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Review Article

Utilizing magnetic resonance techniques to study membrane interactions of amyloid peptides

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Alzheimer's disease (AD) is a common neurodegenerative condition that involves the extracellular accumulation of amyloid plaques predominantly consisting of Aβ peptide aggregates. The amyloid plaques and soluble oligomeric species of Aβ are believed to be the major cause of synaptic dysfunction in AD brain and their cytotoxic mechanisms have been proposed to involve interactions with cell membranes. In this review, we discuss our solid-state nuclear magnetic resonance (ssNMR) studies of Aβ interactions with model membranes.

Introduction

Alzheimer's disease (AD) is a common neurodegenerative condition associated with a progressive cognitive decline, usually in the elderly. AD pathogenesis involves the extracellular accumulation of amyloid plaques predominantly consisting of Aβ peptide aggregates and intracellular accumulation of neurofibrillary tangles of misfolded tau protein. The Aβ peptide is derived from the proteolytic cleavage of an integral type 1 membrane glycoprotein called the amyloid precursor protein (APP), that has a range of important physiological functions in healthy cells [1]. It is mainly processed in the cell membranes via a non-amyloidogenic pathway, which competes with the amyloidogenic pathway that results in the formation of Aβ peptides with 38–43 amino acid residues (Figure 1A) [2,3]. Disruption of the enzymatic equilibrium in the AD affected brain tissue leads to an imbalance of Aβ40 and Aβ42 peptides with Aβ42 the more toxic of the Aβ peptides. Aβ peptides then aggregate to form various types of assemblies including oligomers, protofibrils, fibrils and insoluble amyloid plaques (Figure 1B) [4]. The amyloid plaques and soluble oligomeric species of Aβ are believed to be the major cause of synaptic dysfunction in AD brain [5]. As Aβ peptides are generated in a membrane environment and their pathogenesis is proposed to involve their interactions with cell membranes [6], understanding of Aβ-membrane interactions is important for advancing our understanding of AD. In this review, we focus on our solid-state nuclear magnetic resonance (ssNMR) studies of Aβ interactions with model membranes.

Model membranes

Understanding the interactions between neuronal cell membranes and Aβ peptides together with the impact of these interactions on the toxic mechanisms of the amyloid peptides may be a key milestone in advancing our comprehension of AD pathogenesis. Complex physiological mechanisms are often deduced by comparing *in vivo* observations to structural information obtained by employing high-resolution experimental techniques, such as ssNMR, in simpler and more controllable *in vitro* model environments due to harsh experimental conditions imposed by these techniques (e.g. long acquisition times, lower hydration, mechanical shearing under magic angle spinning, etc. for ssNMR) which may not be well tolerated by the fragile neuronal cells. Although, some progress has been made in facilitating the application of high-resolution techniques to *in vivo* study of physiological processes in cells [7–17], significant advances are still needed, necessitating the use of model systems in current studies.

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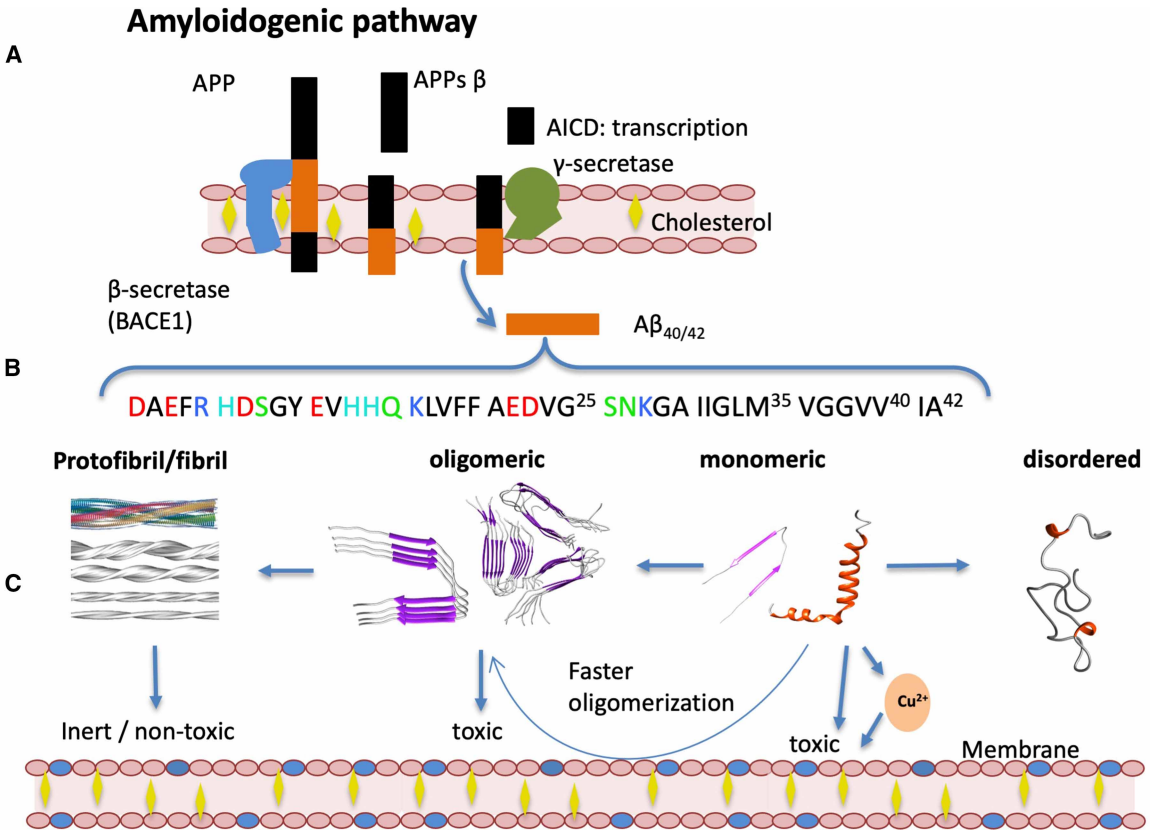

Figure 1. (**A**) The amyloidogenic pathway leading to the production of (**B**) amyloid peptide fragments of the amyloid protein precursor (APP). (**C**) Study of the peptide polymorphism in interaction with lipid membranes has been investigated using solid-state NMR [4].

The composition of natural cell membranes is complex and remarkably diverse, with a large variety of proteins, sterols, glycolipids, gangliosides, phospholipids and sphingomyelin [18,19]. Our understanding of the diverse composition and structure of cell membranes, along with the impact of this diversity/complexity on membrane protein function, has evolved since the original suggestion in 1888 of the presence of lipids in cell membranes and may allow us to craft more relevant models of cell membranes (Figure 1C) [20,21].

Model membranes used mainly for the studies discussed here are composed of phospholipids that are a major component of cell membranes. Phospholipids contain a hydrophilic phosphate headgroup and hydrophobic acyl chains. Typically, two phospholipids, deuterated palmitoyloleoylphosphatidylcholine (d-POPC) and palmitoyloleoylphosphatidylserine (POPS), were co-dissolved in various ratios. POPC is a zwitterionic phospholipid while POPS is anionic and provides negative charge density on the membrane surface. Interactions between Aβ42 and negatively charged POPS are reported to be associated with lipid flip-flop [22], a step that is implicated in apoptosis, and induce folding of the peptides to β-sheet [23] which may relate to Aβ-membrane binding [24]. Furthermore, the effects of including cholesterol (Chol) and monosialotetrahexosylganglioside (GM1) in the model systems were also investigated, as both are key components of natural membrane systems [25] and are reported to play a role in AD pathogenesis with GM1 reported to have a specific interaction with a dye-labelled Aβ peptide [26–32]. These studies were also extended to *polar* (without cholesterol) and brain *total* lipid extracts (BTLE), as these are closer substitutes for the natural systems.

Model membrane bilayer structures may comprise large unilamellar vesicles (LUVs) composed of a single lipid bilayer, multilamellar vesicles (MLVs) that have multiple lipid bilayers or magnetically oriented systems such as bicelles. MLV diameter is typically of the order of 0.5 μm, which is more closely related to biological systems and more conducive to ssNMR studies.

Solid-state NMR

Solid-state NMR is a powerful and uniquely suitable technique frequently used to provide insights into the structure and dynamics of membrane systems and peptide–lipid interactions (Figure 2) [33–37]. As discussed earlier, model membranes are comprised mainly of phospholipids that contain a phosphorus head-group, e.g. phosphatidylcholine (PC) or phosphatidylglycerol (PG), and often deuterated acyl hydrocarbon chains are used.

The NMR-active spin ½ isotope ^{31}P is almost 100% naturally abundant and hence ^{31}P ssNMR is a convenient probe of the effect of peptides on the phospholipid head-groups. The static ^{31}P lineshape, quantified by the chemical shift anisotropy (CSA) and based on the chemical shift interaction, is the main feature. Changes in the lineshape reflect perturbations resulting from either a change in the orientations of the lipid headgroups or increased/decreased disorder in the lipid bilayer (Figure 2B). The ^{31}P lineshape may also undergo broadening due to increased T_2 relaxation, indicative of membrane interactions, or collapse into isotropic 'spikes' due to disintegration of MLVs into small vesicles, akin to the effect of detergents, giving rise to much faster tumbling and thus averaging out the CSA. Relaxation measurements, such as T_1 and T_2, report on motions of molecules on different timescales. For ^{31}P relaxation times, changes in T_1 report primarily on the dynamics of individual lipids while changes in T_2 reflect the collective motions of the bilayer [38].

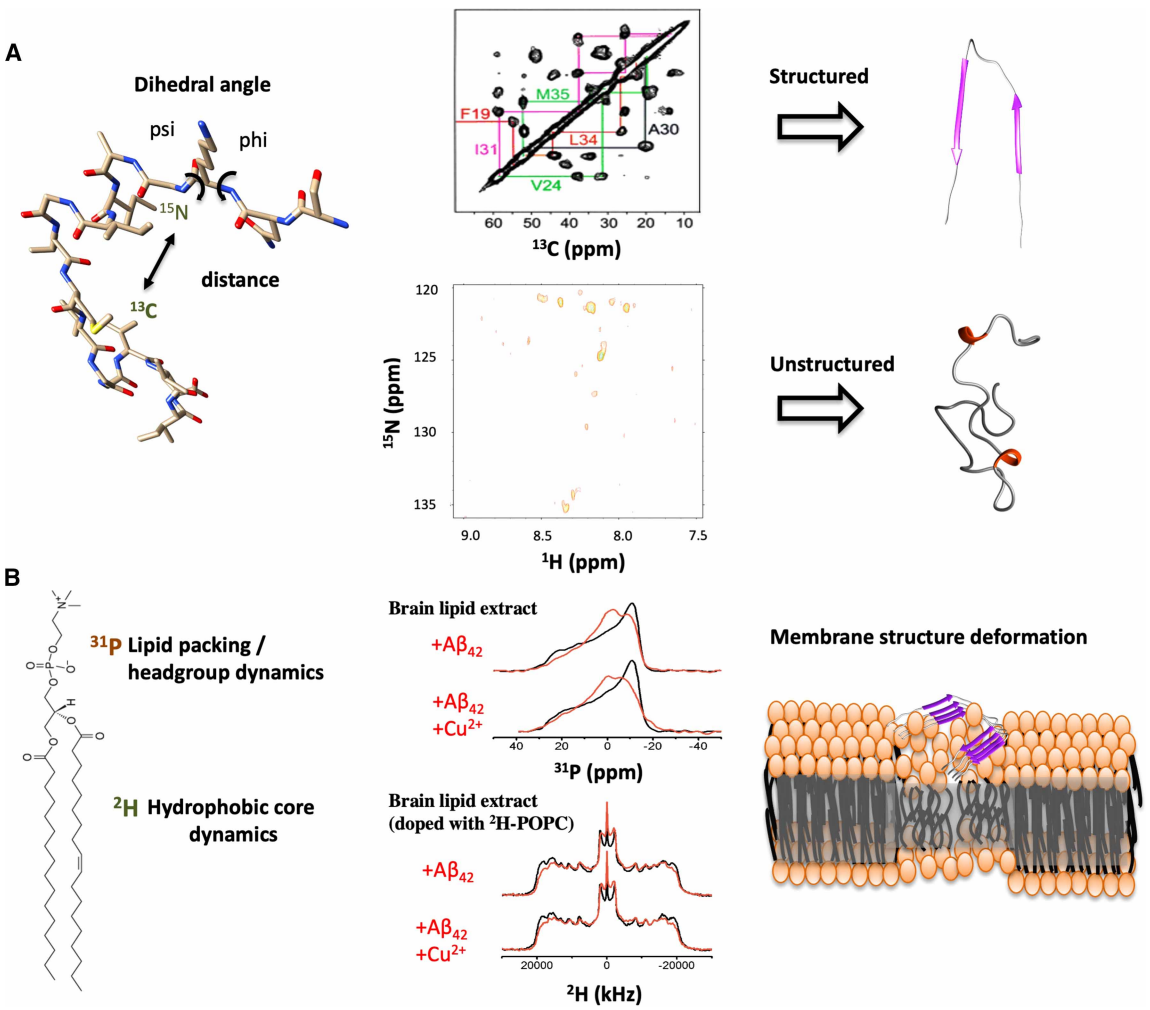

Figure 2. (**A**) ^{13}C and ^{15}N labelled Aβ peptides exhibit beta-sheet fibril [39] or disordered aggregate structure in model membrane environment, respectively. (**B**) The impact of Aβ peptides on the membrane has been monitored using ^{31}P or ^{2}H NMR leading to valuable insights into specific perturbations, such as the presence of copper, on the lipid headgroups and acyl chains, respectively [40].

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Deuterium ssNMR on the other hand provides information on the interaction of peptides with the deuterated acyl chains of phospholipids. The natural abundance of the ^{2}H isotope is negligible, which facilitates the observation of deuterium labelled lipid acyl chains with almost no background signal. Typically, a C-^{2}H bond oriented at an angle φ to the applied magnetic field yields a doublet separated by a quadrupolar splitting dependent on <3cos2φ-1>, which has both an orientational and dynamic component. The quadrupolar splittings are modulated by the order of the C-^{2}H bond, which varies along the lipid acyl chain with increased disorder towards the terminal CD_3 position. A superposition of the resonances observed for all orientations of an acyl chain gives rise to a 'Pake' powder pattern and results in the typical spectrum observed for ^{2}H-ssNMR of phospholipids (Figure 2B). Changes in membrane composition, e.g. inclusion of cholesterol, or addition of peptides perturb the dynamics of the phospholipid acyl chains. These differences in lipid dynamics are reflected by changes in the typically observed ^{2}H-ssNMR pattern, thus resulting in a convenient probe for investigating the effect on the hydrophobic core of membrane bilayers [41].

Furthermore, the peptides can be specifically labelled (e.g. with ^{15}N or ^{13}C) to provide structural information of the peptides (Figure 2A). Spinning of solid or membrane samples about an angle 54.74° to the applied magnetic field is referred to as magic angle spinning (MAS) and can create an effect similar to molecules tumbling rapidly, as in solution-state NMR, by removing or reducing dipolar couplings and CSA [42]. If sufficient speed is used, the resultant spectra are resolved into sharp isotropic resonances that can be used to discern the local structure of backbone nuclei of peptides and proteins, that in turn are characteristic of secondary structures [43–45]. Oriented membrane systems can also provide comparable high resolution lineshapes, provided the peptides are structurally homogenous [37] and these larger systems have more intense cooperative motions and hence shorter T_2 relaxation times, which are reflected in broader linewidths [46].

Aβ effect on bilayer integrity: is lipid composition a key parameter?

Aβ peptides may undergo insertion into the membranes leading to changes in membrane permeability and formation of Aβ aggregates with ion channel like structure or non-specifically associate/bind to the surface of the membranes affecting the membrane stability [47]. The disruption of calcium homeostasis observed in the pathogenesis of AD may be a result of membrane destabilization due to Aβ-membrane interactions, either via formation of multimeric pores by insertion of Aβ into the membrane or destabilization of the membrane by non-specific surface association. Planque et al. used proteoliposomes prepared by hydrating a mixed film of Aβ40 peptides and zwitterionic PC lipids and observed: (i) an irregular 'single channel like' conductance on fusion of Aβ40 containing vesicles with the zwitterionic bilayer, (ii) absence of any channel like topological features in supported bilayers formed from Aβ40/PC vesicles, and (iii) presence of predominantly β-sheet structured Aβ40 in the supported multilayers formed from the same vesicles, which point to a non-specific perturbation of the model membranes by surface association with Aβ40 assemblies [48].

The mode of membrane-Aβ peptide interaction was also investigated for the more toxic Aβ42 peptide [49] and the hydrophobic C-terminal Aβ(25–35) fragment [50] utilizing ^{31}P and ^{2}H ssNMR. The Aβ(25–35) fragment is interesting because, despite being devoid of potential metal binding or cross-linking sites implicated in the proposed cytotoxic mechanisms of the full-length amyloid peptides, it undergoes aggregation readily and is cytotoxic [51–53]. Mixed d-POPC and POPS bilayers were used to investigate how the mode of addition of the peptide modulates the Aβ-membrane interaction so as to mimic the two common *in vivo* environments of the Aβ peptide i.e. peptide found in the extracellular region and within the cell membrane. With exogenously added Aβ42 peptide (peptide added to the already prepared lipid bilayer, mimicking the extracellular peptide environment) only minor changes were observed in the ^{31}P and ^{2}H NMR together with a smoothing or rounding of the spectra, indicative of the association of the peptide to the lipid bilayer surface [49]. Addition of Aβ (25−35) exogenously to the MLVs resulted in a decrease in ^{2}H NMR quadrupolar splittings, i.e. a decrease in the lipid acyl chain order, and a direct perturbation of the phospholipid headgroup with an increase in the ^{31}P CSA and a decrease in the T_2 relaxation time. These observations imply insertion of the hydrophobic Aβ(25–35) fragment into the membrane bilayer, close to the aqueous interface to affect the phospholipid headgroup [50]. On the other hand, incorporation of the full length Aβ42 peptide lead to disintegration of the MLVs into small vesicles as reflected by the appearance of isotropic peaks in both ^{31}P and ^{2}H spectra. The ^{31}P CSA was also notably reduced which suggests increase in headgroup disorder [49]. Comparatively, incorporation of Aβ (25–35) within the lipid bilayer had a much smaller impact on the ^{31}P CSA or T_2 relaxation time. However, an

increase in lipid acyl chain order was observed as reflected by a larger quadrupolar splitting in the ^{2}H NMR, an effect similar to the incorporation of cholesterol into a lipid bilayer [50].

Modulation of Aβ-membrane interactions by other membrane components, e.g. cholesterol, was also investigated. Inclusion of cholesterol in the d-POPS/POPC bilayer increased the lipid acyl chain order and decreased the phospholipid headgroup order, indicting insertion of the sterol into the membrane, as previously reported [54]. The presence of cholesterol mitigated the insertion of Aβ(25–35) into the bilayer, with the likelihood of membrane stabilization by cholesterol, reducing the effect of the amyloid peptide [50]. This study points to the dependence of Aβ-membrane interactions on the choice of lipid composition.

Elucidation of the impact of lipid composition on the Aβ−membrane interactions by directly comparing different studies is difficult due to variations in lipid saturation, cholesterol content, peptide preparation, temperature or salt and buffer compositions among various studies. Therefore, we investigated the effect of Aβ42 on several model systems, i.e. POPC/Chol, POPC/POPS/Chol, POPC/GM1/Chol and BTLE under the same experimental conditions [40]. Although for all the model systems, strong headgroup perturbation was observed with no significant impact on the acyl chains, POPC/Chol and BTLE model membranes showed higher perturbation than the POPC/POPS/Chol and POPC/GM1/Chol systems. This may be attributed to electrostatic interactions with the negatively charged PS or the steric effects of the large GM1 headgroups, resulting in a larger distance between the peptide and the phosphate headgroup of the bilayer [40].

Furthermore, the Aβ42 neurotoxic pathways may involve specific binding to PS lipid [55] and may be impacted by post-translational changes to the peptide such as phosphorylation and methionine oxidation. For example, Aβ42 peptide phosphorylated at serine 8 (pAβ42) has higher β-sheet content and undergoes faster amyloid formation in a lipid environment compared with Aβ42, concomitant with diminished neurotoxicity. Thus, phosphorylation of Aβ42 together with the peptide-membrane interaction may promote the formation of amyloid plaques in the brain, which are not as neurotoxic as the oligomeric species [56]. On the other hand, Aβ peptide with an oxidized methionine residue is more hydrophilic and has higher propensity towards random coil conformation in a lipid environment. It interacts with Cu^{2+} ions to generate reactive oxygen species (ROS) and has been shown to be cytotoxic [57]. Thus, our results show that Aβ peptides disrupt lipid membranes and the effect is modulated by the mode of addition of the peptide, lipid composition of the bilayer, specific binding to lipids and post-translational changes to the peptide.

Another partner in crime: how metals modulate Aβ impact on membranes

Elevated levels of zinc, iron and copper ions by ∼3 times have been observed in the post-mortem analysis of neuritic plaques [58]. The presence of three potential metal-binding histidine residues (positions 6, 13 and 14) in Aβ peptides may point to toxic mechanisms mediated by metal ions [59,60]. Furthermore, Cu^{2+}-Aβ complexes may facilitate the formation of ROS via Fenton-like chemistry, resulting in lipid peroxidation, protein oxidation and dityrosine formation [57,61–65]. Addition of Zn^{2+} to the d-POPC/POPS bilayer produced a slight decrease in the ^{31}P CSA and a slight increase in the CD_3 quadrupolar splitting, which suggest the Zn^{2+} ions are located at the headgroup-chain interface [49]. The addition of Cu^{2+} ions, on the other hand, resulted in the collapse of the ^{31}P CSA and ^{2}H quadrupolar splittings to form an isotropic peak, indicating the formation of small lipid vesicles [49]. Interestingly, addition of Cu^{2+} ions to Aβ42 associated bilayers did not induce MLV disintegration, which may be attributed to a strong affinity of the Cu^{2+} ions to the histidine residues of the Aβ peptide (Figure 2B) [49]. Furthermore, the MLV disintegration observed with incorporated Aβ42 peptide was not enhanced by the addition of Cu^{2+} and Zn^{2+} ions, although relaxation broadening of the ^{31}P CSA was noted concomitant with changes in lipid dynamics [49]. Subsequent addition of Zn^{2+} ions to lipid bilayers with associated Aβ(25–35), which lacks metal coordinating sites, did not produce significant changes in the ssNMR spectra [50]. Addition of Cu^{2+} ions, however, induced changes in the ^{31}P NMR which can be attributed to the paramagnetic nature of the Cu^{2+} ions [50]. Furthermore, the membrane stabilizing effect of cholesterol in the presence of the Aβ(25–35) fragment and Cu^{2+} ions, was revealed by a marked increase in the ^{31}P isotropic peak for the *polar* brain lipid extract (without cholesterol) as compared with the BTLE [50]. Aβ42 fibrillation was accelerated by the addition of Cu^{2+} ions in low concentrations and the resulting Aβ42-Cu^{2+} complex showed a stronger interaction with the phosphate headgroup of the bilayers of various compositions, especially POPC/POPS/Chol and POPC/GM1/Chol systems, possibly reflecting bilayer insertion [40]. Thus,

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metal ions affect the Aβ-membrane interactions and Aβ fibrillation, but the impact of metal ions is also modulated by lipid composition, which reiterates the importance of using relevant mimetic environments.

Effect of lipid bilayers on the Aβ peptides

Aβ-membrane interactions influence the aggregation and structure of the peptides. We examined the formation of Aβ fibrils in various lipid environments and observed significant differences in fibrillation kinetics for different lipid compositions [66]. Furthermore, to determine the impact on the peptide secondary structure in the context of lipid interactions, Aβ42 was specifically ^{13}C-labelled at Ala30, Leu34 and Gly29. The ^{13}C peaks from the carbonyl carbons of Ala30 and Leu34 shifted from 178 ppm to 173 and 171 ppm for the incorporated and associated peptide, respectively [50]. This upfield shift suggests an increased β-sheet conformation for both the incorporated and associated peptide [43]. This conformational change from coiled to extended structures was also observed by circular dichroism for Aβ42 in LUV of the same lipid composition [49] and for Aβ40 in dimyristoylPC/PG LUV [67]. Similar upfield shift, i.e. a conformational change towards β-sheet, was also observed for analogously labelled Aβ(25–35) where the carbonyl carbons from the same residues shifted from 174 to 173 ppm for the incorporated peptide and 171 ppm for the associated peptide. No significant difference was observed for the C-α chemical shift of Gly29 in Aβ(25–35), which could not be resolved for the full-length peptide due to spectral overlap [50]. The association of the observed upfield shift of the carbonyl peaks with a shift towards β-sheet conformation was assessed by changing the labelling to observe the Gly29 and Ala30 carbonyl carbons (which are likely to produce a greater chemical shift change) with same deductions as previously reported [68]. These changes in the peptide structure also were found to be unaffected by the cholesterol content of the bilayer [68]. A shift towards the extended β-sheet structure may be a requisite feature to facilitate the perturbation of the model membranes by the peptides. An ^{15}N labelled Aβ40 peptide in oriented dimyristoyl PC bilayers produced a spectrum indicative of multiple conformations which exposes the high sensitivity to polymorphism influenced by membrane composition (Figure 2A), a difficulty in investigating Aβ peptides in complex cellular environments.

Perspectives

- Solid-state NMR studies have shown that Aβ peptides disrupt lipid model membranes and the Aβ-membrane interactions depend on lipid composition and the presence of metal ions. These interactions also impact the peptide structure, fibrillation kinetics and morphology.
- Recent progress in new NMR methodologies, such as dynamic nuclear polarization (DNP) [69,70], and development of protein expression methods that enable *in situ* labelling could allow in-cell studies of labelled peptides [71–73] to determine the key structural change in physiological environments linked to pathogenic pathways.
- Useful insights into the pathogenesis of AD gained from the application of NMR techniques may pave the way for the development of effective AD therapeutics.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions

F.S. planned the article and discussed with D.W.K., S.R. & M.A.S.; S.R. wrote the manuscript and all authors contributed sections, revised and edited the draft.

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Abbreviations

AD, Alzheimer's disease; APP, amyloid precursor protein; BTLE, brain *total* lipid extracts; CSA, chemical shift anisotropy; d-POPC, deuterated palmitoyloleoylphosphatidylcholine; MLVs, multilamellar vesicles; PC, phosphatidylcholine; PG, phosphatidylglycerol; POPS, palmitoyloleoylphosphatidylserine; ROS, reactive oxygen species.

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