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Spectroscopic analysis of the interaction of a peptide sequence of Hepatitis G virus with bilayers

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Abstract

Merocyanine 540 (MC540) has been used as external probe to determine the interaction of the peptide sequence 125–139 corresponding to the E2 protein of Hepatitis G virus, with lipid bilayers. The probe was incorporated into large unilamellar vesicles (LUVs) or small unilamellar vesicles (SUVs) of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). When incorporated into bilayers, MC540 shows two absorption maxima corresponding to the monomer (570 nm) and dimer (530 nm) form of the probe. Changes in the probe microenvironment are reflected by a modification in the position and/or intensity of these maxima. Addition of increasing amounts of peptide resulted in a slight decrease of the ratio A570/A530 thus indicating a change in MC540 partition into the membrane, going from a hydrophobic to a more hydrophilic environment. This effect was concomitant with an increase in dimer formation as stated from the values of the apparent dimerization constant (K_{app}) obtained. Fluorescence spectra as well as steady state anisotropy measurements were in agreement with the above results indicating that the peptide was able to relocate the probe and displacing MC540 from its initial location into the bilayer. Results with SUVs or LUVs were similar for what curvature does not seem to play any role on peptide activity. These results reflect the ability of peptide to interact with biomimetic membranes in the lipid head group region.

Keywords: Hepatitis G; Peptide; Merocyanine 540; Liposomes

1. Introduction

Merocyanine 540 (MC540) is an amphipathic anionic molecule with a well-defined absorption and fluorescence properties, that has been used as a probe of model and biological membranes [1,2]. The negative charge of the probe determines its location at or near the membrane interface, slightly above the domain of the glycerol backbone of neutral and charged phospholipids as assessed by the extinction coefficient and maximum wavelength [3]. Only its neutralization, as reported by Bilski et al. [4] will allow a deeper probe penetration into the non-polar hydrophobic membrane interior.

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The spectral properties of MC540 depend strongly on the fluidity and polarity of its environment. In aqueous solution, MC540 spectra shows two main maxima centered at ~500 and ~530 nm attributed to the dimer and monomer form of the probe, respectively. In a hydrophobic environment, for instance a micellar solution or a lipid bilayer, these maxima are red-shifted to ~530 and ~570 nm for the dimer and monomer, respectively. Therefore, the absorption intensity as well as the position of these maxima are strongly dependent on the mono or bilayer interfacial packing density. When the probe locates in a more hydrophobic environment, the A570 increases whereas the A530 is slightly reduced. For that reason, the ratio A570/A530 indicates MC540 partitioning into the two media. Consequently, MC540 monomer–dimer equilibrium is relevant to the packing properties of the bilayers and can be used as an indication of lipid head group spacing as well as the surface properties of the membranes [5]. Similar effects are observed in fluorescence measurements, when the probe is located in a hydrophobic environment, its emission maximum is red-shifted and the quantum efficiency is enhanced. Changes in MC540 fluorescence maximum or/and intensity give information about membrane microenvironment [6,7] and for that reason it has been used to report modifications of molecular packing upon the addition of cholesterol [8] or fructans [5]. Fluorescence is higher in fluid membranes above the main phase transition of the membrane lipid(s), than in the gel state.

Most of the studies performed with MC540 were done in presence of model bilayers, mainly liposomes. Quantitative probe incorporation into bilayers is achieved by incubating MC540 with lipid vesicles above the main transition temperature (from gel to liquid crystalline state) of the lipid as a consequence of the lower degree of packing of the bilayer. Below this temperature, the liposomes can be considered approximately as a closed shell for the entrance of the probe. Taking into consideration MC540 properties, we have optimized a membrane model consisting of MC540 incorporated into small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) to study lipid-peptide interaction. The selected synthetic peptide is a potential epitope of the GB virus C/Hepatitis G virus corresponding to the fragment 125–139 of the structural protein E2 of the virus. The peptide was chosen to get insight in its role in the proliferation and infection mechanism of the virus by measuring its effect on the absorption and fluorescence spectra as well as the steady state anisotropy of MC540 incorporated into liposomes.

Results from experiments indicate that the peptide influences the physical state of the membrane lipids in the headgroup and interfacial regions.

2. Experimental

2.1. Materials

DPPC was obtained from Avanti Polar Lipids (Birmingham, AL). MC540 was from Sigma (St. Louis, MO). All other chemicals were of analytical grade and water twice distilled. The peptide E2 (125–139) with a sequence of (CTIAALGSSDRDTVV) was synthesized manually as described elsewhere. It was purified by preparative high performance liquid chromatography (HPLC) on a Shimadzu chromatograph equipped with a C_8-silica column and characterized by analytical HPLC, amino acid analysis and electrospray mass spectrometry.

Concentrations of MC540 and peptide were determined gravimetrically with a high-precision electrobalance (Precisa, Model 40SM-200A, Spain).

2.2. Methods

2.2.1. Preparation of liposomes

Lipids from chloroform solutions were dried in a round bottom flask under vacuum and kept overnight into high vacuum to remove any residual solvent trace. Multilamellar liposomes (MLVs) were prepared by dispersing a known weight of dry lipids in a Tris 10 mM buffer solution at pH 7.3. SUVs were prepared by bath sonication (Lab Supplies, Hickesville, NY; Model

G112SPIT) of a MLV suspension. Sonication was carried out under a Nitrogen stream, at 1 min intervals alternating with 1 min rest, until a clear solution was obtained. LUVs of the same compositions were prepared by extrusion of the coarse MLV dispersion ten times through polycarbonate membrane filters of 100 nm pore (Northern lipids Inc. Canada). Liposome size was checked by dynamic light scattering (Malvern Autosizer) and was found to be 50 ± 0.2 nm for SUVs and 120 ± 3 nm for LUVs. For both samples polydispersity index was about 0.1. In all cases, liposomes were prepared at a temperature above the main transition from gel to liquid crystalline state of the lipids. Vesicles were used within 10 h. Final lipid concentration, assessed by phospholipid analysis [9], was 3.5 mM. Liposome suspensions were adjusted for spectrometric studies to 90 μM phospholipid.

2.2.2. Merocyanine incorporation into liposomes

MC540 was added to the preformed liposomes and the mixture was incubated at ~ 45 °C, above the main phase transition temperature of the phospholipid, during 15 min. Then, the sample was cooled down to room temperature (gel state) and allowed to equilibrate during 30 min at 20 °C. This procedure showed a significant retention of the monomer form of the probe in the gel phase necessary structure for further determinations. Several MC540/DPPC ratios were assayed to get the optimal absorption signal.

2.2.3. Absorption measurements

Absorption spectra were obtained in a Schimadzu UV-2401 PC spectrophotometer. Absorption spectra were recorded between 400 and 650 nm with 1-nm steps. The peptide was added from a stock solution to a cuvette containing LUVs or SUVs with MC540, to reach the desired peptide concentrations. Dye-free liposome suspension served as reference to compensate for turbidity effects. The corresponding spectra were corrected by taking as zero the absorbances at 600 nm of the MC/540 liposomes. The absorbance value at 569 nm obtained after these corrections was used to calculate the apparent dimerization constant (Equation 1 and 2) as described by Bernik and Disalvo [10]:

$$[\text{monomer}] = \frac{A - [\varepsilon^D \times C/2]}{\varepsilon^M - \varepsilon^D/2} \quad (1)$$

$$[\text{dimer}] = \frac{C - [\text{monomer}]}{2} \quad (2)$$

where A is the absorbance at 569 nm, ε^M (1.511×10^5 M^{-1} cm^{-1}) and ε^D (5400 M^{-1} cm^{-1}) are the molar absorption coefficient of the monomer and dimer at 569 nm, respectively [11], and C is the final MC540 concentration into the cuvette. Absorption measurements were recorded in a temperature controlled manner with a circulating water bath. Each sample was submitted to a heating cycle from ~ 20 to ~ 50 °C.

2.2.4. Fluorescence measurements

The fluorescence intensity was measured in an AB-2 spectrofluorimeter SLM-Aminco with constant stirring. MC540 spectra were recorded with an excitation wavelength of 566 nm over an emission range of 570–620 nm with an excitation and emission slit-widths of 4 nm. The sensitivity (PTM voltage) was adjusted to 1% for the Raman peak from the buffer blank at the same excitation wavelength. Each sample was submitted to a heating cycle from ~ 20 up to ~ 50 °C followed by a cooling cycle to the initial temperature, at 1 min intervals allowing for thermal equilibration. All solutions were stirred continuously during the measurements. Cuvette temperature was maintained with a peltier system piloted by a computer program (Microbeam S.A., Barcelona, Spain), and the temperature was registered with a thermocouple inserted into the cuvette. The concentration of MC540 was within the range of linear relation between fluorescence intensity and the concentration of the dye. Spectra were corrected from inner filter effect and scattering by subtracting the contribution from vesicles [12].

2.2.5. Steady-state anisotropy of membrane lipids

The dynamics of lipids in liposome membranes in the presence of different concentrations of peptide was determined by measuring the degree of depolarization of the fluorescence emitted from MC540. Experiments were performed with the

same spectrofluorimeter above described equipped with L-format polarizers. The excitation and emission wavelengths were 560 and 580 nm, respectively. Anisotropy of MC540 labeled vesicles with or without peptide (control) was measured automatically. Fluorescence anisotropy (r) was calculated automatically by the software provided with the instrument, according to:

$$r = (I_{Vv} - GI_{Vh})/(I_{Vv} + 2gI_{Vh})$$

where I_{Vv} and I_{Vh} are the intensities of the emitted polarized light with the emission polarizer parallel or perpendicular to the excitation polarizer, respectively. Anisotropy values were corrected for dependencies in the detection system (G-factor correction, $G = I_{Hv}/I_{Hh}$). Experiments were performed in the same temperature conditions as for fluorescence intensity measurements.

3. Results and discussion

3.1. Peptide selection and MC540 incorporation into liposomes

E2 (125–139) peptide was selected after comparing the structural proteins of GB virus C of several virus isolates of different origin by means of the multiple sequence alignment CLUSTALW program. A 99% of homology was obtained for the above described E2 peptide sequence. Moreover, E2 (125–139) was also selected by determining the exposed regions on the virus surface according to the hydrophilicity and accessibility profiles of Hopp and Woods [13] and Janin [14], respectively. DPPC was the chosen phospholipid to prepare model membranes for being very similar to the most common component of the biological membranes, phosphatidylcholine, and because of its well defined transition temperature (Tm) from the gel to the liquid crystalline state. Several MC540/DPPC ratios:1:25; 1:50; 1:100; 1:200; 1:300 and 1:500 were analyzed in order to get the best absorption signal as explained below.

3.2. Absorption measurements

Before starting the experiments, MC540 incorporation into the bilayer was assessed by recording its absorption spectra. MC540/DPPC ratios lower than 1:200 showed a low absorbance signal fact that was substantially improved by working at the ratio 1:300. In that case, final MC540 concentration into the cuvette was 5×10^{-6} M. Spectra showed clearly two bands, a predominant one centered at 570 nm corresponding to the monomer form, and a less intense at 530 nm, characteristic of the dimer. Peptide addition did not change the shape of the spectra but showed a hypochromic effect as indicated in Fig. 1.

It is also interesting the effect of the temperature on MC540 absorption spectra because it gives information about probe relocation and membrane fluidity. Intensity of the monomer maximum increases with temperature reflecting a more fluid environment and increased head group space or packing defects. As an example, Fig. 2 shows the absorption spectra of MC540-DPPC SUVs incubated with 12 μM (125–139) E2 at different temperatures. The temperature range covers the interval going from the gel to the fluid state of DPPC. In the gel state, the lipid bilayer is rigid and MC540 mobility is restricted. MC540 distributes between the back bone of the bilayer as a monomer and in the surface as a dimer. An increase in temperature up to the main transition from gel to liquid crystalline state (Tm $\sim 41\,^{\circ}C$), makes the bilayer much more flexible as a consequence of an increase of the phospholipid acyl chains mobility. Thus, MC540 can relocate into the backbone of the lipid acylchains [11] changing its partitions into the membrane. The equilibrium monomer⇔dimer reverts towards the direction of the monomer form. The figure shows also an isosbestic point corresponding to the equilibration between the membrane bound monomer and dimer.

Addition of E2 to liposomes decreased the intensity of MC540 maxima and the percent of the monomeric insertion thus indicating that the peptide has an effect on the group packing of the membrane. The possible mechanism of peptide interaction could be explained as a superficial

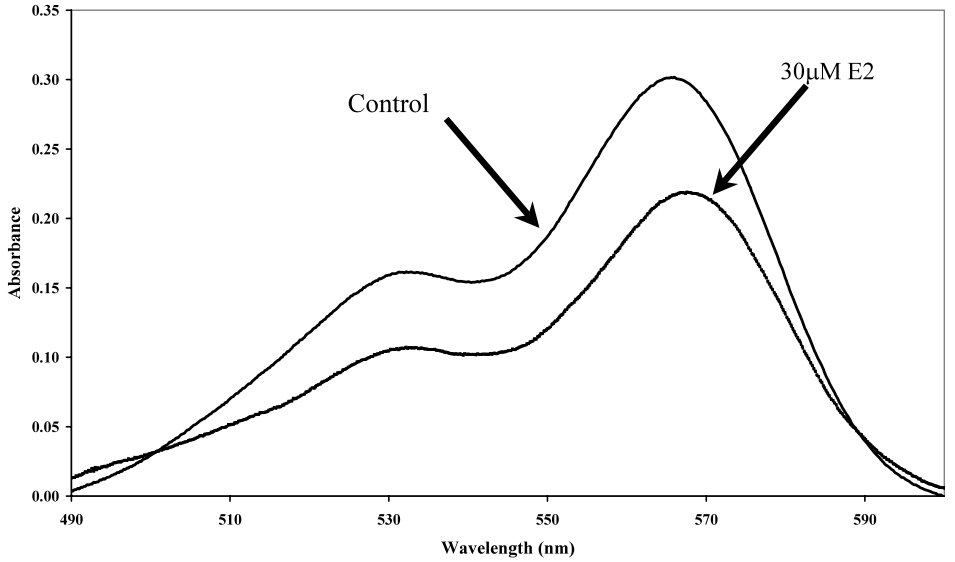

Fig. 1. Visible absorption spectrum of MC540-DPPC SUVs before (control) and after (125–139) E2 addition. Final peptide concentration 30 μM. MC540/DPPC ratio = 1:300. The experiment was performed at room temperature (22 ± 1 °C) under constant stirring.

event in which the peptide displaces MC540 from the lipid chains up to the surface in such a way that dimer formation is favored. This fact is in agreement with previous studies performed with

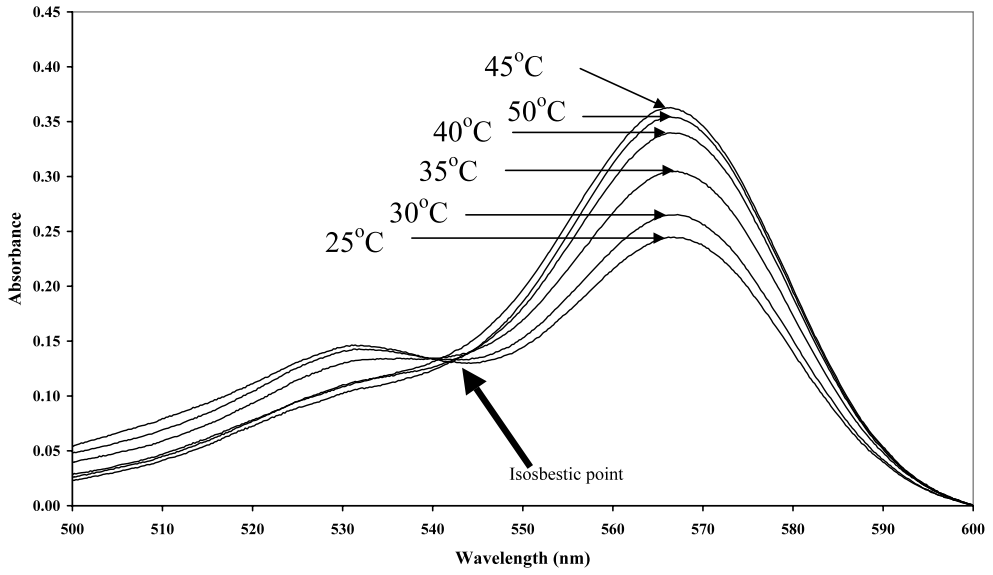

Fig. 2. Visible absorption spectrum of MC540-DPPC SUVs in presence of 12 μM (125–139) E2 in function of the temperature. At temperatures below 41 °C the bilayer is in a rigid state (gel) while above this value it is in a fluid state (liquid crystal). MC540 concentration in the cuvette: 5×10^{-6} M. Probe:DPPC ratio = 1:300. All solutions were stirred continuously during the measurements. Control experiments with MC540-DPPC SUVs gave spectra with the same shape but different intensities in the maximum wavelengths. The arrow indicates the isosbestic point for equilibration between the membrane bound monomer and dimer.

Langmuir–Blodgett technique (results unpublished) that showed not only the ability of the peptide to accumulate into an air/water interface but also its insertion into lipid monolayers.

As indicated before, the degree of partitioning of the probe into membranes can be estimated from the absorbance ratio A570/A530. Fig. 3 shows the ratio as a function of the peptide concentration and temperature. In general, peptide addition results in a slight but significant decrease of the ratio. At temperatures below the Tm, ratio increases with increasing peptide concentration. However, above the Tm there is no clear trend, ratio is always lower than for the control but seems to be independent of peptide concentration. The change on MC540 orientation makes the probe more susceptible to dimer formation as was assessed by calculating the apparent dimerization constant. Table 1 shows the $K_{d(app)}$ values for MC540-SUVs with increasing peptide concentration in a temperature range from 20 to 50 °C. As expected and for all the samples, values are higher at low temperatures. Peptide addition results in a slight increase in the $K_{d(app)}$ confirming the probe relocation into the bilayer making easier dimer formation. According to the table, maximum values are those corresponding to the peptide concentration of 30 μM, thus indicating like a bilayer saturation. However, this fact could be related to the turbidity increase of the sample observed with higher peptide concentrations. Turbidity is a result of liposome fusion or aggregation fact that can also change probe partition into the bilayer. Results obtained with LUVs (data not shown) were very similar to those of SUVs for what curvature does not seem to have any influence on probe relocation under these experimental conditions.

3.3. Fluorescence measurements

As previously described [4,11], the fluorescence intensity of MC540 in the presence of fluid and loosely packed membranes is considerably higher

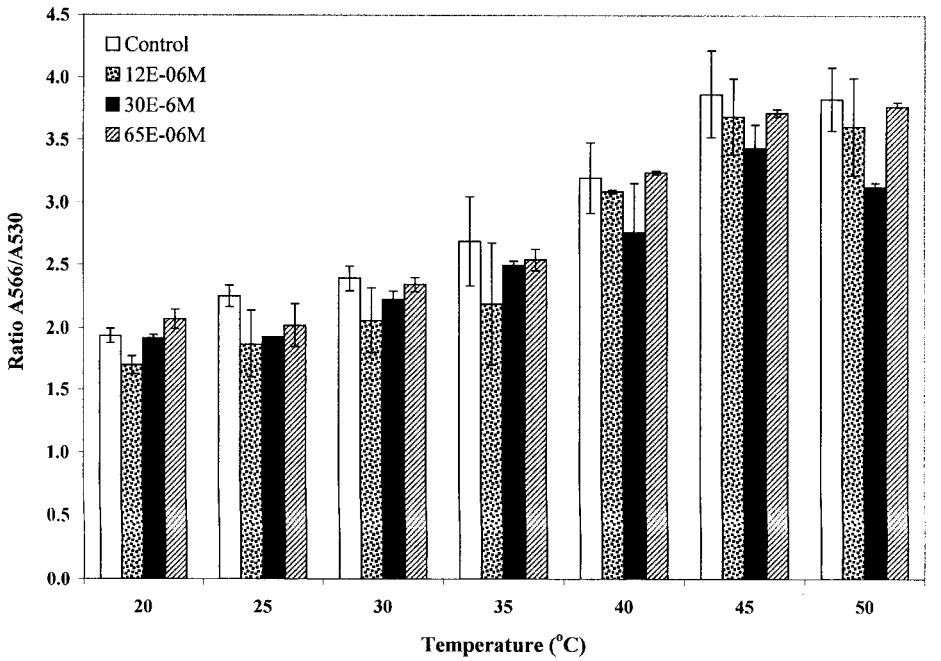

Fig. 3. Effect of various (125–139) E2 peptide concentrations on the partitioning of MC540 between Tris–HCl 10 mM, pH 7.4 and DPPC vesicles in function of the temperature expressed as the ratio A566/A530. A reduction in the absorbance ratio indicates reduced partitioning of the dye into the lipid headgroup region of the membranes. MC540 concentration in the cuvette: 5×10^{-6} M. Probe:DPPC ratio = 1:300. The means ± S.D. of three parallel samples are shown.

Table 1
Effect of (125–139) E2 on the apparent dimerization constant ($K_{d(app)}$) of MC540 in DPPC SUVs

Temperature (°C)	Apparent dimerization constant ($K_{d(app)}$)			
	Control	12 μM E2	30 μM E2	65 μM E2
20	$4.0 \pm 0.10 \times 10^5$	$8.7 \pm 0.09 \times 10^5$	$10 \pm 0.11 \times 10^5$	$9.6 \pm 0.12 \times 10^5$
25	$4.5 \pm 0.09 \times 10^5$	$8.6 \pm 0.13 \times 10^5$	$9.5 \pm 0.09 \times 10^5$	$8.1 \pm 0.13 \times 10^5$
30	$3.5 \pm 0.03 \times 10^5$	$7.5 \pm 0.09 \times 10^5$	$8.6 \pm 0.08 \times 10^5$	$8.8 \pm 0.09 \times 10^5$
35	$2.7 \pm 0.10 \times 10^5$	$5.5 \pm 0.10 \times 10^5$	$6.9 \pm 0.14 \times 10^5$	$6.2 \pm 0.11 \times 10^5$
40	$2.2 \pm 0.08 \times 10^5$	$2.7 \pm 0.03 \times 10^5$	$4.1 \pm 0.05 \times 10^5$	$5.2 \pm 0.07 \times 10^5$
45	$1.8 \pm 0.09 \times 10^5$	$2.6 \pm 0.09 \times 10^5$	$3.6 \pm 0.12 \times 10^5$	$2.9 \pm 0.09 \times 10^5$
50	$2.0 \pm 0.02 \times 10^5$	$2.5 \pm 0.12 \times 10^5$	$3.4 \pm 0.14 \times 10^5$	$2.9 \pm 0.09 \times 10^5$

Values of $K_{d(app)}$ were calculated by means of the expression $K_{d(app)} = [D]/[M]^2$ and are the mean of three independent determinations. [D] and [M] are the dimer and the monomer concentration of MC540 in the membrane phase [8].

than that in the presence of well-organized (gel phase) bilayers. We have measured the intensity of MC540 at constant lipid and dye concentrations but at various temperature. The dependence of fluorescence intensity of MC540 on temperature in the presence of DPPC LUVs with and without peptide is shown in Fig. 4. Maximum emission wavelength is not affected by peptide presence or temperature. However, peptide addition resulted in a decrease in fluorescence intensity at temperatures above 30 °C. These results are one more indication of probe relocation by temperature effect and peptide presence. Fluorescence of the probe in SUVs the same trend although increase was not so relevant and was not significantly affected by E2 (figure not shown).

3.4. Steady-state fluorescence anisotropy of membrane lipids

Up to now there is only one report in the literature describing MC540 fluorescence aniso-

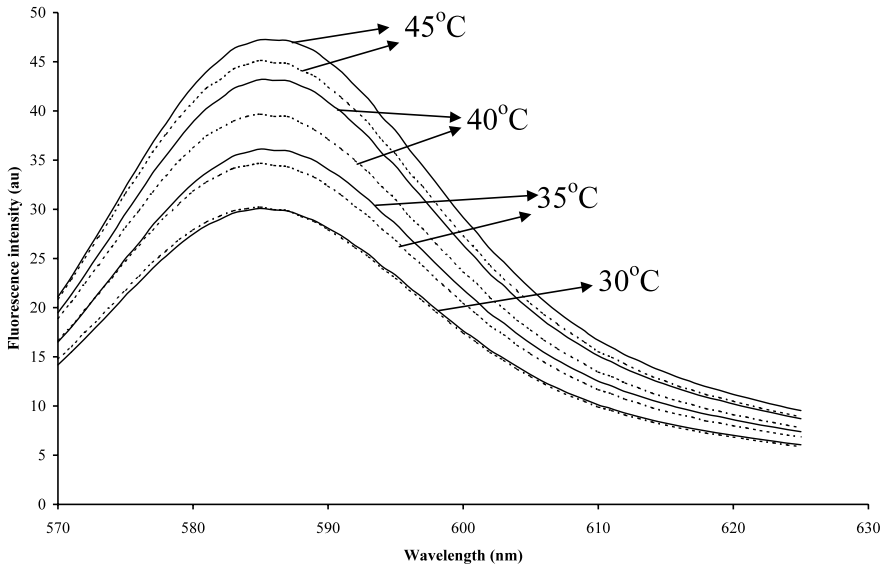

Fig. 4. Fluorescence intensity of MC540-LUVs with (broken line) or without peptide (continuous line) as a function of temperature. Peptide concentration in the cuvette was 12 μM (125–139) E2. $\lambda_{ex} = 568$ nm. All solutions were stirred continuously during the measurements.

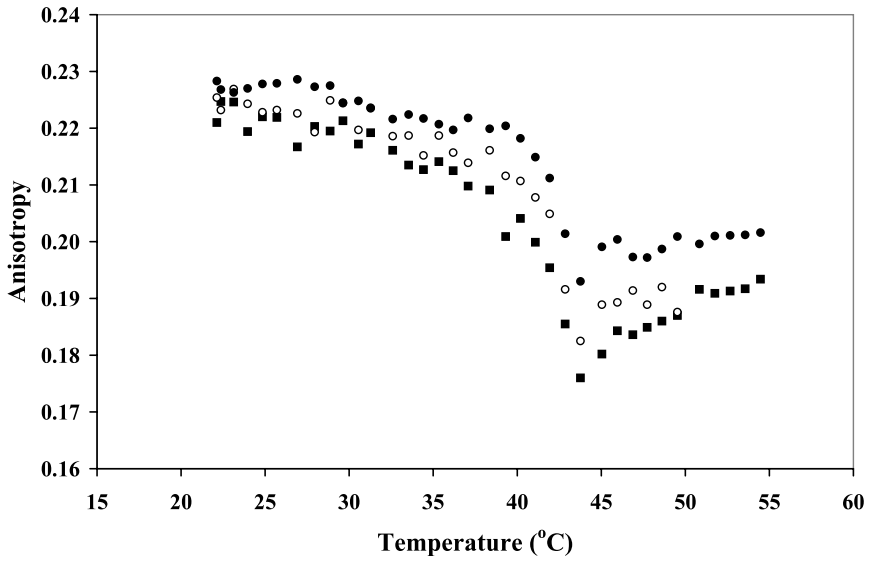

Fig. 5. Temperature dependence of the steady-state fluorescence anisotropy of MC540 labeled DPPC LUVs. Control: ■; 12 μM (125–139) E2: ○ and 30 μM (125–139) E2: ●. MC540 concentration in the cuvette: 5×10^{-6} M. Probe:DPPC ratio = 1:300. All solutions were stirred continuously during the measurements.

tropy decay in polymer-surfactant aggregates [15,16]. However, there are no data about steady-state fluorescence measurements with liposomes. The purpose of this experiment was to measure the ability of the monomer to move into the bilayer upon temperature or/and peptide presence. Fig. 5 shows the temperature dependence of the anisotropy values for LUVs vesicles in absence and presence of 12 and 30 μM peptide. Higher peptide concentrations resulted in very turbid samples for what results were not trustable and are not shown. Although initially anisotropy signal is not very high, there is a clear change in this value in the proximity of Tm ($\sim$41 °C). For that reason we considered the system suitable for study. The anisotropy value, indicative of bilayer rigidity, is higher at temperatures below than above Tm. This is related to probe mobility into the bilayer. At low temperatures, the probe is trapped into the bilayer and its mobility is restricted. As said before, when temperature increases the bilayer becomes more flexible and the molecule is free to move. All the curves follow a similar trend. Initially r has a value between 0.22 and 0.23 and has a tendency to decrease slowly until the vicinity of Tm. Then, there is a sharp decrease followed by an increase. This last change could be explained by the fact that MC540 monomer form concentration increases at high temperatures. Although according to the shape of the plots peptide seems to rigidify the membrane since r values are higher than those of the control, we can not assure that fact. In one hand, peptide causes a relocation of the probe into the bilayer but the remaining MC540 molecules could have a restricted movement by peptide proximity. On another hand, higher r values could simply be due to liposome aggregation. Further studies with other probes that are located at different depth of the membrane are being under study to clarify this result and to get more information about peptide interaction with bilayers. Results with SUVs were practically identical.

Acknowledgements

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