared to pure sterol system. It further suggests that the presence of sterol within the system attenuates the hydrophobic interactions of bolaamphiphile with phospholipid membranes.

5. Conclusion and Perspectives

The interactions of two bolaamphiphiles, composed by two α xylosyls (ααBolaX) or two α rhamnosyls (ααBolaR) connected by a carbon chain of 18 carbon atoms with an unsaturation in the middle of the chain, with membranes were investigated by biophysical tools using different model membranes. Our results show that both bolaamphiphiles were able to insert within phospholipid monolayers or bilayers. No significant differences were observed between ααBolaX and ααBolaR for their membrane-interacting properties, indicating no specific effect of the sugar residues. However, the lipid composition of the model membranes played an important role. The presence of sterol within the lipid system attenuated the insertion of the bolaamphiphiles, even though it remained favorable. The

interactions with membrane lipids were energetically favored and driven by dominant hydrophobic interactions and in a smaller extent by hydrogen bonding. At the molecular level, the bolaamphiphile-phospholipid interactions involve the alkyl chains of phospholipids and their polar phosphate and carbonyl groups. In the presence of sterol, the alkyl chains are less involved but the polar part of the phospholipids including the choline group seems to be more implicated.

Due to their membrane interacting properties, ααBolaX and ααBolaR can be potential molecules of interest for the formulation of drug delivery systems. Our results suggest that the bolaamphiphiles better insert into membranes depleted in sterol and/or enriched in PC. Due to this distinct behavior against lipid systems, bolaamphiphiles could be useful for targeting one type of application or a given membrane, as lipid composition diversity are noticed between cell membranes. For example, Human erythrocytes membranes contain in approximately 50% cholesterol while this percentage is less than 10% for human alveolar macrophages [66–68].

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Acknowledgments

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Table

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Bolaamphiphile Models	Calculated interfacial area (Å²/molecule)
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E-ααBolaX	124
Z-ααBolaR	70
E-ααBolaR	154

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Figure captions

Figure 1: The structure of ααBolaX (left panel) and ααBolaR (right panel).

Figure 2: Determination of the maximal insertion pressure (MIP) of ααBolaX (●, ○) and ααBolaR (■, □) into pure DMPC (●,■) and mixed DMPC-cholesterol (Chol) monolayers (○,□). Inset: Synergy factor of ααBolaX (white bars) and ααBolaR (black bars) towards DMPC or DMPC-cholesterol monolayers.

Figure 3 : (A) and (B) thermodynamic parameters (black bars: $\Delta G_{bol}^{sol\rightarrow lip}$, white bars: $-T\Delta S_{bol}^{sol\rightarrow lip}$, grey bars: $\Delta H_{bol}^{sol\rightarrow lip}$) and binding coefficient (grey bars: ααBolaX and white bars: ααBolaR) for the binding of ααBolaX or ααBolaR to POPC or POPC-Chol vesicles at 25°C.

Figure 4: FTIR spectra of pure POPC MLVs (continuous line) and POPC MLVs containing ααBolaX (small discontinued line) or ααBolaR (large discontinued line). A) 3000-2800 cm^{-1} region, B) 1800-1650 cm^{-1} region, C) 1280-960 cm^{-1} region.

Figure 5: FTIR spectrum of pure POPC/cholesterol MLVs (continuous line) and POPC/chol MLVs with ααBolaX (small discontinued line) or ααBolaR (large discontinued line). A) 3000-2800 cm^{-1} region, B) 1800-1650 cm^{-1} region, C) 1280-960 cm^{-1} region.

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Table

Table 1. Calculated interfacial area of bolaamphiphile models.

Bolaamphiphile Models	Calculated interfacial area (Å²/molecule)
Z-ααBolaX	85
E-ααBolaX	124
Z-ααBolaR	70
E-ααBolaR	154

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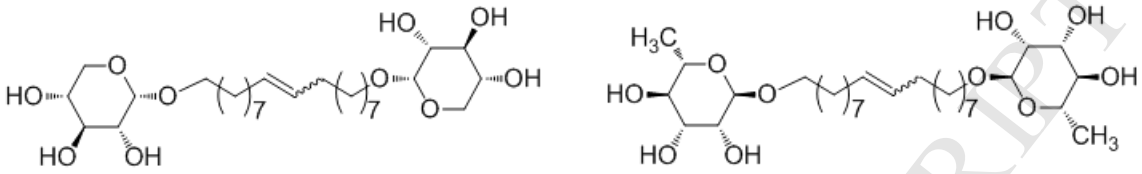

Figure 1

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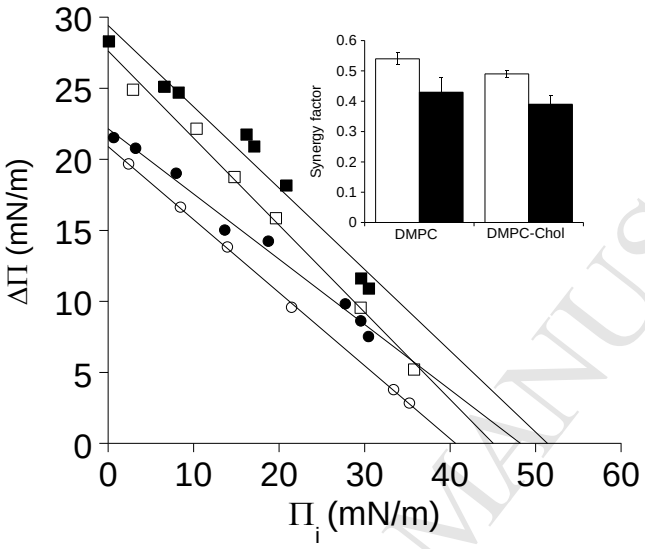

Figure 2

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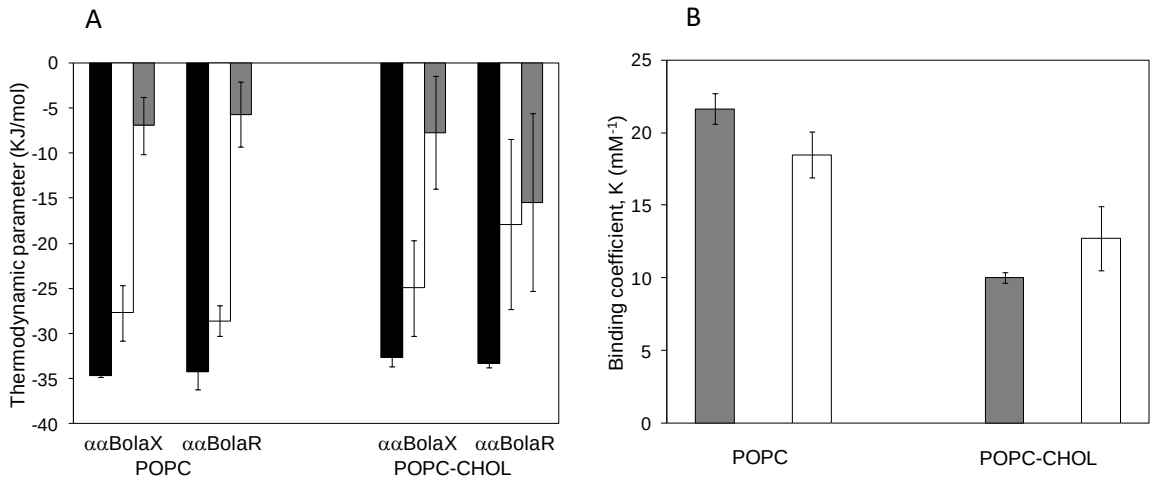

Figure 3

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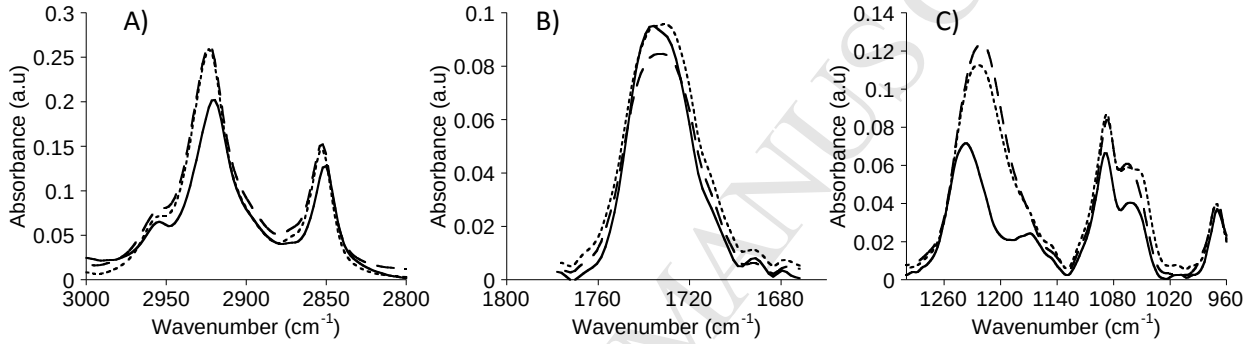

Figure 4

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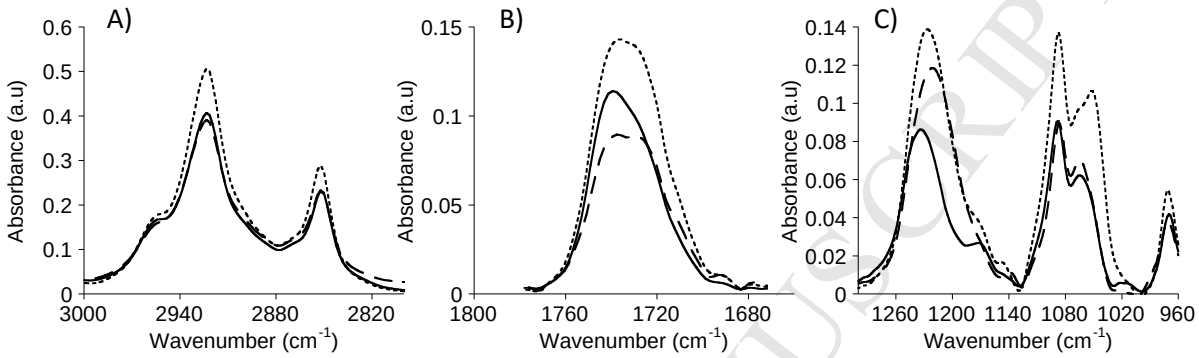

Figure 5

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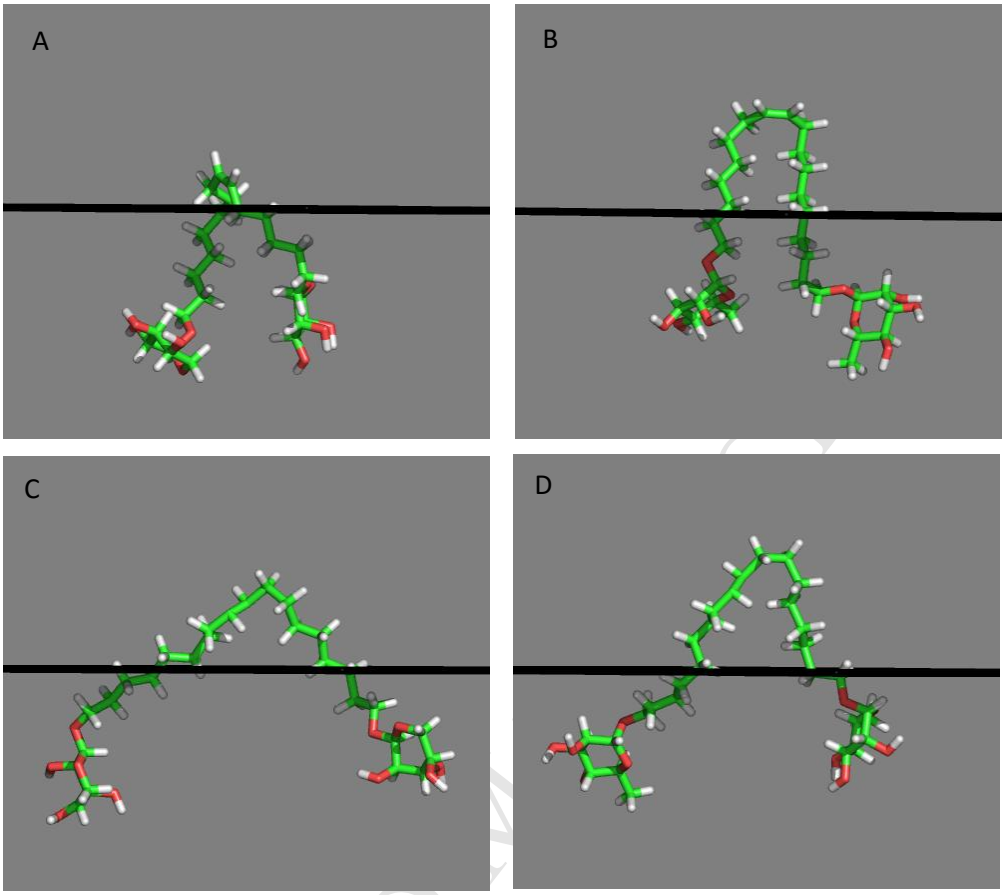

Figure 6

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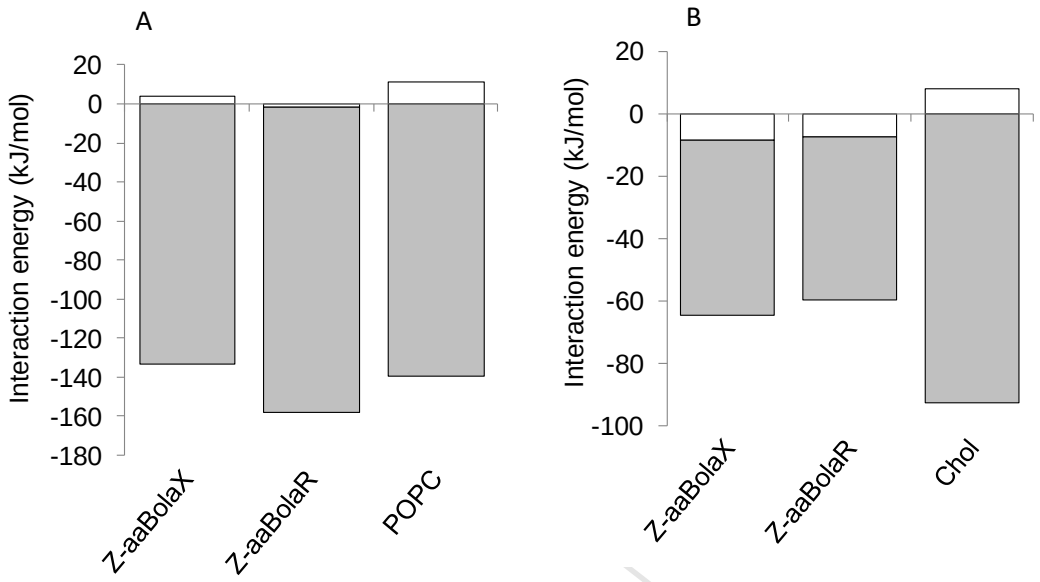

Figure 7

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Highlights

- Hydrophobic interactions are the main forces driven the interactions between bolaamphiphiles and model membranes.
- The polar part of phospholipids plays also a role in the bolaamphiphiles / phospholipid membranes interactions.
- Cholesterol decreases the binding affinity of bolaamphiphiles for model membranes.
- The modification of sugar moeities has no influence on the interactions between bolaamphiphiles and model membranes.

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