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The iAβ5p β-breaker peptide regulates the Aβ(25–35) interaction with lipid bilayers through a cholesterol-mediated mechanism

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ABSTRACT

Alzheimer's disease is characterized by the deposition of aggregates of the β-amyloid peptide (Aβ) in the brain. A potential therapeutic strategy for Alzheimer's disease is the use of synthetic β-sheet breaker peptides, which are capable of binding Aβ but unable to become part of a β-sheet structure, thus inhibiting the peptide aggregation. Many studies suggest that membranes play a key role in the Aβ aggregation; consequently, it is strategic to investigate the interplay between β-sheet breaker peptides and Aβ in the presence of lipid bilayers. In this work, we focused on the effect of the β-sheet breaker peptide acetyl-LPFFD-amide, iAβ5p, on the interaction of the Aβ(25–35) fragment with lipid membranes, studied by Electron Spin Resonance spectroscopy, using spin-labeled membrane components (either phospholipids or cholesterol). The ESR results show that iAβ5p influences the Aβ(25–35) interaction with the bilayer through a cholesterol-mediated mechanism: iAβ5p withholds cholesterol in the inner hydrophobic core of the bilayer, making the interfacial region more fluid and capable to accommodate Aβ(25–35). As a consequence, iAβ5p prevents the Aβ(25–35) release from the lipid membrane, which is the first step of the β-amyloid aggregation process.

1. Introduction

Alzheimer's disease (AD) is one of the most feared neurodegenerative disorder, destroying neuronal tissues in the human brain and, therefore, the consciousness, memory and intellectual activity needed for social and occupational functioning of the individual. AD is characterized by the deposition of insoluble amyloid fibrils as plaques in the extracellular space, as well as the accumulation of neurofibrillary tangles in cell bodies of neurons [1]. The amyloid fibrils are constituted by the β-amyloid (Aβ), a peptide composed of 39–43 amino acids which is produced by an enzymatic cleavage from a larger amyloid precursor protein, APP, with an unknown function [2]. In its native form, Aβ is unfolded but aggregates into a β-sheet structure of ordered fibrils under various conditions [3]. Many initial studies hypothesized that the aggregation of Aβ into fibrils is a prerequisite for its toxicity [4,5]. Indeed, more recent *in vitro* observations have demonstrated that small, soluble and diffusible oligomeric Aβ species are also capable of initiating pathogenic events [6]. This evidence has led several researchers to believe that Aβ oligomeric intermediates, rather than fully formed fibrils, are the predominant toxic species [7].

Several strategies have been proposed to minimize or revert the negative effects of amyloid, including reduction of Aβ production, inhibition of Aβ misfolding and aggregation, enhancement of Aβ clearance. A promising approach is to use short peptides, named β-sheet breakers [8], that present a sequence similar to the middle hydrophobic region of Aβ, but have a very low propensity to adopt a β-sheet conformation, which is responsible for the aggregation properties and the consequent neurotoxicity. Thus, these peptides have the ability to interact specifically with Aβ blocking its β-sheet conformation [9], to disassemble preformed fibrils *in vitro*, to prevent neuronal death induced by fibrils in cell culture, to reduce amyloid β-protein deposition *in vivo* and to block the formation of amyloid fibrils in a rat brain model of amyloidosis [10]. Several β-sheet breaker peptides, from 11 to 5 amino acids long, were generated and tested [11]. One of these, named iAβ5 (LPFFD), was found to be able to inhibit and disassemble amyloid fibrils *in vitro*. Moreover, chemical modifications, such as N-terminal acetylation and C-terminal amidation, yield the end-protected peptide, iAβ5p, which presents a major stability and is rapidly taken up by the brain, reducing *in vivo* amyloid deposition [12,13].

Many studies suggest that lipid membranes are implicated in the mechanisms of β-amyloid fibrillization [14], favoring the Aβ misfolding and aggregation. In particular, the membrane surface

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may act as a two-dimensional template for fibril nucleation seeds [15]. Consequently, the analysis of the β-sheet breaker activity against Aβ fibrillization in the presence of membrane systems appears a strategic condition. In a previous work, we have preliminarily studied the interaction between the iAβ5p peptide and lipid membranes, observing that a significant fraction of peptide molecules insert in the membrane environment [16].

In the present work, we report an ESR investigation on the effect of the β-breaker peptide, iAβ5p, on the interaction between the Aβ(25–35) fragment and lipid bilayers, composed by dilauroyl phosphocholine (DLPC) and cholesterol (CHOL) at 80:20 weight ratio, by using phospholipids spin-labeled on the acyl chain or, alternatively, a radical analogue of CHOL. Aβ(25–35) represents the shortest Aβ fragment that exhibits large β-sheet aggregated structures and retains the toxicity of the full-length peptide [17]. In a previous study, we demonstrated that Aβ(25–35) interacts with DLPC:CHOL bilayer [18]. CHOL is a major component of neuronal cell membranes, influencing their thickness and fluidity [19].

In this framework, here we investigate if iAβ5p and Aβ(25–35) interact in the membrane environment. More generally, we try to elucidate if membrane-interacting iAβ5p contribute to hamper Aβ self-aggregation.

2. Materials and methods

2.1. Materials

Dichloromethane and methanol, HPLC-grade solvents, were obtained from Merck (Darmstadt, Germany), while 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) was obtained from Sigma–Aldrich (St. Louis, MO, USA). The phospholipid dilauroyl phosphatidylcholine (DLPC) and the spin-probe 3β-doxil-5α-cholestane were obtained from Avanti Polar Lipids (Birmingham, AL, USA). Cholesterol (CHOL) was obtained from Sigma. Spin-labeled phosphatidylcholines (*n*-PCSL) with the nitroxide group in the positions 5 and 12 of the acyl chain were synthesized as described by Marsh and Watts [20,21]. The spin-labels were stored at −20 °C in ethanol solutions at a concentration of 1 mg/mL.

2.2. Peptides synthesis

The Aβ(25–35), GSNKGAIIGLM, and the iAβ5p, Ac-LPFFD-NH_2, peptides were manually synthesized by conventional solid-phase chemistry using the Fmoc/tBu strategy and subsequently purified as previously reported [16,18]. The peptides were characterized on a Finningan LCQ-Deca ion trap instrument equipped with an electrospray source (LCQ Deca Finnigan, San José. CA, USA). The samples were directly infused in the ESI source by using a syringe pump at the flow rate of 5 μL/min. Data were analyzed with Xcalibur software. The samples purity was >98%.

2.3. Sample preparation

Multi-Lamellar Vesicles (MLVs) of DLPC:CHOL at 80:20 weight ratio were prepared mixing appropriate amounts of DLPC and CHOL, dissolved in a CH_2Cl_2–methanol mixtures (2:1 v/v, 10 mg/mL lipid concentration), in a round-bottom test tube, and a thin lipid film was produced by evaporating the solvents with dry nitrogen gas. Final traces of solvents were removed by subjecting the sample to vacuum desiccation for at least 3 h. The samples were then hydrated with 20 μL of 10 mM phosphate buffer at pH = 7.4, gently warmed ($T < 35$ °C), and repeatedly vortexed, obtaining a MLV suspension. The suspension of MLV thus obtained was transferred into a 25 μL glass capillary, and immediately sealed.

Samples containing Aβ(25–35) were prepared by the same procedure, adding appropriate amounts of the peptide dissolved in HFIP (10 mg/mL) to the lipid organic solutions. Finally, MLVs containing also the iAβ5p were prepared following the same procedure, in which the β-sheet breaker peptide was dissolved in the buffer solution (10 mg/mL), used to hydrate lipid films. As discussed below, two sets of experiments were performed using (i) spin-labeled phosphatidylcholines and (ii) spin-labeled cholesterol, which were added to the lipid mixture (1% wt./wt. on the total lipid) by mixing appropriate amounts of a spin-label solution in ethanol (1 mg/mL) with the lipid organic mixture. Lipid–iAβ5p peptide ratio was 1:1 wt./wt. (which corresponds to 10:1 mol/mol), while Aβ(25–35)–iAβ5p ratio was 1:1 mol/mol.

2.4. ESR spectroscopy

ESR spectra were recorded with a 9 GHz Bruker Elexys E-500 spectrometer (Bruker, Rheinstetten, Germany). Samples were placed in 25 μL glass capillaries and flame sealed. The capillaries were placed in a standard 4 mm quartz sample tube containing light silicone oil for thermal stability. All the measurements were performed at 25 °C. Spectra were recorded using the following instrumental settings: sweep width, 100 G; resolution, 1024 points; time constant, 20.48 ms; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; incident power, 6.37 mW. Several scans, typically 16, were accumulated to improve the signal-to-noise ratio.

3. Results

The ESR spectroscopy, by using spin-labeled substances (peptides and/or lipids) has been proved to give substantial information on the interaction between peptides and lipid membranes [22–24]. In the present work, the association of the two peptides under investigation with lipid bilayers was investigated by analysing changes in ESR spectra of spin-labeled lipids, as reported in the literature for peptides derived from viral fusion proteins [25–27] as well as for classical water-soluble peripheral membrane proteins [28,29]. Two sets of ESR experiments were performed. In the first one, the samples investigated were phosphatidylcholine spin-labeled at different positions, *n*, in the *sn*-2 chain (*n*-PCSL, $n = 5$, 12) incorporated in membranes of DLPC:CHOL 80:20 wt./wt., in the absence and in the presence of Aβ(25–35), iAβ5p, or both. In the second set of measurements, a radical analogue of CHOL, 3β-doxil-5α-cholestane, was used in order to directly investigate the cholesterol behavior in the systems.

The 5-PCSL and 12-PCSL spectra in the investigated lipid/peptide(s) mixtures are shown in Figs. 1 and 2, respectively. The former spin-label presents the nitroxide reporter group close to the hydrophilic lipid headgroup, while in the latter one the nitroxide group is positioned close to the terminal methyl group. In DLPC:CHOL bilayers, the spectra of both spin-labels present a clearly defined axially anisotropic lineshape, see Figs. 1A and 2A, an evidence that, due to the high cholesterol content, the membrane is in the liquid-ordered state [30]. These ESR spectra were quantitatively analyzed by the determination of the acyl chain order parameter, *S*, calculated according to the relation [31]:

$$S = \frac{(T_{\parallel} - T_{\perp})}{(T_{zz} - T_{xx})} \frac{a_N}{a'_N} \tag{1}$$

where $T_{\parallel}$ and $T_{\perp}$ are two phenomenological hyperfine splitting parameters which can be determined experimentally for each spin-labeled phospholipid as shown in Fig. 1A (note that $2T'_{\perp} = 2T_{\perp} - 1.6$) [32]. T_{xx} and T_{zz} are the principal elements of the real hyperfine splitting tensor in the spin Hamiltonian of the spin-label, which can

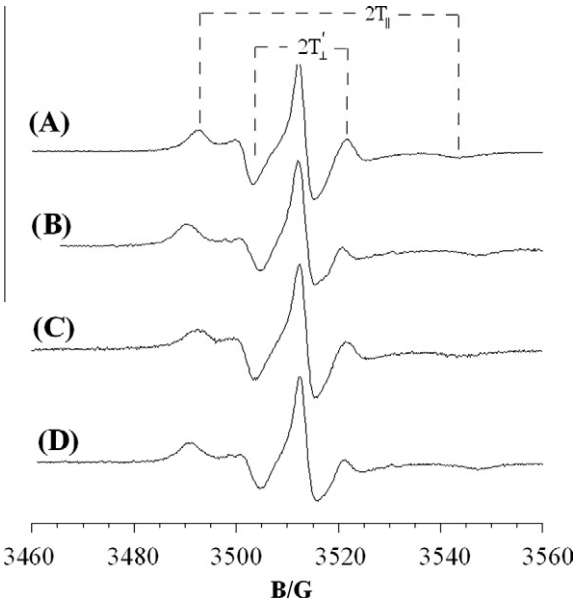

Fig. 1. ESR spectra of 5-PCSL in bilayers of DLPC:CHOL at 80:20 weight ratio: (A) in the absence of peptides; (B) in the presence of Aβ(25–35); (C) in the presence of iAβ5p and (D) in the presence of Aβ(25–35) and iAβ5p at equimolar ratio.

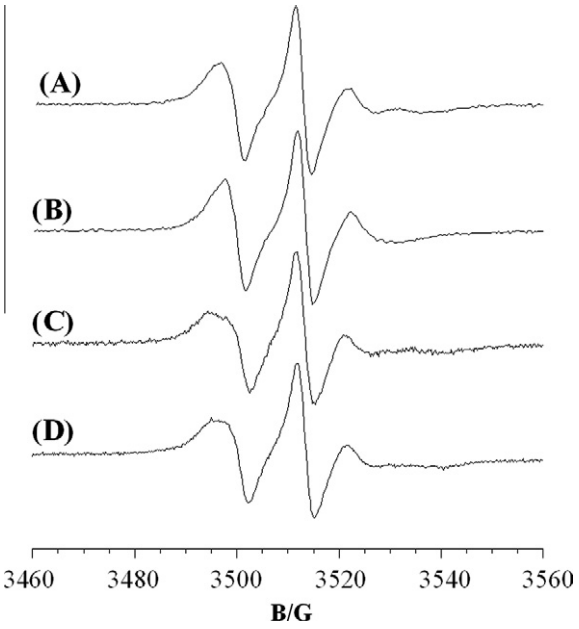

Fig. 2. ESR spectra of 12-PCSL in bilayers of DLPC:CHOL at 80:20 weight ratio: (A) in the absence of peptides; (B) in the presence of Aβ(25–35); (C) in the presence of iAβ5p and (D) in the presence of Aβ(25–35) and iAβ5p at equimolar ratio.

be measured from the corresponding single-crystal ESR spectrum and are reported in the literature (T_{xx} = 6.1 G and T_{zz} = 32.4 G) [33]. a_N and a'_N are the isotropic hyperfine coupling constants for the spin-label in crystal state and in the membrane, respectively, given by:

$$a_N = \frac{1}{3}(T_{zz} + 2T_{xx})$$

$$a'_N = \frac{1}{3}(T_{\parallel} + 2T_{\perp})$$

The isotropic hyperfine coupling constant is an index of the micropolarity experienced by the nitroxide, and the a_N/a'_N ratio in Eq. (1) corrects the order parameter for polarity differences between the crystal state and the membrane. The S and a'_N values reported in Table 1 show that, in the absence of any added peptide, the DLPC:CHOL bilayers are characterized by a decrease of both the micropolarity and the local acyl chain ordering in going from the polar headgroups to the inner hydrophobic core of the bilayer. Particularly, the S reduction indicates that, even in the presence of CHOL, the gradient of segmental chain mobility in DLPC bilayers is preserved [34]. The a'_N decrease is related to the polarity gradient [35], indicating that the hydrophobicity increases as the nitroxide group moves to the center of the bilayer.

Association of peptides to the lipid bilayer causes significant variations in the ESR spectra of spin-labeled phospholipids. Particularly, the anisotropy of the 5-PCSL spectrum clearly increases in samples containing Aβ(25–35), see Fig. 1B and D, while the effect of iAβ5p alone is much lower. In contrast, the 12-PCSL spectrum is much more affected by iAβ5p, alone or in combination, than by Aβ(25–35), see Fig. 2C and D. The S and a'_N values are collected in Table 1. Below, they are commented separately for each peptide or peptides combination.

First, addition of Aβ(25–35) causes a clear S increase of 5-PCSL spectrum, with respect to that registered in the peptide absence, while the S value of 12-PCSL decreases. Moreover, a'_N of 12-PCSL spectrum also decreases indicating a reduction of the local polarity. These evidences indicate that Aβ(25–35) interacts with the external region of DLPC:CHOL bilayers, only partially penetrating in hydrophobic inner core. At the same time, this peptide causes a re-positioning of cholesterol closer to the hydrophilic external layer, making the inner part of the lipid acyl tail more flexible than in the presence of cholesterol alone [18].

Addition of iAβ5p causes a slight S increase for 5-PCSL, while a much stronger effect is observed for 12-PCSL. The a'_N is marginally affected by this peptide, i.e., no evident variation in the local polarity is detectable. The acyl chains tend to assume a more ordered local structure in the vicinity of guest molecules: consequently, these evidences suggest a deeper penetration of iAβ5p in the bilayer with respect to Aβ(25–35).

Finally, in the presence of both Aβ(25–35) and iAβ5p peptides, the S value for 5-PCSL is nearly equal to that obtained in the case of DLPC:CHOL bilayers containing Aβ(25–35) peptide alone. This evidence suggests that Aβ(25–35) location relative to the bilayer is not affected by iAβ5p. Interestingly, for 12-PCSL an S value significantly higher than that registered in the presence of Aβ(25–35) or iAβ5p alone is found. The increase in the order parameter indicates that not only iAβ5p remains deeply inserted in the bilayer, but also that this peptide inhibits the cholesterol re-positioning observed in samples containing Aβ(25–35) alone.

Complementary information on the effect of the peptides on the CHOL positioning in the bilayer has been obtained by using 3β-doxil-5α-cholestane as spin-probe. Fig. 3 shows ESR spectra of the 3β-doxil-5α-cholestane in DLPC:CHOL bilayers, in absence

Table 1
The order parameter, S, and the isotropic hyperfine coupling constant, a'_N of 5-PCSL and 12-PCSL in lipid bilayers of DLPC:CHOL at 80:20 weight ratio, with and without Aβ(25–35) peptide, in the absence and in the presence of the β-sheet breaker iAβ5p.

	S	a'_N/G
5-PCSL		
DLPC:CHOL	0.58 ± 0.01	15.2 ± 0.1
DLPC:CHOL + Aβ(25–35)	0.73 ± 0.01	15.4 ± 0.2
DLPC:CHOL + iAβ5p	0.62 ± 0.02	15.4 ± 0.1
DLPC:CHOL + Aβ(25–35) + iAβ5p	0.72 ± 0.02	15.4 ± 0.2
12-PCSL		
DLPC:CHOL	0.38 ± 0.02	14.2 ± 0.1
DLPC:CHOL + Aβ(25–35)	0.24 ± 0.01	12.8 ± 0.1
DLPC:CHOL + iAβ5p	0.45 ± 0.02	14.3 ± 0.1
DLPC:CHOL + Aβ(25–35) + iAβ5p	0.52 ± 0.01	14.3 ± 0.1

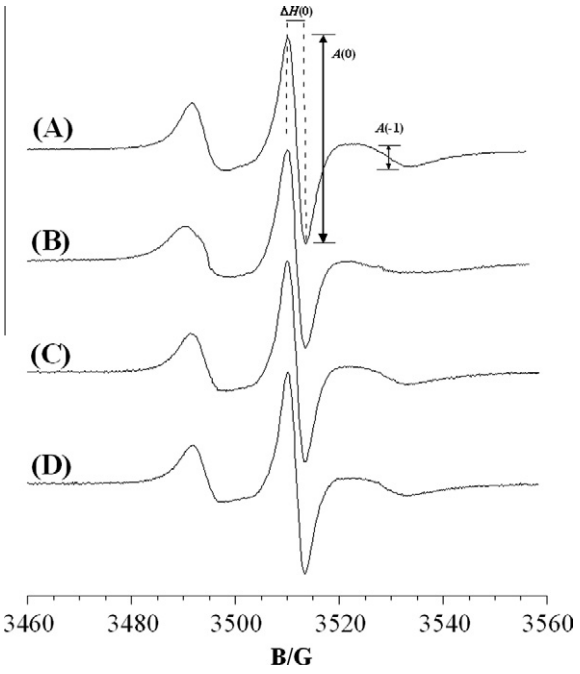

Fig. 3. ESR spectra 3β-doxil-5α-cholestane spin-label in bilayers of DLPC:CHOL at 80:20 weight ratio: (A) in the absence of peptides; (B) in the presence of Aβ(25–35); (C) in the presence of iAβ5p and (D) in the presence of Aβ(25–35) and iAβ5p at equimolar ratio.

Table 2
The isotropic hyperfine coupling constant, a'_N, and the correlation time, τ_c, of the 3β-doxil-5α-cholestane in lipid bilayers of DLPC:CHOL at 80:20 weight ratio, with and without Aβ(25–35) peptide, in the absence and in the presence of the β-sheet breaker iAβ5p.

	a'_N/G	$\tau_c \times 10^9/s$
DLPC:CHOL	21.01 ± 0.01	2.7 ± 0.1
DLPC:CHOL + Aβ(25–35)	23.35 ± 0.02	7.8 ± 0.2
DLPC:CHOL + iAβ5p	20.61 ± 0.01	5.2 ± 0.1
DLPC:CHOL + Aβ(25–35) + iAβ5p	20.49 ± 0.01	5.0 ± 0.1

and presence of Aβ(25–35), iAβ5p, or their equimolar mixture. All the spectra present an almost isotropic slow-motion lineshape. A quantitative analysis of these spectra was realized by determining the isotropic nitrogen hyperfine coupling constant, a'_N, and the tumbling correlation time of the spin-probe, τ_c. These parameters furnish information about the local physicochemical properties of the label. In particular, τ_c variations clearly show changes in the probe rotational mobility, as determined by the microenvironment viscosity and/or by specific interactions. τ_c values were determined according to [36]:

$$\tau_c = (0.65 \times 10^{-9})\, \Delta H_0 [(A_0/A_{-1})^{1/2} - 1] \quad (2)$$

where ΔH_0 is the peak-to-peak width of the center line in Gauss, A_0 is the amplitude of the center line, and A_{-1} is the amplitude of the high-field line. All the a'_N and τ_c values are reported Table 2.

In the presence of Aβ(25–35), a'_N increases indicating that the nitroxide reporter group experiences a more polar environment with respect to that found in the absence of peptide. At the same time τ_c increases, indicating that the motion of the spin probe molecules is hampered by a more viscous and structurally ordered microenvironment. Both evidences agree in suggesting that, in the presence of Aβ(25–35), CHOL molecules move towards the more external region of the bilayer. This part of the bilayer is more hydrated [36] and stiffer with respect of the inner core, as discussed above.

In the presence of iAβ5p, a'_N slightly decreases indicating a deep insertion of CHOL in the bilayer. This evidence indicates that CHOL tends to position close to iAβ5p peptide, which solubilizes in the inner core of DLPC:CHOL bilayers. The presence of the peptide has a rigidifying effect on the bilayer, which causes the concomitant τ_c increase, which however is much lower than that observed for Aβ(25–35).

Finally, a behavior similar is observed in the co-presence of Aβ(25–35) and iAβ5p, indicating that the β-breaker peptide plays a major role in determining the CHOL positioning.

4. Discussion

The end-protected 5-residue synthetic β-sheet breaker peptide, iAβ5p, is able to inhibit and disassemble Aβ fibrils both *in vitro* and *in vivo* [12]. Since several evidences suggest a possible role of biomembrane interface in facilitating the Aβ fibrillogenesis, it is strategic to investigate the effect of this short peptide on the behavior of Aβ in the presence of lipid bilayers. In a previous work, we found that a significant fraction of iAβ5p penetrates deeply in the inner core of the lipid bilayers [16]. Consequently, we wondered if the membrane-internalized iAβ5p molecules are useless or they could also play some role in inhibiting Aβ fibril formation. To clarify this point, we decided to investigate the simultaneous interaction of iAβ5p and Aβ(25–35) with lipid bilayers. Aβ(25–35) is the shorter

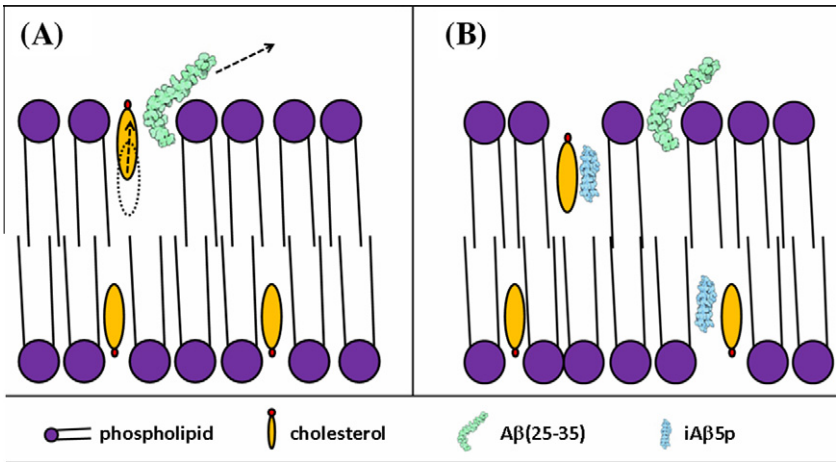

Fig. 4. Schematic representation of DLPC:CHOL bilayers containing the Aβ(25–35) peptide in the absence (A) and in the presence of iAβ5p peptide (B).

bioactive Aβ fragment and tends to reside between the outer part of the hydrophobic core and the external hydrophilic layer of the membrane [18]. Interestingly, the positioning of iAβ5p and Aβ(25–35), if considered separately, with respect to the bilayer is modulated by CHOL: with increasing CHOL content, iAβ5p becomes progressively more deeply buried in the inner hydrophobic core [16], while Aβ(25–35) is released to the external aqueous medium [18]. In the present work, we have investigated the effect of the co-presence of the two peptides on their interaction with liposomes of the DLPC:CHOL at 80:20 wt./wt. ratio considered as biomembrane-mimicking systems.

The ESR results presented above show that iAβ5p influences the Aβ(25–35) interaction with the bilayer through a cholesterol-mediated mechanism, although the two peptides do not directly interact in the membrane environment. Indeed, Aβ(25–35) causes a crowding of CHOL in the more external region of the bilayer (Fig. 4A). As a consequence, this region becomes stiffer, causing the amyloid fragment expulsion to the external medium and favouring the subsequent fibrillization process. The addition of the iAβ5p withholds cholesterol, which tends to place close it, in the inner hydrophobic core of the bilayer. This results in a more fluid bilayer external region, thus disfavoring Aβ(25–35) release (Fig. 4B). In this context we would like to emphasize the CHOL involvement, at molecular level, in finely-tuned membrane processes.

Finally, our results show that iAβ5p peptide prevents the Aβ(25–35) exit from the lipid membrane, that is the first step of the Aβ aggregation process, suggesting a possible mechanism through which membrane-buried β-breaker could help in hampering the β-amyloid self-aggregation.

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