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Cholesterol and Clioquinol modulation of Aβ(1–42) interaction with phospholipid bilayers and metals

Tong-Lay Lau [a], John D. Gehman [a], John D. Wade [b], Colin L. Masters [c], Kevin J. Barnham [c], Frances Separovic [a,*]

[a] School of Chemistry, Bio21 Institute, The University of Melbourne, Victoria 3010, Australia
[b] Howard Florey Institute, The University of Melbourne, Victoria 3010, Australia
[c] Department of Pathology, The University of Melbourne and the Mental Health Research Institute of Victoria, Melbourne, Victoria 3010, Australia

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Abstract

The β-sheet plaques that are the most obvious pathological feature of Alzheimer's disease are composed of amyloid-β peptides and are highly enriched in the metal ions Zn, Fe and Cu. The interaction of the full-length amyloid peptide, Aβ(1–42), with phospholipid lipid bilayers was studied in the presence of the metal-chelating drug, Clioquinol (CQ). The effect of cholesterol and metal ions was also determined using solid-state ^{31}P and ^{2}H NMR. CQ modulated the effect of metal ions on the integrity of the bilayer and although CQ perturbed the phospholipid membrane, the bilayer integrity was maintained. Model membranes enriched in cholesterol were studied under conditions of peptide association and incorporation. Solid-state NMR showed that the bilayer integrity was preserved in cholesterol-enriched membranes in comparison to phosphatidylcholine–phosphatidylserine bilayers. Changes in peptide structure, consistent with an increase in β-sheet, were observed using specifically ^{13}C-labelled Aβ(1–42) by magic angle spinning NMR. Results using aligned phosphatidylcholine bilayers and completely ^{15}N-labelled peptide indicated that the peptide aggregated. The results are consistent with oligomeric β-sheet structured peptides only partially penetrating the bilayer and cholesterol reducing the membrane disruption.

Keywords: Amyloid Aβ; Peptide–lipid interaction; Phospholipid membrane; Solid-state NMR; Structure; Cholesterol; Aligned lipid bilayer

1. Introduction

The cause of neuronal dysfunction in Alzheimer's disease (AD) has yet to be established. Interestingly, there is no correlation between disease progression and the β-sheet plaques that are the most obvious pathological feature of AD. Notably, the plaques are highly enriched in the metals ions Zn, Fe and Cu [1]. Disease progression correlates with soluble Aβ species and evidence is accumulating that soluble oligomeric species of Aβ are responsible for neuronal toxicity [2–4], possibly by affecting membrane stability resulting in cell dysfunction [5]. Soluble oligomeric species of Aβ(1–42), and not the monomeric or fibrillar species of the peptide, were also found to promote lipid release from cells [6], implicating membrane disruption as a phenomenological cytotoxic mechanism for the soluble oligomer. While cholesterol, phospholipids and monosialogangliosides (GM1) were the main components released soon after incubation with oligomeric Aβ, cell death occurred only much later, further suggesting alteration in lipid composition or assembly as a possible mechanism of Aβ toxicity. The formation of oligomeric aggregates was found to be promoted by Aβ binding to membrane vesicles containing phospholipids [7] and gangliosides [8].

The importance of lipid membranes in mediating Aβ toxicity is further demonstrated by several studies with conflicting conclusions involving cholesterol, which orders membrane bilayers [9,10]. An inverse correlation between Aβ toxicity and cholesterol has been suggested [11], where Aβ toxicity was attenuated in cholesterol-enriched cultured PC12 cells and enhanced in cholesterol-depleted cells. *In vivo* studies, by contrast, suggested cholesterol metabolism in the plasma membrane is modulated by Aβ [12,13] and cholesterol lowering (statin) drugs appear to delay AD progression [14]. *In vitro* [15] and epidemiological

* Corresponding author.
E-mail address: fs@unimelb.edu.au (F. Separovic).

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studies [16,17] support this correlation between Aβ toxicity and cholesterol, which requires further study.

The interaction of soluble oligomers and cell membranes is further complicated by the implication of metals in Aβ toxicity. Cu^{2+}-mediated toxicity has been proposed to be a result of a metal ion binding site in the Aβ peptide involving the residues His6, His13 and His14 [18–20]. The redox active metal ions (Fe^{3+} and Cu^{2+}) coordinated to Aβ [18,21–23] are capable of Fenton-like redox reactions [20,24,25] and may be responsible for the generation of reactive oxygen species. The resulting oxidative stress [26] could alter normal neuronal membrane content via lipid oxidation [27,28], including cholesterol [29], and has been found to specifically induce Aβ cross-linking via free radical reaction of the Tyr10 residue resulting in dimers [18,20,25,30,31]. The formation of Aβ dimers may in turn seed higher order oligomers species, although not necessarily along the pathway leading to amyloid aggregates.

Intriguingly, the metal-promoted aggregation of Aβ is reversed by treatment with metal chelators [32,33] such as the quinoline drug Clioquinol (CQ) (Fig. 1). Clinically, CQ has demonstrated encouraging results as a candidate for AD treatment, as it is capable of crossing the blood–brain barrier and inhibited or reversed the accumulation of Aβ deposits in APP2576 transgenic mice [33]. A number of studies have hinted at the action of CQ. Both CQ and Zn^{2+} blocked the toxicity of Aβ(1–42) and Aβ(25–35)-induced Ca^{2+} influx into cultured neuronal astrocytes and preserved the integrity of neuronal membranes [34]. CQ also inhibited the toxicity of Aβ(1–40) and attenuated the Cu^{2+}-dependent toxicity in neuronal cultures of Met(O)Aβ(1–42), an oxidised-Met35 derivative of Aβ(1–42) [24]. H_2O_2 was produced by Met(O)Aβ(1–42) in the presence of Cu^{2+}, and this toxicity was 'rescued' by the addition of catalase. A straightforward explanation attributes the apparent effect of CQ to its metal coordinating properties, ultimately inhibiting the generation of reactive oxygen species and related downstream oxidation effects, as CQ was shown to inhibit co-ordination of Cu^{2+} to Aβ [33] and the subsequent Cu-mediated Aβ redox chemistry [35].

The interplay between CQ and the membrane composition and integrity, correlated with Aβ structural changes, metal interactions and oxidation chemistry, known to underpin toxicity remains uncharacterized. Previously, when associated with a phosphatidylcholine–phosphatidylserine membrane system, Aβ(1–42) was found to bind to phospholipid head-groups leading to a decrease in T_2 relaxation time [36] and appeared to form a peptide–lipid phase [18]. The toxic species of the Aβ(1–42):Cu^{2+} complex when added to similar bilayer systems did not perturb the lipid acyl chains as reported by fluorescence anisotropy measurements, thus implying that lipid acyl chain perturbation is not involved in the toxic mechanism of Aβ [18]. However, amyloidic species of the Aβ(1–42):Cu^{2+} complex did perturb the acyl chains, while not exhibiting any neurotoxicity in cultured primary cortical neurons [18]. NMR spectroscopy and fluorescence anisotropy measurements showed that association of Aβ(1–42) to the phospholipid bilayers and subsequent addition of Cu^{2+} and Zn^{2+} did not cause significant perturbation of the bilayer [36]. Our previous work showed preferential binding of Cu^{2+} to the peptide-associated lipid phase, which suggested that the formation of this new phase is representative of non-amyloidic Aβ(1–42):Cu^{2+} species that exhibited neurotoxicity [18].

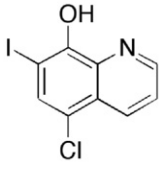

Fig. 1. Structure of 5-chloro-7-iodo-8-hydroxyquinoline, also known as Clioquinol.

Given the positive clinical implications of CQ in relation to Alzheimer's disease, we have extended these previous studies to examine the effect of CQ on membrane integrity both alone and together with Aβ(1–42) and Aβ(25–35) in phospholipid bilayers with and without metal ions (Cu^{2+} and Zn^{2+}). The effect of cholesterol on membrane integrity was also investigated as an extension of our earlier studies on the comparison of Aβ(1–42) to the shorter fragment Aβ(25–35) [37], which is devoid of the metal binding sites. Human peripheral nerve membranes [38] and rat synaptic plasma membranes of the cerebral cortex, cerebellum and hippocampus [39] are rich in cholesterol, ~0.6 and 0.8 molar ratio, respectively, relative to phospholipid. Cholesterol-enriched phospholipid bilayers have a higher transition temperature and an accompanying increase in order or decrease in membrane 'fluidity' of the acyl chain region dependent on the cholesterol content [40], which may effect peptide structure and insertion.

2. Materials and methods

2.1. Materials

Synthetic deuterated 1-palmitoyl(D31)-2-oleoyl-*sn*-glycero-3-phosphocholine (*d*POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS), supplied as dry powders, and 1,2-di-O-hexyl-*sn*-glycero-3-phosphocholine (6-O-PC), and 1,2-di-O-tetradecyl-*sn*-glycero-3-phosphocholine (14-O-PC), dissolved in chloroform, were purchased from Avanti Polar Lipids (Alabaster, USA) and used without further purification. Cholesterol was purchased from Sigma Aldrich (St. Louis, USA). ^{15}N-enriched Aβ(1–42) peptide was purchased from W.M. Keck Laboratory (Yale University, New Haven, USA). Specifically labelled Aβ(1–42) peptide was made using standard FMOC-solid phase peptide synthesis [33] purified by reverse phase HPLC and the product identified by mass spectrometry with *m/z* of 4519.11. The full-length peptide was labelled with sequence, DAEFR HDSGY EVHHQ KLVFF AEDVG SNK[$^{13}C_2$]-G*[$^{13}C_1$]-A* IIG[$^{13}C_1$]-L*M VGGVV IA, and similarly, the shorter peptide Aβ(25–35), GSNK[$^{13}C_2$]-G*[$^{13}C_1$]-A* IIG[$^{13}C_1$]-L*M was synthesized as described [37]. $^{13}C_2$-Gly, $^{13}C_1$-Ala and $^{13}C_1$-Leu were purchased from Cambridge Isotope Laboratories (Andover, USA), FMOC-succinamide (OSu) was purchased from Auspep (Melbourne, Australia) and FMOC-protected amino acids were synthesized and purified for use in peptide synthesis.

2.2. Preparation of multilamellar vesicles

*d*POPC/POPS (1:1) or *d*POPC/POPS/cholesterol (1:1:1) was dissolved in chloroform and methanol (9:1) solution in a round bottom flask and the solvent removed under vacuum resulting in a lipid film. Multilamellar vesicles (MLV) were formed by suspending the lipid film in 0.5 ml of 50 mM Tris–HCl buffer

(pH=7.2). Samples were freeze–thawed 4–6 times following addition of all other components.

2.3. Association of peptides

Aβ(1–42) and Aβ(25–35) were dissolved in HFIP and dried into a thin film. Pre-formed MLVs of either *d*POPC/POPS (1:1) or *d*POPC/POPS/cholesterol (1:1:1), together with other components as appropriate, were added to the dry peptide at a lipid/peptide ratio of 30:1, followed by several freeze–thaw cycles. Each sample typically contained ~1 mg of Aβ(1–42) or ~0.25 mg of Aβ(25–35) and ~5 mg lipid.

2.4. Addition of CQ

As CQ is insoluble in water and many convenient organic solvents at the required stock solution concentrations, it was dissolved at 30 mg/ml in 10 mM NaOH for dilution into MLV suspensions at a total lipid/CQ mol ratio 30:2. pH was adjusted back to 7.2 with 10 mM HCl following addition.

2.5. Addition of metal ions

Copper and zinc ion stock solutions were prepared using 0.1 M copper glycine ($CuGly_2$) solution or 0.1 M zinc glycine solution ($ZnGly_2$), respectively, by dissolving 1:2 mole ratio of metal chloride with glycine in de-ionised water. Copper and zinc glycine solution was added to the phospholipid bilayer samples at lipid/metal mol ratio of 30:1.

The order of addition of peptide, CQ, and metal to MLV was dictated by the need to protect against the disruption of MLV structure by Cu^{2+}, as reported previously [36]. For data presented here, peptide was added first to MLV, followed by CQ, then metals. Other possible permutations of order-of-addition to MLV (e.g., CQ, peptide, then metal) were also performed, but had little effect (data not shown). This invariance to the order in which components were added suggests that the observations represent an equilibrium state.

2.6. Incorporation of Aβ(1–42)

Aβ(1–42) was dissolved in hexafluoroisopropanol (HFIP) (Sigma Aldrich, St. Louis, USA) and added to MLV suspension resulting in approximately 10% v/v HFIP. The mixture was freeze-dried overnight and the film re-suspended with distilled water and freeze–thawed for several cycles. The total lipid to peptide to mole ratio was 30:1.

2.7. Aβ(1–42) in aligned membrane bilayers

Solid-state NMR samples of membrane proteins in magnetically aligned phospholipid bicelles are prepared by dissolving purified, ^{15}N-labelled proteins in a 6-O-PC aqueous solution, and then adding this solution to a dispersion of 14-O-PC in water [41]. Five samples were made each using 1 mg of dry, uniformly ^{15}N-labelled Aβ(1–42), which was first dissolved in trifluoroethanol (TFE) or a mixture of TFE/chloroform and sonicated for 5–30 min in a bath sonicator. The peptide solution was filtered using a 0.2-μm Teflon filter (PTFE Millex, Millipore) to eliminate any undissolved particles. Solvent was evaporated under a stream of N_2 gas to obtain a thin, transparent peptide film, which was placed under high vacuum overnight. The ether lipids were similarly prepared by drying with N_2 gas and placing under high vacuum overnight. Bicelles with ratio of 14-O-PC/6-O-PC (q)=3.2 and 28% w/v with peptide included were prepared by adding 9.5 mg 6-O-PC in 100 μl aqueous solution to the dry peptide film. A dispersion of 14-O-PC in water (46.5 mg in 75 μl) was prepared by vortexing extensively, freeze–thawing three times (liquid nitrogen and warming to 40 °C). The peptide/6-O-PC solution was then added to the 14-O-PC dispersion, warmed to 40 °C, and an additional 25 μl of water used to complete the transfer. The resulting solution was briefly vortexed, freeze–thawed approximately five times and then allowed to equilibrate to room temperature. Upon bicelle formation, the previously white dispersion of 14-O-PC becomes a clear solution between 0 and 10 °C. Since the peptide-bicelle sample was extremely viscous at room temperature, the bicelle solution was pre-chilled in an ice-water bath for a few seconds before transferring to an NMR tube using a chilled glass Pasteur pipette. A small, flat-bottomed NMR tube with 5-mm outer diameter was filled with ~170 μl of the bicelle solution and sealed with a tight-fitting rubber septum, pierced with a thin syringe to remove excess air from the sample and to create a tight seal.

2.8. Solid-state NMR (SS-NMR)

^2H and ^{31}P NMR static and ^{31}P and ^{13}C magic angle spinning (MAS) experiments were conducted on a Varian Inova 300-MHz spectrometer (Palo Alto, CA, USA) operating at a resonance frequency of 300 MHz for ^1H. Static spectra were acquired at 28 °C, using a Doty (Columbia, SC, USA) 5-mm double or triple resonance probe.

^2H static solid or quadrupolar echo experiments [42] on samples without cholesterol were conducted using the double resonance probe with a $\pi/2$ pulse of 6 μs and a spectral width of 250 kHz, with a recycle delay of 0.5 s and line broadening of 100 Hz. The preacquisition delay for wideline ^2H detection for samples with cholesterol was optimised, such that Fourier transform was performed for a FID beginning at the top of the echo, by first constructing an effectual 10-MHz spectral width spectrum. This was accomplished by shortening the second delay of the echo, collecting an array of 500-kHz spectral width (2.0-μs dwell time, the maximum digitisation rate of the receiver) spectra with the preacquisition delay incremented by 0.1 μs, interleaving points from the resulting array of FIDs, left shifting the interleaved FID to the centre/top of the echo, and inspecting the appropriately down-sampled Fourier transform with zero linear phase correction. The required number of left-shifted points was then translated to a preacquisition delay such that a left shift of FID points for a 500-kHz wide spectrum corresponds to the centre/top of the echo in routine 1D ^2H spectra. These routine spectra were collected with audio filter bandwidth set to 70 kHz, and down-sampled by a factor of 4 to a spectral width of 125 kHz. This approach accomplishes a similar optimisation to that performed numerically when spline fitting the FID to interpolate between digitised points [43].

For static ^{31}P experiments using the double resonance probe with proton decoupling, a $\pi/2$ pulse of 4 μs and a spectral width of 62 500 Hz were used, with a recycle delay of 1.5 s and line broadening of 100 Hz. For static ^{31}P experiments using the triple resonance probe, a $\pi/2$ pulse of 5 μs was applied with a recycle delay of 2 s with similar line broadening. Static experiments for samples with cholesterol used a short Hahn-echo pulse sequence with optimised preacquisition delays and a spectral width of 125 kHz, down-sampled by a factor

Table 1
^2H quadrupolar splitting ($\Delta\nu_Q$) and ^{31}P chemical shift anisotropy (CSA, $\Delta\sigma$) for *d*POPC/POPS MLV with Aβ(1–42), Aβ(25–35), CQ, Cu^{2+} and Zn^{2+} at 28 °C and pH=7.2

	Observed nuclei	^2H		^{31}P
		($\Delta\nu_Q$) kHz		($\Delta\sigma$) ppm
	Sample	CD_2 (±0.3)	CD_3 (±0.1)	CSA (±2)
(a)	*d*POPC/POPS lipid (1:1)	25.1	2.4	−43
(b)	Lipid+CQ (30:2)	24.5	2.1	−49
(c)	Lipid+Aβ(1–42) (associated) (30:1)	24.8	2.5	−43
(d)	Lipid+Aβ(1–42)+CQ (30:1:2)	25.5	2.3	−48
(e) [21]	Lipid+Aβ(1–42)+Cu^{2+} (30:1:1)	24.0	2.5	−32
(f)	Lipid+Aβ(1–42)+CQ+Cu^{2+} (30:1:2:1)	25.3	2.4	−45
(g) [21]	Lipid+Aβ(1–42)+Zn^{2+} (30:1:1)	25.0	2.5	−40
(h)	Lipid+Aβ(1–42)+CQ+Zn^{2+} (30:1:2:1)	26.0	2.3	−45
(i)	Lipid+Aβ(25–35) (associated) (30:1)	24.8	2.5	−47
(j)	Lipid+Aβ(25–35)+CQ (30:1:2)	25.5	2.4	−44
(k)	Lipid+Aβ(25–35)+CQ+Cu^{2+} (30:1:2:1)	25.4	2.4	−42
(l)	Lipid+Aβ(25–35)+CQ+Zn^{2+} (30:1:2:1)	24.9	2.3	−44

$\Delta\nu_Q$ was taken from the 90° edges of the powder pattern for the terminal CD_3 and most ordered CD_2. ^{31}P CSA was determined from discontinuities in overall powder pattern.

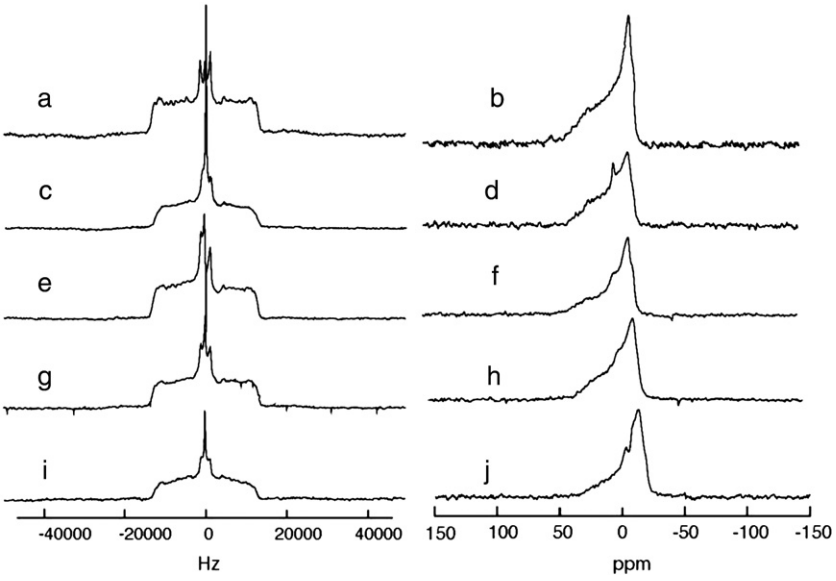

Fig. 2. ^{2}H (left) and ^{31}P NMR (right) static spectra of *d*POPC/POPS (1:1 mol/mol) hydrated dispersions at pH 7: (a, b) with Aβ(1–42); (c, d) with CQ showing an isotropic peak forming; (e, f) with Aβ(1–42) and CQ added; (g, h) with Aβ(1–42), CQ and Cu^{2+} ions; and (i, j) with Aβ(1–42), CQ and Zn^{2+} ions.

of 4, and no apodization. Chemical shifts were externally referenced using 85% phosphoric acid (H_3PO_4) as 0 ppm for ^{31}P experiments. T_2 experiments for ^{31}P were carried out with 6-kHz MAS using a Hahn-echo sequence with $\pi/2$ pulse of 5 μs and variable rotor-synchronized echo delay. T_2 non-linear data were fitted using Gnuplot 4.0 [44].

^{13}C cross polarisation (CP)-MAS experiments [45,46] were carried out at 6 kHz using the 5-mm triple resonance probe and a CP pulse sequence with $\pi/2$=4.5 μs for proton nuclei, 1-ms contact time, 2-s recycle delay, spectral width of 30 kHz, typically 30,000 scans and line broadening of 50 Hz. The ^{13}C chemical shifts were referenced externally to tetramethylsilane (TMS) as 0 ppm and adamantane at 38.48 and 29.46 ppm [47].

^{15}N NMR experiments were conducted on a Bruker (Karlsruhe, Germany) Avance 750-MHz spectrometer using a home-made 5-mm double resonance (PISEMA) [48,49] probe. ^{15}N CP spectra were acquired at 40 °C using a contact time of 1 ms, ^{1}H pulse length of $\pi/2$=5 μs with 50-kHz SPINAL64 decoupling, recycle delay of 6 s, spectral width of 50 kHz and 500-Hz line broadening.

3. Results

3.1. The effect of CQ on membrane integrity in the presence of Aβ(1–42) and Aβ(25–35)

CQ, when added alone to *d*POPC/POPS phospholipid membrane bilayers, decreased the quadrupolar splitting of the most-ordered CD_2 and terminal CD_3 *d*POPC *sn*-1 acyl chain segments by ~10% (Table 1(a) vs. (b)). CQ also caused ~15% increase in the axially symmetric chemical shift anisotropy (CSA) of the phospholipid headgroup relative to MLV bilayers alone, and induced a small fraction of the sample to form an isotropic peak as seen in both the ^{31}P and the ^{2}H spectra (Figs. 2(c, d)). The observation of an isotropic peak suggests that CQ perturbs the integrity of the lipid bilayer [50–52].

Full-length Aβ(1–42) and the hydrophobic segment Aβ(25–35) peptides, when individually associated with *d*POPC/POPS phospholipid membrane bilayers, had similar or less impact than CQ added alone. Each peptide caused a ~10% reduction in quadrupolar splitting at both the terminal CD_3 and the most ordered CD_2 segments of the *d*POPC *sn*-1 acyl chains (Table 1 (c) and (i) vs. (a)). The full-length peptide and hydrophobic subpeptide differ in their effect, however, on the phospholipid headgroups. Aβ(1–42) had no apparent effect, while Aβ(25–35) caused a ~10% increase in the axially symmetric ^{31}P CSA.

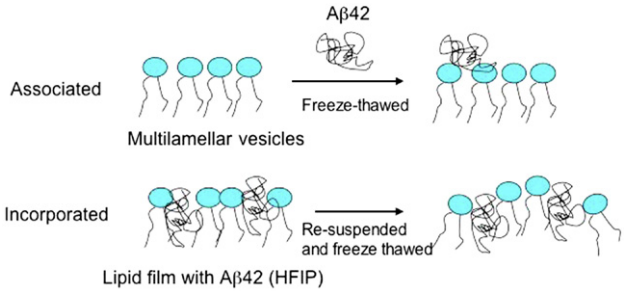

Fig. 3. Schematic showing the difference between 'associated' and 'incorporated' peptides with lipid bilayers.

Table 2
^{2}H quadrupolar splitting ($\Delta\nu_Q$), ^{31}P chemical shift anisotropy (CSA, $\Delta\sigma$) and ^{31}P MAS T_2 relaxation time for dPOPC/POPS/cholesterol MLV with and without Aβ(1–42) 30:1 lipid/peptide ratio at 28 °C, pH=7.2

	Observed nuclei	^{2}H		^{31}P	^{31}P
		($\Delta\nu_Q$) kHz		($\Delta\sigma$) ppm	Isotropic
	Sample	CD_2 (±0.5)	CD_3 (±0.1)	CSA (±2)	T_2 (ms)
(a)	*d*POPC/POPS/Cholesterol lipid (1:1:1)	45.4	3.8	−40	10.6±0.6
(b)	Lipid+Aβ(1–42) (associated)	45.8	4.3	−38	5.2±0.3
(c)	Lipid+Aβ(1–42) (incorporated)	46.7	4.1	−38	4.2±0.2

$\Delta\nu_Q$ was taken from the 90° edges of the powder pattern for the terminal CD_3 and most ordered CD_2. ^{31}P CSA was determined from discontinuities in overall powder pattern.

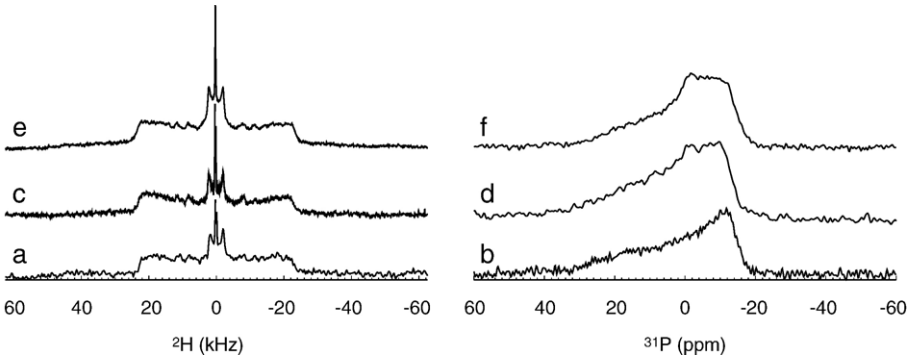

Fig. 4. ^{2}H NMR (left) and ^{31}P NMR (right) spectra of: (a, b) *d*POPC/POPS/cholesterol (1:1:1 mol/mol) hydrated dispersions at pH 7.2; (c, d) *d*POPC/POPS/cholesterol with associated Aβ(1–42); and (e, f) *d*POPC/POPS/cholesterol with incorporated Aβ(1–42).

^{31}P CSA, however, again showed intriguing differences. Addition of CQ to POPC/POPS membranes following Aβ(1–42) peptide increased the ^{31}P CSA (Table 1(d)) similar to CQ alone (Table 1(b)), and in contrast to the lack of effect of Aβ(1–42) alone (Table 1(c)). Further addition of Cu^{2+} or Zn^{2+} ions caused a reduction of the ^{31}P CSA similar to that of Aβ(1–42) alone associated with the phospholipid membrane (Table 1(f, h)). Addition of CQ following Aβ(25–35), on the other hand reduces the CSA (Table 1(j)) to that of the lipid membrane control (Table 1(a)), in contrast to the increased CSA of both the CQ and Aβ(25–35) alone (Table 1(b, i)). The further addition of Cu^{2+} or Zn^{2+} ions had little effect on both the ^{31}P CSA and the ^{2}H splittings (Table 1(k, l)).

3.2. The effect of Aβ(1–42) on phospholipid-cholesterol membranes

Our earlier results with Aβ(1–42) and phospholipid vesicles [36] showed that a difference in bilayer stability is that the peptide was 'associated', i.e., added after vesicle formation, or 'incorporated', i.e., solubilized with the lipid prior to vesicle formation (Fig. 3). Aβ(1–42), whether associated with or incorporated in cholesterol-enriched *d*POPC/POPS MLV bilayers, produced a slight increase in ^{2}H quadrupolar splitting at both ends of the acyl chain and a more significant decrease in ^{31}P CSA (Table 2). In addition to overall narrowing the apparent ^{31}P CSA, the characteristic features of the axially symmetric CSA apparent in the MLV sample without peptide become broader (Fig. 4). The static ^{2}H and ^{31}P measurements did not discriminate significantly, however, between the 'associated' and 'incorporated' methods of addition to MLV bilayers. Measured MAS ^{31}P T_2 relaxation times, however, decreased significantly in the presence of Aβ(1–42) from 10.6 ms in the MLV without peptide to 5.2 ms for 'associated' and 4.2 ms for 'incorporated' peptide (Table 2).

CP-MAS NMR studies of ^{13}C-enriched specific sites in the C-terminal region of the full-length peptide also indicated differences in peptide structure irrespective of 'incorporating' or 'associating' the peptide. After solubilizing the Aβ(1–42) peptide in HFIP and drying it, but before exposure to *d*POPC/POPS/cholesterol MLV bilayers, the Ala30 and Leu34 residues have an isotropic chemical shift of ~177.5 ppm. Upon exposure to *d*POPC/POPS/cholesterol bilayers, the carbonyl resonances move upfield by approximately 4 ppm (Fig. 5).

In order to probe the tertiary fold of the peptide, SS-NMR NMR experiments were performed using fully ^{15}N-labelled Aβ(1–42) with phospholipid bicelles aligned in the magnetic field. Although the ^{15}N spectra of Aβ(1–42) in aligned bicelles initially appeared to show peptide alignment, but for each variation in sample preparation, the peptide appeared to aggregate

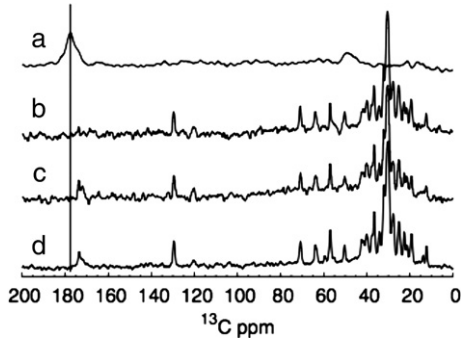

Fig. 5. ^{13}C CP-MAS NMR spectra of: (a) HFIP-dissolved and dried Aβ(1–42), (b) *d*POPC/POPS/cholesterol, (c) *d*POPC/POPS/cholesterol with associated Aβ(1–42), and (d) *d*POPC/POPS/cholesterol with incorporated Aβ(1–42). Carbonyl chemical shifts for HFIP-treated Aβ(1–42) are ~177.5, versus ~173.6 and ~172.8 ppm when either associated or incorporated with *d*POPC/POPS/cholesterol MLV bilayers.

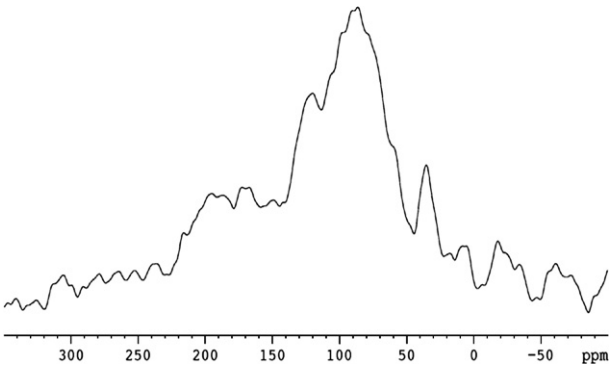

Fig. 6. ^{15}N CP NMR spectrum of uniformly labelled ^{15}N-Aβ(1–42) in aligned bicelles of 14-O-PC/6-O-PC (P/L ~1:300) at 40 °C after 4096 scans.

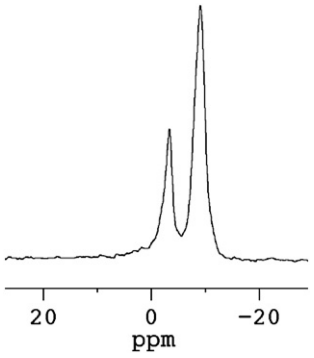

Fig. 7. ^{31}P NMR spectrum of aligned bicelles of 14-O-PC/6-O-PC with uniformly labelled ^{15}N-Aβ(1–42) in (P/L ~1:300) at 40 °C, with the two ^{31}P resonances peaks due to the short and long chain phospholipids [41].

within a few hours and an overlapping powder pattern was evident (Fig. 6). The ^{31}P NMR spectra at 40 °C (Fig. 7) showed that the phospholipids were aligned whereas the ^{15}N spectra indicated that the peptide was not.

4. Discussion

Our earlier studies of *d*POPC/POPS MLV with associated Aβ(1–42) showed a small disordering effect on the lipid acyl chains of the *sn*-1-palmitoyl chain as indicated by only slightly reduced maximum CD_2 and CD_3 quadrupolar splittings [36]. Although a number of models could be used to explain this reduction in order parameter, including a change in wobble or tilt angle of the lipids, a similar reduction in order parameter has been attributed to an 1 Å thinning of the membrane [53]. There was little effect of the peptide on the phosphate headgroups as indicated by the similar axially symmetric "powder" patterns. In the presence of Cu^{2+} ions, however, there was a dramatic reduction in ^{31}P CSA of the peptide-associated MLV, and an additional slight disordering effect in the upper region of the acyl chains [36] (Table 1(e)). Aβ(1–42) with Zn^{2+} had a similar but less dramatic effect on headgroups, and almost no change in the order of the palmitoyl chain. Notably, in the absence of Aβ(1–42) peptide, Cu^{2+} destabilizes the MLV structures, causing much of the phospholipid to form relatively small, isotropically tumbling structures [36].

Clearly, metals and Aβ(1–42) mutually modulate their interaction with *d*POPC/POPS phospholipid membranes. If the mode of action of CQ is solely the chelation of metals, then the data for Aβ(1–42) associated alone with MLV should resemble that when CQ is included together with both Aβ(1–42) and metal ions, and CQ itself should not perturb membrane structure. Surprisingly, however, CQ added alone to MLV had the effect of disordering the terminal *sn*-1 CD_3 of *d*POPC, while increasing the ^{31}P CSA (from −43 to −49 ppm). For typical phospholipid ^{31}P chemical shift tensors undergoing fast axial rotation about the long axis of the lipid and other modes of disorder [54–59], this increase in CSA upon addition of CQ may correspond to headgroup orientations extending more upright from the plane of the lipid bilayer, as previously reported for another heterocyclic pharmaceutical [60], and a possible consequence of CQ occupancy in the more polar headgroup area rather than further down into the interfacial/lipid carbonyl area. CQ, when added to the bilayer alone also caused the appearance of an isotropic peak, indicating a loss in integrity of the bilayer (Figs. 2(c, d)). However, the overall ^{2}H quadrupolar splitting was not reduced with the formation of the isotropic peak, suggesting the persistency of large bilayer structures. The isotropic peak was small in the ^{31}P spectra, which suggested the extent of CQ perturbation to be sufficient only for averaging the CD_3 splittings but insufficient for averaging the ^{31}P CSA [36]. The amyloidogenic human Islet Amyloid Polypeptide, which forms small aggregates that kill cells by disrupting the cellular membrane, also produces an isotropic ^{31}P chemical shift signal indicative of membrane fragmentation [61].

In the presence of Aβ(1–42), but still without metals, the terminal *sn*-1 CD_3 regained the order typical of unperturbed MLV with the addition of CQ, but the headgroups ^{31}P anisotropy remained almost as broad (−48 ppm) as with CQ alone. The headgroup appeared to be more restricted when CQ was added after Aβ(1–42) to the membrane bilayers as seen by the increase in ^{31}P CSA, while an opposite effect was seen for Aβ(25–35) possibly due to Aβ(25–35) penetrating the bilayer [62]. Taken together, this effect on model membranes without metal ions suggests that CQ may potentially have an additional mode of action in modulating cytotoxic activity of Aβ(1–42).

The effect of CQ on membranes with Aβ(1–42) and Aβ(25–35) peptides was examined in the presence of Cu^{2+} and Zn^{2+}. Subsequent addition of Cu^{2+} ions after CQ and Aβ(1–42) gave lipid ^{31}P CSA and ^{2}H quadrupolar splittings similar to the results seen when Aβ(1–42) was associated with the phospholipid bilayers alone, as if CQ has reduced the effect of metal ions. In contrast to Aβ(1–42), the C-terminal mostly hydrophobic subpeptide Aβ(25–35) increased the ^{31}P CSA on its own, but prevented the increase in CSA by CQ, whether metal was present or not. If CQ counteracts Aβ(1–42) toxicity by abolishing the formation of Cu^{2+} coordination required for peptide toxicity [21], then the toxicity of Aβ(25–35) which excludes histidine–metal binding, would not be 'rescued' by CQ. The differences in lipid changes in the presence of Aβ(1–42) compared to Aβ(25–35) further substantiate the latter having a different toxicity mechanism to Aβ(1–42).

CQ bears some structural similarity to tryptophan and cholesterol, which are both known for their tendency to locate at the interfacial region of the phospholipid bilayers [9,10,63], and as cholesterol occurs naturally in cell membranes with some correlations to Aβ cytotoxicity [11–13], we examined the interaction between Aβ(1–42) and cholesterol-containing MLV. Although CQ and cholesterol are both aromatic compounds with a relationship to the progression of AD [33], their effects on lipid bilayers in the presence of Aβ(1–42) are rather different. Cholesterol increases segmental order at both the top and bottom of the *sn*-1 chain [10,40,42] and causes a reduced ^{31}P CSA [37,40], opposite to the effect seen with CQ. Our earlier studies of cholesterol-enriched membranes in the presence of the fibril forming Aβ(25–35) showed a further increase in the ^{2}H splittings [37]. Both associated and incorporated Aβ(1–42) also caused a further ordering of the *sn*-1 acyl chain, and even

more reduction in the ^{31}P CSA in cholesterol–phospholipid bilayers. T_2 relaxation measurements, in contrast, did not obviously follow suit: T_2 for *d*POPC/POPS MLV increased from 6.2 ms [37] to 10.6 ms with cholesterol, but decreased again to 5.2 and 4.2 ms upon association and incorporation of Aβ(1–42), respectively. Conversely to the effect observed upon addition of CQ to MLV, the decrease in phospholipid ^{31}P CSA when cholesterol was included may correspond to a more lateral orientation of the headgroup as lipids are spaced further apart from one another [64,65], an effect made more so by the presence of Aβ(1–42). The increase in T_2 relaxation time with cholesterol indicates a decrease in intensity of slow modes of bilayer disorder [57,59]. The decrease in T_2 upon addition of Aβ(1–42), as well as the corresponding broadening of ^{31}P static lineshape features in Figs. 3(d) and (f) relative to (b), similar to spectral effects noted in aligned lipid–peptide systems, [66] indicates that slow-motion headgroup disorder is reintroduced, in spite of the presence of cholesterol. The lack of significant impact on acyl chain order suggests that at least a part of the Aβ(1–42) occupies the interfacial regions of the MLV bilayers. Both the full-length peptide and the fragment are toxic to cells and the opposite effects observed for the ^{31}P CSA for Aβ(25–35) in comparison to Aβ(1–42) indicate that their membrane interactions are rather different.

The ^{13}C-detected CP-MAS spectra (Fig. 5) provide evidence for the structural conformation of Aβ(1–42). Organic solvents such as TFE [63] or HFIP used here to initially solubilize Aβ(1–42) prior to introducing MLV lipid bilayers induced α-helical structure, as noted by circular dichroism (data not shown) and correlation of the ~177.5 ppm isotropic chemical shift of the ^{13}C=O (Fig. 5) to secondary structures observed by solid-state NMR [67,68]. Notably, upon association or incorporation of HFIP-treated Aβ(1–42) with *d*POPC/POPS/cholesterol MLV, the carbonyl resonances of Ala30 and Leu34 moved upfield to isotropic chemical shifts characteristic of extended β-strand conformations [67], similar to previous experience with Aβ(25–35) interacting with *d*POPC/POPS MLV lipid bilayers [37]. The two chemical shifts may correspond to Ala (173.6 ppm) and Leu (172.8 ppm), which were not resolved in the dried peptide because the linewidths were broader than in the membrane-associated samples. Alternatively, the two peaks could be due to: (i) Aβ(1–42) inserted deeper into the bilayer, and (ii) Aβ(1–42) unable to penetrate the bilayer due to the higher membrane order due to cholesterol. Peptide aggregation may have occurred, possibly due to peptides being unable to fold/unfold in the bilayer or peptide aggregating in the bilayer and being expelled, as reported for Aβ40 with saturated PC bilayers [69]. Interestingly, cholesterol also inhibited the insertion of the peptide fragment Aβ(25–35) in phospholipid bilayers [70].

Significantly, when attempting incorporation of Aβ(1–42) with the zwitterionic PC ether lipids, the peptide may have aggregated in the bicelles with a broad distribution of orientations but, since the lipid was well aligned (Fig. 7), the aggregates are more likely to be in the aqueous phase as similarly observed in PC vesicles [69]. On the other hand, the interactions between lipid and peptide persist through the experimental time required to collect the ^{2}H and ^{31}P static and ^{31}P and ^{13}C MAS spectra in our anionic membrane systems, even at ten times greater concentration than in the bicelle samples, as detailed above. While there are several clear differences between the ether–PC bicelle and MLV bilayer lipids, the apparent surface interactions of the peptide suggest that the variation in phospholipid headgroup composition between the two systems provides the principal difference.

Our previous studies have shown that Aβ(1–42) interactions with phospholipid bilayers result in an oligomeric β-sheet structure as a toxic species [18], and result in no significant membrane perturbation [36]. In the present study, Aβ(1–42) when 'associated' with cholesterol-enriched bilayers was found to perturb the phospholipid acyl chain region and retain a β-sheet structure. Aβ42 appeared to aggregate with aligned PC bicelles, even at one tenth of the concentration in PC/PS bilayers, and may explain the lack of significant membrane disruption observed by NMR. A possible mode of Aβ(1–42) cell toxicity that does not involve considerable membrane perturbation may depend on peptide electrostatic interactions with the membrane bilayer, e.g., with charged phospholipids such as PS, which is associated with apoptosis [71]. The histidine residues in particular may play a major role in membrane binding since N-methylation of the His residues has been shown to inhibit Aβ–membrane interactions [21].

Cholesterol appeared to preserve the bilayer stability when 'incorporated' Aβ(1–42) was included. Earlier results for Aβ(1–42) incorporated into PC/PS bilayers [36] show a loss of bilayer stability, which is consistent with cholesterol playing a protective role in Aβ toxicity. Comparison of 'associated' Aβ(1–42) with phospholipid bilayers [18] and cholesterol-enriched bilayers suggests that association of Aβ(1–42) alone does not significantly perturb the membrane integrity. However, with the addition of cholesterol, an increase in ^{2}H quadrupolar splitting and a decrease in ^{31}P CSA were seen, together with a decrease in T_2. The peptide seemed to maintain an interaction with the phospholipid headgroup but, since cholesterol decreases membrane fluidity and sits in the acyl chain region, the lipid headgroups are further apart with the addition of cholesterol, which may affect the peptide interaction with the membrane. The ^{13}C NMR data suggest that incorporated and associated Aβ(1–42) appeared to have differences in secondary structure, and differences in membrane association have previously been reported for Aβ(1–40) [72]. Our ^{15}N results showing unoriented peptide in aligned bicelles supports previously reported results for Aβ(1–42) in PC/PS [18] and Aβ40 with PC vesicles [69] that aggregation may be taking place on the membrane surface and preventing transmembrane insertion. The 'associated' Aβ(1–42) would then have little effect on the order of the ^{2}H acyl chains and decrease that of the ^{31}P headgroups. Possibly the associated species has a greater effect on the cholesterol containing phospholipid bilayers, which represent a more biologically relevant model system, by allowing a greater lipid–peptide interaction as reflected by the resulting decrease in ^{31}P T_2.

Incorporated Aβ(1–42) appeared to enhance the effect of cholesterol by further ordering the lipid acyl chain while

disordering the headgroup (Table 2). The decrease in the ^{31}P headgroup relaxation time from 10.6 ms to ~5 ms (Table 2) also indicated an increase in low-frequency motions, consistent with 'immobilisation' of the lipid in the presence of the peptide [73]. Broadening of the characteristic ^{31}P axially symmetric features for cholesterol-enriched PC/PS MLV upon addition of peptide was greater than that which would be consistent with the decreased T_2 relaxation times as measured by ^{31}P MAS. This may result from the interaction of the phospholipids with aggregated species of Aβ peptides, which is modulated by the fluidity of the bilayer [70,74]. The results suggest that Aβ(1–42) inserted into the cholesterol enriched bilayer as reflected by the increase in ^{2}H quadrupolar splitting of the chains and the simultaneous decrease in the ^{31}P CSA of the headgroup, as a result of an increase in lipid headgroup area caused by peptide insertion. Incorporated had a similar or greater effect than associated peptide, which can be explained by each having the same fundamental interaction, but that the incorporated method is simply more thorough in establishing the interaction. The similar ^{13}C shifts also is consistent with the peptide having similar structure and lipid interaction. Also, since cholesterol decreases the fluidity and enhances the stability of membranes, the effect is less dramatic than that seen for phospholipid bilayers without cholesterol [36], where the membrane integrity was disrupted by Aβ(1–42) incorporation. A possible explanation, which accounts for the solid-state NMR data presented here and an interfacial location of Aβ(1–42), is that bilayer order influences the depth of insertion of the peptide, as reported for the antimicrobial peptide LL-37 [75]. Although Aβ peptides and metals interact with model membranes, their effects are modulated by the composition of the lipid bilayer and the presence of metal-chelating drugs, which together can maintain bilayer stability.

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