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Interaction of the β Amyloid - A β (25-35) - Peptide with Zwitterionic and Negatively Charged Vesicles with and without Cholesterol

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Abstract

The interactions of the Alzheimer's β -amyloid peptide, A β (25–35), with 18:1 (9-Cis) PC 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), L- α -phosphatidylcholine (EPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (DOPG), and L- α -phosphatidylglycerol (EPG) phospholipid vesicles with and without cholesterol (Ch) are studied by the nitroxide spin probe electron paramagnetic resonance (EPR) method. Two nitroxide spin probes, 2,2,6,6-tetramethyl-piperidin-1-oxyl-4-yl hexadecanoate (TP, TEMPO-Palmitate) and 2-Ethyl-2-(15-methoxy-15-oxopentadecyl)-4,4-dimethyl-3-oxazolidinyloxy (16-DSE), are utilized in the study. TEMPO-Palmitate has the reporting EPR moiety located at the top of this spin probe, while 16-DSE has the reporting EPR moiety located at the tail of the spin probe. These two probes enable us to sample the surface and the middle of the phospholipid bilayer, respectively. All EPR measurements are done above the melting points of all four phospholipids when the bilayer is in the liquid crystal phase, the physiologically relevant phase. Due to non-linear spectral line fitting, the EPR spectral parameters are extracted with high precision. The results show that there are two populations of A β (25–35) and that one of them is located in the hydrophobic phospholipid layer below the hydrophilic headgroup region. The second population appears to be weakly coupled to the surface of the bilayer. Both hydrophobic and electrostatic interactions affect the insertion of A β (25–35) in the bilayer. Also, there is strong evidence for an interaction between cholesterol and A β (25–35), which affects the dielectric and dynamic properties of the bilayer.

Graphical abstract

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Author contributions

J.S. contributed with the acquisition of data, drafting and revising of the manuscript, and final approval of the article. M.P. contributed with conception and design of the study, analysis, and interpretation of data, drafting and revising, and final approval of the article.

Conflicts of interest

None.

Supplementary material

(I) Amino acid polarity composition of A β (25–35), (II) Composition of Egg PG and Egg PC and (III) Additional figures of the hyperfine splitting and rotational correlation time of the spin probes TP and 16-DSE as a function of temperature

Keywords

Alzheimer disease; β -amyloid peptide; Phospholipids; Cholesterol; Electron paramagnetic resonance (EPR)

1. Introduction

Alzheimer's disease (AD), a chronic neurodegenerative disease for which there is still no effective treatment, is the most common cause of dementia in the elderly.(Huang and Mucke, 2012; Hertel et al., 1997; Anon., 2010; Tiraboschi et al., 2004; del Mar Martinez-Senac et al., 1999) Due to the ever-increasing life expectancy, AD, whose symptoms usually first appear in adults after age 60, has become one of the scourges of modern times. AD is a complex disease which is caused by a wide range of biochemical and biophysical interactions between genetic, epigenetic and environmental factors.(Huang and Mucke, 2012) Pathologically, the brain of AD patients has intracellular neurofibrillary tangles consisting of abnormally phosphorylated tau proteins and extracellular neuritic amyloid plaques made of β -amyloid peptides ($\text{A}\beta$). (D'Errico et al., 2008a; Kremer and Murphy, 2003; Wood et al., 2003) β -amyloid peptides, such as $\text{A}\beta(1-40)$ (40 amino acids in length) and $\text{A}\beta(1-42)$ (42 amino acids in length), are cleaved from the larger transmembrane amyloid precursor protein (APP) in the membrane.

The causes of AD are still not well understood. There are several competing hypotheses of the causes of the disease, of which one is the cascade amyloid hypothesis.(D'Errico et al., 2008a; Ballard et al., 2011; Barrett et al., 2015) According to this hypothesis, the amyloid plaques, due to their neurotoxicity, cause neuronal dysfunction and death in the brain. (Ballard et al., 2011) Recently, it has been proposed that the neurotoxicity may not be caused by amyloid plaques but rather by membrane active monomers and oligomers.(Barrett et al., 2015; McLean and Cherny, 1999; Ono et al., 2009; Williams and Serpell, 2011) A number of studies suggest that $\text{A}\beta$ may affect the physicochemical properties of membranes such as membrane structure, (Dante et al., 2002) permeability (Friedman et al., 2009), fluidity (Muller et al., 1995; Yip et al., 2002; Zubenko et al., 1999) and dielectric properties (Rodrigues et al., 2001). These findings indicate that the direct physiochemical interactions between $\text{A}\beta$ and membrane phospholipid may initiate both neurotoxicity and plaque formation. For that reason, it is important to explore the basis of molecular interactions between $\text{A}\beta$ and phospholipid membranes. The segment of $\text{A}\beta$ from 25 to 35 is the

transmembrane segment of APP, and as such it is used to study the A β interactions and location in the membrane (Hertel et al., 1997; Dante et al., 2006; Dante et al., 2002; Dies et al., 2014; Konno, 2001; Mason et al., 1996; Tedeschi et al., 2010). Frozza et al. (Frozza et al., 2009) have presented strong evidence that A β (25–35) and A β (1–42) peptides induce similar neural injury, and thereby they recommended A β (25–35) as a convenient alternative for the investigation of neurotoxic mechanisms involved in AD. Also, several characteristics of A β (25–35) such as its amphiphilic nature, aggregation, neurotoxicity and ability to form β -sheet-rich fibrils are similar to those of the full-length A β peptide, (Tsai et al., 2014; Smith and Klimov, 2018) and therefore A β (25–35) is a biologically active region of the full-length peptide (Smith and Klimov, 2018; Cuco et al., 2016). Ultimately, an understanding of how the A β (25–35) peptides change the fluidity and dielectric properties of the membrane may provide new insights on the mechanisms of the neurodegenerative process associated with AD.

The phospholipid bilayers dielectric and permeability properties, which depend on their phospholipid composition, affect the partitioning and folding of membrane proteins (Leventis and Grinstein, 2010). Hertel et al. (1997) have shown that the dipolar compounds phloretin and exifone decrease the effective negative charge of membranes. This reduced effective negative charge prevented association of A β (1–40) and A β (25–35) to negatively charged lipid vesicles, which in turn, was correlated to the reduced toxicity of A β peptides on neuron-like PC12 cells. Based on these results Hertel et al. suggested that the toxic effect of A β on neuronal cell membranes might be caused by a nonspecific physicochemical interaction between A β and negatively charged lipid membranes (Hertel et al., 1997). It has been shown that A β (1–40) binds more preferentially to negatively charged lipid membranes than to zwitterionic lipid membranes (Williams and Serpell, 2011). Also, according to Tofoleanu and Buchete (2012) the electrostatic attraction between charged A β residues and the lipid headgroups drives the C-termini of A β peptide into the hydrophobic region of the lipid membrane. Since the electrostatic interaction between A β peptides and negatively charged lipid membranes have been suggested as a cause of cytotoxicity, (Hertel et al., 1997; Williams and Serpell, 2011; Tofoleanu and Buchete, 2012; Bonev et al., 2001) measuring the changes of dielectric properties of the phospholipid membranes resulting from membrane association of charged A β peptides might be important (Bonev et al., 2001).

Electron Paramagnetic Resonance (EPR) spectroscopy of nitroxide spin labels has been successfully utilized to study the polarity (Marsh, 2001, 2002; Marsh and Toniolo, 2008; Dzikovski et al., 2009) and fluidity (Severcan and Cannistraro, 1988; Peric et al., 2005; Alves et al., 2008; D'Errico et al., 2008) of lipid membranes. D'Errico and colleagues (D'Errico et al., 2008), using phospholipids spin labeled at two different positions on the hydrocarbon chain, have investigated the interaction between A β (25–35) and negatively charged and zwitterionic phospholipid bilayers with and without cholesterol by EPR spectroscopy. They found out that A β (25–35) penetrated the phospholipid bilayer and was located below the hydration layer of the membrane. Also, according to D'Errico and colleagues the charge of the phospholipid did not affect the insertion, so they concluded that the interaction was driven by hydrophobic interactions.

The goal of this work is to study the interactions of A β (25–35) with 18:1 (9-Cis) PC 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), L- α -phosphatidylcholine (EPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) (DOPG), and L- α -phosphatidylglycerol (EPG) phospholipid vesicles with and without cholesterol (Ch). The melting points of all four phospholipids are low, implying that the vesicles in the measured temperature range are in the liquid crystal phase, in other words, the physiologically relevant phase. The guiding principles for choosing these phospholipids were:

(I) head group charge, where EPG and DOPG are negatively charged whereas EPC and DOPC are zwitterionic, and

(II) vesicle order, where DOPC and DOPG vesicles are more homogeneously ordered one component lipid bilayers, whereas EPC and EPG vesicles are mixtures of several different chain length phospholipids (see Supplemental material).

We expect that these choices will allow us to obtain the effects of the head group charge and bilayer order on the interactions of A β (25–35) and phospholipid vesicles with and without cholesterol.

2. Materials and Methods

The phospholipids DOPC (18:1 (9-Cis) PC 1,2-dioleoyl-*sn*-glycero-3-phosphocholine), EPC (Egg PC (95%) L- α -phosphatidylcholine (95%) (Egg, Chicken)), DOPG (18:1 (9-Cis) PG 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt)) and EPG (Egg PG L- α -phosphatidylglycerol (Egg, Chicken) (sodium salt)) were obtained from Avanti Polar Lipids (Birmingham, AL, USA). Cholesterol (grade, minimum 99%) was bought from Sigma-Aldrich (Saint Louis, MO, USA). A β (Beta Amyloid (25–35), human) was from California Peptide Research, Inc. (Napa, CA, USA). The phospholipid composition of EPC and EPG is presented in Figure S1 in Supplemental material. TP (TEMPO-palmitate – 2,2,6,6-tetramethyl-piperidin-1-oxyl-4-yl hexadecanoate) was a gift from the late Prof Joseph Hajdu (Department of Chemistry, CSUN, CA, USA), and 16-DSE, 2-Ethyl-2-(15-methoxy-15-oxopentadecyl)-4,4-dimethyl-3-oxazolidinyloxy, free radical was obtained from Sigma-Aldrich (Saint Louis, MO, USA). All chemicals are used as received.

The appropriate amount of EPR spin probe (TEMPO palmitate or 16-DSE) ethanol solution was added to phospholipid (EPG, EPC, DOPG, and DOPC) powder to produce [lipid]/ [spin probe] mole ratio of 250. The ethanol solution was vortexed thoroughly to obtain a clear solution which was then dried under dry N₂ flux to produce a thin film of lipid and spin probe. To prepare samples with cholesterol, the calculated amount of cholesterol solution in ethanol was also added to the clear solution of the lipid-spin probe before preparing the dry film. The dried films were kept under reduced pressure overnight to ensure the complete absence of ethanol. Thereafter, the required amount of the Hepes buffer (pH = 7.4) was added to the dry film to achieve the final lipid concentration of 50mM. The solutions were then vortexed for 10 minutes, which produced an opaque suspension of multi lamellar vesicles (MLV). The lipid-BA mixture was prepared by weighing the calculated amount of BA powder into a glass vial. MLV suspension was then added to the vial, and the

contents were vortexed for 10 minutes. The final concentration of BA and cholesterol in the mixture was 5mM and 10mM, respectively.

EPR spectra were recorded on a Bruker ESP 300 spectrometer which was controlled and run by EWWIN 2012 EPR software from Scientific Software Services (Northville, MI, USA). The spectrometer was equipped with a Bruker variable temperature unit (Model B-VT-3000), which was controlled manually. To ensure that the temperature of the sample is stable within ± 0.5 K, the temperature equilibration time at each temperature was at least five minutes. The sample temperature was measured with a copper-constantan thermocouple connected to temperature (Model DP41-TC-S2A) from Omega (Stamford, CT, USA). The thermocouple tip was positioned close to the sample just outside of the active region of the EPR cavity. Five first-harmonic EPR spectra were recorded in the temperature interval from 25 to 60 C with five-degree intervals.

Experimental EPR spectra are fitted by the homemade computer program Lowfit as explained previously. (Bales and Peric, 1997, 2002) Since the inhomogeneously broadened EPR lines are well represented by a Voigt line shape, (Bales, 1989) which in turn can be approximated by the sum of a Gaussian and Lorentzian, (Halpern et al., 1993) Lowfit uses nonlinear least-squares fitting to fit the experimental EPR spectrum to the Gaussian-Lorentzian sum function (Halpern et al., 1993; Smirnov and Belford, 1995; Robinson et al., 1999; Peric and Halpern, 1994). Figure 1 shows a typical EPR spectrum (blue trace) and its fit (red trace) of TP in EPG-A β vesicles at 35 $^{\circ}$ C. One can easily see in Figure 1, the fit is indistinguishable from the EPR spectrum, except for the ^{13}C EPR lines flanking each line, which can be observed as wiggles in the residual (flat black line below). The fitting output parameters, such as the resonance fields of the lines, are then used to evaluate the hyperfine splitting, while the observed peak-to-peak line widths and Lorentzian-Gaussian mixing parameters are used for calculating the rotational correlation time.

In the fast motion regime, the peak-to-peak Lorentzian line width of an individual hyperfine line $\Delta B_{pp}^L(m)$ is given by

$$\Delta B_{pp}^L(m) = A + Bm + Cm^2 \quad (1)$$

where m is the m -th component of the nitrogen nuclear spin, that is $m = +1, 0$ and -1 . Term A is the Lorentzian line width of the central EPR line. Terms B and C can be found from $\Delta B_{pp}^L(m)$, which can be extracted from the observed peak-to-peak linewidths as detailed in ref (Bales, 1989), or from the EPR peak-to-peak line heights $V_{pp}(m)$, which can be obtained with more precision

$$B = \frac{1}{2} \Delta B_{pp}(0) \left\{ \sqrt{\frac{V_{pp}(0)}{V_{pp}(+1)}} - \sqrt{\frac{V_{pp}(0)}{V_{pp}(-1)}} \right\} \quad (2)$$

and

$$C = \frac{1}{2} \Delta B_{pp}(0) \left\{ \sqrt{\frac{V_{pp}(0)}{V_{pp}(+1)}} + \sqrt{\frac{V_{pp}(0)}{V_{pp}(-1)}} - 2 \right\} \quad (3)$$

where $B_{pp}(0)$ is the peak-to-peak line width of the central EPR line. As the experimental EPR lines have a Voigt shape, the terms B and C are additionally corrected by using the procedure from ref (Bales, 1989). Since the terms B and C are affected by the rotational diffusion of the spin probe, they can be used to calculate the rotational correlation time as follows (Schreier et al., 1978; Marsh, 1989)

$$B = 0.103 \omega_e [\Delta g \Delta A + 3(\delta g)(\delta A)] \tau_B \quad (4)$$

$$C = 1.81 * 10^6 [(\Delta A)^2 + 3(\delta A)^2] \frac{5}{8} \tau_C \quad (5)$$

where $\Delta A = A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})$, $\delta A = \frac{1}{2}(A_{xx} - A_{yy})$, where A_{xx} , A_{yy} and A_{zz} are the principal values of the hyperfine tensor. Similar expressions hold for the g -tensor. $\omega_N = 6.8 \cdot 10^6 A_N$, where A_N is the isotropic hyperfine splitting and ω_e is the EPR spectrometer frequency. We used the following values for 16-DSE: $A_{xx} = 5.5$ G, $A_{yy} = 5.5$ G, $A_{zz} = 33.0$ G, $g_{xx} = 2.0094$, $g_{yy} = 2.006$, and $g_{zz} = 2.0023$; and for TP: $A_{xx} = 5.3$ G, $A_{yy} = 7.0$ G, $A_{zz} = 35.0$ G, $g_{xx} = 2.0099$, $g_{yy} = 2.0061$, and $g_{zz} = 2.0024$. Finally, from τ_B and τ_C , we find the mean rotational correlation time $\tau_R = (\tau_B \tau_C)^{1/2}$.

The nitrogen hyperfine splitting A_+ is sensitive to the polarity of the surroundings of the nitroxide (Marsh and Toniolo, 2008; Peric et al., 2005; Mukerjee et al., 1982; Bales et al., 1998). Mukerjee et al., (1982) have shown that the hyperfine splitting of di-tert-butyl nitroxide is a linear function of dielectric constant with a correlation coefficient of 0.995. In this work, we intend to use the changes in the hyperfine splitting of TP and 16-DSE as an indication of the change in the dielectric properties around the nitroxide moiety. To minimize the effects of the second order dynamic shift we used the nitrogen hyperfine splitting A_+ , Figure 1, which is the distance between the field positions of central- and low-field EPR lines, not the nitrogen hyperfine splitting A_0 , which is half the distance between the field positions of low- and high-field EPR lines. (Bales et al., 1998)

3. Experimental Results and Discussion

The EPR spectra of nitroxide spin probes are sensitive to the molecular motions and the nature and composition of the environment in which they reside (Berliner, 1976). The EPR spectral parameters are very sensitive to the electronic distribution in the probe, which is in turn affected by the perturbations in the probe's surroundings. In the polar environment, the lone pairs of electrons on the oxygen atom interact with the surrounding polar molecules, which lowers the energy of nonbonded electrons and increases the oxygen atom electron

affinity (Berliner, 1976). Due to these polar interactions, the unpaired electron density shifts toward the nitrogen atom in the N-O group, which increases A_z (Marsh and Toniolo, 2008; Griffith et al., 1974)

The spin probe EPR method is a probe method in which the probe is distributed uniformly and sparsely throughout the vesicle. The uniform distribution of the spin probes in the vesicle without Heisenberg spin exchange interactions between them is experimentally confirmed by the absence of the dispersion spectral component in the fit of the observed EPR spectra of TP and 16-DSE (Bales and Peric, 1997; Peric et al., 2012). The uniformity and low spin probe concentration ensures that the probe minimally disturbs the bilayer and that it reports the average values of the polarity and fluidity of its surrounding.

3.1 Hyperfine Splittings of the Spin Probes TP and 16-DSE

For comparison of the effect of different phospholipids on A_z , Figures 2a and 2b show the hyperfine splittings A_z of the spin probes TP and 16-DSE, respectively, in pure EPG, EPC, DOPG, and DOPC vesicles. These A_z values will be used as reference values for the changes in A_z induced by A β and Ch. In those figures, it can be noticed that the temperature functional dependence of A_z is the same for each spin probe. In the case of TP, the values of A_z in the charged PG vesicles (Figure 2a – blue symbols) are greater than in the zwitterionic PC vesicles (Figure 2b – red symbols). They are also greater in the more ordered DOPG and DOPC vesicles (Figure 2a – open symbols) than in the respective same phospholipid head group EPG and EPC vesicles (Figure 2a – closed symbols). The behavior of the values of A_z of 16-DSE is similar with an exception that the values of A_z of 16-DSE in the DOPC vesicles (Figure 2b – open red squares) are greater than in the EPG containing vesicles (Figure 2b – blue closed circles). One can notice that in the case of TP that the spacing between the solid/open symbols in Figure 2a (i.e., closed (open) blue circles versus closed (open) red squares) is greater than the spacing between the circle/square symbols in Figure 2a (i.e., blue versus red symbols of the same shape). These relationships mean that the charge of the phospholipid group affects the value of A_z of TP more than the bilayer order. Whereas in the case of 16-DSE this trend appears reversed, such that the spacing between the same color symbols (blue versus red) is greater than the spacing between the same shape symbols (circles versus squares), which implies that the value of A_z of 16-DSE is more affected by the vesicle homogeneity, Figure 2b.

3.1.1. Effect of Cholesterol—Figures 3 and 4 show the temperature dependence of the hyperfine splitting A_z of the two nitroxide spin probes used in pure phospholipid, phospholipid-A β , phospholipid-cholesterol (Ch) and phospholipid-Ch-A β vesicles. The spin probe in Figures 3a and 4a is TP, and in Figures 3b and 4b is 16-DSE, while the phospholipids in Figure 3 are EPG and EPC, and Figure 4 are DOPG and DOPC. Note that the scale of A_z is the same for each probe, 0.4 G.

In all cases, the spacings between the closed and open symbols of the same color and shape for each phospholipid are bigger than the spacings between the different closed (open) shapes for each phospholipid (i.e. circles versus squares for EPG and DOPG; and diamonds versus triangles for EPC and DOPC) in Figures 3 and 4. These observations indicate that the

insertion of cholesterol into the phospholipid bilayer produces the largest change in A_+ . Note that each shape has its own color so that either descriptor is sufficient. In the case of TP, the values of A_+ increase (the open symbols are above the closed symbols for each color in Figures 3a and 4a), which implies that the hydration layer of the vesicles becomes more polar. Since the open symbols, which indicate the presence of cholesterol, are below the closed symbols for each shape in Figures 3b and 4b, the values of A_+ for 16-DSE in all samples decrease indicating the decrease in polarity in the middle of the bilayer. The behavior of A_+ for both probes is in agreement with the way cholesterol inserts into the lipid bilayer. Since the cholesterol's hydrophilic end is positioned close to the polar shell (Dies et al., 2014; Bhattacharya and Haldar, 2000), the TP nitroxide senses its polarity through the increased value of A_+ , blue and red symbols in Figures 3a and 4a. The increase in the polarity of the vesicle surface, which can be clearly observed in Figure S3 (blue and red symbols) in Supplemental material, is in agreement with a recently molecular dynamics calculated cholesterol-driven increase in membrane surface charge density. (Doktorova et al., 2017) On the other hand, the hydrophobic tail buried deep in the bilayer reduces the polarity of the middle bilayer as sensed by the doxyl nitroxide, green and brown symbols Figures 3b and 4b and Figure S4. The change in A_+ of 16-DSE is about two to three times larger than the change A_+ of TP, which is a strong indication that cholesterol is located below the polar shell deeper in the bilayer.

3.1.2. Effect of A β (25–53)—Looking at the insertion of A β into both the pure phospholipid and mixed cholesterol lipid vesicles, one can immediately notice that the A β insertion does not affect the A_+ value of TP (i.e., solid (open) circles versus solid (open) squares for EPG and DOPG, and solid (open) diamonds versus solid (open) triangles for EPC and DOPC in Figures 3a to 4a) as much as the A_+ value of 16-DSE (i.e., the same symbols as before in Figures 3b to 4b). This result can be even better observed in Figure 5 where the differences in hyperfine splitting A_+ for both probes between the A β -lipid and lipid vesicles, $A_+(A\beta) - A_+(L)$, are presented. Firstly, looking at the effect of A β on the A_+ values of 16-DSE in EPG, DOPG, EPC and DOPC vesicles with and without cholesterol, green and brown symbols in Figures 5 and 6, one can observe, in both cases, noticeable changes in A_+ . The insertion of A β lowers the value of A_+ of 16-DSE in the same way as cholesterol, green and brown symbols in Figure 5. The change in A_+ in EPG (Figure 5 – closed diamond), DOPG (Figure 5 – open diamond) and DOPC (Figure 5 – open triangle) vesicles, is about the same and is greater than the change in A_+ in EPC (Figure 5 – closed diamond) vesicles. In the presence of cholesterol in the vesicles, the change in A_+ of 16-DSE within the experimental errors follows the same trend as the change in A_+ in the pure phospholipid vesicles, Figure 6. Our results indicate that some of the A β peptides are located in the bilayer of the vesicle and that the presence of cholesterol does not change their location. Secondly, it can be seen that the values of $A_+(A\beta) - A_+(L)$ of TP, blue and red symbols in Figure 5, and the values $A_+(Ch+A\beta) - A_+(Ch)$, blue and red symbols in Figure 6 are less affected by A β (25–35), which means the insertion of A β into the bilayer does not noticeably change the surface polarity. Also, one can conclude that at the lower temperatures the values of $A_+(A\beta) - A_+(L)$, although small, are different from zero, while at the higher temperatures they are closer to zero within the standard deviation, Figure 5.

Dies et al. (2014) found one population of A β (25–35) on the surface of mixed dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphoserine (DMPS) vesicles, and another embedded in the bilayer center. Similarly, Dante et al. (2002) using neutron diffraction with the use of selectively deuterated amino acids, have been able to determine the position of A β (25–35) in the POPC and POPC/POPS bilayers, where POPS is 1-palmitoyl-2-oleoyl-phosphatidylserine. They have detected two populations of the peptide, one in the aqueous phase close to the vesicle surface and the other population intercalated deep inside the bilayer, with the C terminus close to the middle of the bilayer. Buchsteiner et al. (2012, 2010) have investigated the interaction of A β (25–35) with DMPC/DMPS model membranes by means of quasi-elastic neutron scattering. They have found that the A β (25–35) inserted in the bilayer affects the local dynamics of phospholipid molecules, as well as their lateral diffusion. Using a combination of microscopic and spectroscopic techniques such as transmission electron microscopy, circular dichroism, transmission-FTIR spectroscopy, single reflection horizontal FTIR-ATR spectroscopy and solid-state NMR spectroscopy (^{13}C , ^2H and ^{31}P), Labbe et al. (Labbé et al., 2013) have studied the interactions between the A β (25–35) peptide and model DMPC/DMPG membranes. The results of Labbe et al. indicate that A β (25–35) seems to intercalate within the bilayer altering the lipid chain order (Labbé et al., 2013). Replica exchange with solute tempering (REST) molecular dynamics (MD) simulations (Smith and Klimov, 2018) have been used to study the molecular mechanism of A β (25–35) to the zwitterionic DMPC bilayer. According to these MD simulations, there are two populations of A β (25–35): a dominant surface-bound population and a less stable inserted population. The N-terminus of all peptides is anchored to the bilayer, while the C terminus of the surface-bound peptides points away from the bilayer and the C terminus of the inserted peptides reside deep in the bilayer hydrophobic core.

As already mentioned, Figure 5 can be used as a good indicator of the location of A β (25–35) in the bilayer. The change in hyperfine splitting $A_+(\text{A}\beta) - A_+(\text{L})$ of 16-DSE of about 30 mG (Figure 5 – diamonds and triangles) in all four phospholipids unambiguously indicate that there is a population of A β (25–35) deep in the bilayer. This result agrees with the findings of Mason et al. (Mason et al., 1996) who observed by X-ray diffraction that A β (25–53) inserts into the bilayer hydrocarbon core of 1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC) vesicles, as well as with the results of Dante et al. (Dante et al., 2002), Dies et al. (Dies et al., 2014) Labbe et al. (Labbé et al., 2013) and Smith et al. (Smith and Klimov, 2018) which are discussed above. The circle and square symbols at the lower temperatures in Figure 5 point to the second population of A β (25–35). The weak effect A β (25–35) on the surface of the vesicles can be explained by the MD simulation results of Smith et al. (Smith and Klimov, 2018) in which the authors suggest that the surface-bound peptides point away from the vesicle surface. The temperature behavior of $A_+(\text{A}\beta) - A_+(\text{L})$ of TP (blue and red symbols in Figure 5) supports this interpretation, since the peptide conformation very likely becomes more extended with increasing temperature, which causes the decrease in the magnitude of $A_+(\text{A}\beta) - A_+(\text{L})$, as observed in Figure 5.

In the case of polar EPG and DOPG vesicles with cholesterol, the value of A_+ of TP is affected when A β (25–35) is added to the vesicles, Figure 6. The value of $A_+(\text{Ch+A}\beta) - A_+(\text{Ch})$ for EPG (closed circles) is clearly different from zero and is positive, while the value $A_+(\text{Ch+A}\beta) - A_+(\text{Ch})$ for DOPG (open circles) is of the same magnitude, but in the negative

direction, Figure 6. These changes indicate that the interaction between the surface population of A β (25–35) with the vesicle surface might depend on both polarity and surface roughness. In the case of zwitterionic EPC and DOPC vesicles, no conclusion can be drawn.

3.2 Rotational Correlation Time of the Spin Probes TP and 16-DSE

The reciprocal of the rotational correlation time τ_R of a spin probe can give us an indication the fluidity of the local environment of the spin probe. (Schreier et al., 1978) Figures 7 and 8 show the inverse temperature dependence of the rotational correlation time τ_R of the two nitroxide probes dissolved in pure phospholipid, phospholipid-A β , phospholipid-Ch and phospholipid-Ch-A β vesicles. The nitroxide in Figures 7a and 8a is TP, and in Figures 7b and 8b is 16-DSE, while the phospholipids in Figure 7 are EPG and EPC, and Figure 8 are DOPG and DOPC. Note that the τ_R scale is the same for each spin probe. Figure S2 in Supplemental material show the rotational correlation time τ_R of TP and 16-DSE, respectively, in pure EPG, EPC, DOPG and DOPC vesicles. The solid lines in Figures 7 and 8 are the fits to the Arrhenius equation. All the fits are excellent with correlation coefficients being larger than 0.997, Table 1.

As in the case of $A_{\parallel}$, the insertion of cholesterol into the bilayer causes the largest change in the values of τ_R of both spin probes in all four phospholipid vesicles, Figures 7 and 8 (closed versus open symbols of the same shape for each phospholipid), and Figure S4. Figure S4 clearly show that the τ_R of TP and 16-DSE in the mixed phospholipid-Ch vesicles, respectively, become quite longer than those measured in the pure phospholipid vesicles. These results are expected due to the condensation effect of cholesterol in the phospholipid bilayer, which reduces the fluidity of the bilayer, as well as, rigidifies it (Bhattacharya and Halder, 2000; Rubenstein et al., 1979; Mainali et al., 2011). Since the interaction of cholesterol and the phospholipid bilayer has been well studied and established, and our spin probe results are in full agreement with the expected knowledge of the cholesterol bilayer interaction, we believe that these results support the validity of our experimental approach.

Addition of A β to the phospholipid vesicles without cholesterol does not change the rotational correlation time τ_R of either TP or 16-DSE, Figures 7 and 8 (closed versus open symbols of different shapes for each phospholipid), and Figure S5. The differences in rotational correlation time τ_R for both probes between the A β -lipid and lipid vesicles $\tau_R(A\beta) - \tau_R(L)$, Figure S5, are close to zero (dashed line) within the standard deviation. These results imply that A β does not affect the fluidity of the studied phospholipid vesicles. When A β is added to the phospholipid vesicles with cholesterol, the rotational correlation time of TP changes only slightly, Figure 9 – circle and square symbols. One can argue that the values of $\tau_R(A\beta) - \tau_R(L)$ for the EPG vesicles (circles) are noticeably different from zero, but only at the four lowest temperatures. On the other side, τ_R of 16-DSE does change in the case of EPG (solid diamonds), EPC (solid triangles) and DOPC (open triangles) vesicles, Figure 9, while in the case of DOPG (open diamonds) vesicles, the change in τ_R of 16-DSE is minimal. Also, one can notice that τ_R decreases for the zwitterionic vesicles (Figure 9 – open and closed triangles), while it increases noticeably in the case of the negatively charged EPG vesicles (Figure 9 – closed diamonds) and only slightly in the case

of DOPG vesicles (Figure 9 – open diamonds). These changes indicate that there is very likely some kind of electrostatic interactions between the cholesterol and A β molecules in the bilayer, which affects the rotation of the nitroxide. The interaction between cholesterol and A β is also supported by the data presented in Figure 6, which show a synergetic effect of cholesterol and A β on the hyperfine splitting. This conclusion agrees with the well-established affinity of cholesterol for A β .(Di Scala et al., 2014; Drolle et al., 2012)

3.3 Activation Energy of the Rotational Correlation Time

As mentioned above, the rotational correlation times of both spin probes can be almost perfectly fitted to the Arrhenius equation

$$\tau_R = \tau_{R0} e^{-\frac{E_a}{RT}} \quad (6)$$

where E_a is the activation energy of rotation, R is the universal gas constant and τ_{R0} is τ_R at $T = \infty$. We can think of the activation energy of the rotation of a nitroxide molecule as the minimum energy supplied by the environment needed for the nitroxide molecule to start rotating. Thereby, it is sensitive to the structure and dynamics of the surrounding environment. Table 1 and Figure 10 show the activation energies for the rotation of TP and 16 DSE in pure and mixed phospholipid vesicles. Within the experimental error, one can notice that the activation energy is the highest in the pure phospholipid vesicles, and it becomes lower after addition of A β or/and cholesterol. As in all the other measured parameters, the biggest change in E_a is produced by cholesterol, which implies that bilayer structure and dynamics more affected by cholesterol than A β , which supports the already reached conclusions from the $A_\perp$ and τ_R results. As expected, the values of E_a for 16-DSE are lower than those for TP, Figure 10, due to the higher fluidity of the middle of the bilayer. The same can be stated for the EPC based vesicles, which are more loosely packed compared to the other vesicles. Despite slightly larger error bars, one still can observe that the changes in E_a of 16-DSE from pure lipid vesicles to lipid-Ch-A β vesicles are about two times larger than those of TP for all the vesicles except the EPC based vesicles, for which the change is about the same, and appears to be mostly due to cholesterol insertion. This agrees with the minimal change in $A_\perp$ of 16-DSE in the EPC based vesicles due to the addition of A β , Figures 5 and 6 – solid triangles. These results indicate that the structural and dielectric properties of the EPC vesicles are least affected by the insertion of A β .

4. Conclusions

Due to its dual polar-hydrophobic nature, Scheme S1, one can expect that the interaction of A β with the phospholipid bilayer is quite complex. The presented results indicate that although there are some complexities, there is also some well-defined behavior. The experimental data indicate that there are likely two different populations of A β (25–35) in the studied phospholipid vesicles. One population of A β (25–35), regardless of the charge nature of the vesicle surface, unambiguously inserts in the phospholipid bilayer below the headgroups as has been shown in previous studies.(Dante et al., 2002; Dies et al., 2014;

Smith and Klimov, 2018; Labbé et al., 2013) Contrary to D'Errico et al., (2008) the changes in $A_{\perp}$ in the case of negatively charged DOPG and EPG phospholipid vesicles indicate that not only are the hydrophobic interactions important for the insertion A β (25–35) into the bilayer, but also the electrostatic interaction between A β and the negatively charged phospholipids. This finding agrees with the conclusion of Esposito et al.(2006) According to our results, the second population A β (25–35) weakly interacts with the surface of vesicles. The reduction in $A_{\perp}$ of 16-DSE after addition of A β (25–35) to all four pure phospholipid vesicles indicates that A β (25–35) changes dielectric properties in its vicinity in the bilayer. The EPC vesicles are least affected by the insertion of A β , which is likely due to their heterogeneous and zwitterionic nature. At the same time, A β (25–35) does not affect the rotational motion of the spin probe, which might mean that the insertion of A β (25–35) does not introduce any structural changes. Both the hyperfine splitting and rotational correlation time of 16-DSE show that there is an interaction between cholesterol and A β (25–35), which affects the dielectric and dynamic properties of the bilayer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

EPR spectrum (blue trace) and its fit (red trace) of TP in EPG-A β vesicles at 35 °C. The line below is the residual (the difference between the spectrum and fit); the wiggles at the location of each EPR line are due to ^{13}C lines, which are not included in the fit function. One can observe that the fit is indistinguishable from the EPR spectrum. The definitions of the hyperfine splitting A_+ and the observed EPR line width B_{pp} are also shown.

Figure 2. Hyperfine splittings A_+ of the spin probes TP (a) and 16-DSE (b) as a function of temperature in EPG vesicles ($\bullet$), EPC vesicles ($\blacksquare$), DOPG vesicles ($\circ$) and DOPC vesicles ($\square$). The lines are to guide the eye.

Figure 3.

Hyperfine splitting A_+ of the spin probes TP (a) and 16-DSE (b) as a function of temperature in EPG vesicles (●), EPG- $\text{A}\beta$ vesicles (■), EPG-Ch vesicles (○), EPG-Ch- $\text{A}\beta$ vesicles (□), EPC vesicles (◆), EPC- $\text{A}\beta$ vesicles (▲), EPC-Ch vesicles (◇), and EPC-Ch- $\text{A}\beta$ vesicles (△). The error bars are the standard deviations of five consecutive measurements and less than the size of the symbols. The lines are to guide the eye.

Figure 4.

Hyperfine splitting A_+ of the spin probes TP (a) and 16-DSE (b) as a function of temperature in DOPG vesicles (●), DOPG- $\text{A}\beta$ vesicles (■), DOPG-Ch vesicles (○), DOPG-Ch- $\text{A}\beta$ vesicles (□), DOPC vesicles (◆), DOPC- $\text{A}\beta$ vesicles (▲), DOPC-Ch vesicles (◇), and DOPC-Ch- $\text{A}\beta$ vesicles (△). The error bars are the standard deviations of five consecutive measurements and less than the size of the symbols. The lines are to guide the eye.

Figure 5.

Hyperfine splitting difference $A_+(A\beta) - A_+(L)$ of the spin probes TP and 16-DSE as a function of temperature in lipid vesicles; where L is EPG (●), EPC (■), DOPG (○) and DOPC (□) for TP, and EPG (◆), EPC (▲), DOPG (◇), and DOPC (△) for 16-DSE. The error bars are the standard deviations of five consecutive measurements. The dashed line represents $A_+(A\beta) - A_+(L) = 0$.

Figure 6.

Hyperfine splitting difference $A_+(Ch+A\beta) - A_+(Ch)$ of the spin probes TP and 16-DSE as a function of temperature in lipid vesicles, where L is EPG (●), EPC (■), DOPG (○) and DOPC (□) for TP, and EPG (◆), EPC (▲), DOPG (◇), and DOPC (△) for 16-DSE. The error bars are the standard deviations of five consecutive measurements. The dashed line represents $A_+(Ch+A\beta) - A_+(Ch) = 0$.

Figure 7.

Rotational correlation time τ_R of the spin probes TP (a) and 16- DSE (b) as a function of inverse temperature in EPG vesicles (●), EPG-A β vesicles (■), EPG-Ch vesicles (○), EPG-Ch-A β vesicles (□), EPC vesicles (◆), EPC-A β vesicles (▲), EPC-Ch vesicles (◇), and EPC-Ch-A β vesicles (△). The error bars are the standard deviations of five consecutive measurements. The lines are fits to the Arrhenius equation.

Figure 8.

Rotational correlation time τ_R of the spin probes TP (a) and 16-DSE (b) as a function of inverse temperature in DOPG vesicles (●), DOPG-A β vesicles (■), DOPG-Ch vesicles (○), DOPG-Ch-A β vesicles (□), DOPC vesicles (◆), DOPC-A β vesicles (▲), DOPC-Ch vesicles (◇), and DOPC-Ch-A β vesicles (△). The lines are fits to the Arrhenius equation.

Figure 9.

Rotational correlation time difference $\tau_R(\text{Ch}+\text{A}\beta) - \tau_R(\text{Ch})$ of the spin probes TP and 16-DSE as a function of temperature in lipid vesicles, where L is EPG (●), EPC (■), DOPG (○) and DOPC (□) for TP, and EPG (◆), EPC (▲), DOPG (◇), and DOPC (△) for 16-DSE. The error bars are the standard deviations of five consecutive measurements. The Dashed line represents $\tau_R(\text{Ch}+\text{A}\beta) - \tau_R(\text{Ch}) = 0$.

Figure 10.

The activation energy of rotation of the spin probes TP (upper lines) and 16-DSE (lower lines) as a function of composition in lipid vesicles; where L is EPG (●), EPC (■), DOPG (○) and DOPC (□). The lines are to guide the eye.

Table 1

Activation energy of the rotation of the spin probes TP and 16 DSE in pure and mixed phospholipid vesicles.

Vesicles	E_a TP /JK ⁻¹ mol ⁻¹	R	E_a 16-DSE/ JK ⁻¹ mol ⁻¹	R
DOPC	35.7 ± 0.3	0.999	30.8 ± 0.4	0.999
DOPC-Aβ	35.8 ± 0.3	0.999	29.4 ± 0.5	0.999
DOPC-Ch	31.6 ± 0.3	0.999	24.3 ± 0.7	0.998
DOPC-Ch-Aβ	32.1 ± 0.3	0.999	23.6 ± 0.6	0.998
DOPG	35.3 ± 0.3	0.999	31.4 ± 0.6	0.999
DOPG-Aβ	35.1 ± 0.3	0.999	29.9 ± 0.6	0.999
DOPG-Ch	33.7 ± 0.5	0.998	26.1 ± 0.7	0.997
DOPG-Ch-Aβ	33.7 ± 0.4	0.998	25.2 ± 0.8	0.998
EPC	35.7 ± 0.5	0.999	26.7 ± 0.6	0.999
EPC-Aβ	34.4 ± 0.4	0.999	27.0 ± 0.5	0.999
EPC-Ch	31.3 ± 0.5	0.999	22.6 ± 0.3	0.999
EPC-Ch-Aβ	30.8 ± 0.7	0.999	22.3 ± 0.3	0.999
EPG	37.3 ± 0.5	0.999	31.3 ± 1.1	0.997
EPG-Aβ	35.9 ± 0.7	0.999	28.9 ± 0.7	0.999
EPG-Ch	34.0 ± 0.5	0.999	23.4 ± 0.5	0.999
EPG- Ch-Aβ	33.7 ± 0.5	0.999	23.8 ± 0.6	0.998