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Characterization of channel-forming peptide nanostructures

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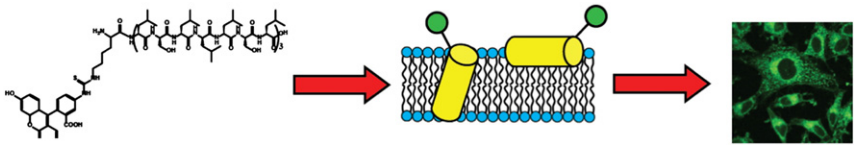
HIGHLIGHTS

- We prepared fluorescently labeled analogs of ion-channel-forming peptide nanostructures.
- The compounds retain their propensity to adopt a helical conformation within lipid membranes.
- Adding a fluorescent probe decreases the nanostructures' ability to insert in a transmembrane orientation.
- The peptide nanostructures enter living cells and localize into both organelle and cell membranes.

GRAPHICAL ABSTRACT



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ABSTRACT

We have prepared fluorescent analogs of known ion-channel-forming synthetic peptide nanostructures. These analogs were designed as probes to gain insight about the mechanism by which self-assembling amphiphilic peptides interact with lipid membranes. Conformational studies demonstrated that the labeled analogs retain their propensity to adopt a strong helical conformation in 2,2,2-trifluoroethanol and lipid bilayers. Attenuated total reflectance results indicated that the fluorescent peptide nanostructures are under an incorporation equilibrium between two forms, adsorbed at the surface or incorporated within the bilayer, similar to their unlabeled counterparts. However, when using a HeLa mimicking membrane, the proportion of peptide nanostructures in the transmembrane orientation decreases significantly. Finally, we were able to show by confocal microscopy studies that fluorescent analogs internalized into HeLa cells and localized into both the membranes of inner organelles and the cell membrane.

1. Introduction

Understanding the fine interactions between the transmembrane helices of ion channels has been the focus of extensive work over the past two decades [1,2]. While considerable knowledge has been acquired in this field, mastering the self-assembly of α-helices remains a daunting challenge. The inherent complexity of channel proteins calls for the use of simplified model peptides of nanoscale dimensions [3,4]. The best-known of all the reported peptide artificial ion channels are the ones developed by DeGrado and co-workers [5,6]. These (named LS2 and LS3) are 21-residue peptides mimicking alamethicin-type [7,8] structures, designed to self-assemble into an α-helix bundle pore in the hydrophobic regime of the lipid bilayer. These peptides consist of three repeating heptads of leucines (Leu or L) and serines (Ser or S) to provide the final sequences $(LSLLLSL)_3$ for LS2 and $(LSSLLSL)_3$ for LS3 (Fig. 1). In a helical conformation, the spacing of Ser residues gives rise to a polar face where all the hydroxyl groups line up one above the other. These amphipathic helices are believed to orient themselves in such a way that the inner surface of the channel is a hydrophilic environment. This allows the passage of partially hydrated ions. The outer surface of the supramolecular assembly is hydrophobic since it is composed of all the side chains from the leucines; this stabilizes the entire supramolecular structure in the aliphatic portion of a membrane.

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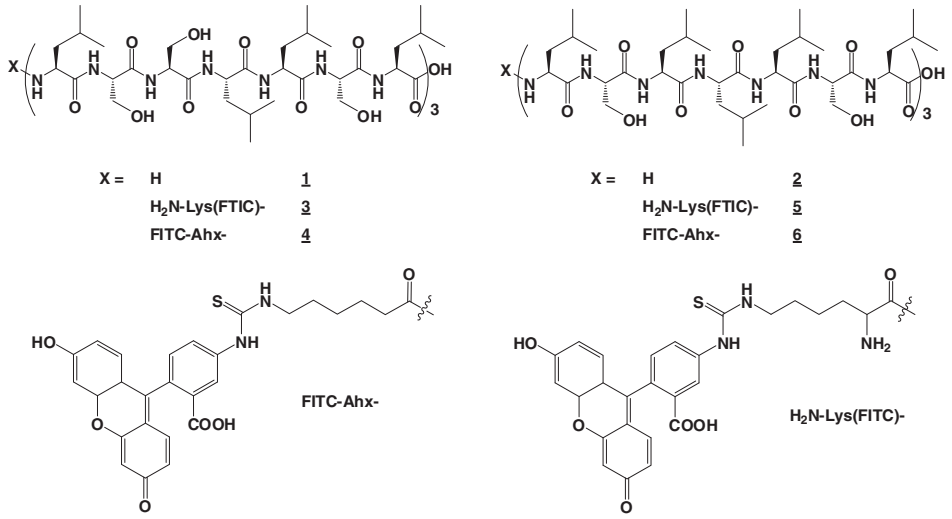

Fig. 1. The sequence of peptide nanostructures LS3 (top left) and LS2 (top right) forming self-assembling ion channels in lipid bilayer membranes and the structure of their four fluorescent analogs prepared and used in this study.

DeGrado et al. characterized LS2 and LS3 extensively [5,6,9–11]. They first carried out conductivity experiments that proved that these peptides can indeed transport ions through a membrane by a channel mechanism. They also showed that LS3 allows the passage of ions of various sizes, while LS2 is proton-specific. DeGrado et al. explained this difference through bioinformatic studies. They proposed that the pore formed by LS3 was larger due to the fact that it was composed of six helices instead of LS2's four. The origin of this difference would be the larger hydrophilic face of LS3. In a tetramer arrangement, some hydroxyl groups would be trapped in a hydrophobic environment, disfavouring this type of super-structure. DeGrado also performed circular dichroism (CD) measurements, showing that both peptides are α-helical in 2,2,2-trifluoroethanol (TFE). Theoretical simulations have been aimed toward understanding the molecular dynamics of the LS2 and LS3 pores, and have improved in their degree of precision and duration throughout the years, notably in the level of details of the bilayer membrane. Last, but not least, DeGrado and more recently, Bechinger [12] proposed that the transmembrane orientations of LS2 and LS3 are in constant equilibrium with the closed state where at least one peptide molecule is laying flat on the bilayer surface (Fig. 2).

To complement the informative work done on LS2 and LS3 under controlled conditions or *in silico* [5,6,9,10,13], we wanted to explore the behavior of these peptide nanostructures under biologically relevant conditions. To do so, we report here the synthesis of fluorescent analogs of LS2 and LS3 so that we could locate them within artificial and living cells. Here, we report the synthesis of four novel fluorescent analogs of LS2 and LS3, and their characterization by CD and polarized IR attenuated total reflectance (ATR) spectroscopy. We also report *in vitro* localization studies with living cells using confocal fluorescence microscopy.

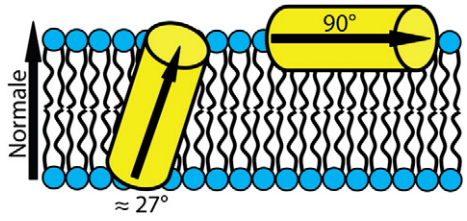

Fig. 2. Two possible orientations of the peptide nanostructures LS2 and LS3 in a lipid bilayer membrane. Transmembrane is on the left side and surface orientation on the right.

2. Experimental section

2.1. Materials

All lipids came from Avanti Polar-Lipids (Alabaster, AL, USA). Wang resin (0.69 mmol/g) was purchased from Advanced Chemtech (Louisville, KY, USA). All amino acids were bought from Matrix Innovation (Québec, QC, Canada) as well as hydroxybenzotriazole (HOBt). Fluorescein isothiocyanate (FITC) was purchased from Toronto Reasearch Chemicals (Toronto, ON, Canada) and piperidine was bought from American Chemicals Ltd. (Montreal, QC). All other chemicals were purchased from either Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA) and were used without further purification. All solvents were Reagent, Spectro, or HPLC grade quality purchased commercially and used without any further purification except for DMF (degassed with N_2), dichloromethane (distilled), and diethyl ether (distilled from sodium and benzophenone). Water used throughout these studies was doubly distilled and deionized using a Barnstead NANOpurII system (Boston, MA, USA) with four purification columns. Solid-phase peptide synthesis was performed manually using solid-phase reaction vessels equipped with a coarse glass frit (ChemGlass, Vineland, NJ, USA). Determination of peptide purity was performed by reverse phase HPLC with a C-4 or C-18 Vydac analytical column (Phenomenex, Torrance, CA, USA). HPLC analyses were done using an Agilent 1050 chromatograph. All solvents were degassed and gradients of A (89.9% H_2O/5% CH_3CN/5% isopropanol/0.1% TFA) to B (50% CH_3CN/50% isopropanol) were used over 60 min. ^1H NMR spectra were recorded on a Varian 400 MHz spectrometer. Sonication was done using a Branson water bath model 3510. Mass spectra were obtained from the Mass Spectrometry Laboratory of the University of Toronto (Toronto, ON, Canada).

2.2. Peptide synthesis

Five equivalents (eq.) of Fmoc-leucine were first dissolved in a minimal amount of DMF at 0 °C. It was activated with 5 eq. of diisopropylcarbodiimide (DIC) and stirred for 5 min. HOBt (5 eq.) was then added to prevent racemization via the formation of oxazolone. This solution was stirred for 30 min before adding it to 1 eq. of Wang resin, previously swelled in DCM. This mixture was stirred at room temperature for 24 h and then washed 3 times with DMF, 3 times with MeOH, again 3 times with DMF and then another 3 times with MeOH, before being dried under vacuum for 1 h. A quantitative ninhydrin test was performed to find out what proportion of the resin

sites was substituted. The remaining sites were capped with an acetyl group to prevent the growth of shorter sequences during the subsequent couplings. To do so, the resin bearing the first leucine was stirred with a 50:50 solution of acetic anhydride and DMF for 1 h then washed as described above. The Fmoc group on the leucine was removed with a solution of piperidine in DMF (20:80) stirred for 15 min, and submitted to the same washing sequence. Following amino acids were activated as described above. The Fmoc group at the end of the growing peptide was always removed as described above. Every coupling reaction lasted 2 h and a qualitative ninhydrin test was performed after each one. If needed, a second solution of activated amino acid was added and stirred for another 2 h. The purity of all peptides was checked by RP-HPLC and was over 90% in every case. The molecular weights were confirmed by mass spectrometry.

1 LS3 MW calc. 2160; obt. MALDI MS 2161 $(M+H^+)$, 2183 $(M+Na^+)$
2 LS2 MW calc. 2237; obt. MALDI 2238 $(M+H^+)$, 2261 $(M+Na^+)$

2.3. Fluorescent labeling

Two strategies were used to provide fluorescently-tagged peptides. From either the LS2 or LS3 peptides still bound to the resin, the Fmoc group was removed with piperidine, then previously-activated Boc-Lys(Fmoc)-OH was added to provide 22 residue peptides. FMOC deprotection and reaction with FITC in DMF in the presence of DIPEA as base led to fluorescent peptides with fluorophores linked to the amine on the side chain of the terminal lysine.

Alternatively, LS2 or LS3 was deprotected and FMOC-6-aminohexanoic acid was coupled as described above. FMOC deprotection followed by FITC coupling yielded fluorescent analogs with an alkyl spacer at the N-terminal. The purity of each peptide was checked by RP-HPLC, and was over 90% in every case. The structure was confirmed by mass spectrometry.

3 H_2N-Lys(FITC)-LS3-OH MW calc. 2677; obt. MALDI 2678 $(M+H^+)$
4 FITC-Ahx-LS3 MW calc. 2662; obt. MALDI 2663 $(M+H^+)$
5 H_2N-Lys(FITC)-LS2-OH MW calc. 2755 obt. ES 2755.5838 $(M+H^+)$
6 FITC-Ahx-LS2 MW calc. 2743; obt. MALDI 2744 $(M+H^+)$

2.4. Conformational studies

Circular dichroism spectropolarimetry (CD) samples for solution studies were prepared by mixing 0.5 mg of peptide **3**, **4**, **5** or **6** in 1 mL of TFE for a final concentration of 18–20 mM. For vesicle studies, the first step in vesicle sample preparation was mixing 1 mg of peptide and the appropriate amount of Egg Yolk Lecithin (EYL) in chloroform. The solvent was evaporated and dried under vacuum. The resulting mixture was then re-suspended in 2.5 mL of a 0.01 M HCl solution and sonicated until a seemingly uniform solution was obtained. The analysis of CD data has been performed using two algorithms, CONTIN and CDSSTR, available in CDPro package and six protein reference datasets. Data with a normalized root mean square deviation higher than 0.1 have been rejected.

2.5. ATR studies

All ATR samples were prepared with the following procedure: 2.5 mg of peptide and the corresponding quantity of lipid were dissolved together in chloroform and vigorously stirred. Chloroform was then evaporated and the mixture further dried under vacuum. 1.5 mL of D_2O was then added to the dry mix and shaken until a seemingly uniform solution was obtained. This solution was then submitted to five freeze-thaw cycles. Each cycle started by freezing the sample in liquid N_2 and quickly warming it in a water bath at 65 °C for 3 min. The cycle ended with a vigorous stirring of the sample before re-freezing it. Around 0.5 mL of this solution was then poured on an ATR germanium crystal, gently spread, and dried with an N_2 stream. Measurements were made on a Magna-760 E.S.P. (Nicolet) with 256 scans at a 2 cm^{-1} resolution. The spectra were analyzed with O.M.N.I.C. 5.2 and then Dichroic module in GRAMS V8.0.

2.6. Live cell culture and confocal microscopy experiments

HeLa cells were cultivated in a 2 cm Petri dish. The following quantities were used for every cell culture described herein: 2 mL of Iscove's Modified Dulbecco's Medium (IMDM) from Invitrogen, 0.2 mL of Fetal Bovin Serum (FBS) from Hyclone and 75 μL of Penicillin–Streptomycin mix from Invitrogen. A sterilized microscope cover glass was placed in the Petri dish. Prior to transfer, cells were washed with phosphate-buffered saline (PBS) hybridome and then trypsin was used to transfer the cells from the previous culture dish. The cells were then centrifuged at 1000 rpm for 3 min. The supernatant was removed and replaced with 5 mL of IMDM. After shaking the solution thoroughly to resuspend cells, 0.2 mL of the resulting solution was added to every Petri dish used. The cells were incubated for 24 h. A solution of TFE containing either Peptide **3**, **4**, **5** or **6** (20 μL) was dissolved in IMDM (180 μL). The resulting peptide solution was vigorously shaken for several minutes. An aliquot of the peptide solution was then added to the Petri dish to obtain a final peptide concentration of 20 μM in the Petri dish. Cells were incubated for another 24 to 48 h.

The fixation procedure started with the removal of IMDM and gentle washing of the cells with PBS. Cells were then fixed with formaldehyde for 20 min and then washed again with PBS. A solution containing 0.5% of Bovin Serum Albumin (BSA) in PBS was used to treat the cells for 30 min, followed by another washing with PBS. Nuclei staining was done with a DAPI solution in PBS for 20 min. Finally, the cells were treated with a dilute Triton-X100 solution for 30 min to wash off any cellular debris. The cells were washed with PBS three times before mounting the cover glass on microscope slides. Typically, a drop of a mix of 90% glycerol and 10% p-phenylenediamine (Aldrich) was put on a conventional microscope slide. This mixture is used for its refraction index, which suits microscope observations. Then, the small glass square cover plate, on which the cells were sitting, was gently lifted, flipped and slowly put on a top of the glycerol drop. To avoid cell deterioration, by sliding of the two glass plates against one another, sides of the smaller plate were glued to the larger one with common nail polish.

Phase contrast and fluorescence microscopy were both performed using a Leitz Aristoplan epifluorescence microscope (Leica Microsystems Canada, Richmond Hill, Ontario, Canada) with a 488-nm filter for fluorescein. Representative cell fields were captured using a black-and-white digital camera (Dage-MTI, Michigan City, IN) and Bioquant NOVA software (Bioquant-R&M Biometrics, Nashville, TN). All images reported here were captured at 400× zoom. Confocal microscopy was performed on a CarlZeiss LSM310 microscope.

3. Results and discussion

3.1. Design and synthesis of fluorescent analogs of LS2 and LS3

We achieved the synthesis of both LS2 and LS3 peptides through solid phase synthesis using Fmoc chemistry on Wang Resin. All residues were activated with diisopropylcarbodiimide (DIC) followed by hydroxybenzotriazole (HOBt). Each peptidic coupling was asserted by a qualitative Kaiser test and a MALDI-TOF HRMS every seven residues.

In order to be able to localize our peptides within living cells, we needed to tag them with a fluorescent marker. Among the many fluorophores available, we chose fluorescein isothiocyanate (FITC). This choice was motivated by its low cell toxicity and because it is

widely used in cell localization experiments. FITC chemistry is also well known. However, to avoid Edman Degradation during the attachment step, an alkyl spacer was added between the thiourea and the final amino acid. We first investigated the use of the side chain of lysine to link the reporter group by adding Boc-Lys(Fmoc)-OH as the 22nd residue. After selective deprotection of the Fmoc group, we coupled FITC on the side chain amino group. This last step was done using FITC in DMF with diisopropylethylamine (DIPEA).

Since the polarity of both *N* and *C*-terminal amino acids has been shown to influence the interaction of α-helices with bilayer membranes [14], we wanted to substitute the spacing lysine with the amino hexanoic acid (Ahx). This amino acid is well suited for solid phase peptide synthesis and can be used as any other residue after being protected with an Fmoc group. FITC was also added to the terminal Ahx using the same chemistry. The only difference between lysine and Ahx is the absence of the terminal amino group on the latter. Our synthetic efforts resulted in four related fluorescent analogs (**3**–**6**), depicted in Fig. 1.

3.2. Circular dichroism studies

To assess the conformational behavior of peptide nanostructures **3**–**6**, CD studies were conducted in solution and in vesicles. All measurements in solution were performed in 2,2,2-trifluoroethanol (TFE) at the same concentration in the range of 18–20 mM. The vesicles used were made of Egg Yolk Lecithin (EYL) and prepared by sonication. As seen in Figs. 3 and 4, every analog retains a strong α-helical character. They each possess the expected maximum near 192 nm and the two strong minima at 208 and 222 nm. In TFE, adding a FITC-modified lysine at the N-terminal in the case of **3** has no effect on the helicity, as noted by the superimposable curve with the one from **1** (Fig. 3). However, incorporation of an aminohexanoic acid spacer in **4** led to a slight decrease in its helical content. This could be explained by the loss of a positive charge at the N-terminus. On the other hand, LS2 and its analogs behave differently. A significant increase in the helical content is found for 5 and 6 in TFE (Fig. 3). We can hypothesize that LS2 is more sensitive to a helix capping effect due to the presence of an additional carbonyl group at the N-terminus.

Table 1 reports the different percentages of secondary structures derived from CD data. Results confirm the above conclusion that the helical conformation is the most favorable form in solution for all peptides.

In lipid vesicles, LS3 (**1**) retains its helical content as in TFE. But both fluorescent analogs **3** and **4** experienced a decrease in their helical content when incorporated in a lipid bilayer membrane as noted by a less significant 222 nm band. The effect is more pronounced with **4**. The absence of a positive charge at the N-terminus of the latter could be responsible for the enhanced sensitivity of **4** to the environment.

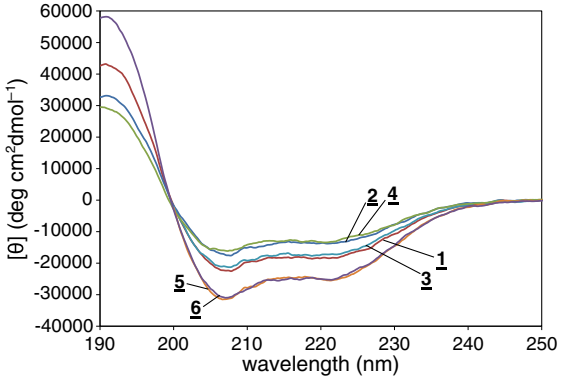

Fig. 3. CD curves for peptides **1** to **6** in TFE solutions (18–20 mM).

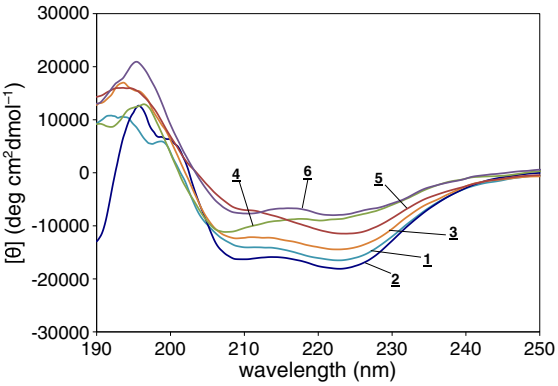

Fig. 4. CD curves for peptides **1** to **6** in Egg Yolk Lecithin (EYL) vesicles.

The decrease in the 222 nm band of fluorescent peptides **3** and **4** could also be due to a lower tendency to self-assemble or to an orientation phenomenon as described previously [15].

For LS2 (**2**), its helicity in the membrane and TFE are almost identical (Fig. 4). On the contrary, fluorescent analogs **5** and **6** experienced a notable decrease in their helical content in the lipid membrane, characterized by lower ellipticities at 208 and 222 nm. Here also, these observations can be explained in part by some peptide orientation effects that are more significant with N-terminal modified peptides, as well as by electrostatic effects as **6** lacking the positive charge is the least helical of the LS2 peptides studied. Also, it is important to note that CD results obtained with vesicles are dependent on the incorporation equilibrium as helicity may vary when peptide structures incorporate the low polarity environment of the lipid membrane.

Nevertheless, results from conformational studies by CD confirmed that adding a fluorescent probe on LS2 and LS3 peptides does not affect their helical character significantly in TFE or within phospholipid bilayers (Figs. 3 and 4), although slight increases or decreases are observed upon attaching the fluorophores to **1** and **2** respectively. The absence of minima at 215 nm points toward the absence of ß-sheet type aggregation, both in solution and in the vesicles. On the other hand, the high absorbance of the solution-containing vesicles accounts for the inability to measure accurately the maximum at 192 nm for these four samples.

3.3. ATR studies

DeGrado et al. demonstrated by fluorescence that LS2 and LS3 main orientation is not transmembranar, as in the active state [11,16]. The peptides rather lie on the surface of the bilayer, the backbone of the α-helix perpendicular to the normal of the membrane as illustrated in Fig. 2. This orientation is in equilibrium

Table 1
Secondary structure fractions of peptides **1**–**6** determined from CD spectrum*.

		% α-helix	% β-sheet	% Turn	% Unordered
In TFE	**1**	60 ± 4	6 ± 3	8 ± 4	25 ± 5
	2	48 ± 4	10 ± 3	15 ± 2	26 ± 4
	3	55 ± 4	8 ± 3	12 ± 3	25 ± 4
	4	44 ± 5	13 ± 4	16 ± 3	27 ± 5
	5	78 ± 8	3 ± 2	3 ± 3	15 ± 5
	6	76 ± 6	3 ± 2	4 ± 3	16 ± 4
In EYL	**1**	47 ± 5	9 ± 6	19 ± 5	24 ± 7
	2	67 ± 15	7 ± 7	13 ± 10	14 ± 7
	3	47 ± 4	7 ± 5	20 ± 5	26 ± 5
	4	38 ± 8	15 ± 4	20 ± 5	26 ± 5
	5	35 ± 6	16 ± 6	20 ± 4	29 ± 4
	6	35 ± 5	21 ± 3	20 ± 3	24 ± 4

* EYL: Egg Yolk Lecithin.

Table 2
Results of ATR studies on peptides LS2 **2** and **5** in different bilayer membranes at a ratio of lipid/peptide of 40 to 1.

	Spectral bands				Transmembrane orientation
	$\nu_{C\text{-}H}$ *sym*		$\nu_{C=O}$ *amide I*		
Samples	*R*	*Θ*	*R*	*Θ*	%
DMPG	1.13	28			
LS2 (**2**)	1.05	24	3.2	34	75
H_2N-Lys(FITC)-LS2-OH (**5**)	1.11	27	3.0	38	64
Simulated HeLa	1.46	40			
LS2 (**2**)	1.51	42	1.7	63	15
H_2N-Lys(FITC)-LS2-OH (**5**)	1.45	40	1.5	71	8
POPC	1.23	32			
LS2 (**2**)	1.57	44	1.5	70	8

with the transmembrane one; this leads to the channel self-assembly. It was also shown that it is possible to exert control over this equilibrium by changing the experimental settings such as the bilayer nature and hydration [12]. Accordingly, to verify the effect of the N-terminal fluorophore on such peptides, we investigated the effects of the bilayer composition on the membrane incorporation of peptidic nanostructures **2** and **5** by polarized ATR spectroscopy (ATR) [17]. This technique was shown to be useful to study this type of equilibrium [15,18–21]. Considering the wide range of possible experimental conditions possible to study our peptides, we selected experimental conditions that are the most relevant for *in vivo* studies. We chose to study the incorporation behavior of **2** and **5** with different lipid compositions used to prepare the bilayer.

We used a lipid-to-peptide ratio of 40 to 1, performing freeze-thaw cycles on a water solution containing DMPG and LS2 (**2**) or **5** for the initial studies, as DMPG is known to approximately mimic the bilayer of mammalian cells. Polarized ATR measurements were recorded after spreading the resulting mixture on a crystal of Ge. As shown in Table 2, there is no significant difference between the two peptides. Whether one looks at the estimated amount of transmembrane peptide (75% for the LS2 (**2**) against 64% for its fluorescent counter-part (**5**)) or the mean angle of the aliphatic chains on the lipids (24° for compound **2** against 27° for compound **5**), the fluorescent probe does not appear to have a significant effect on the peptide incorporation equilibrium and does not impede the transport of Peptide **5** to reach its transmembrane state.

To investigate how our fluorescent peptides would incorporate themselves into a HeLa cell plasma membrane, we used a model system. The outer membrane composition of a living cell is very complex so we decided to use a bilayer composed of a mix of lipids representing those found in the membrane of HeLa cells. All other components containing amines were omitted as they would overlap with the infrared absorption of LS2 and LS3, rendering measurement impossible. That last point is a limitation of our model membrane, since it has been reported that such small charged compounds could have an influence on the permeability of the membrane [22,23]. Our Hela-mimicking bilayer is therefore made of four lipids illustrated in Fig. 5 with their respective proportion based on the work of Bosmann [24] and Keegan [25].

Table 2 also illustrates that **2** and its fluorescent analog **5** behave similarly in a HeLa-mimicking membrane. Indeed, there is no significant difference in the tilt angle and the dichroic ratios of the lipid chains upon addition of the peptides. However, the proportion of peptides in a transmembrane orientation is substantially less, 15% and 8%, respectively for **2** and **5**. Both peptides are predominantly not in the transmembrane, but rather adsorbed at the surface of the bilayer membrane. To confirm this result, we performed a comparative study with LS2 in POPC, the model's major constituent. Interestingly, the behavior of LS2 with POPC alone was similar to the model membrane (Table 2). This suggests that one of the key factors influencing the transmembrane orientation is the length of the aliphatic chains. The size of the peptide nanostructures studied probably matches well with the hydrophobic core of the bilayer formed by lipids bearing 14-carbon aliphatic chains. However, there is a hydrophobic mismatch between the peptide helical structures and the thickness of the bilayer formed; lipids have longer chains. It is important to mention that the values reported here carry a significant margin of error, and should only be used for qualitative comparison.

3.4. In vivo localization studies

To study the incorporation of our fluorescent peptide analogs into live cells, we chose HeLa cells for their general ease of use and their resilience. This last point was crucial since we had to deliver our peptides from TFE solutions. All the other common solvents used for delivery of chemicals into a cell culture were not able to dissolve the fluorescent analogs, probably due to their high hydrophobicity. The high-concentration TFE solutions were diluted in culture media before being injected into the Petri dish. Extensive qualitative toxicity studies were then carried out to ensure that TFE volumes were diluted enough as to not harm the cell. The toxicity threshold is about 5 μL for a 2 cm diameter Petri dish. After a 24 h incubation with either **3**, **4**, **5** or **6**, cells were washed with PBS and fixed with a dilute formaldehyde solution. Washing cells with dilute Triton X-100 proved to be necessary to remove any excess of peptides. The small glass slide upon which the cells were grown was stained with 4′,6-diamidino-2-phenylindole (DAPI) and finally mounted on a microscope glass plate. Cells that had received a fluorescent analog were compared with cells incubated with pure FITC. Negative controls were performed, one with TFE and the other with dimethylsulfoxide (DMSO), as FITC alone is not soluble in TFE at the equivalent concentration used for our fluorescent peptides. A negative control was performed by incubating cells with a FITC solution in TFE. However, FITC alone is not as soluble in TFE as the fluorescent analogs. Therefore, a second control experiment, this time in DMSO, was conducted with the right concentration of FITC. In both case, microscopy studies showed no FITC uptake by the cells.

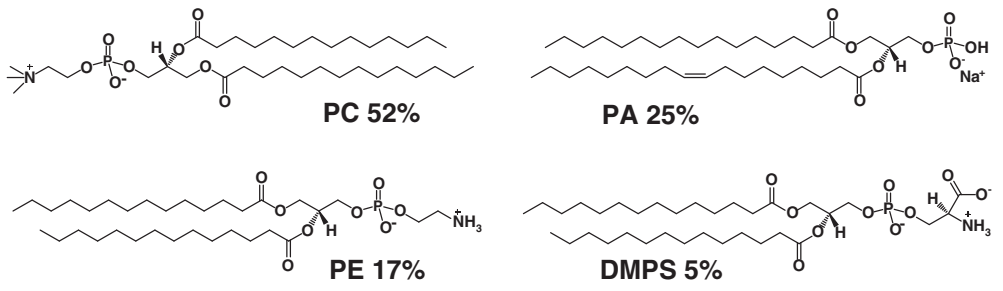

Fig. 5. Lipids used to mimick a HeLa plasma membrane with their respective proportions. L-α-phosphatidylcholine (POPC or PC), phosphatidic acid (PA), phosphoethanolamine (PE) and 1,2-Dimyristoyl-*sn*-Glycero-3-phospho-L-serine (PS).

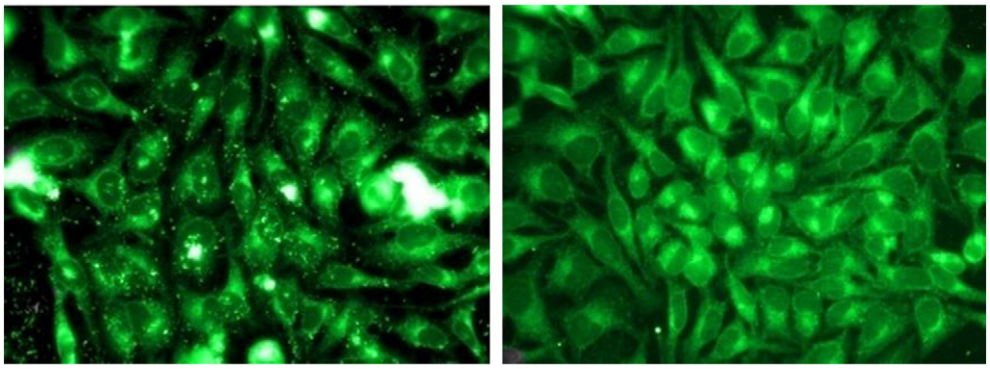

Fig. 6. Fluorescence microscopy images of HeLa cells treated with (left) H_2N-Lys(FITC)-LS3-OH (**3**) and (right) with FITC-Ahx-LS3-OH (**4**). Bright spots represent overexposure or air bubbles.

Figs. 6 and 7 present fluorescence microscopy images with the FITC cut-off for all four fluorescent peptide analogs. It can clearly be seen that each of them incorporate readily into cells, without any noticeable difference. It is crucial to note that peptide nanostructures incorporate into not only the plasma membrane, but also into the membranes of inner organelles as well. These results are in sharp contrast with the incorporation results obtained by ATR spectroscopy with a HeLa-mimicking membrane. They indicate that amphipatic peptide structures **3** to **6** can enter cells rather easily even though their incorporation equilibrium is disfavored in model membrane systems.

Confocal microscopy studies were also performed on the same samples in order to map the localization of each peptide in three dimensions. Representative results obtained are shown in Fig. 8 for fluorescent peptide **6**. Z-series sixteen images are numbered from top to bottom. As seen, cells appear on the first layers as filled green circles, indicating that fluorescent peptides are localized on the outer and organelle membranes as well. As the focal plan descends into the sample, the outline of each cell appears as well as various internal structures. Several layers also show hollow parts of the cells. This indicates that peptide **6** readily incorporates itself into both soft and plasma membranes, while remaining absent from the nucleus and the cytoplasm. The last few layers show a filled circle, corresponding to the bottom of each cell.

As previously mentioned, under the conditions we used, all of our peptides adopt mainly an α-helical conformation. The mechanism by which the fluorescent analogs of LS2 are internalized by HeLa cells, as seen in the confocal microscopy studies, is still unclear. It is possible that, at a critical concentration necessary to form a well-defined supramolecular assembly, the peptide diffuses into the cell and get adsorbed or incorporated in the inner membranes. This hypothesis would imply a different mechanism then the carpet-like mechanism, as the latter would be fatal to the cell, and all stained cells were perfectly healthy throughout the entire experiment. It is also possible that the organelle membrane lipid composition is different enough from the cell membrane that fluorescent peptides remain under a non-channel-forming structure, lying on the surface of each membrane they bind to. Indeed, as the open state would lead to a lethal ion transport. This view is supported by the low ratio of trans-membrane incorporation observed in ATR studies with the simulated HeLa membrane.

It is interesting to note the similarities between the results of our localization studies with peptide **6** and a similar experiment done with Sarcolipin, a transmembrane protein associated with the ion channel phospholamban [26]. In both cases, the cells lines are labeled by the fluorescent species pointing to a specific marking of the membranous environment, whether it is the plasma membrane, the nuclear membrane, or the various organelles.

4. Conclusions

It is understood that any structural modification alters somehow the properties of peptide structures. However, the biophysical studies reported here showed that it is possible to label short channel-forming peptides with a fluorescent probe without altering significantly showed that the incorporation equilibrium is highly dependent on the lipid composition, with a lower incorporation level when lipids have longer alkyl chains. The cellular localization experiments demonstrated that LS2 and LS3 fluorescent analogs internalize readily into HeLa cells, spreading to the inner organelle membranes. To the best of our knowledge, this constitutes one of the first descriptions of an artificial channel-forming peptide localizing on the organelle membrane of a living cell. The results also demonstrate the power of using model peptide nanostructures for studying complex biomolecular processes [27]. We are currently exploiting the fluorescent

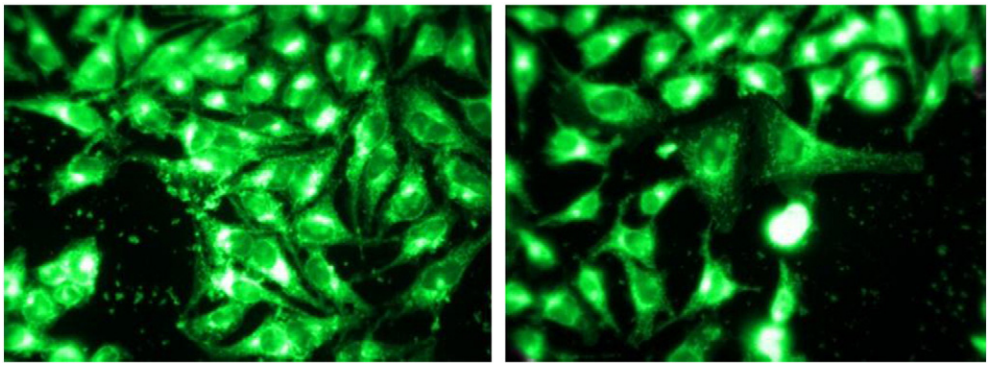

Fig. 7. Fluorescence microscopy images of HeLa cells treated with (left) H_2N-Lys(FITC)-LS2-OH (**5**) and (right) with FITC-Ahx-LS2-OH (**6**).

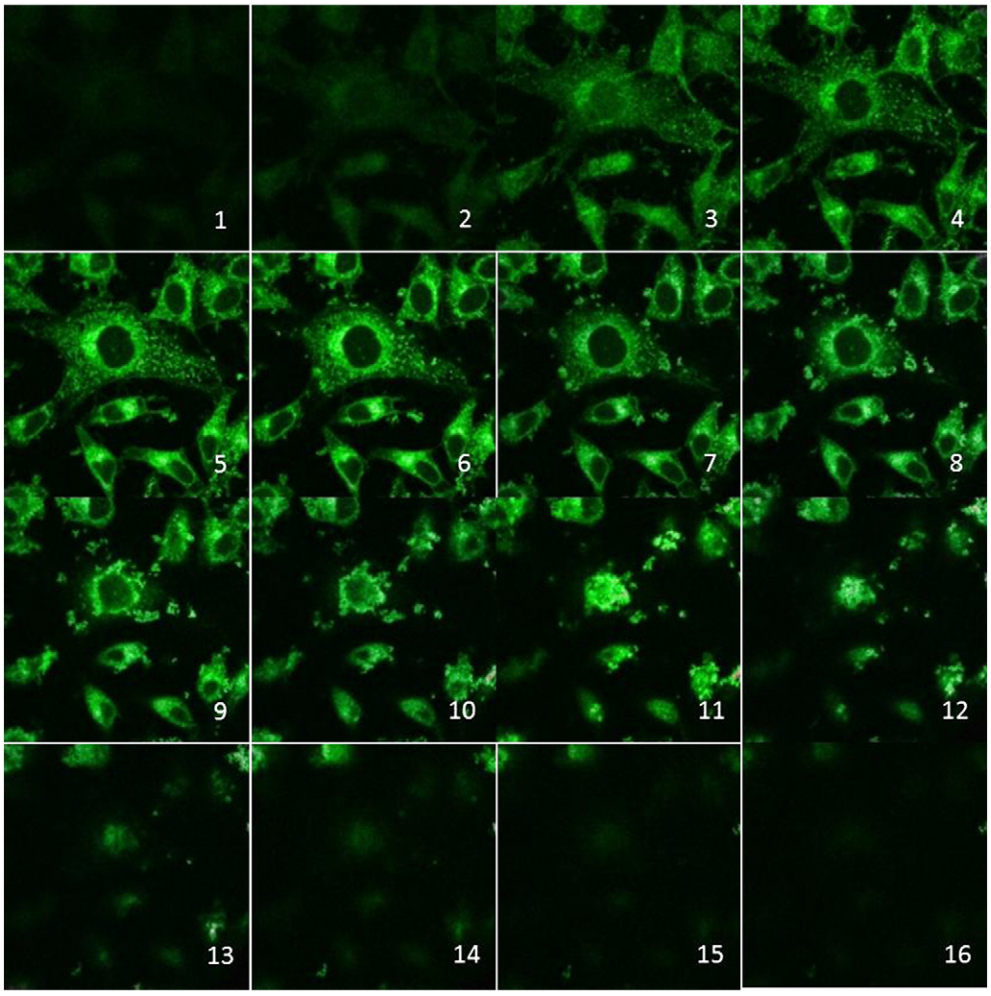

Fig. 8. Z-series confocal microscopy images of HeLa cells treated with FITC-Ahx-LS2-OH (**6**). Images are numbered from 1 to 16 going from top to bottom.

analogs described herein to elucidate the underlying mechanism of the internalization process.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bpc.2011.12.001.

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