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Interaction of E2 and NS3 synthetic peptides of GB virus C/hepatitis G virus with model lipid membranes

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Abstract

The membrane-interacting properties of two potential epitopes of the GB virus C/Hepatitis G virus, located respectively at the regions (99–118) of the E2 structural protein and (440–460) of the NS3 non-structural protein were studied. Changes in the intrinsic fluorescence of Trp and Tyr residues after the addition of DPPC–LUV revealed that the peptide–membrane interaction was optimal above the gel–liquid crystalline transition temperature of the lipid. Differential scanning calorimetry studies showed that the E2 peptide incorporated into lipid bilayers perturbs the packing of lipids and affects their thermotropic properties. Moreover, the 20-mer structural peptide induced a slow leakage of vesicular contents at 55 °C.

Keywords: GB virus C/Hepatitis G virus; Synthetic peptides; Differential scanning calorimetry; Fluorescence

1. Introduction

All life processes during which cellular contents pass through a membrane barrier, such as viral infection, neurotransmission, fertilization and intracellular protein traffic, require the fusion of membranes [1]. One of the most studied membrane fusion processes is the fusion of enveloped viruses with target membranes [2–4]. Enveloped viruses acquire their lipid membranes by budding through host cellular membranes [5]. The GB virus C (GBV-C) and the hepatitis G virus (HGV) are strain variants of a recently discovered enveloped RNA virus belonging to the Flaviviridae family which is transmitted by contaminated blood and/or blood products, intravenous drug use, from mother to child and via sexual intercourse. The natural history of the GBV-C/HGV infection is at present not fully understood and its potential to cause hepatitis in humans is questionable [6]. Following infection, about 80% of people clear their viremia, concomitantly developing antibody to the GBV-C E2 protein [7,8]. GBV-C/HGV is the most closely related human virus to the hepatitis C

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virus (HCV), a major worldwide pathogen [9]. Recently, a stretch of conserved, hydrophobic amino acids within the envelop glycoprotein of HCV has been proposed as the virus fusion peptide [10]. However, the mode of entry of GBV-C/HGV into target cells is at present unknown.

Liposomes have been used primarily as a model system for studying biological membranes. Numerous chemical, biochemical and biophysical methods have been used to elucidate the various aspects of the interaction between proteins or peptides and phospholipids during the last years [11]. Having in mind the potential use of synthetic peptides as antiviral therapies and aiming for a better understanding of the molecular interaction of the E2 envelop of the GBV-C with liposomes as model membranes, a potential epitope of GBV-C located at the (99–118) region (VSWFASTGGRDSKIDVWSLV) of this structural protein and a peptide belonging to the non-structural protein NS3 of the same virus (sequence 440–460: AIAYYRGKDSSIIKDGDLVVC) were selected. The main aim of the present study was to get insight into the membrane-interacting properties of the above-described synthetic peptides, using fluorescence spectroscopy for the study of phospholipid–peptide interactions. Moreover, differential scanning calorimetry was used to follow the thermotropic behaviour of phospholipid bilayers in the presence of GBV-C/HGV synthetic peptides.

2. Experimental

2.1. Peptides

Experimental details of the synthetic procedures will be described elsewhere. To briefly summarize the procedures: NS3(440–460): AIAYYRGKDSSIIKDGDLVVC and E2(99–118): VSWFASTGGRDSKIDVWSLV peptide sequences were obtained by solid-phase peptide synthesis following a Fmoc/tBu strategy essentially by means of a *N*-*N*′ diisopropylcarbodiimide/1-hydroxybenzotriazole activation.

Crude peptides were purified by preparative high performance liquid chromatography (HPLC) on a Shimadzu chromatograph equipped with a C8-silica column. The samples were eluted with a linear gradient of 60% H_2O (0.05% TFA)/40% acetonitrile (0.05% TFA) to 40% H_2O (0.05% TFA)/60% acetonitrile (0.05% TFA) in 30 min at a flow rate of 5 ml min^{-1} and detected at 220 nm. Purified peptides were successfully characterized by analytical HPLC, amino acid analysis and electrospray mass spectrometry (Table 1).

2.2. Liposomes

L-α-dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar-Lipids, Inc. and was used without further purification. Lipid vesicles for differential scanning calorimetry and fluorescence measurements were prepared as follows.

Pure DPPC was dissolved in a chloroform/methanol (2/1) mixture and the lipid solution was dried by slow evaporation under a constant flow of nitrogen. The last traces of solvents were removed under vacuum at 50 °C. Finally Hepes buffer (5 mM, pH 7.4) was added to the lipids to give a final concentration of 0.2 mM. Multilamellar vesicles (MLVs) were prepared by vortexing the lipid suspension during 10 min.

Large unilamellar vesicles (LUVs) of the same lipid composition were prepared by hydration of the lipid film with Hepes buffer followed by 10 freeze–thaw cycles [12]. This preparation was extruded 10 times through two 100 nm pore-size polycarbonate filters (Nucleopore, Pleasanton, CA) in a high pressure extruder (Lipex, Biomembranes, Vancouver, Canada) above the transition temperature of DPPC (50 °C).

For the 8-aminonaphtalene-1,3,6-trisulfonic acid–*N*,*N*′-p-xylenebis(pyridinium bromide) (ANTS–DPX) leakage assay [13] about 15 mg of DPPC were dissolved in a mixture of chloroform and methanol (3:1) that was subsequently removed under a stream of nitrogen. About 1 ml of buffer containing 12.5 mM of ANTS and 45 mM of DPX from molecular probes (Eugene, OR) and 20 mM NaCl and 5 mM Hepes was added to the dry lipid. The osmolarity of the ANTS/DPX solution was

Table 1
Peptides characterization by amino acid analysis, HPLC and mass spectrometry

Peptide	Amino acid analysis [a]	HPLC	HPLC/ES-MS
NS3(440–460)	$S=1.2$ (2); $G=2.1$(2); $R=1.0$ (1); $D=2.8$ (3); $K=2.0$ (2); $A=2.1$ (2); $L=1.1$ (1); $Y=1.4$ (2); $V=2.0$ (2); $I=3.0$ (3); C n.d.	$k'=7$ [b]	$[M^+]$: 2286.4
E2(99–118)	$T=0.7$ (1); $S=2.8$ (4); $G=1.6$ (2); $A=0.8$ (1); $D=1.7$ (2); $V=2.8$ (3); $I=0.6$ (1); $L=0.8$ (1); $F=0.6$ (1); $K=1.4$ (1); $R=0.5$(1)	$k'=9.9$ [b]	$[M^+]$: 2208.8

[a] Theoretical values in parenthesis. Cys and Trp not determined.
[b] (A) water (0.05%TFA); (B) acetonitrile (0.05% TFA). Flow, 1 ml min^{-1}. Detection, 215 and 280 nm. Gradient elution, 50% A to 5% in 30 min.

adjusted to be equal to that of the buffer in a cryoscopic osmometer (Fiske One-ten). The suspension was frozen and thawed 10 times to assure maximum entrapment prior to extrusion. The final lipid concentration was 10 mM. A stock solution of LUV of approximately 0.1 μm in diameter was formed by extrusion pressure through Nucleopore polycarbonate membranes. The vesicles were separated from unencapsulated material on Sephadex G-75 (Pharmacia, Uppsala, Sweden), equilibrated with 100 mM NaCl/5 mM Hepes buffer (pH 7.4).

Lipid concentration of liposome suspensions were determined by phosphate analysis [14]. The vesicles' size was determined from the measurement of the sample diffusion coefficient by photon correlation spectroscopy.

2.3. Differential scanning calorimetry

DSC experiments of DPPC MLVs were performed using a DSC 821E Mettler Toledo calorimeter (Greifensee, Switzerland). Hermetically sealed aluminium references and samples containing pans were used. Sample pans were loaded by adding 30 μl of DPPC vesicle suspension, corresponding to approximately 0.13 mg of phospholipid. Differences in the heat capacity between the sample and the reference cell were obtained by raising the temperature at a constant rate of 5 °C min^{-1} over the range from 0 to 60 °C. All samples were submitted to three heating/cooling cycles. Data from the first scan were always discarded to avoid false results coming to the possible lipid–peptide mixing in the sample under heating. The endothermal peak coming from the second scan of the control sample was used as a reference template. The instrument was calibrated with Indium. To ensure scan-to-scan reproducibility three consecutive scans of the same sample were performed. DSC runs were carried out within the same day of liposome preparation. Molar enthalpies of transition (ΔH) were calculated from peak areas by means of a START[e] Mettler Toledo system software.

2.4. Tryptophan and tyrosine fluorescence measurements

Fluorescence experiments were carried out on a Perkin-Elmer (Beaconsfield, Bucks, UK) spectrofluorimeter LS 50. Cells of 1-cm optical path were used.

The NS3 peptide (that contain Tyr) and the E2 peptide (that contain Trp) were respectively excited at 280 and 285 nm. Emission spectra were recorded from 310 to 340 nm. Slits widths a nominal band-pass of 5 nm were used for both excitation and emission beams.

Emission fluorescence spectra were recorded at a peptide concentration of 1 μM in HEPES 5 mM, pH 7.4, at 20 and 55 °C temperatures by incremental addition of 4–16 μl aliquots of a 10 mg ml^{-1} DPPC solution into the initial 2 ml solution of 1 μM of peptides. After each addition, the emission spectrum was recorded. Peptide–phospholipid interactions were assessed by monitoring the changes in the fluorescence spectra when LUV–DPPC liposomes were incubated with 1 μM peptide concentration of each sequence in lipid to peptide molar ratios of 25:1, 50:1, 100:1, 200:1, 300:1 and 400:1. Suspensions were continu-

ously stirred, and in the case of the experiments at 55 °C, it was left to equilibrate for 5 min before recording the spectrum. Fluorescence intensities were corrected for contribution of light scattering, by subtraction of the appropriate vesicle blank. The last correction was obtained from a parallel lipid titration of *N*-acetyl-tryptophanamide, which is known not to interact with lipids [15].

The absorbance of peptide samples was measured by using LKB-Biochrom Ultrospec II Spectrophotometer at 280, 285, 310 and 340 nm.

2.5. ANTS/DPX leakage assay

Dequenching of co-encapsulated ANTS and DPX fluorescence resulting from dilution was measured to assess the leakage of aqueous contents [13]. LUV vesicles containing ANTS/DPX were obtained as described in Section 2.2. The medium in the cuvettes was continuously stirred to allow the rapid mixing of peptide and vesicles. Fluorescence measurements were performed by setting the ANTS emission at 520 nm as a function of time and the excitation at 355 nm. A lipid suspension was diluted in buffer to give a final lipid concentration of 100 μM; the fluorescence level of this preparation did not vary with time and was set as 0% leakage. Hundred percentage leakage was the fluorescence value obtained after the addition of 10% (vol/vol) Triton X-100.

Leakage of ANTS–DPX from LUV of DPPC induced by GBV-C synthetic peptides was calculated by using the following equation:

$$\%\text{Leakage} = [F_f - F_0]/[F_{100} - F_0] \times 100$$

where F_f is the fluorescence value after adding the peptide, F_0 the initial fluorescence and F_{100} the measured fluorescence after the Triton X-100 treatment.

3. Results and discussion

Figs. 1 and 2 show respectively, the amphipathic character (Kyte and Doolittle hydropathy scale) [16] and the accessibility (Janin scale) [17] of the sequences belonging to NS3 and E2 proteins of GBV-C/HGV used in this work. As shown, clearly the E2(99–118) peptide is more accessible than the non-structural sequence, NS3(440–460). On the other hand the estimation of the side chain hydrophobicity by Kite and Doolittle hydropathy index of each amino acid, only renders the NS3 domain on the hydrophobic side above the zero midpoint line.

It has been described that classical hydrophobicity scales such as the Kyte–Doolittle scale might not identify protein sequences that show a marked tendency to partition into membrane interfaces by experimental assays [18]. This hydropathy index is based on bulk-phase partitioning and, therefore, estimates side chain hydrophobicity alone underscoring the peptide bound partitioning and, moreover, does not take into account the effect of the membrane interface on partitioning.

Although the scales based on normalized hydrophobicity values have widely been used to detect membranes interacting sequences in polypeptides chains, further biophysical assays using model membranes are required in order to detect putative regions involved in membrane partitioning within sequences of viral proteins.

3.1. Differential scanning calorimetry studies

Transition of lipid bilayers occurs from an ordered gel state at lower temperature to a more disordered liquid crystalline phase at higher temperature. The temperature at which the transition occurs is called the main transition temperature (Tm).

DPPC is a lipid with a bulky polar head group that has its hydrocarbon chains titled relative to the phase of the membrane. This chain tilt is expressed by a ripple phase. In fact, the transition from gel to liquid crystal in DPPC involves two transitions, the main transition above described and the pre-transition ($\sim 5^\circ$ below the main transition) at which a change in headgroups orientation occurs.

Accordingly, our results show that DPPC pure bilayers display two endothermic transitions: at 35 °C (pretransition between the gel phase and ripple phase) and at 41 °C (corresponding to the gel to liquid crystalline main transition). The enthalpy, ΔH, of the main transition obtained

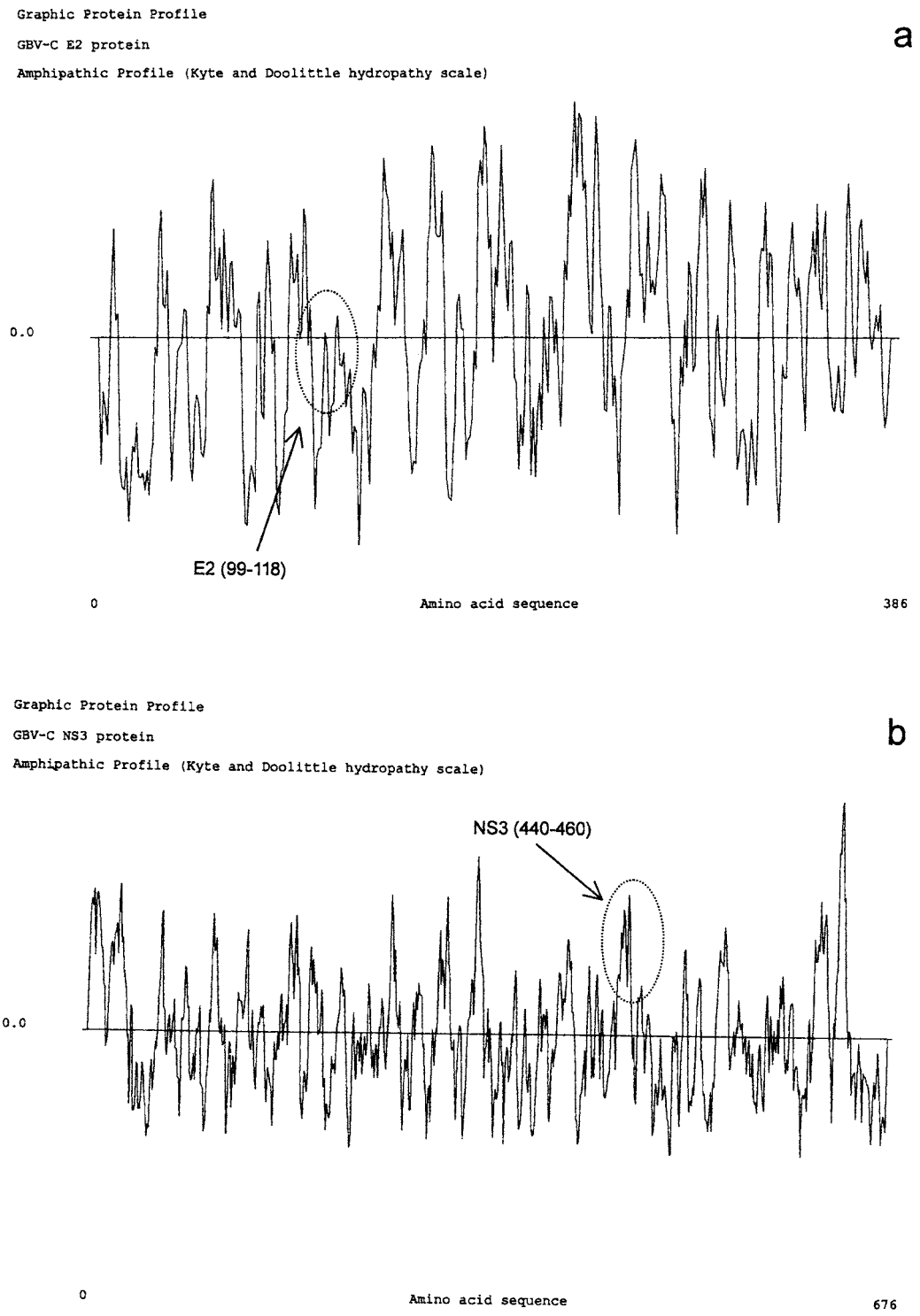

Fig. 1. Hydropathy plots (mean values for a window of 7 amino acids) corresponding to E2 and NS3 GBV-C/HGV proteins, elaborated using the Kyte–Doolite hydropathy index. The location of E2(99–118) and NS3(440–460) sequences is indicated.

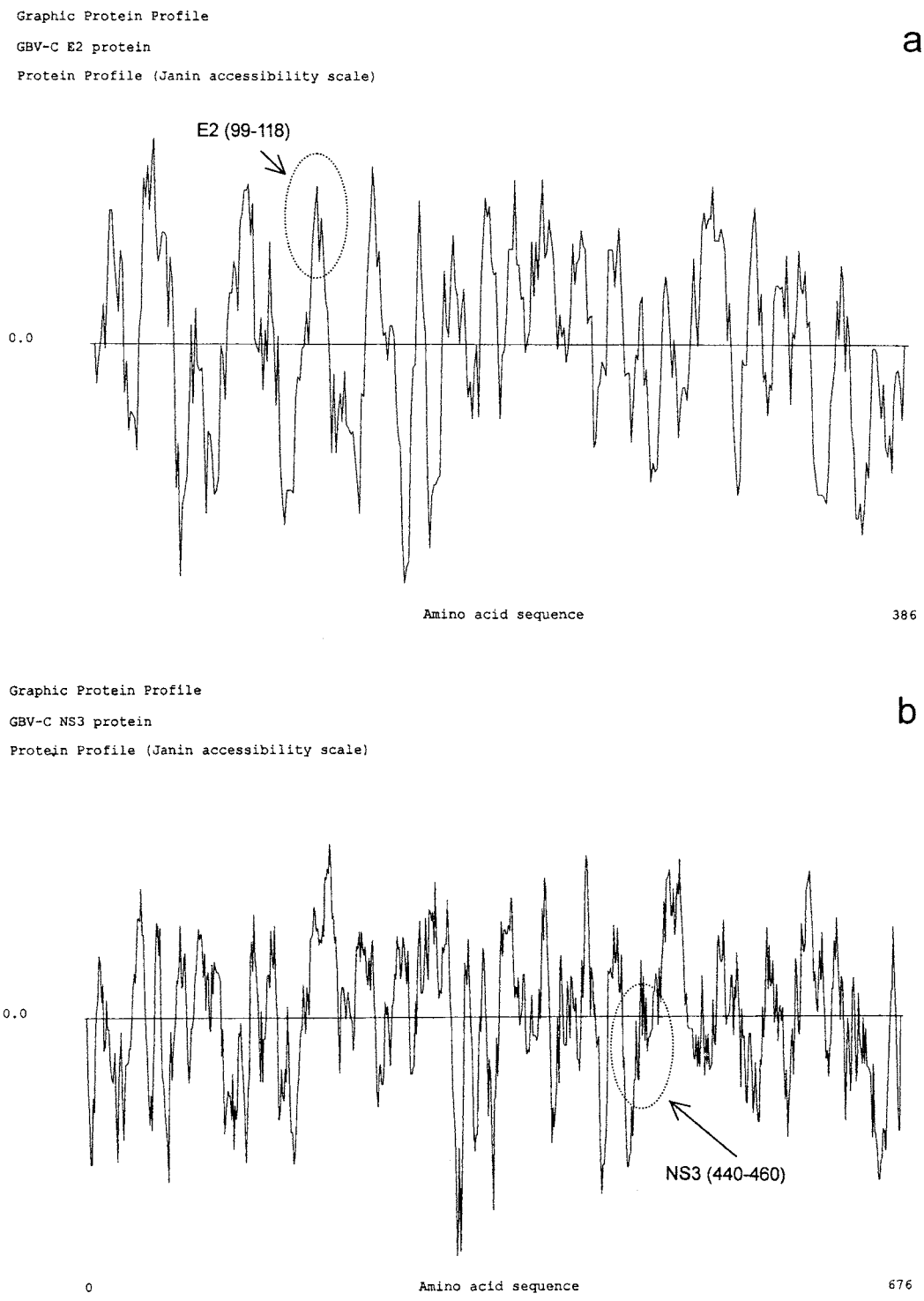

Fig. 2. Protein profile (mean values for a window of 7 amino acids) corresponding to E2 and NS3 GBV-C/HGV proteins, elaborated using the Janin accessibility scale. The location of E2(99–118) and NS3(440–460) sequences is indicated.

was 32.2 kJ mol^{-1} in good agreement with literature values [19,20]. The shape of the thermograms corresponding to the second heating of the DPPC vesicles does not change thus suggesting that the phase transition of DPPC is reversible at all lipid/peptide ratios.

The thermotropic phase behaviour of zwitterionic DPPC vesicles was studied by DSC, in absence and in the presence of increasing concentrations of GBV-C peptides. At high lipid-to peptide ratios, the chain melting transition of DPPC was not affected by the NS3 peptide. Contrarily, for the E2 peptide low quantities (1–2%) clearly induced a peak reduction. (Fig. 3). These observations suggest, on one hand, that the interaction of small amounts of the NS3 synthetic peptide reported in this paper does not alter significantly the packing of hydrocarbon chains in the gel and liquid–crystalline states. On the other hand, as previously obtained for other synthetic peptides [21] the addition of low concentrations of the E2 peptide caused the complete disappearance of the DPPC pretransition, and also the enthalpy of the main transition peak was considerably diminished.

The effects of peptides on gel/liquid-crystalline transition enthalpy (ΔH), the corresponding Tm, and the temperature width at half-height of the heat absorption peak ($\Delta T_{\frac{1}{2}}$) for pure DPPC vesicles and at different percentage of E2 and NS3-peptides as obtained from DSC is shown in Table 2. Although E2 and NS3 peptides did not show a significant displacement of the phase transition midpoint of the DPPC, the broadening of the transition profile of DPPC bilayers, described as peak width at half-height ($\Delta T_{\frac{1}{2}}$), was considerable with increasing amounts of peptides. The higher the percentage of peptide in the bilayer, the greater the broading of the transition. A different non-linear trend with increasing peptide concentration was observed for the two peptides.

In summary, the studied peptides incorporate into lipid bilayers, perturb the packing of lipids and affect their thermotropic properties. From our results and according Papahadjopoulos et al. [22], it is tentatively suggested that the studied peptides preferentially locate at the outer regions of the bilayer extending the interactions to the polar headgroups followed by a partial penetration and deformation of the bilayer.

3.2. GBV-C peptides: intrinsic fluorescence

The interaction of GBV-C peptides with the zwitterionic phospholipid DPPC was investigated by measuring the fluorescence quantum yield and the wavelength of the emission maximum in presence of different concentrations of LUVs. To study the effect of membrane fluidity on lipid–peptide interactions, the fluorescence titration was performed both below (25 °C) and above (55 °C) the gel/liquid crystalline phase transition temperature.

E2 and NS3 peptides have Trp and Tyr residues whose fluorescence is highly sensitive to its environment. Fluorescence spectra at 25 °C showed that in the presence of lipids there was a blue shift in the emission maximum of the E2(99–118) peptide and an increase in fluorescence quantum yield. Both effects suggest that the Trp residue in the presence of DPPC vesicles feels the effects of a less polar environment, which is indicative of binding to and subsequent penetration of the peptide into the hydrophobic core of the bilayer. Besides, for NS3(440–460) a highly moderate fluorescence and no blue shift was detected for the phenol ring of Tyr.

Titration of E2 and NS3 peptides with DPPC–LUVs in gel and in liquid-crystalline states is shown in Fig. 4. Binding results are represented as F/F_0, F_0 being the fluorescence corresponding to peptide solutions at the same dilution of titrated samples (F). As shown in Fig. 4, the partitioning isotherms corresponding to NS3 and E2 peptides at a temperature below (25 °C) and above (55 °C) the gel/liquid crystalline phase transition show a lower affinity towards neutral membranes in gel phase, thus suggesting that the strength packing of DPPC in the gel phase renders the binding of the GBV-C peptides more difficult.

The apparent mole fraction partition coefficients [23] were determined by fitting the binding curves to the equation $I = f_{\text{bound}} I_{\max} + (1 - f_{\text{bound}}) I_0$, for which I is the relative fluorescence intensity, I_0 is the intensity in the absence of lipid, and

$f_{bound} = KxL/(W+KxL)$, where Kx is the mole-fraction partition coefficient, L the lipid concentration and W the molar concentration of water (55.3 M at 25 °C).

When analysing their binding isotherms as partition equilibria, the obtained partition coefficients were of the same order ($Kx = 2.1(\pm 0.09) \times 10^6$ and $Kx = 1.6\ (\pm 0.08)\ \times 10^6$) for E2 and NS3 peptides assayed at 55 °C, respectively) thus indicating that both peptides have similar affinities for DPPC membranes.

3.3. Assessment of vesicle leakage

To further explore the possible interaction of both peptides with phospholipid model membranes, the effect of the peptide on the release of the encapsulated fluorophores ANTS/DPX was monitored by dequenching of the ANTS.

E2 and NS3 peptides did not cause leakage in LUVs–DPPC vesicles at gel phase (room temperature), even at concentration up to 40% molar (data not shown).

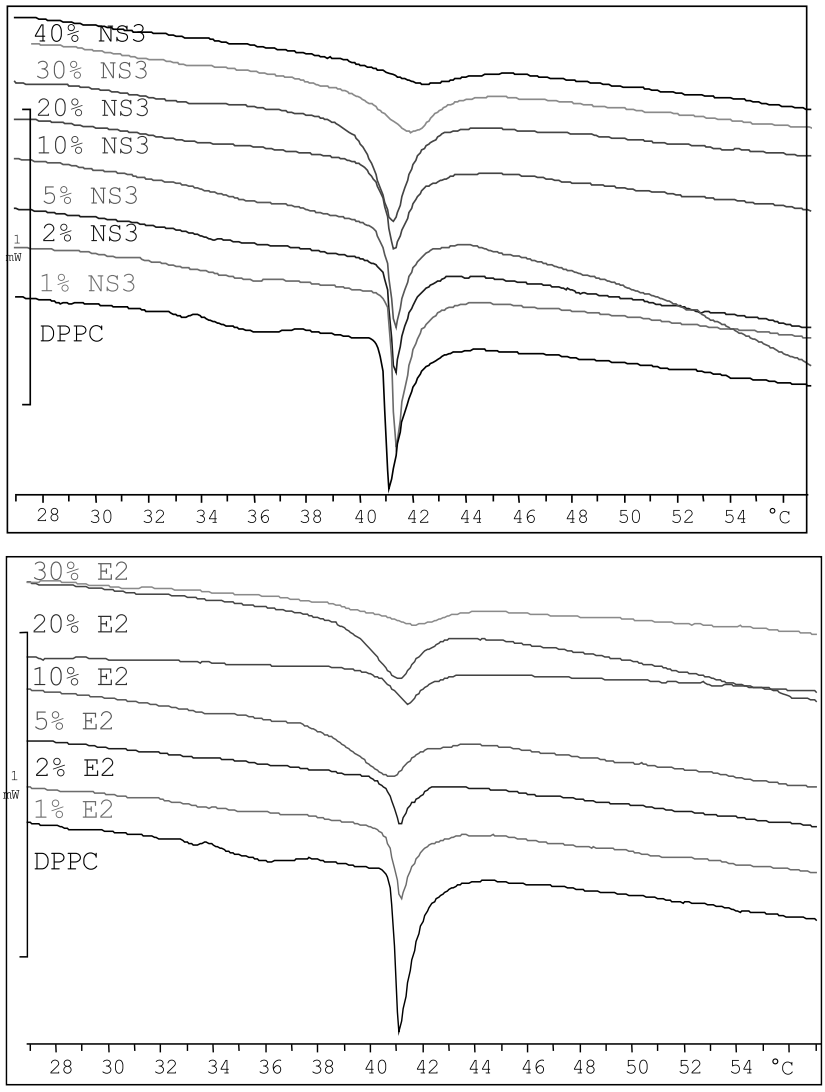

Fig. 3. DSC thermograms of DPPC bilayers in the absence and in the presence of variable concentrations of NS3(440–460) and E2(99–118) peptide sequences. The peptides concentrations given in mol.% with respect to lipid are indicated on the curves.

Table 2
Gel/liquid-crystalline transition enthalpies, ΔH, the corresponding transition temperatures, Tm, and the temperature width at half-height of the heat absorption peak, $\Delta T_{\frac{1}{2}}$, for pure DPPC vesicles and at different percentage of E2 and NS3-peptides as obtained from DSC

Peptide	ΔH (kJ mol^{-1})	Tm (°C)	$\Delta T_{\frac{1}{2}}$ (°C)
DPPC	32.2 (0.7)	41.1 (0.04)	0.7 (0.1)
NS3 (%)			
1	29.0 (0.8)	41.2 (0.03)	0.6 (0.05)
2	22.7 (1.3)	41.2 (0.09)	0.6 (0.09)
5	22.3 (1.1)	41.2 (0.05)	0.7 (0.06)
10	27.8 (1.7)	41.2 (0.05)	1.0 (0.07)
20	27.5 (0.9)	41.2 (0.08)	1.4 (0.09)
30	27.8 (0.8)	41.1 (0.04)	2.4 (0.07)
E2 (%)			
1	17.0 (1.2)	41.1 (0.05)	0.8 (0.09)
2	8.8 (2.8)	41.0 (0.05)	0.8 (0.05)
5	18.0 (1.7)	41.1 (0.04)	1.1 (0.06)
10	11.0 (0.8)	41.4 (0.03)	1.3 (0.1)
20	13.4 (2.6)	41.0 (0.02)	1.9 (0.3)
30	–	–	–

S.D. values in parenthesis.

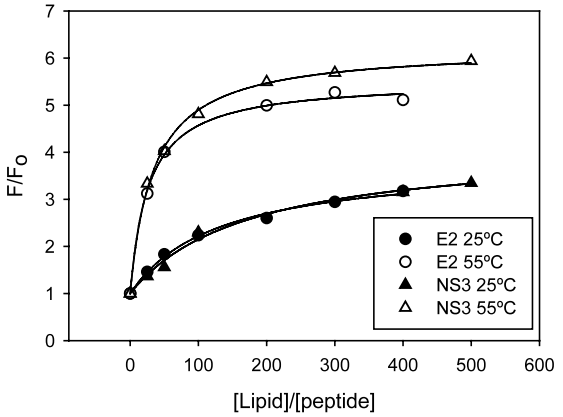

Fig. 4. Partition isotherms corresponding to NS3(440–460) and E2(99–118) peptide sequences at a temperature below (25 °C) and above (55 °C) the gel–liquid crystalline phase transition of DPPC.

Fig. 5 shows the dependence of leakage on the lipid to peptide molar ratio at the liquid crystalline phase (55 °C). Above the gel-to-liquid crystalline transition it can be clearly observed that whereas the E2 peptide permeabilized vesicles efficiently, the NS3 peptide hardly exerted any effect on DPPC liposomes. Although the hydrophaty scale of Kyte and Doolittle indicates that the NS3 peptide has a higher tendency to interact with membranes, it seems that the interaction of the E2 peptide with membranes might result in bilayer perturbations as is inferred by DSC and fluorescence results.

According to this, the difference in the capacity of both peptides to perturb the integrity of the lipid bilayer is not only a consequence of a different binding ability. The oligomeric state, conformation, orientation and depth of penetration of peptides into model membranes might also be invoked to explain the membrane permeation process. In consequence further conformational and biophysical studies using polarized attenuated total reflectance Fourier transform infrared spectroscopy and fluorescence energy transfer assays will be performed in order to characterize the putative E2 peptide interaction with membranes. These studies will allow to investigate if this E2 peptide could play a role in the intermolecular interaction of the E2 structural protein of GBV-C with the lipids of the target membrane.

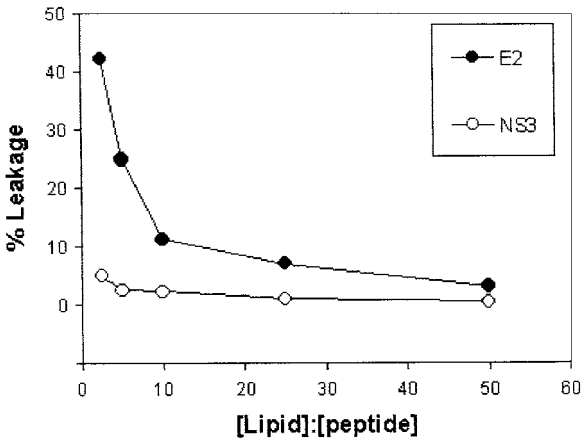

Fig. 5. Leakage of ANTS–DPX from LUVs of DPPC induced by E2(99–118) and NS3(440–460) peptide sequences at different Peptide:Lipid ratios after 30 min of incubation at 55 °C.

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