Rigid Multilamellar Bilayer Cooperativity is Modified by Non Covalently Linked Neuraminic-5-acid: a Spectrophotometric Determination

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Abstract. By means of recording a simple serie of merocyanine 540 spectra, we present a method to calculate the value proportional to co-operative unit size of membranes (n). Our calculations, applied to different liposomal samples processed in the presence or absence of sugars, in high or low ionic strength showed two main results. First, that any temperature cycling in high ionic strength of rigid DPPC bilayers will modify the membrane cooperativity. Second, the presence of polysaccharide Neu-5-ac in solution will always produce a strong drop in co-operativity of a rigid membrane of DPPC, whenever the negative charge is fully exposed. This last result indicates a differential ability of charged Neu-5-ac to disrupt a rigid membrane structure, even in the absence of a covalent linkage and – remarkably – in fully hydrated media.

Key words: Neu-5-Ac — MC_{540} — Multilamellar vesicles — Co-operative unit size

Abbreviations: DPPC – dipalmitoyl phosphatidylcholine; MC_{540} – merocyanine 540; DSC – differential scanning calorimetry; A – absorbance; MLV – multilamellar vesicle; EPR – electronic paramagnetic resonance; NMR – neutron magnetic resonance; FTIR – Fourier transform infrared spectroscopy; ε – molar absorbance; LC – liquid cristaline phase; G – gel phase

Introduction

The presence of carbohydrates affects interactions between membranes in two alternative ways: by exposure on the surface of the lipid bilayer or by binding or insertion at different membrane depths. In the former case, oligosaccharides from bilayer glycolipids or glycoproteins are recognised by a variety of proteins with lectin activity, which belong to serum or to other cell membranes (Cook 1994). Usually, a cascade

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of intracellular events is triggered as a response to this sugar-mediated recognition. Many toxins or microorganisms are able to enter *via* a sugar-specific site expressed in the surface of their host cells. Similarly, as an example of trans-interaction only mediated by a mechanical property of sugars at the surface, the cell-cell distances in nervous tissue are determined by the presence of long polysaccharides, which is the case of the α (2, 8) polysialic acid (a linear polymer of Neu-5-Ac) in the N-CAM molecules (Troy 1992; Rougon 1993). Neu-5-Ac is an anionic 9-carbon sugar which possesses an unusually low pK carboxylic group. Its negative charge, the glycerol lateral chain and the rigidity of its six-member chair ring structure, confer it special biological properties, mainly related to the regulation of cellular and molecular interactions. In the nature, Neu-5-Ac plays a dual role: it can either mask recognition sites like subterminal carbohydrate structures on proteins, or it can serve as recognition determinant (Schauer 1985; Hajela et al. 1994; Mukhopadhyay and Bush 1994; Kelm and Schauer 1997; Varki 1997). In model systems, sugars are exposed on the bilayer surface as a part of gangliosides or are covalently attached to cholesterol or phospholipid polar heads in order to resemble naturally occurring membrane disposition. However, as is the case with binding or insertion into the lipid bilayer, there is a direct perturbation of lipid arrangement of a given phase (Crowe 1988). This has been determined, for example, in studies of water replacement in dry media by trehalose (Crowe et al. 1984a; Lee et al. 1989; Goodrich et al. 1991). Because of the biological relevance of Neu-5-Ac, we looked for the existence of a direct but non-covalent perturbation on lipid membrane structure caused by the presence of Neu-5-Ac. To this aim, we prepared rigid multilamellar liposomes (MLVs) in the presence of high concentrations of monomers of Neu-5-Ac. Vesicles were submitted to a concentration process in order to reinforce sugar lipid interaction, followed by rehydration. Finally, the effects caused by Neu-5-Ac on lipid arrangement of the membrane were determined by DSC and Raman spectroscopy; alternatively, the same results were achieved by a colorimetric method by using absorbance properties of MC_{540}.

Materials and Methods

1. Materials

L-αDPPC, Merocyanine 540 (MC_{540}) and sialic acid (Neu-5-Ac) were purchased from Sigma Chemical CO, St Louis, U.S.A. D (+) Trehalose dihydrate was from Fluka Chemie AG (Buchs, Switzerland). All reagents employed were analytical grade.

2. Sample preparation

The most common method for incorporation of sugars to a phospholipid bilayer without employing covalent bonds is the dehydration process (D), by which a given sugar replaces water molecules associated with the phospholipid polar head. It is well known however, that the last 4–5 water molecules linked to the head moiety

are extremely hard to remove. As stated by some authors (Crowe et al. 1987), an overnight lyophilization from a phospholipid methanol-benzene solution is sufficient to obtain the dihydrate form. More extreme conditions, like applying vacuum combined with temperatures above the phase transition yield the monohydrate form, and from that on, stronger treatments produce molecular damage (Crowe et al. 1984b). In contrast, other authors (Bush et al. 1980) consider that an evacuation process of 1.33×10^{-3} Pa for more than 48 hrs is enough to completely dehydrate a phospholipid molecule, as shown by the absence of FTIR water bands at 3400–1620 cm^{-1}. Due to this, we decided to replace the dehydration step by an alternative approach, in order to incorporate sugar molecules to the bilayer. Basically, sample preparation consisted of concentrating a liposomal suspension in a 0.1 $mol \cdot l^{-1}$ sugar solution; in this way, the water content from the bulk phase is reduced, without modifying the polar head hydration. Briefly, chloroform solutions of L-αDPPC were evaporated at 50 °C in a rotary evaporator until a thin phospholipid film was formed on the wall of a 250 ml glass balloon. Solvent traces were eliminated by N_2 stream. The films were suspended at 50 °C, stirring for 10 min. in different solutions: solution a) Neu-5-Ac 0.1 $mol \cdot l^{-1}$ in PBS buffer, solution b) Neu-5-Ac 0.1 $mol \cdot l^{-1}$ in deionized water, solution c) trehalose 0.1 $mol \cdot l^{-1}$ in PBS buffer, and solution d) trehalose 0.1 $mol \cdot l^{-1}$ in deionized water, at 1 : 10 lipid : sugar monomer molar ratio. After this, the concentration process (C) was performed. Through this process, most of the bulk water of the liposomal suspension was eliminated at 50 °C within ca. 30 min. (a condition soft enough to prevent polar head dehydration, as stated before) by rotary evaporation until a semisolid residue formed. The rehydration process (R) was then performed on these residua, be it with PBS (HIS samples) or deionized water (DW samples) at 50 °C, up to a final phospholipid concentration of 7.35 $\mu g/\mu l$. Samples obtained were designed as follows: a) HIS C/R Neu-5-Ac MLV, b) DW C/R Neu-5-Ac MLV, c) HIS C/R trehalose MLV and d) DW C/R trehalose MLV.

To show the effect of successive steps to which membranes were submitted, we also prepared four samples – representing the four different contributions to membrane processing – ending at HIS C/R MLV, the more complex preparation. Those contributions are summarised as follows:

I. Common MLV: film suspended in deionized water.

II. High ionic strength MLV (HIS MLV): film suspended in PBS.

III. Deionized water C/R MLV (DW C/R MLV): film suspended in deionized water and submitted to 2 consecutive passages through phase transition temperature (temperature cycling in deionized water). First, a raise of temperature is produced in order to eliminate most of the bulk solvent, until semi-solid residuum forms. Second, a temperature raise is produced when bulk solvent is added to reconstitute the original sample volume. In this case, bulk water is first eliminated and then restored.

IV. High ionic strength C/R MLV (HIS C/R MLV): film suspended in PBS and sub-

mitted to 2 subsequent passages through the phase transition temperature (temperature cycling in high ionic strength medium). During both temperature transition passages, the solvent of PBS buffer is consecutively eliminated and then restored up to the final original volume. Nevertheless, non-volatile ionic content of PBS buffer is obviously not eliminated together with the bulk water. Then, an increased ionic concentration in the external bulk compartment is produced at the end of the process.

3. Size measurements

Size measurements were carried out in a Nicomp Analyzer, solid particles mode.

4. Raman spectroscopy

A Spec Ramalog with scamp microprocessor, and 488.0 nm Krypton 165-11 Spectra Physics laser line excitation at 500 mW were used. 100 μl liposomal suspensions (7.35 g DPPC/l) in 500 μl Pyrex tubes were measured. A previous calibration with Ne emission line was done. Measurement accuracy was ± 4 cm^{-1}.

5. DSC determinations

Thermal analysis of the samples was carried out in a Perkin Elmer-DSC 7 Series Thermal Analysis System. Identical mass of Common MLV and HIS C/R MLV (110.25 ± 0.02 μg) were measured. First run was discarded.

6. Colorimetric assays

6. 1. Phase transition temperature as measured by MC_{540} in the external medium

Liposomal suspensions prepared as stated above in 2, were diluted in 1500 μl PBS buffer (HIS C/R samples) or deionized water (DW C/R samples), up to a final concentration of 0.34 mmol·l^{-1} DPPC. MC_{540} was added to external medium from a fresh day ethanolic stock solution of 1 mg.ml^{-1}, to a final concentration < 3 μmol·l^{-1}. The molar DPPC : MC_{540} ratio was 100 : 1. Spectra were run from 400 to 600 nm and fluidity was recorded as [A (570 nm)/A (550 nm)], from 25 °C to 50 °C at a rate of one data point/(0.5–1) °C. Temperature measurement accuracy was of ± 0.5 °C. A Shimadzu double beam UV-160 A, with jacket cells connected to a thermosthatized Lauda water bath was used. Phase transition temperatures were determined by applying a sigmoidal fit program (Micro Cal Origin 2.86, Micro Cal Software, Inc.), and expressed as average of 4 measurements. In order to determine whether or not the presence of sugars altered the signals obtained MC_{540} spectra of PBS buffer or deionized water Neu-5-Ac 0.1 mol·l^{-1} and trehalose 0.1 mol·l^{-1} solutions were run in the absence of vesicles. The obtained spectra showed no difference when compared with those obtained in the absence of sugars. This probe can be found as a monomer, dimer or even greater aggregates, depending on its concentration and the presence of lipid surfaces in the dye vicinity. Working conditions must be carefully fixed; otherwise obtained spectra are hardly reproducible.

6 2 Phase transition amplitude as a rough estimate of membrane phase transition entropy (ΔS_T)

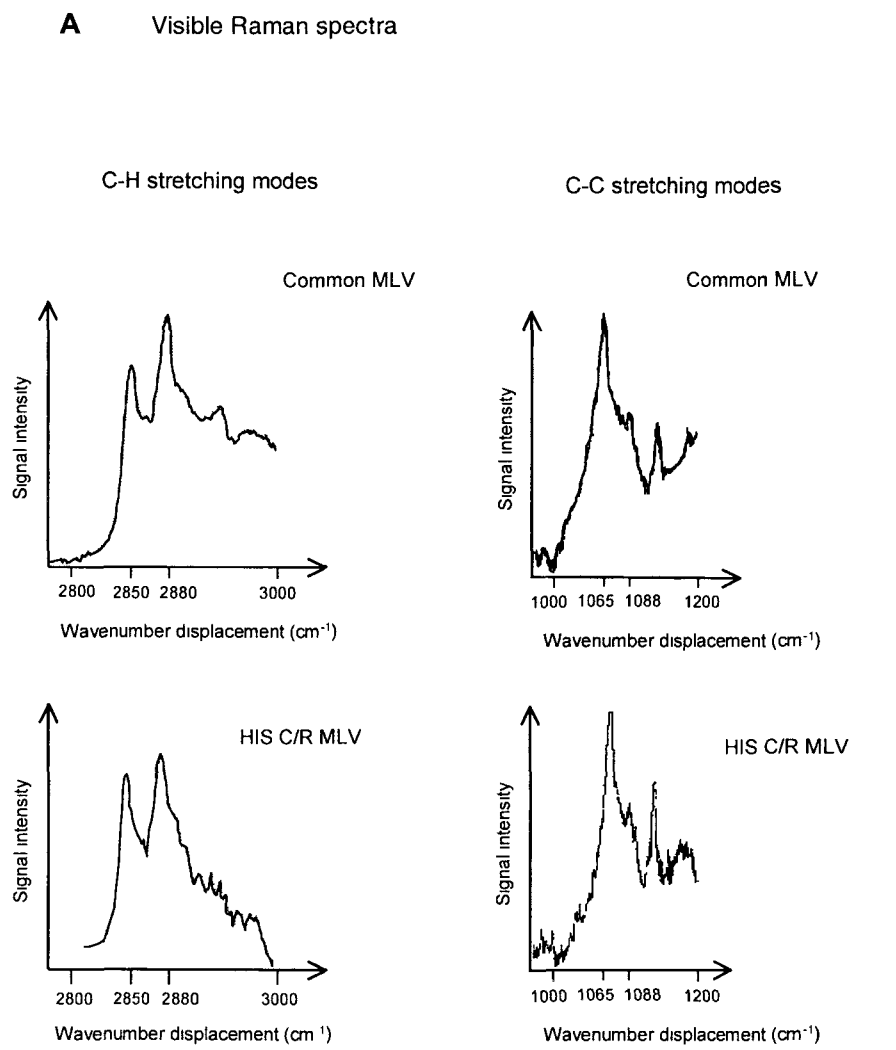
To carry out the colorimetric determination of phase transition entropy, we made use of a relation previously studied by Raman spectroscopy (Lapides and Levin 1982), between phase transition amplitude (ΔI_r, measured as change in peak height of C-H stretching mode ratio) and the phase transition entropy (ΔS_T) According to this, ΔI_r of a homologous series of phosphatidylcholines and ΔS_T are linearly related In this work, we calculated the term ΔI_r from spectral parameters produced by the interfacial dye MC_{540} instead of using Raman spectroscopy To justify data replacement from Raman spectroscopy by MC_{540} colorimetric signals, it is necessary to point out that the former technique does not consider the contribution of water molecules gathering associated with gel to liquid crystalline phase transition Those water molecules are responsible for the negative component of the total ΔS_T, which is defined as $\Delta S_T = (\Delta S_{IC} + \Delta S_{IF} + \Delta S_{GW})$ (IC inter-chain, IF intra-chain interactions, GW gathering of water molecules) Similarly as with Raman spectroscopy, MC_{540} does not report any additional order introduced by the incorporation of water molecules into the bilayer during the phase transition Because of its interfacial location, the dye is sensitive only to changes in dielectric constant related to the separation of the phospholipid acyl chains In this special case then, data obtained by Raman spectroscopy and MC_{540} provide the same type of information, although their origins are quite different In order to estimate the phase transition amplitude, $[A(570\text{ nm})/A(550\text{ nm})_{LC}]/[A(570\text{ nm})/A(550\text{ nm})_G]$ (LC liquid crystalline phase, G gel phase) the ratio was obtained from phase transition temperature curves (see 6 1)

Results

The obtained results can be divided in two parts, one providing direct structural data about liposomal membranes (Raman spectroscopy, size and calorimetric measurements), and the other one, corresponding to the colorimetric phase transition data, measured with Neu-5-Ac MLV MC_{540} Those colorimetric data were used to calculate the resulting spectrophotometric parameters, on which we will base our further discussion

1 Raman spectroscopy

Figure 1a shows the Raman spectra of sugar-free multilamellar liposomal suspensions, common MLV and HIS C/R MLV, in the region of the C-C and the C-H stretching modes of the acyl chains Using the results presented in Figure 1a, we calculated the intra- and inter-chain order parameters for the two samples The calculations were performed as follows intensity ratios ($I_{1088\,cm^{-1}}/I_{1062\,cm^{-1}}$) and ($I_{2850\,cm^{-1}}/I_{2880\,cm^{-1}}$), both are shown in Figure 1b and Figure 1c We can see that there is no alteration in the intra-chain order parameter but a significant increase in the inter-chain order parameter, i e a higher disorder, after the liposomal sample is submitted to the HIS C/R process



2. Size measurements

This value express the vesicles mean diameter which ranged from 7 ± 3 μm (common MLV and HIS C/R MLV), 16 ± 5 μm (HIS C/R Neu-5-Ac MLV), 12 ± 4 μm (HIS and DW C/R trehalose MLV), and 600 ± 150 nm (DW C/R Neu-5-Ac MLV). Liposomal populations showed a unimodal distribution. Results are expressed as mean value of 3 independent measurements, corresponding to 3 different batches runned. The large size of DW C/R Neu-5-Ac MLV can be interpreted as resulting from swelling, due to the presence in the membrane of negatively charged Neu-5-Ac molecules, which are not screened in deionized water.

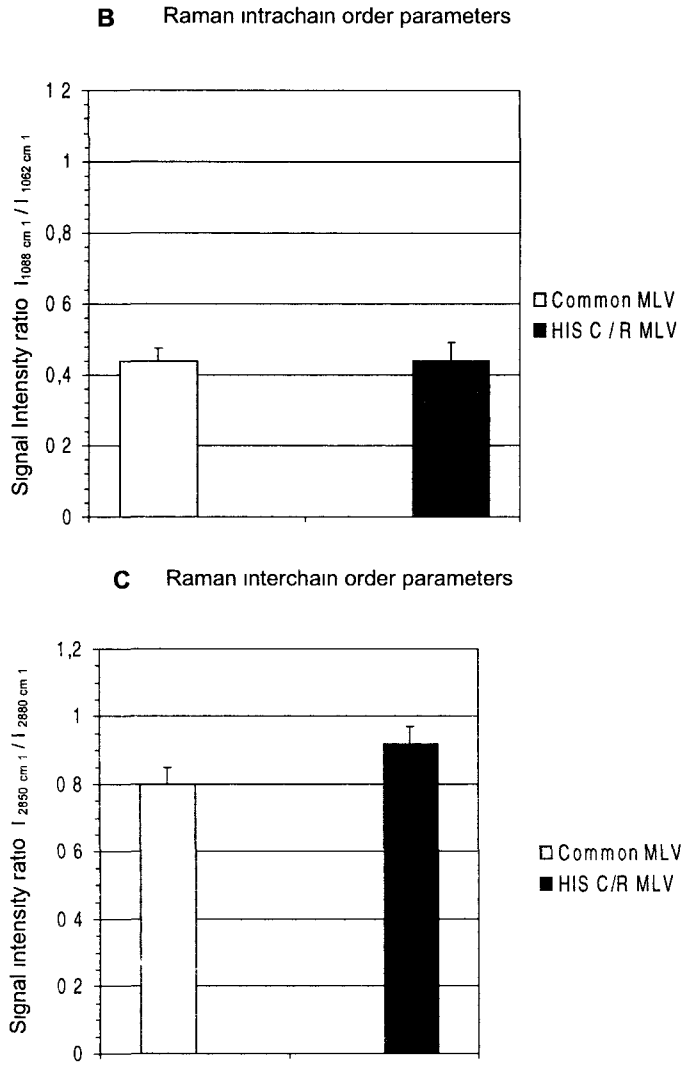


Figure 1. A. Visible Raman spectra of liposomal samples without sugar Each spectrum is the result of four scans **B.** Temperature cycling + high ionic strength do not modify Raman intrachain order parameters Mean common MLVs band intensities $I_{1088\,cm^{-1}}$ 2 1, $I_{1064\,cm^{-1}}$ 4 5 Mean HIS C/R MLVs band intensities $I_{1088\,cm^{-1}}$ 2 3, $I_{1064\,cm^{-1}}$ 4 6 Standard deviations shown in the Figure correspond to intensities ratio as a result of 2 independent measurements Intensities were calculated after baseline correction (not shown) **C.** Temperature cycling + high ionic strength do increase Raman interchain order parameters Mean common MLVs band intensities $I_{2850\,cm^{-1}}$ 14, $I_{2880\,cm^{-1}}$ 16 2 Mean HIS C/R MLVs band intensities $I_{2850\,cm^{-1}}$ 10 6, $I_{2880\,cm^{-1}}$ 11 5 Standard deviations in the Figure correspond to intensities ratio as a result of 2 independent measurements Intensities were calculated after baseline correction (not shown)

3. DSC measurements

Thermograms of common MLV and HIS C/R MLV are shown in Figure 2. We determined them in order to obtain a direct estimate of the transition enthalpy per gram of sample, which is proportional to the area under the curve (Yang et al. 1988) and inversely proportional to the van't Hoff change of enthalpy of the transition (McMullen and McElhaney 1995). We can see that the area under the curve of common MLVs is around twice as bigger than the corresponding area derived from the HIS C/R MLV samples. Areas under each thermogram result from three independent runs corresponding to 3 different batches, for each type of sample. The three runs did not show appreciable differences between each other.

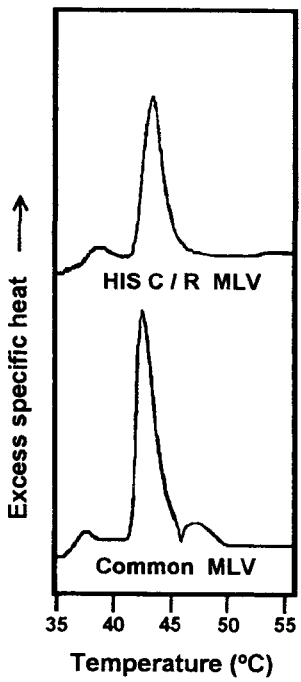


Figure 2. Membrane thermograms in the absence of sugars

4. Colorimetric measurements with MC_{540}

By employing MC_{540} to measure the width of phase transition temperature curves, phase transition amplitude (ΔI_r) and phase transition temperature, it is possible to obtain a value proportional to the co-operative unit size, expressed as n. Assuming that for any system undergoing a first-order phase transition at T_T, the free energy does not change, then:

$$0 = \Delta G_T = \Delta H_T - T_T \Delta S_T$$
$$\Delta H_T = T_T \Delta S_T$$
$$\Delta H_{VH}/\Delta H_T = n$$
$$\Delta H_{VH}/T_T \Delta S_T = n$$

Since ΔH_{VH} (van't Hoff width of the phase transition) is proportional to the slope of $\ln[A(570\text{ nm})/A(550\text{ nm})]$ plotted *vs* $1/T$ (Biltonen 1990), $\Delta S_T \propto \Delta I_r \propto [A(570\text{ nm})/(550\text{ nm})_{LC}/A(570\text{ nm})/A(550\text{ nm})_G]$ and T_T is feasible to be calculated from the slope of the fluidity curve (measured as $A(570\text{ nm})/A(550\text{ nm})$ *vs.* temperature, it is possible to estimate n only from MC_{540}. The bigger the value of n, the more cooperative the process is, whereas a sharper phase transition is produced.

Figure 3 shows a typical plot of $[A(570\text{ nm})/A(550\text{ nm})]$ ratio *vs.* temperature. The non-dimensional spectrophotometric parameters obtained from this plot, recorded for each type of samples, are shown in Table 1. The resulting n^*, calculated from non-dimensional parameters of vesicles in the presence and absence of sugars, are shown in Figures 4, 5 and 6 Figure 4 clearly shows the increase of n^* corresponding to HIS C/R MLV as compared with the other samples processed in the absence of sugars. In contrast, Figure 5 shows the decrease of n^* calculated for DW Neu-5-Ac C/R MLV with respect to the other samples prepared in deionized water, a condition where charge screening is maximized. Finally, Figure 6 compares the value of n^* for HIS C/R MLV with n^* HIS C/R samples processed in the presence of sugars; in this high ionic strength medium, Neu-5-Ac charge is supposed to be partly or totally screened

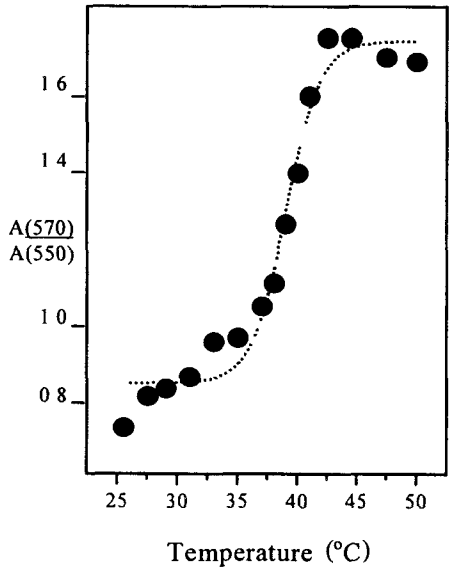


Figure 3. Phase transition temperature curve

Table 1. Non-dimensional spectrophotometric parameters

Samples	T_T[(1)]	ΔS_T[(2)]	$\Delta S_T T_T$[(3)]	ΔH_{VH}[(4)]	n[(5)]
Common MLV	315 6	4 1	1293 8	−104	0 08
HIS MLV	315	3 2	1007 8	−74	0 07
DW C/R MLV	315 5	3 5	1104 1	−93	0 08
HIS C/R MLV	314 3	2 1	659 9	−113	0 17
DW trehalose C/R MLV	314 4	2 4	754 4	−74	0 1
HIS trehalose C/R MLV	315 3	2 4	757	−62	0 08
DW Neu-5-Ac C/R MLV	309 4	2	619	−32	0 05
HIS Neu-5-Ac C/R MLV	312 9	2 5	782 1	−69	0 09

(1) Phase transition temperature (K) (2) Proportional to the phase transition amplitude determined as $[A(570\text{ nm})/A(550\text{ nm})_{\text{liquid crystalline phase}}/(A(570\text{ nm})/A(550\text{ nm})]_{\text{gel phase}}$ (3) Proportional to ΔH_T (4) Corresponds to slope In $[A(570\text{ nm})/A(550\text{ nm})]$ *vs* $1/T$ (K) (5) Corresponds to $(\Delta H_{VH}/\Delta S_T T_T) = n$ (proportional to the cooperative unit size)

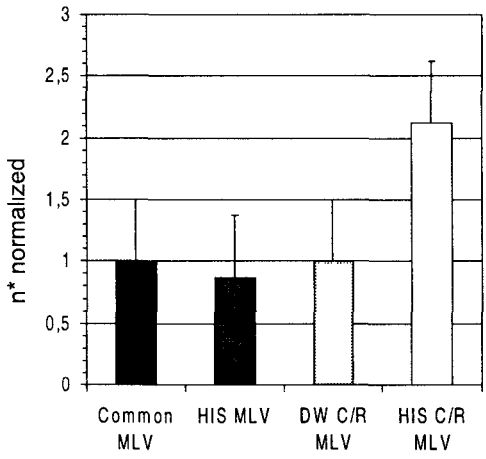


Figure 4. Bilayer n^* in the absence of sugars, n^* are normalized against common MLV n^* value $n^*_X/n^*_{\text{Common MLV}}$ (X sample)

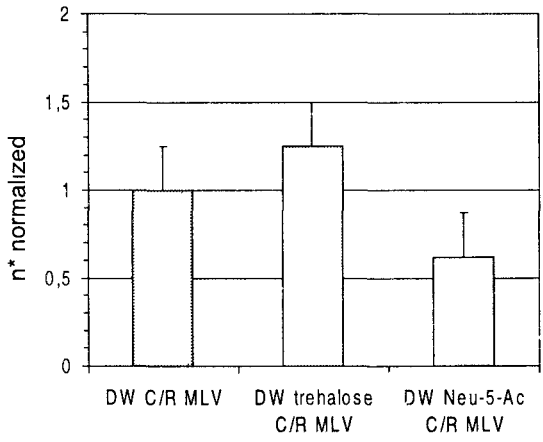


Figure 5. Effect of sugars on n^*, deionized water (DW) medium

Discussion

In this work, we present a simple approach to determine the effect caused on bilayer arrangement by non-covalently linked sugar molecules. Provided that a simple relation between width, amplitude and phase transition temperature allows the calculation of $\Delta H_{vH}/\Delta S_T \cdot T_T = n^*$ (proportional to the size of the co-operative unit) we show that only by using a colorimetric probe it is possible to obtain results accurate enough to assess the co-operative unit size in multilamellar liposomes This avoids the use of any other complex or expensive methodology intended to determine n^*. Secondly, we applied this approach to rigid multilamellar liposomes suspended in highly concentrated sialic acid solution with the purpose of finding

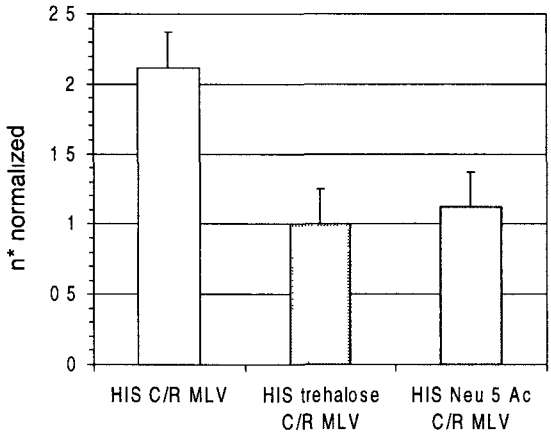


Figure 6. Effect of sugars on n^*, high ionic strength (HIS) medium

out whether there is an alteration or not in membrane co-operative unit size, related to the presence of this sugar

1 MC_{540} as a colorimetric tool for assessing bilayer co-operative unit size

MC_{540} spectral properties are dependent on the solvent polarity, specifically, they are a function of its dielectric constant In pure water, at concentrations below 10^{-6} mol l^{-1} the dye is considered to be completely in the monomer form (Ehrenberg and Pevzner 1993) The water monomer spectrum shows two peaks at 500 and 532 nm, with an ε of $0\,6 \times 10^5$ mol^{-1} l cm^{-1} for both bands Those bands undergo a red shift when the solvent polarity decreases, the same as for other cyanine dyes (West and Pierce 1965) At concentrations higher than 60×10^{-6} mol l^{-1}, MC_{540} molecules are fully in the dimer form (Ehrenberg and Pevzner 1993) The dimer in water shows a 500-nm band and a hypochromic blue shift of the 532-nm band Between 10^{-6} mol l^{-1} and 60×10^{-6} mol l^{-1}, MC_{540} is found as a mixture of dimers and monomers At the dye concentrations used in this work, ionic strength does not interfere with MC_{540} spectra (Šikurová et al 1995), signals obtained at high ionic strength are not different from spectra in distilled water (Lelkes and Miller 1980) MC_{540} undergoes additional dimerization in the presence of lipid bilayers in the gel phase A 525-nm peak corresponding to a dimer form appears specifically associated with membranes in the gel phase Conversely, when the membrane is in the fluid phase, the dimer-monomer equilibrium is displaced to the monomer form, which shows a characteristic 567-nm peak (Ehrenberg and Pevzner 1993) This 567-nm peak corresponds to an environment with a dielectric constant of 6 8, associated with the glycerol backbone of the phospholipids Above the phase transition temperature, MC_{540} is placed at membrane interface, as inferred from the shift observed in the membrane-dimer spectra (500–525 nm) to the in-membrane monomer spectra (525 567 nm) (Waggoner and Grinvald 1977) Since this probe locates at bilayer interface, local perturbations caused on the membrane structure

can be strong enough to mask differences of signals corresponding to phospholipid bilayers, with different arrangements and polarity of environments (Lelkes and Miller 1980). Due to this, to diminish local perturbations, the final MC_{540} concentration used in this work ranged between 2–3 $\mu mol \cdot l^{-1}$. It is assumed also that the dye molecules are fully bound to the membrane for a 100 : 1 PC : MC_{540} molar ratio (Dodin and Dupont 1987). According to the properties mentioned above, the dependence of signals on the polarity of the microenvironment is used to check membrane fluidity, usually expressed as $[A(570\ nm)_{lipid-monomer}/A(500\ nm)_{water\ peak}]$ ratio (Lelkes and Miller 1980), since the dye distribution between water and membrane changes abruptly at phase transition temperature. We choose, however, the $A(570\ nm)_{lipid-monomer}/A(550\ nm)$ ratio to express membrane fluidity, with 550 nm being the average of the two isosbestic points of the dye (555 nm in water and 545 nm in the lipid phase) (Verkman and Frosch 1985; Ehrenberg and Pevzner 1993). The isosbestic point is a signal only dependent on the total amount of absorbing species (dimer and monomer form) rather than on their relative proportion. The 550-nm point is an appropriate reference then, since absorbance at this medium wavelength is practically not modified by membrane phase fluidity.

2. Acyl chain order parameters as determined by Raman spectroscopy

The C-C stretching modes ($I_{1050-1150\,cm^{-1}}$) of Raman spectra are used for assessing intramolecular acylic chains order. Characteristic bands of this region are the out-of-phase skeletal stretching motion at $I_{1062\,cm^{-1}}$, and the in-phase skeletal stretching motions at $I_{1100\,cm^{-1}}$ and $I_{1029\,cm^{-1}}$. The former is both chain length and temperature insensitive, whereas the two others are dependent on temperature and chain length. Upon chain melting, the intensity of the all-*trans* features decreases, due to the presence of *gauche* conformers. This type of intramolecular disorder is indicated by the increase of the $I_{1088\,cm^{-1}}/I_{1062\,cm^{-1}}$ ratio. The C-H acyl chains stretching modes ($I_{2800-3100\,cm^{-1}}$) are used to check the acylic chain lateral packing. Characteristic peaks of this zone are the methylene symmetric stretching band at $I_{2850\,cm^{-1}}$ and the methylene asymmetric stretching band at $I_{2880\,cm^{-1}}$.

There are two situations responsible for the raise of chain interaction (equivalent to chain order): the shortening of inter-chain distance or the increase of all-*trans* conformer's content. In both cases, the $I_{2850\,cm^{-1}}/I_{2880\,cm^{-1}}$ ratio diminishes. Nevertheless, the $I_{2880\,cm^{-1}}$ band raises if *trans* conformer's content increases, whereas the $I_{2850\,cm^{-1}}$ signal only gets lower and wider, if a tighter chain packing increases the interchain order. It is also assumed that all-*trans* conformer content doesn't change as long as the temperature is kept constant (Kint et al. 1992; Batenjani et al. 1994).

a. MLVs without sugar

If the non covalent inclusion of sugar molecules in lipid bilayers requires C/R steps, we considered necessary to find out whether or not the C/R process itself modifies the membrane structure. To gain insight into the effect of this process on bilayer cooperativity, we determined n^* in common MLV, HIS MLV, C/R MLV

and HIS C/R MLV As shown in Figure 4, n^* for HIS C/R MLV increased only 2 fold as compared with the other of the membrane processes Raman spectra of common MLV and HIS C/R MLV showed no modifications in the intra-chain order parameter, as determined by the constancy of $I_{1088\,cm^{-1}}/I_{1062\,cm^{-1}}$ ratio (Fig 1b) Since *trans* rotamer's content is constant (due to the constancy of temperature), we ascribe the increase of the $I_{2850\,cm^{-1}}/I_{2880\,cm^{-1}}$ ratio to a longer intra-chain distance (Fig 1c) in HIS C/R MLV as compared with common MLV

Taken together, Raman spectroscopy and spectrophotometric data from MC_{540} indicate that bigger domains (increased n^*) with larger inter-chain distance are produced as a result of temperature cycling in high ionic strength According to this, final structural modifications of sample HIS C/R MLV cannot be considered as being the result of separated contributions only temperature cycling in a high ionic strenght medium considerably affects the membrane cooperativity, in spite of the absence of sugars It can be stated that liposomal bilayers do alter their lipid arrangement as a consequence of the treatment to incorporate sugars

Now we will compare the results obtained by our colorimetric method with those obtained by DSC, in vesicles with the same phospholipid mass, average size and size distribution Remarkably, the area under the thermogram curve, corresponding to the change of enthalpy (ΔH_T per gram) of common MLVs is almost 2-fold bigger (0 51/g) than the corresponding parameter for HIS C/R MLV (0 30/g) Clearly, these results can also be achieved by our colorimetric determinations (see Table 1), where $\Delta H_T = \Delta S_T\ T_T$ for common MLV (1293 4) is around twice the value for HIS C/R MLV (659 9) It must be stressed that numeric values obtained by our colorimetric method are relative and non-dimensional, in order to establish a comparison between different samples Although the enthalpy values obtained by DSC and MC_{540} are numerically different, one has to keep in mind that the degree of difference between common MLV and HIS C/R MLV remains the same (two times) for both methods

b MLVs with sugars

Provided that results obtained by DSC are supporting the alternative use of spectrophotometric data, we applied the colorimetric approach to samples containing sugar, in order to determine their respective n^*

HIS Neu-5-Ac C/R MLV and HIS Trehalose C/R MLV a comparable effect caused by the presence of sugar in PBS buffer is observed in DPPC membranes after the C/R process It is expected that the carboxylate charge of Neu-5-Ac is fully screened in PBS, so trehalose and Neu-5-ac can be considered non-charged molecules Both sugars have similar molecular weights (ca 310–320), and as determined by n^* calculations, they produce similar effect on membrane cooperativity (Fig 5)

DW Neu-5-Ac C/R MLV and DW Trehalose C/R MLV a striking contrast is observed between effects exerted by charged and non-charged sugars In Neu-5-Ac containing samples n^* is decreased about 30% in respect to the control sample (DW C/R MLVs) The phase transition temperature is also diminished 6°C On the

other hand, trehalose-containing samples do not modify n^* or the phase transition temperature (Fig 6)

It is well known that a given bilayer exhibits domains (Datta 1987, Gennis 1989, Israelachvili and Wennerstrom 1992) which are lipid arrangements with the same packing and orientation, separated by defect zones (e g , grain boundaries, hydrophobic defects) where lipid concentration is very low Whether in the gel or in the liquid crystalline phase, the existence of domains has been predicted by molecular dynamic simulation and detected by freeze etching electronic microscopy An important feature is that the domain size is directly proportional to the co-operative unit size of membranes (Mouritzen and Kinnunen 1996) The co-operative unit size, a theoretical concept, is usually calculated from data obtained by DSC, and it is inversely proportional to the width of the phase transition, it can also be expressed as $\Delta H_{\mathrm{VH}}/T_{\mathrm{T}}\ \Delta S_{\mathrm{T}}$ Complex and expensive equipment is routinely used to characterise bilayer-sugar interactions, such as NMR, FTIR, EPR, DSC, fluorescence spectroscopy, (Dubnickova et al 1997, Ondrias et al 1997), etc In the present work, aiming at avoiding the traditional employment of these techniques, we used colorimetry as an alternative way to calculate n^*, a value proportional to the co-operative unit size n

The size of lamellar domains can be reversibly varied, e g , by temperature cycling or through impurity content This can be observed by fluorescence microscopy upon increasing the impurities concentration, there is an increase in domain density, domain shape becomes carved and does not anneal for hours (Losche and Mohwald 1984, Florsheimer and Mohwald 1989) It is also known that solute molecules (mainly of hydrophobic nature) are prone to become incorporated into those defect zones, causing minimal membrane packing distortion, but diminishing domain size, i e , decreasing bilayer cooperativity Particularly in our system, if sugar molecules behave like impurities, they should probably occupy lipid-depleted zones of the membrane, i e , hydrophobic defects In this respect, it is conceivable that at the beginning of the temperature cycling in PBS, DPPC membranes present a certain amount of hydrophobic defects which are further reduced along the membrane processing, until a more co-operative (bigger n^*) and loosely packed bilayer is produced But when this process is accomplished in the presence of sugars, n^* does not increase and remains at its initial value The concordance of n^* obtained for HIS Neu-5-Ac C/R MLVs and HIS trehalose C/R MLVs, with the n^* obtained with common MLVs, indicates that both Neu-5-Ac and trehalose accommodate on the hydrophobic defects initially exposed, blocking the annealing process usually occurring during temperature cycling in PBS and impairing membrane homogenisation (higher n^*) In high ionic strength, trehalose and Neu-5-Ac behave similarly (although having different molecular structures), probably because hydrophilicity of Neu-5-Ac is decreased in PBS, due to the screening of the carboxylate charge But when placed in deionized water, Neu-5-Ac produces a further decrease in n^*, while the effect of trehalose on membrane is negligible This indicates the permanence of trehalose on hydrophobic defects without significantly modifying the lipid packing, and is accomplished by a further reduction in phase transition temperature Briefly,

our experimental evidence suggests that even when water molecules are not eliminated from phospholipid polar heads (due to the employment of a concentration process instead of real dehydration), both trehalose and Neu-5-Ac interact with the membrane mediated by sugar occupation of hydrophobic binding sites in the bilayer But probably the charge exposure of Neu-5-Ac produces a superimposed ionic type of interaction, since in deionized water this carbohydrate is unable to fit on hydrophobic defects without causing further packing distortion. Remarkably, in spite of the absence of a covalent linkage, Neu-5-Ac molecules produce an additional reduction in overall membrane cooperativity when its carboxylate charge is fully exposed.

The biological role of Neu-5-Ac has been systematically associated to surface recognition. This sugar usually points out to the extracellular medium and is always linked to an oligosaccharide core. Considering the ability of Neu-5-Ac to change lipid arrangement of these rigid bilayers, perhaps another effect not straightly associated with a specific ligand-receptor interaction, but involved in direct changes in lipid structure of a biomembrane has to be considered

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