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Unravelling the molecular basis of the selectivity of the HIV-1 fusion inhibitor sifuvirtide towards phosphatidylcholine-rich rigid membranes

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ABSTRACT

Sifuvirtide, a 36 amino acid negatively charged peptide, is a novel HIV-1 fusion inhibitor with improved antiretroviral activity. In this work we evaluated the physical chemistry foundation of the interaction of sifuvirtide with biomembrane model systems. Since this peptide has aromatic residues, fluorescence spectroscopy techniques were mostly used. The interaction was assessed by partition and quenching experiments. Results showed no significant interaction with large unilamellar vesicles composed by sphingomyelin and ceramide. In contrast, sifuvirtide presented selectivity towards vesicles composed by phosphatidylcholines (PC) in the gel phase, in opposition to fluid phase PC vesicles. The interaction of this peptide with gel phase PC membranes ($K_p = 1.2 \times 10^2$) is dependent on the ionic strength, which indicates the mediation of electrostatic interactions at an interfacial level. The effects of sifuvirtide on the lipid membranes' structural properties were further evaluated using dipole-potential membrane probes, zeta-potential, dynamic light scattering and atomic force microscopy measurements. The results show that sifuvirtide does not cause a noticeable effect on lipid bilayer structure, except for membranes composed by cationic phospholipids. Altogether, we can conclude that sifuvirtide presents a specific affinity towards rigid PC membranes, and the interaction is mediated by electrostatic factors, not affecting the membrane architecture.

1. Introduction

The human immunodeficiency virus type 1 (HIV-1) is the causative agent of the acquired immunodeficiency syndrome (AIDS). One of the crucial steps of the HIV-1 infection cycle is the binding and entry of the virus into the target $CD4^+$ host cells, mediated by the viral envelope proteins gp120 and gp41. After binding of the surface protein gp120 to CD4 and CCR5 (or CXCR4) receptors, a cascade of conformational changes on the transmembrane protein gp41 is triggered. Following the exposure and insertion of the fusion peptide into the target cell membrane, the two helical domains of gp41, N-terminal and C-terminal heptad repeat (HR1 and HR2), are allowed to interact and form a 6-helix bundle (a stabilized trimer of hairpins structure), bringing in close proximity the viral envelope and cellular membrane and thus enabling both membranes to fuse [1–3].

Inhibitors acting on the fusion process are one of the most novel and promising anti-HIV therapies [3–6]. Synthetic peptides are being developed based on the gp41 HR2 to target the viral HR1/HR2 interaction. Enfuvirtide (formerly T-20, the only FDA-approved drug in this class) and T-1249 are two fusion inhibitor peptides presenting high therapeutic potency [3,5,6]. Nonetheless, the emergence of undesired resistant strains of the virus and the natural side effects due to a prolonged antiretroviral drug administration are common; therefore, it is important to keep the development of more potent drugs a top priority.

The development of new fusion inhibitors has been focused on the improvement of the affinity to the HR1 region of gp41 by means of the increase in the helical content and stability of the peptides [7–11]. To achieve this, most of the newly designed peptides include the deep pocket binding domain essential for an improved anti-HIV activity [12–14].

Besides the competitive binding to HR1, the interaction of fusion inhibitors with biological membranes is important for their mode of action and activity, since the inhibition process must occur in extreme confinement between both viral and cellular membranes [15]. For instance it was shown that enfuvirtide [16] and T-1249 [17] interact with lipid membranes. Enfuvirtide inserts preferentially in fluid phase lipid membranes, whereas T-1249 may also adsorb on the surface of cholesterol-rich membranes. Membranes can therefore serve as catalysts [18] of the inhibition process, by providing an increased concentration of peptide near the fusion site.

Sifuvirtide is a novel HIV fusion inhibitor presently in phase II clinical trials [19]. It possesses a higher activity and lower toxicity when compared to enfuvirtide, profiling itself as a suitable and

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promising alternative [19–22]. This 36-amino acid residue peptide (Fig. 1) presents amphipathic segments, increased helicity, a global negative net charge (−6) and two tryptophan amino acid residues, rendering it suitable to be studied by fluorescence spectroscopy without the need of further labelling. We previously reported a selective adsorption of this peptide on rigid dipalmitoylphosphatidylcholine (DPPC) membranes [23], which may help to explain its improved efficiency by screening the more rigid domains of the membrane. Nonetheless the nature of this interaction and the dependence on rigid lipid composition, such as sphingomyelin (SM) and ceramide (Cer), and also other saturated phosphatidylcholines (PC), characteristic of lipid rafts and viral envelopes [24–27], are still not fully understood. For instance, in HIV membranes there is a 3.2-fold increase in SM and a 3.6-fold increase in saturated PC compared to eukaryotic plasma membranes [24]. Due to the increased concentration of these lipid components in the viral membrane and lipid rafts, it is important to have a better insight on their affinity with HIV fusion inhibitors, especially in the case of sifuvirtide. The aim of this work is, therefore, to study the interaction of sifuvirtide with rigid membrane models, using mainly fluorescence spectroscopy techniques. Atomic force microscopy (AFM), dynamic light scattering (DLS) and zeta-potential measurements were additionally used throughout this work. Its ultimate goal is to clarify the specific molecular mode of action of sifuvirtide at the membrane level, demonstrating the importance of membrane interactions and interfacial properties of lipids in the improved efficiency of sifuvirtide as a novel HIV fusion inhibitor peptide.

2. Materials and methods

2.1. Materials

Sifuvirtide, was a kind gift from FusoGen Pharmaceuticals Inc. (Tianjin, P. R. China). 1-Palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleyl-*sn*-glycero-3-ethylphosphocholine (EPOPC), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), N-palmitoyl-D-*erythro*-sphingosine (C16 Ceramide, Cer) and chicken egg yolk sphingomyelin (SM) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol) was obtained from Sigma (St. Louis, MO), whereas 1-anilinonaphthalene-8-sulfonic acid (ANS) and 4-[2-[6-(dioctylamino)-2-naphthalenyl]ethenyl]-1-(3-sulfopropyl)-pyridinum (Di-8-ANEPPS) were purchased from Molecular Probes (Eugene, OR). L-tryptophan (Trp), acrylamide, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), ethylenedinitrilotetraacetic acid (EDTA), NaOH, NaCl, $CaCl_2 \cdot 2H_2O$, chloroform and methanol (both with spectroscopic grade) were purchased from Merck (Darmstadt, Germany).

2.2. Sample preparation and instrumentation

Sifuvirtide and Trp stock solutions were prepared in 10 mM HEPES buffer pH 7.4, containing 10 mM or 150 mM NaCl (low or physiologic ionic strength, respectively). Concentrated suspensions of large and small unilamellar vesicles (LUV and SUV, respectively) were prepared by extrusion or sonication methods, as described elsewhere [28,29]. Several lipid mixtures were used throughout this work. For spectroscopy assays, LUV (prepared by extrusion through 100 nm pore size filters) were used as models for biological membranes. POPC, POPC:EPOPC (1:1), DLPC, DPPC, DSPC, SM, SM:Chol (2:1) and SM:Cer (9:1 and 7:3) mixtures were mainly analyzed. For AFM experiments, supported lipid bilayers were obtained by fusion of SUV deposited on mica [30]. POPC:DPPC (1:1) and POPC:EPOPC:DPPC (4:1:3) mixtures were chosen. All the experiments were performed at 22 °C, and, unless otherwise stated, all the solutions were prepared using the same buffers as for the sifuvirtide and Trp stock solutions.

Absorption measurements were performed in a UV–vis spectrometer Jasco V-560 (Easton, MD), fluorescence assays were carried out in a Varian Cary Eclipse spectrofluorometer (Mulgrave, Australia) and a Malvern Instruments Nano ZS (Worcestershire, UK) was used for DLS and zeta-potential measurements. Disposable polystyrene and capillary cells were used in DLS and zeta-potential measurements, respectively, whereas 0.5 cm length quartz cells were used for adsorption and fluorescence measurements. To perform AFM experiments, a JPK Nanowizard II (Berlin, Germany) coupled to

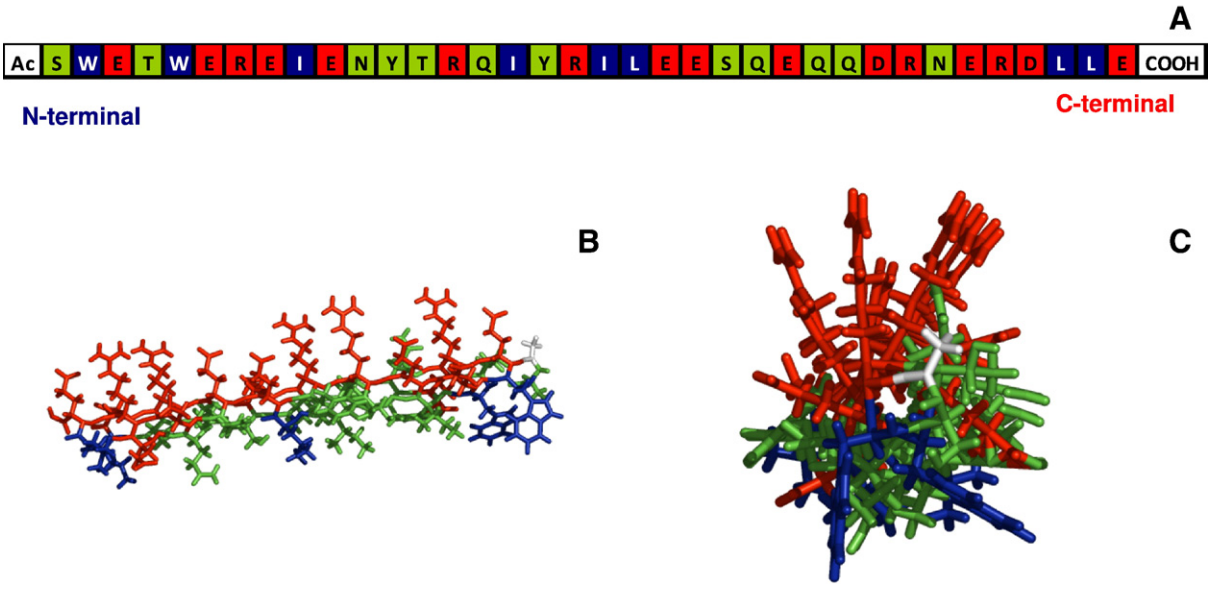

Fig. 1. (A) Amino acid sequence of HIV fusion inhibitor sifuvirtide. (B) Lateral and (C) frontal views of sifuvirtide in α-helical arrangement. It should be noticed that charged residues in this arrangement are projected towards one side of the peptide helix. Hydrophobic residues are represented in blue, noncharged polar in green, and charged polar in red.

a Zeiss Axiovert 200 inverted microscope (Göttingen, Germany) was utilized.

2.3. Photophysical characterization of sifuvirtide in aqueous solution

Based on the presence of Trp residues on the sequence of sifuvirtide, the spectral characteristics in aqueous and lipidic environments of this peptide were studied by fluorescence spectroscopy at low and physiologic ionic strengths. Emission spectra were collected in the 300–450 nm wavelength range, at an excitation wavelength (λ_{exc}) of 280 nm. The dependence of the emission fluorescence intensity of sifuvirtide on its concentration was studied in the 0.5–15 μM range. All the spectra were corrected for inner filter effect [31]. Whenever a fixed concentration of peptide was needed, sifuvirtide 15 μM was used. In the case of the Trp solutions (used as controls), a fixed concentration of 36 μM was chosen. In both cases, the absorbance of the solutions is below 0.1, ensuring minimal inner filter effects [31]. The accessibility of the Trp residues in the peptide structure was assessed by quenching assays using acrylamide, as described in Section 2.5. The relative fluorescence quantum yield for sifuvirtide at both low and physiologic ionic strengths was determined as indicated in Ref. [32] by choosing a Trp solution as standard. To study the effect of $CaCl_2$ in the structural properties of sifuvirtide, the fluorescence emission spectra of the 15 μM peptide were recorded in the 0–10 mM $CaCl_2$ concentration range in a 10 mM HEPES pH 7.4 150 mM NaCl buffer. To further complement the previous studies, the fluorescence of ANS 26 μM (λ_{exc} = 344 nm; λ_{em}: [430,630 nm]) was also evaluated at the same peptide and $CaCl_2$ concentrations.

2.4. Membrane interaction studies

Membrane partition studies were carried by successive additions of small amounts of 15 mM lipid suspensions (LUV) to a sifuvirtide 15 μM solution, with a 10 min incubation between each addition. Samples were prepared in 10 mM HEPES buffer pH 7.4 with low (NaCl 10 mM) or physiologic (NaCl 150 mM) ionic strength, or in the presence of $CaCl_2$ at concentration ranging from 0 to 12.5 mM. The membrane interactions were assessed by steady-state fluorescence (according to Ref. [33]), recording the emission in the 300–450 nm range with an excitation wavelength of 280 nm. The fluorescence intensities were corrected for successive dilutions, background intensities and scatter [34]. To study the effect of NaCl concentration in the specific interaction of sifuvirtide with DPPC, the concentrations of peptide and lipid were kept constant (15 μM and 3 mM, respectively), varying the NaCl concentration from 0.01 M to 1 M by adding small amounts of a 2.5 M concentrated NaCl to our samples.

2.5. Quenching experiments

Fluorescence quenching of sifuvirtide was assessed using the quencher soluble in aqueous solution, acrylamide. The assays were performed using sifuvirtide 15 μM in low or physiologic ionic strength buffer, in the presence or absence of 3 mM of lipid (LUV). Small aliquots of acrylamide were successively added to the samples. The acrylamide concentrations were within the 0–60 mM range [23]. For every addition a minimal 10 min incubation time was allowed before measurement. To minimize the relative quencher/fluorophore light-adsorption ratios, the excitation wavelength used was 290 nm. Regarding the emission, it was recorded at a fixed wavelength of 360 nm. Quenching data were corrected both for inner filter effect and light-adsorption [35], before being analyzed using the Stern–Volmer equation [36] (Eq. (1)) or the Lehrer equation [37,38] (Eq. (2)), when a negative deviation to the Stern–Volmer relationship is observed.

$$\frac{I_0}{I} = 1 + K_{SV}[Q] \tag{1}$$

$$\frac{I_0}{I} = \frac{1 + K_{SV}[Q]}{(1 + K_{SV}[Q](1-f_B) + f_B} \tag{2}$$

I and I_0 are the fluorescence intensities of the sample in the presence and absence of quencher, respectively, K_{SV} is the Stern–Volmer constant, [Q] the quencher concentration and f_B is the fraction of peptide accessible to the quencher. Additionally, when a f_B value is retrieved, an apparent partition coefficient value, K_p, can be calculated using Eq. (3):

$$K_p = \frac{1-f_B}{f_B} \times \frac{1}{\gamma_L[L]}, \tag{3}$$

valid for $\gamma_L[L] \ll 1$, where [L] is the lipid concentration and γ_L the molar volume of a lipid bilayer [39].

2.6. Dipole potential membrane assays

Changes on the membrane dipole potential magnitude, driven by membrane binding and insertion of the peptide, were monitored by means of fluorescence excitation spectral shifts of the dye di-8-ANEPPS [40,41]. Small volumes of di-8-ANEPPS dissolved in ethanol (less than 2%) [42] were added to LUV. To allow proper incorporation of the probe within the membranes, the mixtures were incubated overnight. For these assays, the final concentrations used were 200 μM for lipids, 5 μM for di-8-ANEPPS and 15 μM for sifuvirtide [43,44]. Fluorescence difference spectra of di-8-ANEPPS-labelled vesicles were obtained by subtracting the excitation spectrum before the addition of peptide from the excitation spectrum after the addition of sifuvirtide 15 μM. Before subtraction, the spectra were normalized to the integrated areas to reflect only the spectral shifts. The emission wavelengths were fixed at the maximum for each vesicle composition used. All the experiments in this assay were performed using a 10 mM HEPES buffer pH 7.4 in NaCl 150 mM.

2.7. Zeta-potential and dynamic light scattering spectroscopy (DLS)

DLS experiments [45–47] of lipid vesicles (LUV) in the presence of peptide were carried out with a backscattering detection at 173° using an incident wavelength of 632.8 nm, at 25 °C, with an incubation time of 15 min. Before measurements, the samples were filtered with 0.45 μm pore nylon membranes (Whatman, Kent, UK). Lipid concentration was fixed at 50 or 200 μM and the sifuvirtide concentration varied from 0 to 15 μM. Normalized intensity autocorrelation functions were analyzed using the CONTIN method [48,49] yielding a distribution of diffusion coefficients (D) through multiexponential fits. The measured D was used for the calculation of the hydrodynamic radius of the vesicles through the Stokes–Einstein relationship [50].

Zeta-potential experiments of lipid vesicles (LUV) in the presence of peptide were based on the laser-Doppler electrophoresis [46,47]. The scattered light detection during zeta-potential measurements was made at the forward angle of 12°. Lipid concentration was fixed at 200 μM and the sifuvirtide concentration varied from 0 to 40 μM. The values of zeta-potential of the liposomes were calculated from their electrophoretic mobility at 25 °C (15 min pre-incubation times) by means of the Henry correction of Smoluchowski's equation [51,52]. Results are the average of 9 to 15 measurements at the stationary level. When significant changes of the zeta-potential occurred as a function of the peptide concentration, results were fitted using Eq. (4):

$$\zeta = \frac{\zeta_0 + \zeta_{lim}K_B[P]}{1 + K_B[P]} \tag{4}$$

where ζ_0 is the zeta-potential value of the vesicles in the absence of peptide, ζ_{lim} corresponds to the zeta-potential value of the vesicles in

the presence of saturated concentrations of peptide, [*P*] the peptide concentration and K_B the apparent binding constant.

All the samples at this study were prepared using a 10 mM HEPES buffer pH 7.4 in NaCl 150 mM.

2.8. Atomic force microscopy (AFM)

AFM [53–55] was used to visualize the sifuvirtide-induced topographical perturbations on phase separated supported lipid bilayers. A 1.5 mM SUV suspension (10 mM HEPES buffer pH 7.4 in NaCl 150 mM) was deposited in the presence of $CaCl_2$ 50 mM on a freshly cleaved mica substrate previously glued to a glass coverslip. The lipid sample was then incubated for 30 min at 25 °C. After incubation, the sample was rinsed at least 4 times with EDTA 1 mM and afterwards at least 10 times with buffer.

AFM imaging was performed using uncoated silicon cantilevers CSC38 from MikroMasch (Tallinn, Estonia) with a typical spring constant of 0.01–0.2 N/m for contact mode and uncoated silicon nitride cantilevers OMCL-TR800PSA-1 from Olympus (Tokyo, Japan) with a typical stiffness of 0.57 N/m for intermittent contact mode. The scan rate was set to less than 1 Hz for intermittent contact mode and between 1–2.5 Hz for contact mode. The force applied on the sample was maintained at the lowest possible value by continuously adjusting the set-point and gain during the imaging. For intermittent contact mode the cantilever oscillation was turned to a frequency of 15–20 kHz. Height, deflection and phase-shift signals were collected and images were line-fitted as required. Tip artefacts were ruled out by image analysis.

3. Results and discussion

3.1. Characterization of sifuvirtide in aqueous solution

Fluorescence spectra of sifuvirtide were recorded in solution under physiological pH and under low (NaCl 10 mM) and physiological (NaCl 150 mM) ionic strength conditions. Structural properties of this peptide can be retrieved from the spectral characteristics of its Trp residues [36,56]. At a peptide concentration of 15 μM, the peptide presented the same excitation and emission maximum at both ionic strength conditions (Fig. S1). An approximate value of 0.07 was recovered for the fluorescence quantum yield (Φ) of the peptide under both ionic strengths. The dependence of the fluorescence quantum yield on peptide concentration was studied at both ionic strength conditions, to evaluate possible changes in the supramolecular structure of sifuvirtide. No significant deviations of linearity were observed, indicating the absence of aggregation in the analyzed concentration range (0–15 μM) (Fig. S1). Fluorescence quenching of the Trp residues of the peptide by acrylamide was also studied at both conditions to assess the accessibility of the fluorophores. Linear Stern–Volmer plots were obtained (Fig. S1), revealing that the Trp residues are accessible to the quencher and therefore not inserted in hydrophobic pockets [23].

Additionally, as a calcium-binding domain was identified on the original HR2 sequence of gp41 from which sifuvirtide is derived [57,58], the effect of Ca^{2+} ions on the supramolecular organization of sifuvirtide was evaluated. We intended to evaluate if the peptide could sequester Ca^{2+} ions, contributing to neutralize its global negative net charge, promoting a putative aggregation of peptide monomers. The emission spectra of sifuvirtide (under physiological pH and ionic strength) were registered upon successive additions of calcium chloride (0–10 mM). No significant changes in the fluorescence intensities neither spectral shifts were observed (data not shown), providing evidence of no significant aggregation. ANS was also used as a reporter for supramolecular aggregation, since it is conventionally considered to bind to pre-existing hydrophobic (nonpolar) surfaces of proteins and peptides, being this binding concomitantly followed by an increase in its fluorescence intensity [56,59]. ANS emission spectra were recorded upon addition of calcium chloride (0–10 mM) and no significant increase of the ANS fluorescence intensity was recovered (data not shown).

All these results suggest that the Trp residues of this peptide are considerably exposed to the aqueous medium [36,56] and that the ionic strength and Ca^{2+} concentration do not contribute significantly to major molecular changes on the supramolecular organization of the peptide.

3.2. Interaction of sifuvirtide with rigid membrane models

Previous studies performed by our group revealed the importance of lipid phase in the interaction of sifuvirtide with zwitterionic lipid membrane models [23]. Sifuvirtide adsorbs on the surface of DPPC gel phase (S_o) membranes, but does not interact significantly, for instance, with POPC liquid disordered (l_d) or POPC:Chol liquid ordered (l_o) membranes. Lipid rafts [26], where the receptors needed for HIV entry are located upon viral binding [27], and the viral membrane [24,25] are enriched in rigid lipids such as sphingolipids and saturated PC. Furthermore the specificity of the interaction of sifuvirtide within these rigid membrane domains would be a key factor for its improved efficacy, since the inhibition process occurs in extreme confinement between those membranes [23]. Therefore, we were prompted to study in more detail the interaction of sifuvirtide with rigid bilayer vesicles of several lipid compositions.

The interaction of sifuvirtide (15 μM) with rigid membrane models at physiological pH and ionic strength conditions was studied using LUV of DPPC, DSPC, SM, SM:Chol (2:1) and SM:Cer (9:1 and 7:3). POPC and DLPC were used as liquid disordered membrane models for the sake of comparison.

No significant changes in the fluorescence intensity (Fig. 2A) or spectral shifts (data not shown) were observed for the fluid phase POPC and DLPC vesicles. Nonetheless, for the gel phase DPPC and DSPC bilayers a significant increase in the fluorescence intensity (Fig. 2A), as well a significant blue-shift of the emission spectra (7 nm), was observed. This indicates that sifuvirtide binds specifically to gel phase PC bilayers, such as DPPC (gel-liquid phase transition temperature, $T_m = 41.4 \pm 0.5$ °C [60]) or DSPC ($T_m = 55.1 \pm 1.5$ °C [60]), but not towards PC with disaturated acyl chains presenting a fluid phase organization, such as DLPC ($T_m = -1.1 \pm 0.8$ °C [60]). It is to be noticed that the addition of cholesterol and POPC to DPPC vesicles would reduce the affinity of sifuvirtide towards lipids [23].

Concerning the interaction of sifuvirtide with vesicles containing sphingolipids, no significant affinity of the peptides toward these membranes was observed. In the case of SM and SM:Cer, which present similar or stronger gel phase properties at room temperature, when compared to DPPC [61,62], no significant changes in the fluorescence intensities (Fig. 2A) nor spectral-blue shifts (data not shown) were observed. At this point, this would indicate that the interaction of sifuvirtide with membranes is specific towards rigid PC membranes.

To further elucidate and quantify this interaction, quenching experiments in the presence of lipid vesicles, using acrylamide as a quencher of the peptide's fluorescence, were performed. Acrylamide is a soluble quencher in aqueous solution that presents a low penetration into lipid bilayers capacity [63]. If a peptide interacts with lipid bilayers, it will be less accessible to the quencher in solution and therefore its fluorescence will be less quenched [64,65]. A characteristic negative deviation to the linear Stern–Volmer relationship can then be observed and, by fitting the Lehrer equation to the data, it is possible to obtain the fraction of peptide that is accessible to the quencher in the solution (f_B) [23], and subsequently the fraction of peptide interacting with the lipid bilayers. In addition, a partition coefficient value (K_p) for this interaction can be calculated [33]. A comparison of the quenching profiles in the presence and in the

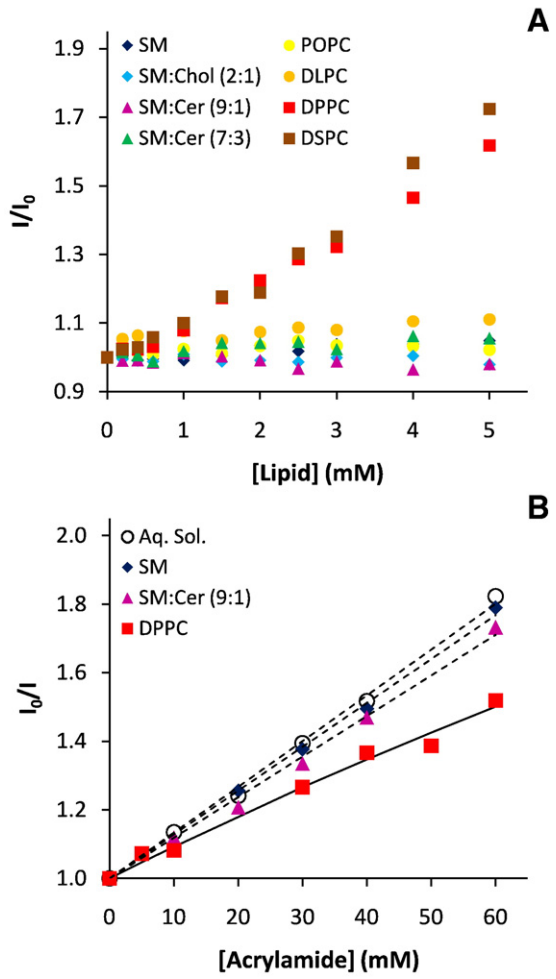

Fig. 2. Sifuvirtide interaction with lipid membranes. (A) Titration of sifuvirtide 15 μM with LUV. (B) Fluorescence quenching of sifuvirtide 15 μM in the presence of 3 mM lipid vesicles. The dashed lines are fittings of the Stern–Volmer equation (Eq. (1)) whereas the solid line is a fitting of the Lehrer equation (Eq. (2)) to experimental data. All the assays were performed in 10 mM HEPES buffer pH 7.4, containing NaCl 150 mM. (I — fluorescence intensity of sifuvirtide at 360 nm; I_0 — fluorescence intensity in the absence of lipid (A) or acrylamide (B)).

absence of 3 mM of DPPC, SM or SM:Cer (9:1) (Fig. 2B), reveals that for all the studied lipid compositions, except DPPC, a linear quenching profile similar to the peptide in the absence of lipid was obtained. Similar K_{SV} values were obtained (Table 1). In the case of DPPC, a negative deviation to the Stern–Volmer relationship was observed, indicating that a fraction of peptide is interacting with those vesicles. Applying the Lehrer equation, we could retrieve a value of $f_B = 0.8$, similar to the value previously obtained by time-resolved fluorescence ($f_B = 0.84$). This yields a value of $K_p = 1.2 \times 10^2$, similar to the one previously obtained using a Förster resonance energy transfer approach ($K_p = 1.2 \times 10^2$) [23].

Table 1
Parameters obtained for the quenching of fluorescence of sifuvirtide by acrylamide in aqueous and lipidic environment.

		K_{SV} (M^{-1})	f_B
Aq. sol	NaCl 150 mM	13.1 ± 0.7	1[§]
	NaCl 10 mM	13.3 ± 0.3	1[§]
Lipid	SM	12.8 ± 0.3	1[§]
	SM:Chol (2:1)	15.1 ± 0.1	1[§]
	SM:Cer (9:1)	11.8 ± 0.2	1[§]
	SM:Cer (7:3)	14.5 ± 0.5	1[§]
	DPPC	11.7 ± 4.5	0.8 ± 0.2[#]

Assays were performed in 10 mM HEPES buffer pH 7.4, containing 10 or 150 mM NaCl. K_{SV} and f_B were obtained using Eqs. (1)[§] and (2)[#]. All experiments with lipid were conducted with 3 mM lipid concentration and 150 mM NaCl buffer.

Altogether, the partition and quenching data indicate a selective affinity of sifuvirtide towards gel phase PC lipids, but not towards SM.

3.3. Effect of salt concentration on the interaction of sifuvirtide with membranes

Salt concentration can affect both peptides and lipids. The increase in the ionic strength affects the Debye screening length, diminishing for instance the electrostatic repulsion among charged peptide molecules or, in the case of neutral lipids, the repulsive forces between the polar parts of lipid molecules [66]. Furthermore, the dependence on the salt concentration can give us valuable information about the electrostatic or non-electrostatic nature of peptide–lipid interactions.

Since sifuvirtide presented a selective affinity towards gel phase PC membranes, we were prompted to study the effect of ionic strength in the interaction of sifuvirtide with DPPC vesicles. We studied the effect of NaCl salt concentration on the interaction of sifuvirtide with DPPC vesicles. A significant decrease in the fluorescence intensity of sifuvirtide in the presence of DPPC vesicles at [NaCl] = 10 mM, when compared to [NaCl] = 150 mM, was observed (Fig. 3A). At physiological ionic strength, the increase in the fluorescence intensities was concomitant with a blue-shift of the emission spectra. In contrast, at

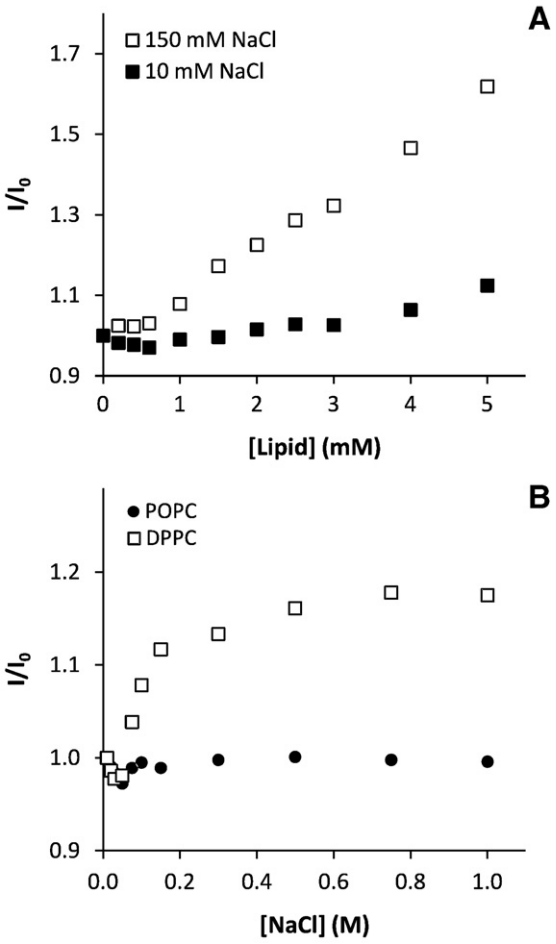

Fig. 3. Effect of ionic strength in the interaction of sifuvirtide with DPPC vesicles. (A) Titration of sifuvirtide 15 μM with DPPC LUV under different ionic strengths. (B) Interaction of sifuvirtide 15 μM with DPPC and POPC vesicles (3 mM lipid concentration) as a function of the concentration of NaCl. (I — fluorescence intensity of sifuvirtide at 360 nm; I_0 — fluorescence intensity in the absence of lipid (A) or at 10 mM NaCl (B)).

low ionic strength no significant changes in the peptide fluorescence intensity or blue-shifts were observed.

The fluorescence intensity of sifuvirtide in the presence of DPPC and POPC membranes was measured in the NaCl concentration range 10 mM–1 M (Fig. 3B). POPC served as an initial negative control in this experiment, since there is no interaction of the peptide with this lipid. For POPC no significant changes in fluorescence parameters within the analyzed NaCl concentration range occurred. For DPPC, between 10 and 50 mM of NaCl, no significant changes in the peptide's fluorescence emission intensity or spectrum were observed. For salt concentrations in the 50–300 mM range, a significant increase in the fluorescence intensity of the peptide, accompanied by a significant blue-shift of the emission spectra is observed. At higher concentration, a lower increase in the fluorescence intensities and emission blue-shift were obtained.

Besides the effect of Na^+, we assessed the effect of Ca^{2+} ions on the interaction of sifuvirtide with POPC and DPPC LUV. For the fluid phase POPC vesicles, as expected, the addition of $CaCl_2$ did not cause any significant increase in the fluorescence intensity of the peptide (Fig. S2) nor spectral shifts (data not shown). Nevertheless, in the case of the gel phase DPPC vesicles, small increases in the fluorescence intensity of the peptide were observed as the calcium chloride concentration was increased (Fig. S2).

Since the affinity of sifuvirtide towards DPPC gel phase membranes is affected by the ionic strength, the results obtained indicate a dependence on electrostatic factors. The nature of this interaction should be related to interfacial specificities of rigid PC membranes at high salt concentrations, more precisely at the choline headgroup level [67,68].

3.4. Evaluation of the membrane dipole-potential of lipid bilayers upon interaction with sifuvirtide

The use of intrinsic fluorescence properties of a peptide to evaluate its interaction with lipid bilayers is important to determine structure, dynamics and location within the membranes [36,56]. However, instead of focusing the measurements on the peptide, changes in membrane properties can also serve as a valuable sensor in peptide–lipid interactions [40]. Thus, to further elucidate the action of sifuvirtide at the membrane level, we studied changes in the membrane dipole potential and zeta-potential, as well as the aggregation susceptibility of the lipid vesicles upon addition of peptide.

The membrane dipole potential is originated from the alignment of dipolar residues of lipids (polar headgroups and glycerol-ester regions) and oriented water molecules hydrating the outer surface of the membrane [40,41]. The magnitude of the membrane dipole is affected by membrane binding and by the insertion or adsorption of molecules (including peptides) [43,69]. Changes on the dipole potential magnitude may be monitored through spectral shifts of the fluorescence dye di-8-ANEPPS, sensing the local electric field derived from the dipoles of the lipid headgroup region [40,41]. No significant spectral shifts promoted by the peptide occur in POPC (Fig. 4), POPC:Chol (2:1) and SM (data not shown). The exceptions are DPPC and POPC:EPOPC vesicles (positive control) (Fig. 4), that presented spectral shifts to the red indicative of decreases in the dipole potential. Sifuvirtide interacts strongly with vesicles containing EPOPC and this peptide inserts into these membranes due to electrostatic attraction of the negatively charged sifuvirtide towards the EPOPC cationic headgroups [23]. In the case of DPPC vesicles, only a slight decrease of the membrane dipole potential was recovered, in accordance with the very low K_p previously estimated.

3.5. Evaluation of the zeta-potential and dynamic light scattering (DLS) parameters of lipid bilayers upon interaction with sifuvirtide

The zeta-potential of a particle is related to its surface charge potential, corresponding to the potential at the hydrodynamic shear surface [51,52]. This potential can be calculated by the electrophoretic mobility of the particles in solution by laser-Doppler velocimetry in a zeta-sizer device [46]. First of all, we evaluated the mean zeta-potential value (ζ) of a concentrated solution of sifuvirtide (560 μM) under physiological pH and ionic strength. In agreement with the negative charge of the peptide, we found $\zeta = -12.6 \pm 2.4$ mV. ζ values for POPC:EPOPC, POPC and DPPC vesicles (200 μM lipid concentration) in the presence of several concentration of peptide (0–40 μM) were measured under the same conditions (Fig. 5). Upon addition of increasing concentrations of peptide, no extensive changes in the ζ values in DPPC and POPC vesicles were recovered. This is expected for POPC, but in the case of DPPC vesicles these results could indicate a desorption of the peptide during the electrophoretic process. In contrast, we obtained a significant decrease in the zeta-potential upon addition of peptide to vesicles containing EPOPC that we could fit using Eq. (4), obtaining an apparent binding coefficient (K_B) of $(2.0 \pm 0.8) \times 10^2$ mM^{-1}. It should be noticed that this value does not have direct correspondence to the partition constant values, determined by fluorescence spectroscopy. Furthermore, regarding the zeta-potential of those vesicles at saturating peptide concentrations (ζ_{lim}), we

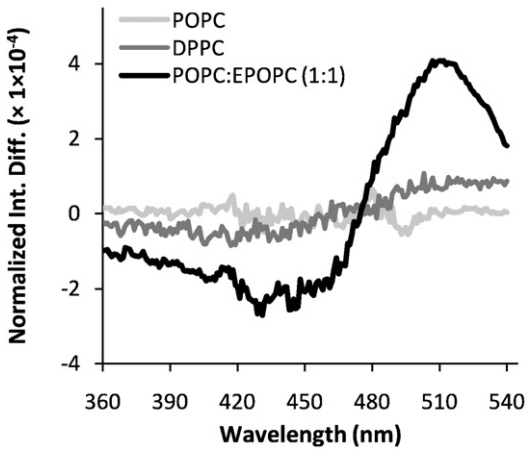

Fig. 4. Dipole membrane-dependent differential excitation fluorescence spectra of di-8-ANEPPS-labelled vesicles in the presence of sifuvirtide 15 μM. Lipid concentration and di-8-ANEPPS were kept constant at 200 μM and 5 μM, respectively. All the assays were performed in 10 mM HEPES buffer pH 7.4, containing NaCl 150 mM.

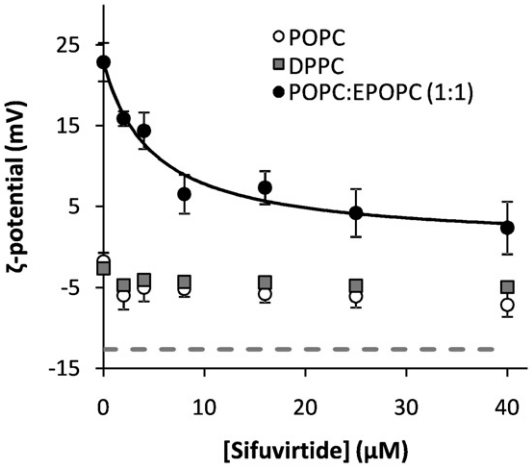

Fig. 5. Zeta-potential dependence of LUV on sifuvirtide concentration. Lipid concentration was kept constant at 200 μM. Each point corresponds to an average of 9–15 measurements. Error bars represent standard deviation. Solid line is the fitting of Eq. (4) to POPC:EPOPC (1:1) data. Dashed line corresponds to the zeta-potential value of 560 μM sifuvirtide. All the assays were performed in 10 mM HEPES buffer pH 7.4, containing NaCl 150 mM.

obtained a value of 0.45 ± 2.27, indicating that the vesicles are reaching electroneutrality.

DLS [46] of POPC:EPOPC vesicles at 200 μM lipid concentration and in the presence and absence of 15 μM sifuvirtide, reveals an increase in the hydrodynamic radius of the vesicles in the presence of the peptide (data not shown), possibly due to the initiation of the aggregation of the POPC:EPOPC vesicles. Zeta-potential values in these conditions show that almost all the positive charges on the EPOPC moieties are being neutralized by the negatively charged peptide, and therefore electroneutrality is being reached. At low peptide concentrations no changes in the vesicles hydrodynamic radius were detected. We also tested the aggregation tendency of DPPC vesicles upon addition of peptide and no significant changes of the hydrodynamic radius of the vesicles were retrieved (as seen for POPC). The hydrodynamic radius of DPPC vesicles remained constant regardless of peptide concentration. Altogether, the results show that sifuvirtide does not cause a noticeable effect on lipid bilayer structure in DPPC vesicles.

3.6. Evaluation of membrane topography upon interaction with sifuvirtide by atomic force microscopy (AFM)

Given the selectivity of sifuvirtide to rigid membranes and its ability to adsorb to bilayers without extensive perturbation, we were interested in visualizing the effect of sifuvirtide on the topography and stability of phase separated planar lipid bilayers. AFM was used for this purpose. This technique uses a sharp tip to scan over a surface, being the signal detected through an optic lever mechanism, enabling the acquisition of high-resolution images [53]. It is to be noticed that this technique was successfully applied in the study of the fusion activity of the HIV gp41 ectodomain on lipid bilayers [70–72].

In this part of the work, POPC:DPPC (1:1) and POPC:EPOPC:DPPC (4:1:3) lipid bilayers were imaged before and after the incubation with sifuvirtide 15 μM. Controls adding buffer instead of peptide solution were performed with the same incubation times and measurement conditions. The peptide does not cause significant changes on the overall POPC:DPPC membrane topography (Fig. 6), which is in agreement with the results obtained in the zeta-potential and membrane dipole potential measurements. AFM, therefore, reinforces the conclusion that the low but specific affinity of sifuvirtide towards DPPC (reported by fluorescence spectroscopy) does not significantly perturb the surface of these membranes. In contrast, in the POPC:EPOPC:DPPC (4:3:1) membrane system (Fig. 7), upon addition of peptide and after a 2 h incubation, small defects spread over the membrane in the fluid matrix were observed. These defects, typically less than 1–2 nm in depth, were not as deep as a hole in the lipid bilayer (4–6 nm). Therefore, they can be interpreted as a local thinning or partial disruption of the membrane matrix, most likely involving the EPOPC cationic phospholipids, caused by the action of the anionic peptide. Similar defects were reported in the action of an amylin peptide in fluid anionic lipid bilayers [73], for instance.

4. Conclusions

Sifuvirtide is a second generation HIV fusion inhibitor peptide with an improved efficacy and promising preclinical results [19,20,22]. It was previously revealed that lipid membranes play an important role in the mode of action of sifuvirtide and other HIV fusion inhibitors, such as enfuvirtide and T-1249 [16,17,23,74,75]. Since the inhibition of the membrane fusion process must occur in extreme confinement between the cellular plasma and viral membranes, the lipid bilayers may be enhancers of the process (sometimes referred to as a catalytic-

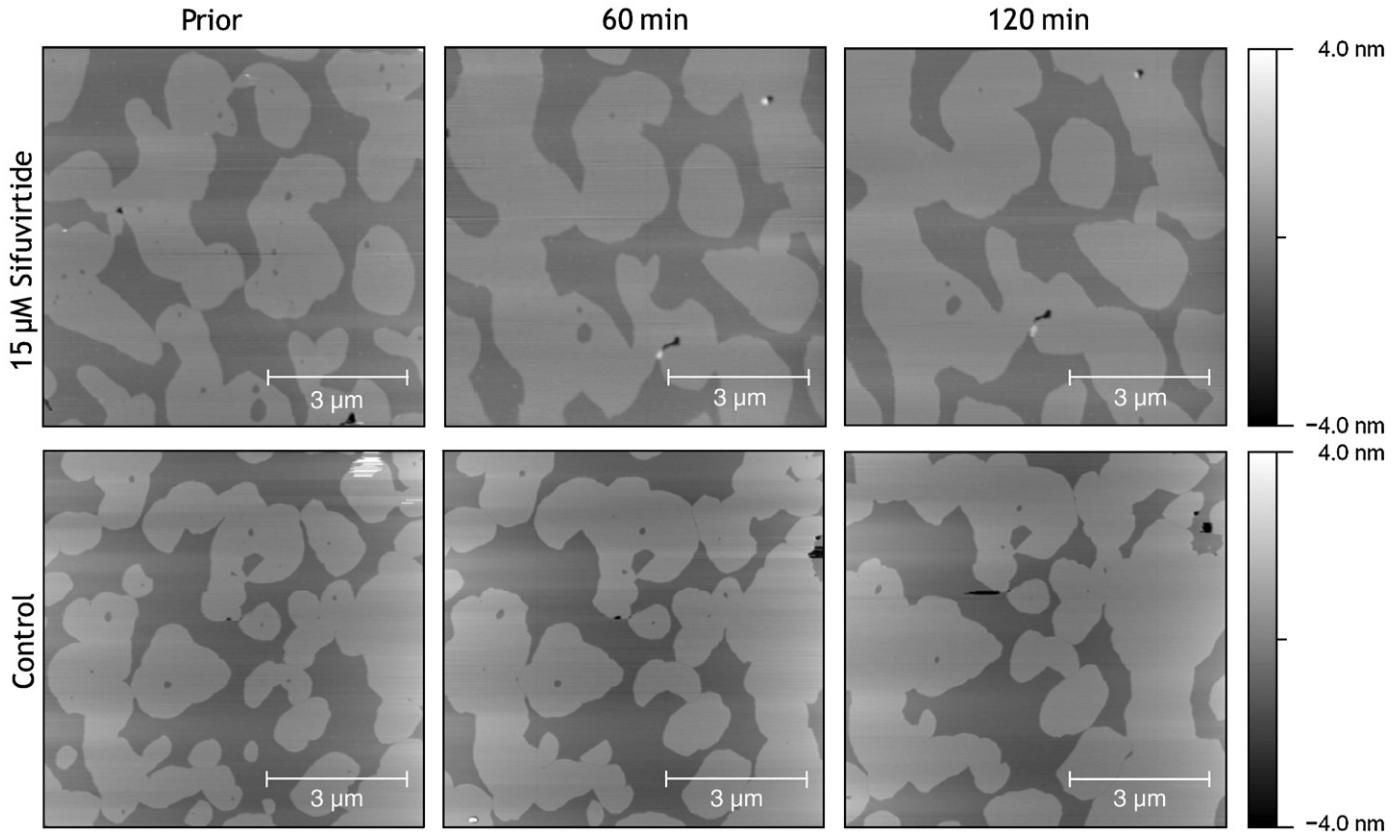

Fig. 6. Effect of sifuvirtide on the topography of zwitterionic supported lipid bilayers with phase separation. AFM measurements on POPC:DPPC (1:1) lipid bilayers were performed in the absence and in the presence of sifuvirtide 15 μM. Control measurements were performed by adding buffer instead of peptide. Images were recovered at intermittent contact mode prior and after addition of peptide. 10 mM HEPES buffer pH 7.4 containing NaCl 150 mM was used. The brighter (and higher) domains correspond to the DPPC rigid domains, whereas the darker parts correspond to the POPC fluid phase.

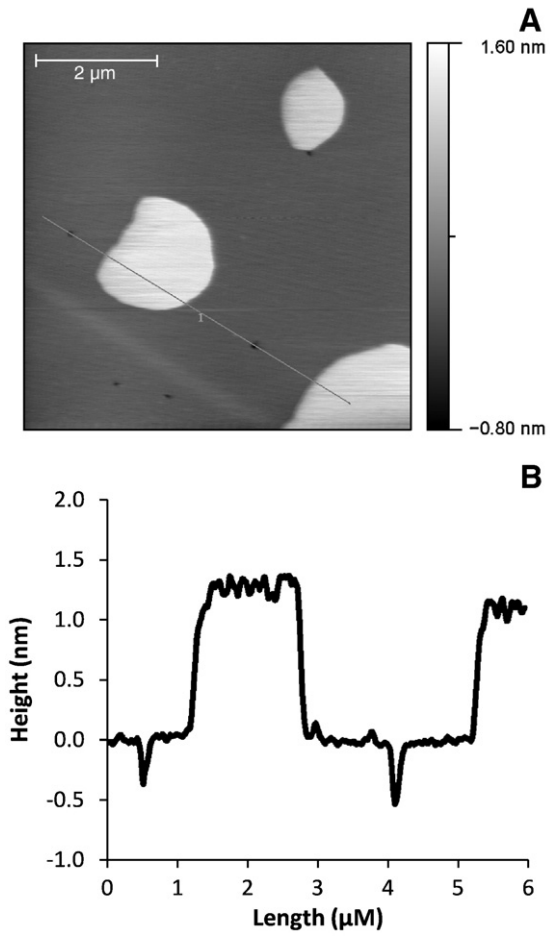

Fig. 7. Effect of sifuvirtide 15 μM on the topography of POPC:EPOPC:DPPC (4:1:3) supported lipid bilayers after a 2 h incubation. Image obtained in contact mode in a 10 mM HEPES buffer pH 7.4, containing NaCl 150 mM. The brighter (higher) domains correspond to the DPPC rigid domains, whereas the darker parts correspond to the POPC:EPOPC fluid matrix. (B) Height profile of the cross-section (in grey) presented in (A). As seen in (A) and (B), the presence of small defects (less than 1 nm in height) on the lipid bilayer fluid matrix caused by the peptide is to be noticed.

like activity [18]) by concentrating the inhibitor peptides near the fusion sites. Besides the interaction of enfuvirtide, T-1249 and sifuvirtide with membranes, the interaction of peptides such as P1 (gp41 residues 649-683) and P5 (628-683) derived from the gp41 CHR with galactosylceramide (GalCer) in biological membranes was also reported [57,58,76,77]. Furthermore, fusion inhibitors linked to lipids and sterols to improve the affinity to biological membranes have increased anti-HIV activity, further demonstrating the importance of biological membranes in the mechanism of action of these drugs [78,79]. Nevertheless, the interaction with membranes may differ depending on the structural properties of the peptides. Compared with the sequence of enfuvirtide, sifuvirtide has the deep pocket binding domain [19], but lacks the Trp-rich domains of the membrane proximal region (MPR) region of HIV gp41, which is assumed to be important for the interaction with membranes [19]. In opposition to sifuvirtide, enfuvirtide interacts strongly with POPC vesicles and this may be due to the Trp-rich region in enfuvirtide's C-terminus. On the other hand, sifuvirtide is able to adsorb at the surface of DPPC gel phase membranes [23]. These structural differences may be responsible for the distinct and more selective mode of action of sifuvirtide. In this work, we revealed a unique selectivity of sifuvirtide towards PC gel phase lipids. We demonstrated that this peptide would interact with DPPC and DSPC gel phase membranes, but not to SM or SM:Cer rigid membranes. Despite having a low K_p, sifuvirtide is remarkably efficient in screening rigid PC lipid bilayers. Nonetheless, no significant changes on the membrane dipole potential, zeta-potential and membrane topography were reported for DPPC. This is indicative of a selective yet weak adsorption on the surface of DPPC. An insertion into the membrane would promote larger effects over these parameters, as seen for the POPC:EPOPC controls. We can therefore hypothesize a low toxicity of this peptide towards its targeted membranes, which may be clinically important.

The selectively towards rigid PC membranes in contrast with SM, may be explained by the interfacial differences that both membranes present. Although both SM and PC contain a phosphocholine as the polar headgroup, their hydrophobic backbone is different. Whereas SM contains both hydrogen bond donating and accepting groups (due to the sphingosine backbone), PC only has hydrogen bond accepting groups [61]. These differences at the interfacial level do not affect extensively the rigidity of both membranes (DPPC and 16:0-SM have similar T_m [60]), but have a pronounced effect on the polarity, water exposure and accessibility to salt at the headgroup level.

Moreover, for PC vesicles the conformation of the polar headgroups depends on the binding of cations to the phosphate group [66]. Binding of positively charged solutes can repel the N^+-end of the choline moiety toward the aqueous phase [66]. According to a model for the gel phase PC membrane [67], the configuration of the polar heads depends on the ionic strength. With the increase in salt concentration, a rising number of standing polar heads was revealed, exposing the choline moieties and increasing the accessibility of ions and solutes [67]. This explains the dependence of the interaction of sifuvirtide with DPPC vesicles on the ionic strength observed in this work. At higher salt concentrations, the negatively charged sifuvirtide is likely to bind to the positively charged choline moieties, closely arranged and fully exposed at the surfaces of tightly packed PC bilayers. This tendency to selectively interact with rigid PC membranes is not limited to this peptide. Aβ peptides, for instance, interact preferentially towards gel phase PC membranes [80–82].

Additionally to Na^+ ions, Ca^{2+} ions can also contribute to improve the interaction of sifuvirtide with membranes. Ca^{2+} cations are important in the HIV membrane fusion process: 1) Ca^{2+} was reported to be essential for post-binding steps of the fusion process [83,84]; and 2) a calcium-binding site was identified in gp41 CHR [57,58]. Sifuvirtide is not extensively affected by the presence of Ca^{2+} in the solution and no aggregation was observed. Moreover, the presence of high concentrations of Ca^{2+} slightly improved the interaction with DPPC vesicles. This may be due to a bridging by Ca^{2+} between the phosphate headgroups and the negative charges of the peptide [85].

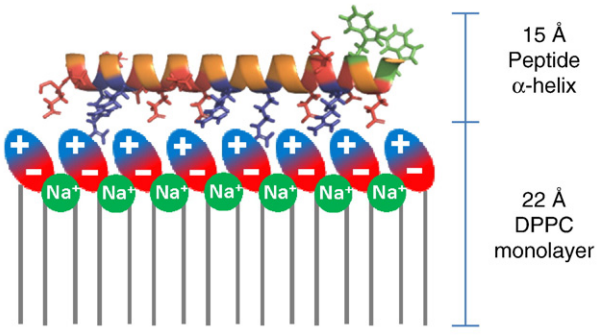

Fig. 8. Schematic representation of a putative mode of action of sifuvirtide on the surface of DPPC gel phase membranes in the presence of high NaCl concentration. Na^+ cations stabilize the anionic charge of the phosphate moiety (red) in the PC headgroups, leaving the choline moieties (cationic — blue) more exposed. This enables the anionic peptide to adsorb on the membrane with its charged residues (blue — cationic residues, red — anionic residues) oriented towards the surface, leaving the Trp residues (green) in a more exposed and less protected orientation.

To summarize, this study reveals the selectivity of the peptide sifuvirtide towards PC gel membranes and unravels the molecular basis of such selectivity. By complementing the results here presented with those from the FRET experiment, where we validated the hypothesis of the adsorption of sifuvirtide on the surface of DPPC bilayers [23], we disclose a putative mode of action for this peptide on PC gel phase membranes at physiological salt concentrations (Fig. 8 and Appendix B — Supporting information). The interaction of sifuvirtide through its charged residues with gel phase PC membranes would explain the interplanar distances obtained by FRET in Ref. [23], the higher values of interaction at higher ionic strengths and the low K_p values for the partition of sifuvirtide in gel phase PC vesicles.

From a biological point of view, since saturated PC lipids are found in high concentration in lipid rafts [26,27], but mainly in the viral envelope (for instance DPPC represents approximately 20% of the PC content in the viral membrane) [24,25], the efficacy of sifuvirtide may be related to its screening ability towards both these regions, allowing the peptide to have an increased concentration at the fusion site. Furthermore, several glycolipids and proteins are associated within lipid rafts and viral membranes; thus, we can expect more possibilities for transient electrostatic peptide–lipid and/or peptide–protein interactions on the surface of those lipid domains. In the end, the importance of membrane rigidity and selective electrostatic interactions were demonstrated for the mode of action of sifuvirtide at the molecular level and may help the understanding of its improved efficacy when compared with other drugs from the same class.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2010.02.010.

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