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ARTICLE

NMR study of the interaction of fluorescent 3-hydroxy-4-pyridinone chelators with DMPC liposomes

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In the present study we discuss the interaction of two fluorescent 3-hydroxy-4-pyridinone chelators (MRB7 and MRB8) of different lipophilicity with DMPC liposomes based on the analysis of the shifts of the resonance NMR signals and changes in the translational diffusion of both species. The analysis of the variation of the resonance signals of the chelators indicates that both MRB7 and MRB8 strongly interact with the lipossomes and that such interaction occurs through both the fluorophore and the chelating moieties of the chelator's framework. Analysis of the variations in the characteristic resonance signals of the lipid provides evidence that MRB7 is able to reach the hydrophobic zone of the bilayer independently of the chelator concentration. The present results corroborate the fact that ethyl substituents in the amino groups of the xanthene ring and the thiourea link are important to the chelator's ability to diffuse across the lipid bilayer.

Introduction

The design of metal ion chelators based on 3-hydroxy-4-pyridinone (3,4-HPO) bidentate units is a central topic in our research group. In view of the field of application we synthesized ligands of different denticity and modified the basic molecular framework with substituents that allow tuning the hydrophilic/lipophilic balance and the spectroscopic properties of the chelators.[1-11] By conjugating 3,4-HPO chelating units with fluorophores, derived from different families of molecules such as naphthalene[6], naphtalimide[7], fluorescein[8] and rhodamine[8-11], we prepared fluorescent chelators with different fluorescence emission spectrum and quantum yield, variable charge at physiological pH and distinct hydrophilic/lipophilic balance as a consequence of the different nature of the fluorophore bound to the chelating unit.

Iron is an essential nutrient for most living organisms, namely bacteria, fungi and protozoa. Numerous examples of the use of iron chelators showed the improvement in infection susceptibility of several pathogens.[12-21]

Aiming the development of new strategies to fight infection based on the concept of iron deprivation we designed fluorescent bidentate and hexadentate 3,4-HPO iron (III) chelators that proved to limit the access of iron to bacteria and have a significant inhibitory effect in the intramacrophagic growth of *Mycobacterium avium*.[8, 10, 22] The results showed that the activity of the chelators is strongly dependent on the presence of the xanthene fluorophore, its type and on the linker binding the fluorophore and the chelating unit.[10]

In a more recent study[23] we confirmed the relevance of the N-ethyl substituents on the rhodamine framework and the thiourea linkage for the biological effect. Moreover, co-administration of one of the chelators with a classic antimycobacterial antibiotic (Ethambutol) was advantageous for the control of *M. avium* infection.

We found that a differential interaction of the fluorescent chelators with membranes seems to be determinant for their differential cellular uptake and distribution. Moreover, we hypothesize that such distinctive interaction may ultimately be related with the observed antimycobacterial effect. To gain insight into structure activity relationships we performed Molecular Dynamic simulations and NMR studies of the interactions of the synthesized fluorescent 3,4-HPO chelators with DMPC liposomes as model membranes.[24] The results brought evidence about the capacity of the chelators to interact with lipid bilayers of liposomes and showed that different chelators interact with the lipid phases at different levels of the bilayer. The data also suggest that the interaction is strengthened for the rhodamine B isothiocyanate labelled compounds, which interestingly are those exhibiting the highest biological activity.[10]

Our previous study was performed using chelator concentrations identical to those used in the biological studies thus implying that the NMR analysis was only based on the alterations of the resonance signals of the lipid protons. In the

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ESI:_Figure 1; ESI_Figure 2. See DOI: 10.1039/x0xx00000x

present work, a more extensive and detailed NMR analysis is performed using a higher concentration of chelators MRB7 and MRB8 thus allowing generation of NMR signals of appropriate intensity for measurements based on the chelators NMR resonance signals. The mode of chelator/liposome interaction and binding efficacy of the chelators is assessed by trace analysis of their NMR spectroscopic behaviour and response upon incubation with liposomes through a certain time interval. NMR spectroscopy is particularly sensitive to structural and dynamical changes and well known as useful methodology for the study of inter-molecular interactions, such as the association processes between drugs and cellular membrane.[25-27] Diffusion ordered NMR spectroscopy (DOSY) has been established as a powerful technique for study of molecular interactions, binding and aggregation phenomena in solution. The self-diffusion coefficients of particular species are related to their size, shape and weight and may give valuable information on the specific interactions of these species with the molecular environment. [28, 29] DOSY is used to directly measure the mobility of lipids and trapped molecules in fluid lipid membranes giving information about the drug and liposome behaviour, and drug-membrane interactions. [24, 30-32]

Experimental

Materials

Chemicals were obtained from Sigma–Aldrich (grade puriss, p.a.) and were used as received unless otherwise specified. The lipid 1,2-dimyristoyl-sn-glycero-3-phosphocholine, (DMPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Methods

Liposome Preparation

DMPC liposomes were prepared by evaporation to dryness, using a stream of nitrogen, of a lipid solution in chloroform. The film was maintained under vacuum, for a minimum of 4 h, to remove all traces of the organic solvent. The resulting dried lipid film was dispersed with D_2O (≈ 2×10^{-2} M) and the mixture was vortexed above the phase-transition temperature (37 ± 0.1ºC) to produce multilamellar liposomes (MLVs). The multilamellar liposomes were then exposed to the following cycle five times: freeze vesicles in liquid nitrogen and thaw in a water bath at 37 ± 0.1 ºC. Lipid suspensions were equilibrated at 37 ± 0.1 ºC for 30 min and extruded ten times through polycarbonate filters (100 nm) to produce large unilamellar vesicles (LUVs). Extrusion of liposomes was performed with a Lipex Biomembranes (Vancouver, Canada) extruder attached to a circulating water bath. The size distribution of extruded DMPC liposomes was determined by dynamic light scattering analysis using a Malvern Instruments Zetasizer nano ZS.

Chelator Stock Solutions and Chelator/Liposome Mixtures for NMR Analysis.

The DMPC liposome suspensions were degassed with a stream of nitrogen for 20 min prior to the addition of the chelator stock solution.

Stock solutions of compounds MRB7 and MRB8 were obtained by preparing a solution of the compound in dimethylsulfoxide (DMSO-d6). For the preparation of NMR samples, 15μL of the chelator DMSO stock solution was added to the DMPC liposome suspension (≈ 2×10^{-2} M), to lead to a final concentration of chelator in the samples of 3×10^{-3} or 3×10^{-5} M, respectively. The studies with a chelator concentration of 3×10^{-3} M are designated as high concentration and samples denoted with a star (*) and a chelator concentration of 3×10^{-5} M, is designated as low concentration conditions. After addition of the chelator solution to the liposome suspensions, the mixtures were vigorously agitated for 5 min producing the samples labelled DMPC-MRB7*(2×10^{-2} M : 3×10^{-3} M), DPMC-MRB8*(2×10^{-2} M : 3×10^{-3} M), DMPC-MRB7(2×10^{-2} M : 3×10^{-5} M), DMPC-MRB8(2×10^{-2} M : 3×10^{-5} M).

The chelators MRB7 and MRB8 used in this work were previously synthetized and characterized in our laboratory.[10]

NMR Spectroscopy

NMR experiments were recorded on a Bruker Avance III 600 HD spectrometer, operating at 600.13 MHz for ^{1}H, equipped with 5 mm CryoProbe Prodigy and pulse gradient units, capable of producing magnetic field pulsed gradients in the z-direction of 50 G cm^{-1}. The NMR measurements were carried out in deuterium oxide (D_2O), at 37 °C and spectral width of 10000 Hz. ^{1}H NMR experiments were performed with water suppression using excitation sculpting with gradients.[33] The chemical shifts of the ^{1}H NMR signals were referred to the absorption frequency of the solvent deuteron monitored as the lock signal and further scaled to the resonance frequency of dimethylsulfoxide-d_6 (DMSO- d_6) added in equal amount (15 μL) to all samples.

Diffusion-ordered NMR (^{1}H DOSY) experiments were acquired using the bipolar longitudinal eddy current delay (BPPLED – Bipolar Pulsed Field Gradient Longitudinal Eddy Delay) pulse sequence.[34] The experiments were carried out using the same conditions, i.e. chelators and liposome concentration, temperature, no sample rotation and air flow of 500 L h-1. Before starting the NMR experiments, the temperature was equilibrated and maintained at 37 °C, as measured using the spectrometer thermocouple system. Typically, in each experiment a number of 16 spectra of 16K data points and 64 scans were collected, with values for the duration of the magnetic field pulse gradients (δ) of 4 ms, diffusion times (Δ) of 400 ms and an eddy current delay set to 5 ms. The pulse gradient (g) was incremented from 2 to 98% of the maximum gradient strength in a linear ramp. The spectra were processed with the Bruker Topspin software package (version 3.2). The diffusion coefficients (D) were determined from the resonance signals of the N-methyl protons of the choline residues of DMPC (γ in Figure 1) and were all scaled to the absolute D value obtained for DMSO-d6 (15 μL/per sample; 1.73×10^{-9} m^2 s^{-1}, calculated standard deviation of 1.1×10^{-3}) used as an internal reference. For each sample at least two independent assays were performed, using freshly prepared liposomes.

Results and discussion

The formulae and abbreviations of the fluorescent chelators under study in this work are depicted in Figure 1, together with the formula of the lipid DMPC. The numbering of the protons is included to facilitate the chemical shift assignment.

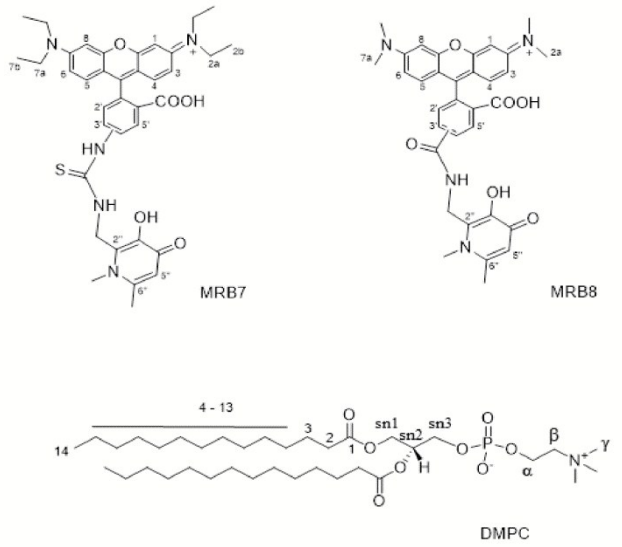

Figure 1. Formulae of MRB7 and MRB8 chelators and of the lipid 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC).

The two fluorescent chelators bear the same 3,4-HPO chelating unit and two rhodamine fluorophores that differ on the substituents of the xanthene amino groups, ethyl groups in MRB7 and methyl groups in MRB8, and on the type of linkage, a thiourea in MRB7 and an amide in MRB8. It is well known that the ethyl groups provide a higher lipophilicity than methyl ones and thiourea groups have been identified with antibacterial activity against Gram(+) and Gram(−) bacteria, due to their capacity to disturb the bacterial membrane.[35-38]

In the present work we compare the results obtained with two lipid:chelator molar ratios: (a) DMPC-MRB7*(2×10^{-2} M : 3×10^{-3} M); (b) DPMC-MRB8*(2×10^{-2} M : 3×10^{-3} M); (c) DMPC-MRB7(2×10^{-2} M : 3×10^{-5} M and DMPC-MRB8 (2×10^{-2} M : 3×10^{-5} M), the star denoting the higher concentration of chelator. The higher molar ratio has been used in order to allow for the minimum amount of chelator to generate NMR signals of appropriate intensity for measurements based on the chelators NMR spectra and the experiments using the lower molar ratio DMPC-MRB (2×10^{-2} M : 3×10^{-5} M) were repeated for consistency with the previous studies.[10, 24]

Induced chemical shifts alterations.

In the spectra obtained for the high concentration samples, DMPC-MRB7* and DMPC-MRB8*, it was possible to identify and register alterations on the resonance signals belonging to the chelators MRB7 and MRB8. Representative ^{1}H NMR spectra of DMPC-MRB7* and DMPC-MRB8* in D_2O recorded at 37 °C are shown in Figure 2. The ^{1}H NMR spectra of MRB7, MRB8 and DMPC liposomes acquired at the same experimental conditions with the assignment of characteristic resonance signals in the molecule formulae are included for comparison.

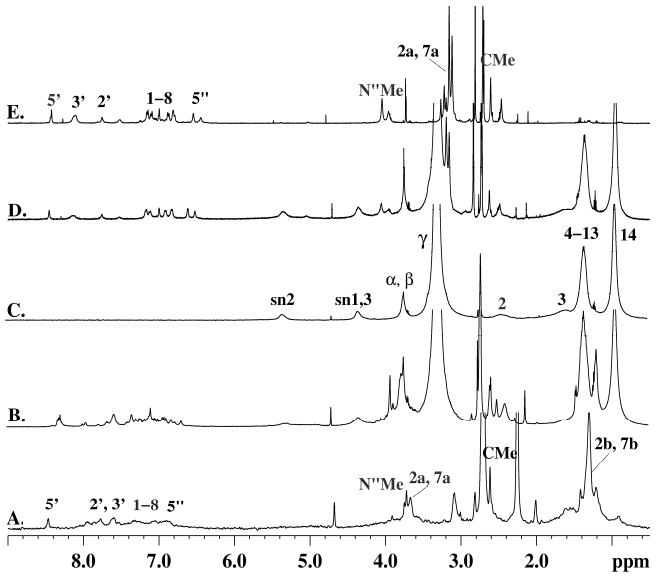

Figure 2. 600.13 MHz ^{1}H NMR spectra in D_2O at 37 °C of: A. MRB7; B. DMPC-MRB7* C. DMPC; D. DMPC-MRB8* and E. MRB8.

Analysis of the ^{1}H NMR spectra of the DMPC-MRB7* and DMPC-MRB8* samples shows that incubation of the chelators in DMPC liposomes induces shifts on the resonance signals of MRB7 and MRB8 thus implying the existence of an interaction chelator/liposome. The detailed assignment of the resonance signals of the chelators and the induced chemical shift changes recorded for the time range from 30 min to 60 h after addition of the chelators are registered in ESI Table 1. The analysis of the values shows that the interaction of the chelator occurs through both the fluorophore and the chelating unit parts of the molecule as revealed in the computational studies.[24] The proton resonance signals of the N-alkyl groups of rhodamine moieties and those of H5" and H5' of the 3,4-HPO chelating unit of MRB7 and MRB8 were found to be predominantly affected. Much stronger changes were registered for MRB7 almost immediately (in 30 min) after mixing with the liposomes. The observed chemical shift changes for the time interval between 30 min and 60 hours after mixing of MRB7 and MRB8 with liposomes reveal the dynamic nature of the interaction and much higher binding activity of the chelator MRB7.

Additional valuable information concerning the interaction and distribution of the chelators into liposomes was gained from the analysis of the response of the lipid resonance signals in ^{1}H NMR spectra after the addition of MRB7 and MBR8. NMR spectra of the samples prepared at low and high concentration of the chelators were measured in the time interval between 30 min and 60 hours and the data analysed. The results obtained for the choline protons N-$(CH_3)_3$, and -CH_2- and CH_3 protons of the acyl lipid chains of liposomes are shown in the graphics depicted in Figure 3 (and ESI: Figure 1). The corresponding chemical shift values for pure DPMC liposomes (without chelators) measured

at the same experimental conditions are included for comparison (time 0 h)

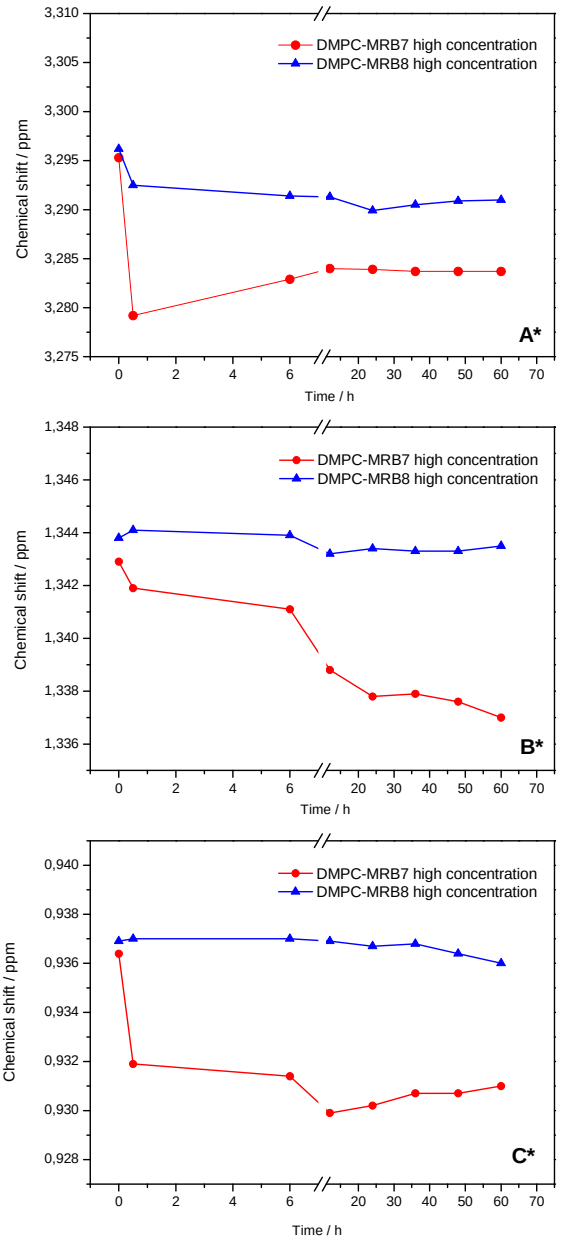

Figure 3. Chemical shift of protons of the choline (A*), -CH_2- of acyl chain (B*) and -CH_3 of acyl chain (C*) groups in DMPC structure in the presence of MRB7 (red circles) or MRB8 (blue triangles), at high (*) concentration.

The comparison of the results obtained for the two concentrations values demonstrates that the induced chemical shift alterations of liposome proton resonances depend not only on the structure but also on the concentration of the chelator being applied.

In the ^{1}H NMR spectra of DMPC-MRB7 and DMPC-MRB7* an up-field shift of the resonance signals of N-$(CH_3)_3$, -CH_2- and CH_3 protons of phospholipid chains was observed, the effect being stronger at higher MRB7 concentration. Regarding the N-$(CH_3)_3$ choline protons a significant drop in the chemical shift is observed in the first measurement upon 30 min after adding of MRB7 for both concentrations, with a further increase along the period between 6 and 12 hours and maintenance of the attained values up to 60 hours. The pattern is similar for both concentrations although the chemical shift drops are bigger for the DMPC-MRB7* samples. Contrastingly, for the -CH_2- and CH_3 resonances of the acyl chains, a first drop is observed upon incubation and significant alterations were registered after a period of 6 hours upon incubation of MRB7 with liposomes in the higher concentration samples. In fact, while for the DMPC-MRB7 samples the effect of the presence of chelator induces a drop that is more or less maintained for the DMPC-MRB7* a greater change is observed upon 6 hours of incubation, in particular for the -CH_2- protons of the acyl chain.

The results are indicative of an interaction of MRB7 molecules with the choline head groups from the outer leaflet shortly after adding of the chelator and further penetration and interaction with the lipid acyl chains from the interior of liposomes.

Through the time of the experiment, no change in the spectral line shape of the choline resonance was observed as a result of a preferred accumulation of MRB7 molecules at the surface of liposomes. This could be an indication for the transport and crossing of MRB7 molecules through the membrane, and ability for an interaction with the choline head groups of the inner layer of liposomes.

The analysis of the more concentrated samples provided additional information regarding the interaction of MRB7 with liposomes. In the region of the choline protons of the ^{1}H NMR spectra of DMPC-MRB7* recorded after 36 hours, we observed the appearance of an additional narrow signal, slightly shifted towards higher magnetic field values (3.246 ppm), and which exhibits a gradually increasing intensity along the time (Fig. 4, left). A resonance signal with the same NMR characteristics was registered in NMR spectra of DMPC liposomes recorded before performing an extrusion procedure (ESI: Fig. 2).

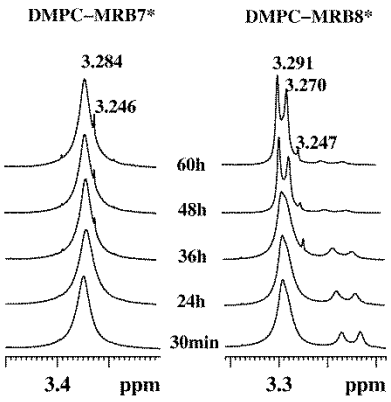

Figure 4. Choline resonance signals in 600.13 MHz ^{1}H NMR spectra of DMPC-MRB7* (left) and DMPC-MRB8*(right).

Considering the specific line shape and chemical shift, the resonance signal at 3.246 ppm in the ^{1}H NMR spectra of DMPC-MRB7* we hypothesize that this signal may be due to choline methyl protons of lipid acyl chains generated in a process of

damage of part of the DMPC vesicles in the presence of MRB7 at high concentration.[14, 39] In fact this signal is only visible after 36 hours in the concentrated samples in which the concentration of MRB7 is 100 times bigger.

The results obtained herein for MRB8 confirm the differential interaction of the two chelators with liposomes. In the ^{1}H NMR spectra of DMPC-MRB8 and DMPC-MRB8* chemical shift changes of the proton resonances from the choline methyl groups but no changes of those from the interior of the lipid bilayer (-CH_2- and -CH_3) were detected in the time interval of the experiment. According to our expectations, much stronger effect on the chemical shift of the N-$(CH_3)_3$ protons was observed in the spectra of DMPC-MRB8* due to the higher MRB8 concentration.

Moreover, in the ^{1}H NMR spectra of DMPC-MRB8* a broadening (in 36 h) and further splitting (in 48 h) of the choline resonance signal was observed (Figure 4). This splitting can be related to the accumulation of MRB8 molecules associated to the polar outer leaflet of DMPC liposomes, which allows the resonance signals of protons from the phospholipid head groups of the outer and inner layers to be distinguished. Our assumption was verified by analysis of ^{1}H NMR spectra of DMPC-MRB8* recorded in the presence of $SmCl_3$. The addition of samarium ions induced significant chemical shift (ESI: Fig. 3) of the choline resonance signal at 3.291 ppm and this signal was assigned to protons from the phospholipid head groups of the outer layer of liposomes. The observed chemical shift changes due to the pseudo contact shifts produced by shift reagents from the group of lanthanides, such as samarium ions.[40] In the ^{1}H NMR spectra of DMPC-MRB8* recorded in 36 hours, we also observed the appearance of weak signal at 3.246 ppm related to the generation of lipid acyl chains under the influence of MRB8 (Fig. 4, right).

The changes observed in the resonance frequency of protons belonging to specific functional groups of phospholipid bilayer offer direct evidence for the preferred distribution and location of the chelators within DMPC.[25] The magnitude of these changes can be related to the binding affinity of the chelators with respect to DMPC. Our data clearly indicate that MRB7 and MRB8 interact with liposomes but exhibit quite different permeation properties and binding affinity. The chemical shift changes in ^{1}H NMR spectra of DMPC-MRB8 and DMPC-MRB8* coincide with an interaction of MRB8 molecules with the polar surface of liposomes and preferred distribution between the polar interface and ester groups of lipid bilayer. Therefore, no perturbations of the proton resonances belonging to the lipid acyl chains were registered.

The significant changes in the ^{1}H NMR spectra of DMPC-MRB7 and DMPC-MRB7* indicate strong interactions of MRB7 with the choline head-groups at the surface and further deep penetration and distribution across the lipid bilayer of liposomes. The results exhibit the higher permeation and binding affinity of MRB7. We believe the presence of thiourea and N-diethyl functional groups in the structure of MRB7 facilitate the transport of molecules from the bulk water environment to the liposomes surface and their penetration and distribution within the phospholipid bilayer of liposomes. The deep penetration of MRB7 could be a reason for a distortion of the well-organized structure of liposomes, resulting in a generation of a certain amount of lipid acyl chains.[39] The same effect but much less displayed was observed for the chelator MRB8 added at high concentration (Figure 4).

Diffusion NMR spectroscopy

The diffusion coefficients (D) of the chelators in DMPC-MRB7* and DMPC-MRB8* and those of DMPC in all samples studied, i.e. DMPC-MRB7, DMPC-MRB7*, DMPC-MRB8 and DMPC-MRB8* were measured and compared with the corresponding values obtained for the free MRB7, MRB8 and DMPC. Proton registered DOSY spectra were recorded for the time interval between 30 min and 60 hours of incubation of MRB7 and MRB8 with liposomes. The diffusion coefficients (D) of the chelators and liposomes were determined from the resonance signals in aromatic spectral area and N-methyl protons of the choline residues, respectively, with estimated standard deviations $\leq 3.3 \times 10^{-3}$. All D values were scaled to the D value obtained for DMSO-d_6. The results are presented in Figure 5.

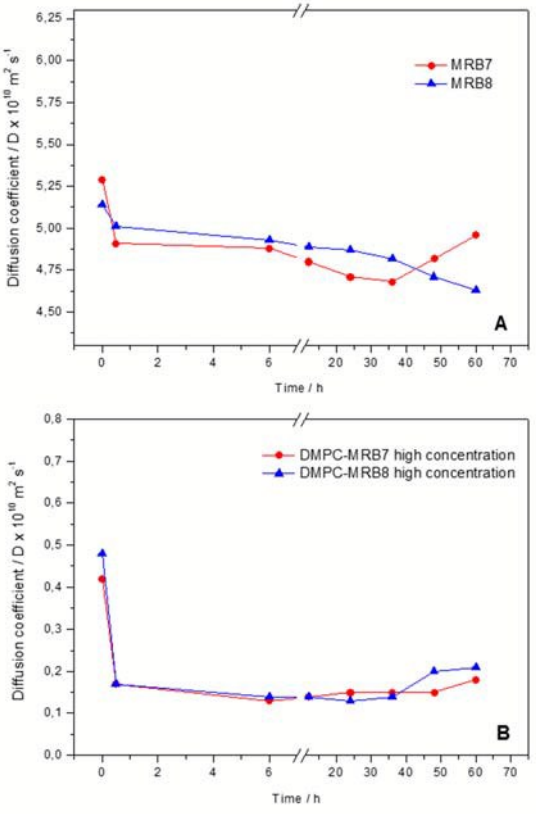

Figure 5. Diffusion coefficients (D x 10^{10} m^2 s^{-1}) obtained for (A) the chelators MRB7 and MRB8 and for (B) DMPC in the presence of MRB7 (red circles) or MRB8 (blue triangles).

Upon incubation of MRB7 and MRB8 in DMPC-MRB7* and DMPC-MRB8* samples we observed a decrease of the diffusion

coefficient values when compared to the corresponding values determined for MRB7 and MRB8 in aqueous solution. This result is an indication of the existence of an association process between the chelators and DMPC liposomes. The registered more significant reduction of MRB7 diffusion almost immediately (in 30 min) after mixing with the liposomes revels the more efficient binding and interaction of the chelator with phospholipid bilayer of liposomes. For the following period of 36 hours upon incubation with liposomes, both chelators, MRB7 and MRB8, exhibit similar translational diffusion behaviour with diffusion rates always slightly lower for the former (Figure 5A). The systematic lower diffusion of MRB7 in the liposomal matrix with respect to MRB8 suggests a more effective association and deeper penetration of MRB7 in the phospholipid bilayer of liposomes. Along the time interval between 36 and 60 hours a gradual increase of the diffusion rate of MRB7 was observed. The latter result supports the assumption made above that the presence of high concentration of MRB7 could damage liposomes thus provoking the release of not only lipid acyl chains but also release of MRB7 molecules.

Analysis of figure 5B shows that significant changes in the diffusion of liposomes occurs shortly after incubation of the chelators thus suggesting a transfer of the chelators from the bulk water environment to the liposome vesicles. The D coefficient of the liposomes in the all samples was found to gradually increase after a period of 36 hours of incubation with the chelators. The observed diffusion behaviour could due to partial damage of DMPC liposomes under the influence of both chelators and accumulation of smaller lipid species that contribute to the D values measured. In this case, the diffusion coefficients should be considered as average values between the phospholipid species presented in the system. The diffusion data (Figure 5B) suggest partial damage of liposomes after 36 hours of incubation with the chelators. The results coincide with the observed appearance of additional signals in the ^{1}H NMR spectra of DMPC-MRB7* and DMPC-MRB8* (Figure 4) and further support the assumption that the chelators MRB7 and MRB8 initiate a degradation of a certain amount liposomes resulting in a generation of lipid acyl chains or smaller liposomal vehicles.

Under the influence of higher concentrations of MRB7 and MRB8 similar diffusion coefficients were measured contrasting with the result obtained for a concentration of chelators 100 times lower.[24] Such a different behaviour is probably justified by the fact that in the high concentration conditions the number of chelator molecules is too high to allow distinction between the two chelators.

The overall results obtained from our previous and present study corroborate the fact that the presence of the ethyl substituents in the amino groups of the xanthene ring and the thiourea link are fundamental to the ability of the chelator to diffuse across the lipid bilayer. In Figure 6 we outline a possible distribution of the rhodamine labelled 3,4-HPO chelators in the membrane of liposomes vesicles, in which we emphasize the ability of MRB7 to cross the bilayer, a property that may be responsible for its higher ability to reach the infection targets.

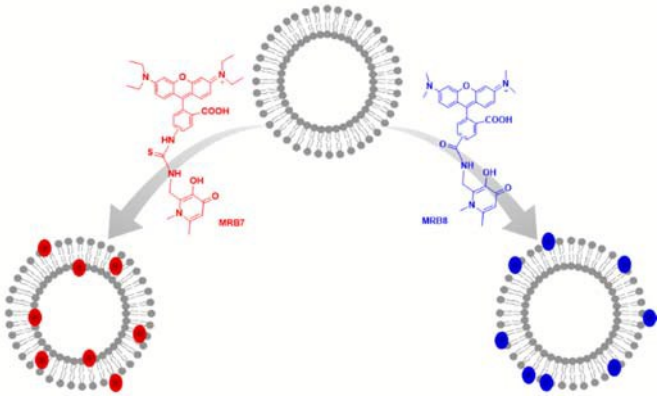

Figure 6: Outline of a hypothetical distribution of the rhodamine labelled 3,4-HPO chelators, MRB7 (red circles) and MRB8 (blue circles) in the membrane of liposomes vesicles.

Conclusion

In this work, we established convenient experimental conditions that allow the study of the interaction of rhodamine-labelled 3,4-HPO chelators with liposomes.

This study reveals that in high concentration, such as the one deliberately used in the present work, the chelators, in particular MRB7, may induce alterations in the liposome structure.

Taking into consideration the acquired knowledge, we expect to be able: (a) to implement these methodologies to the study of newly synthesized ligands and get insight on structure-activity relationships and (b) to correlate the relevance of several functional groups in the chelator structure with their ability to interact with biological membranes.

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References

1 A. Leite, A. M. G. Silva, A. Nunes, M. Andrade, C. Sousa, L. Cunha-Silva, P. Gameiro, B. De Castro and M. Rangel, *Tetrahedron*, 2011, **67**, 4009-4016.

2 R. B. R. Mesquita, R. Suárez, V. Cerdà, M. Rangel and A. O. S. S. Rangel, *Talanta*, 2013, **108**, 38-45.

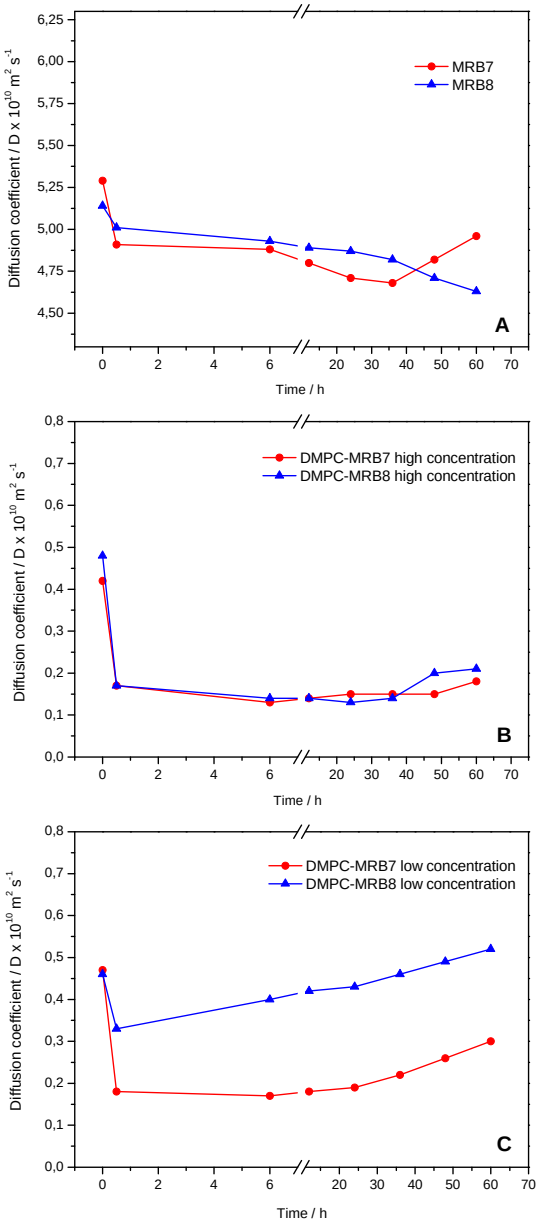
3 T. Moniz, M. J. Amorim, R. Ferreira, A. Nunes, A. Silva, C. Queirós, A. Leite, P. Gameiro, B. Sarmento, F. Remião, Y. Yoshikawa, H. Sakurai and M. Rangel, *Journal of Inorganic Biochemistry*, 2011, **105**, 1675-1682.
4 M. Rangel, A. Leite, A. M. N. Silva, T. Moniz, A. Nunes, M. J. Amorim, C. Queiros, L. Cunha-Silva, P. Gameiro and J. Burgess, *Dalton Transactions*, 2014, **43**, 9722-9731.
5 R. Suarez, R. B. Mesquita, M. Rangel, V. Cerda and A. O. Rangel, *Talanta*, 2015, **133**, 15-20.
6 A. M. G. Silva, A. Leite, M. Andrade, P. Gameiro, P. Brandão, V. Felix, B. de Castro and M. Rangel, *Tetrahedron*, 2010, **66**, 8544-8550.
7 T. Moniz, C. Queirós, R. Ferreira, A. Leite, P. Gameiro, A. M. G. Silva and M. Rangel, *Dyes and Pigments*, 2013, **98**, 201-211.
8 A. Nunes, M. Podinovskaia, A. Leite, P. Gameiro, T. Zhou, Y. Ma, X. Kong, U. E. Schaible, R. C. Hider and M. Rangel, *Journal of Biological Inorganic Chemistry*, 2010, **15**, 861–877.
9 A. Leite, A. M. Silva, L. Cunha-Silva, B. de Castro, P. Gameiro and M. Rangel, *Dalton transactions (Cambridge, England : 2003)*, 2013, **42**, 6110-6118.
10 T. Moniz, A. Nunes, A. M. G. Silva, C. Queirós, G. Ivanova, M. S. Gomes and M. Rangel, *Journal of Inorganic Biochemistry*, 2013, **121**, 156-166.
11 C. Queirós, A. Leite, M. G. M. Couto, T. Moniz, L. Cunha-Silva, P. Gameiro, A. M. G. Silva and M. Rangel, *Dyes and Pigments*, 2014, **110**, 193-202.
12 G. C. Chan, S. Chan, P. L. Ho and S. Y. Ha, *Hemoglobin*, 2009, **33**, 352-360.
13 B. D. Corbin, E. H. Seeley, A. Raab, J. Feldmann, M. R. Miller, V. J. Torres, K. L. Anderson, B. M. Dattilo, P. M. Dunman, R. Gerads, R. M. Caprioli, W. Nacken, W. J. Chazin and E. P. Skaar, *Science (New York, N.Y.)*, 2008, **319**, 962-965.
14 M. Luckey, *Membrane Structural Biology: With Biochemical and Biophysiscal foundations*, Cambridge University Press, United Kingdom, 2014.
15 D.-H. Qiu, Z.-L. Huang, T. Zhou, C. Shen and R. C. Hider, *FEMS Microbiology Letters*, 2011, **314**, 107-111.
16 C. Ratledge and L. G. Dover, *Annual Review of Microbiology*, 2000, **54**, 881–941.
17 R. J. Ward, R. R. Crichton, D. L. Taylor, L. D. Corte, S. K. Srai and D. T. Dexter, *Journal of Neural Transmission*, 2011, **118**, 315-328.
18 Y.-Y. Xie, M.-S. Liu, P.-P. Hu, X.-L. Kong, D.-H. Qiu, J.-L. Xu, R. Hider and T. Zhou, *Med Chem Res*, 2013, **22**, 2351-2359.
19 B. Xu, X. L. Kong, T. Zhou, D. H. Qiu, Y. L. Chen, M. S. Liu, R. H. Yang and R. C. Hider, *Bioorganic and Medicinal Chemistry Letters*, 2011, **21**, 6376-6380.
20 M. X. Zhang, C. F. Zhu, Y. J. Zhou, X. Le Kong, R. C. Hider and T. Zhou, *Chemical biology & drug design*, 2014, **84**, 659-668.
21 T. Zhou, G. Winkelmann, Z.-Y. Dai and R. C. Hider, *Journal of Pharmacy and Pharmacology*, 2011, **63**, 893-903.
22 S. S. Fernandes, A. Nunes, A. R. Gomes, B. de Castro, R. C. Hider, M. Rangel, R. Appelberg and M. S. Gomes, *Microbes and Infection*, 2010, **12**, 287-294.
23 T. Moniz, D. Silva, T. Silva, M. -S. Gomes and M. Rangel, *MedChemComm*, 2015, in press. DOI:10.1039/C5MD00456J
24 J. T. S. Coimbra, T. Moniz, N. F. Brás, G. Ivanova, P. A. Fernandes, M. J. Ramos and M. Rangel, *The Journal of Physical Chemistry B*, 2014, **118**, 14590-14601.
25 J. K. Seydel and M. Wiese, *Drug-Membrane Interactions: Analysis, Drug Distribution, Modeling.*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2002.
26 A. M. Seddon, D. Casey, R. V. Law, A. Gee, R. H. Templer and O. Ces, *Chemical Society reviews*, 2009, **38**, 2509-2519.
27 Y. Xu, V. E. Yushmanov and P. Tang, *Bioscience reports*, 2002, **22**, 175-196.
28 T. Brand, E. J. Cabrita and S. Berger, Prog. Nucl. Magn. Reson. Spectrosc., 2005, 46, 159-196.
29 Y. Cohen, L. Avram and L. Frish, Angew. Chem., Int. Ed., 2005, 44, 520-554.
30 N. Yoshii and E. Okamura, *The Journal of Physical Chemistry B*, 2011, **115**, 11074-11080.
31 E. Okamura and N. Yoshii, *The Journal of chemical physics*, 2008, **129**, 215102.
32 S. Lopes, M. Simeonova, P. Gameiro, M. Rangel and G. Ivanova, *The journal of physical chemistry. B*, 2012, **116**, 667-675.
33 T. L. Hwang and A. J. Shaka, *Journal of Magnetic Resonance, Series A*, 1995, **112**, 275-279.
34 D. H. Wu, A. D. Chen and C. S. Johnson, *Journal of Magnetic Resonance, Series A*, 1995, **115**, 260-264.
35 H. Kim, C. Lee, M.-G. Kim and H.-S. Lee, *Journal of the Korean Society for Applied Biological Chemistry*, 2009, **52**, 555-559.
36 M. Jang, E. Hong and G.-H. Kim, *Journal of Food Science*, 2010, **75**, M412-M416.
37 Z. Ngaini, Mohd Arif, M. Asyikin, H. Hussain, E. S. Mei, D. Tang and D. H. A. Kamaluddin, *Phosphorus, Sulfur, and Silicon and the Related Elements*, 2012, **187**, 1-7.
38 A. Sofrata, E. M. Santangelo, M. Azeem, A.-K. Borg-Karlson, A. Gustafsson and K. Pütsep, *PLoS ONE*, 2011, **6**, e23045.
39 S. Schreier, S. V. P. Malheiros and E. de Paula, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 2000, **1508**, 210-234.
40 G. Royden, A. Hunt and L. R. H. Tipping, *Journal of Inorganic Biochemistry*, 1980, **12**, 17-36.

Electronic Supplementary Information

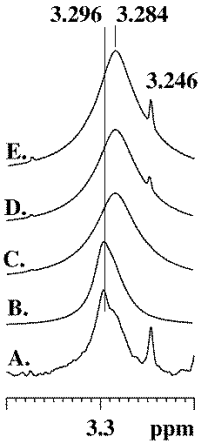
Table 1. Chemical shifts of protons in 600.13 MHz ^{1}H NMR spectra of MRB7 and MRB8 (0h) and the induced chemical shifts alterations after incubation with DMPC liposomes for 30 min, 24 h, 48h and 60 h.

	Chemical shifts and DMPC induced alterations (in ppm)									
^{1}H	MRB7		DMPC/MRB7*			MRB8		DMPC/MRB8*		
	0h	30min	24h	48h	60h	0h	30min	24h	48h	60h
1,8	7.044	+0.031	+0.031	+0.024	+0.023	6.986	- 0.010	0	+0.012	+0.015
3,6	6.963	-*	-*	-*	-*	6.898	-0.006	+0.004	+0.018	+0.018
4,5	7.297	+0.026	+0.026	+0.026	+0.026	7.155	0	+0.004	+0.011	+0.013
2a, 7a	3.622	+0.139	+0.139	+0.139	+0.140	3.175	0	+0.006	+0.022	+0.026
2b, 7b	1.254	-*	-*	-*	-*	-	-	-	-	-
H2'	7.728	-*	-*	-*	-*	7.736	0	+0.003	+0.008	+0.009
H3',4'	8.033	-*	-*	-*	-*	8.124	0	0	0	0
H5'	8.191	+0.099	+0.084	+0.084	+0.084	8.439	-0.006	-0.002	+0.017	+0.019
H5"	6.531	+0.137	+0.252	+0.252	+0.251	6.609	-0.011	+0.013	+0.045	+0.051
>NCH_3	3.947	- 0.042	- 0.044	- 0.061	- 0.061	4.041	- 0.005	- 0.005	- 0.001	- 0.003
>CCH_3	2.605	- 0.021	- 0.021	- 0.031	- 0.031	2.606	- 0.002	- 0.002	- 0.005	- 0.005

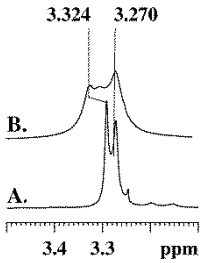
*Overlapped signals



ESI_Figure 1. Chemical shift of protons of the choline (A), -CH_2- of acyl chain (B) and -CH_3 of acyl chain (C) groups in DMPC structure in the presence of MRB7 (red circles) or MRB8 (blue triangles), at low concentration.



ESI_Figure 2. Choline resonance signals in 600 MHz ^{1}H NMR spectra (in D_2O, 310 K of DMPC liposomes before (A) and after (B) extrusion; and of DMPC-MRB7* after 24 h (C), 48 h (D) and 60 h (E) of incubation of DMPC liposomes with MRB7, respectively.



ESI_Figure 3. Choline resonance signals in 600 MHz ^{1}H NMR spectra of DMPC-MRB8* (in 60 h; in D_2O; at 310 K) before (A) and after (B) addition of $SmCl_3$.

ARTICLE Journal Name

Graphical Abstract

NMR study of the interaction of fluorescent 3-hydroxy-4-pyridinone chelators with DMPC liposomes

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The NMR results corroborate the fact that the presence of the ethyl substituents in the amino groups of the xanthene ring and the thiourea link are fundamental to the ability of the chelator to diffuse across the lipid bilayer and consequently relevant for their enhanced biological activity.