Amyloid Peptide Self Assembly in Protic Ionic Liquids

Presented By

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Centre for Chemistry and Biotechnology
Faculty of Science, Engineering and Built Environment
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October 2014
I am the author of the thesis entitled

‘Amyloid Peptide Self Assembly in Protic Ionic Liquids’

submitted for the degree of

Doctor of Philosophy

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‘Amyloid Peptide Self Assembly in Protonic Ionic Liquids’

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is the result of my own work and that where reference is made to the work of others, due acknowledgment is given.

I also certify that any material in the thesis which has been accepted for a degree or diploma by any university or institution is identified in the text.

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Full Name: .................................................................(Please Print)

Signed: .................................................................

Date: .................................................................

9th January, 2015
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<tr>
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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Aβ</td>
<td>amyloid-beta</td>
</tr>
<tr>
<td>Aβ1-40</td>
<td>Aβ fragment between aspartate 1 and valine 40</td>
</tr>
<tr>
<td>Aβ1-42</td>
<td>Aβ fragment between aspartate 1 and alanine 42</td>
</tr>
<tr>
<td>Aβ16-22</td>
<td>Aβ fragment between lysine 16 and glutamate 22</td>
</tr>
<tr>
<td>Aβ3pE-40</td>
<td>pyroglutamate Aβ fragment between glutamate 3 and valine 40</td>
</tr>
<tr>
<td>Aβ11pE-40</td>
<td>pyroglutamate Aβ fragment between glutamate 11 and valine 40</td>
</tr>
<tr>
<td>ANS</td>
<td>8-anilino-1-naphthalene-sulfonic acid</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>attenuated total reflectance Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>BaLa</td>
<td>butylammonium lactate</td>
</tr>
<tr>
<td>BMIMAc</td>
<td>1-butyl-3-methylimidazolium acetate</td>
</tr>
<tr>
<td>BMIMCl</td>
<td>1-butyl-3-methylimidazolium chloride</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>Choline dhp</td>
<td>choline dihydrogen phosphate</td>
</tr>
<tr>
<td>ChoLa</td>
<td>choline lactate</td>
</tr>
<tr>
<td>ChoMa</td>
<td>choline malate</td>
</tr>
<tr>
<td>CR</td>
<td>Congo red</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DeaLa</td>
<td>diethylammonium lactate</td>
</tr>
<tr>
<td>DeaMs</td>
<td>diethylammonium mesylate</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EaLa</td>
<td>ethylammonium lactate</td>
</tr>
<tr>
<td>EaMs</td>
<td>ethylammonium mesylate</td>
</tr>
<tr>
<td>EAN</td>
<td>ethylammonium nitrate</td>
</tr>
<tr>
<td>EMIMSO₃</td>
<td>1-ethyl-3-methylimidazolium sulfate</td>
</tr>
<tr>
<td>ETALa</td>
<td>ethanolammonium lactate</td>
</tr>
<tr>
<td>ETAMa</td>
<td>ethanolammonium malate</td>
</tr>
<tr>
<td>FA</td>
<td>formic acid</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>HBTU</td>
<td>O-(benzotriazol-1-yl)N,N,N,N,N’tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HDO</td>
<td>deuterium oxide</td>
</tr>
<tr>
<td>HFIP</td>
<td>1,1,1,3,3,3-hexafluoro-2-propanol</td>
</tr>
<tr>
<td>HOBt</td>
<td>1-hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>LCST</td>
<td>lower critical solution temperature</td>
</tr>
<tr>
<td>TOF-MS</td>
<td>time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance (spectroscopy)</td>
</tr>
<tr>
<td>NR</td>
<td>Nile Red</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PNIPAM</td>
<td>poly(N-isopropylacrylamide)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>pH</td>
<td>power of hydrogen</td>
</tr>
<tr>
<td>pKₐ</td>
<td>acid dissociation constant</td>
</tr>
<tr>
<td>PIL</td>
<td>protic ionic liquid</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>TeaBF₄</td>
<td>triethylammonium tetrafluoroborate</td>
</tr>
<tr>
<td>TeaH₂PO₄</td>
<td>triethylammonium dihydrogen phosphate</td>
</tr>
<tr>
<td>TeaHSO₄</td>
<td>triethylammonium hydrogen sulfate</td>
</tr>
<tr>
<td>TeaLa</td>
<td>triethylammonium lactate</td>
</tr>
<tr>
<td>TeaMa</td>
<td>triethylammonium malate</td>
</tr>
<tr>
<td>TeaMs</td>
<td>triethylammonium mesylate</td>
</tr>
<tr>
<td>TeaTf</td>
<td>triethylammonium triflate</td>
</tr>
<tr>
<td>TeaTfac</td>
<td>triethylammonium trifluoroacetate</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TFE</td>
<td>2,2,2-trifluoroethanol</td>
</tr>
<tr>
<td>ThT</td>
<td>thioflavin T</td>
</tr>
<tr>
<td>TMGLa</td>
<td>1,1,3,3-tetramethylguanidinium lactate</td>
</tr>
<tr>
<td>TMS</td>
<td>tetramethylsilane</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>ultraviolet-visible</td>
</tr>
<tr>
<td>XRD</td>
<td>x-ray diffraction</td>
</tr>
</tbody>
</table>
SYMBOLS

2θ  angle
Å  Ångstrom
$N_A$  Avogadro’s number
$^{13}\text{C}$  carbon-13
$\delta$  chemical shift
$^\circ\text{C}$  degrees Celsius
$\Delta$  difference
$^1\text{H}$  hydrogen-1
$\alpha$  Kamlet-Taft hydrogen-bonding acidity
$\beta$  Kamlet-Taft hydrogen-bonding basicity
$\pi^*$  Kamlet-Taft dipolarity/polarizability
K  kelvin
$m/z$  mass to charge ratio
$\mu\text{L}$  microlitre
$E_{\text{NR}}$  molar transition energy of Nile Red
$E_T^N$  normalized transition energy
$^{31}\text{P}$  phosphorus-31
$\pi$  pi-orbital
$h$  Planck’s constant
$c$  speed of light
$T_1$  spin-lattice (longitudinal) relaxation
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_t(30)$</td>
<td>transition energy of Reichardt’s dye</td>
</tr>
<tr>
<td>$\phi$</td>
<td>volume fraction</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$</td>
<td>wavelength maximum</td>
</tr>
<tr>
<td>$\nu$</td>
<td>wavenumber</td>
</tr>
<tr>
<td>wt%</td>
<td>weight percent</td>
</tr>
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ABSTRACT

Alzheimer’s disease (AD) is one of many debilitating human neurodegenerative disorders characterized by the accumulation of misfolded protein into insoluble amyloid deposits. The widespread implication of amyloid fibrils in this disease has inspired intense research efforts towards understanding the mechanisms of amyloid self-assembly, including the early stages of fibril formation, toxic amyloid species and possible methods for fibril inhibition in vivo.

Inspired by the growing field of ionic liquids in protein research, initial investigations were aimed towards controlling the kinetics of Aβ self-assembly in protic ionic liquids (PILs). Thorough experimentation revealed that PIL anion kosmotropicity, and the ability to form extensive intermolecular hydrogen bonds with water molecules, was critical in the rapid generation of Aβ16-22 amyloid fibrils. Reversal of these anion properties was shown to lead to long-term solution stability and a marked inhibition of fibril formation. PIL cations were further tailored to accelerate or inhibit the fibrilization of Aβ16-22, and comprehensive circular dichroism analysis identified that peptide-PIL interactions in these PILs facilitated changes in the secondary structure of various Aβ isoforms.

In an expansion of this work, further investigations into the fibrilization of full-length Aβ (Aβ1-40) showed that helical-inducing PILs could inhibit amyloid fibril formation. Pre-formed Aβ1-40 amyloid fibrils could be dissolved in the presence of the neat PIL triethylammonium mesylate (TeaMs) via the formation of intermolecular hydrogen bonds.
bonds between the solvent and peptide, promoting solubilization of Aβ1-40 monomers, in a similar manner to helical-inducing solvents such as TFE and HFIP, but providing an alternative to these solvents for manipulating Aβ1-40 solubilisation and also amyloid formation.

Many amyloidogenic proteins display conformational or structural polymorphism during self-assembly as the result of changes to the secondary structure of the protein or various environmental stimuli. In order to understand this process, the central Aβ fragment (NH2-FF-COOH) was utilized as a model of the events in amyloid fibril formation. It was found that various polymorphs including crystalline fibrils and flat sheets could be self-assembled by simply altering the concentration of ethylammonium nitrate (EAN) in aqueous solution. The formation of these polymorphs was attributed to the increased exposure of hydrophobic residues on NH2-FF-COOH as the result of increasing EAN concentration.

Finally, the effect of other key solvent parameters (such as acidity, basicity, polarity and polarizability) which are associated with protein folding in PILs were explored, using the thermoresponsive polymer poly(N-isopropylacrylamide) (PNIPAM) as a protein model. After extensive analysis of Kamlet-Taft solvatochromic parameters, the PIL cation basicity (reflected through the choice of anion), and the relative polarizability of the anion (resulting from its kosmotropicity), were identified as the critical properties for the rapid generation of amyloid fibrils.
Overall, this thesis presents the novel approach of using carefully selected PILs to both control amyloid fibril formation and inhibit/dissolve Aβ fibrils through induced helical transitions. These results provide insights into future PIL-mediated protein studies and the potential application of PILs in AD pathogenesis.
“Science never solves a problem, without creating ten more.”

- George Bernard Shaw
CHAPTER 1: Introduction

1.1 Amyloidosis - Protein misfolding and disease

The correct folding of a protein into its native conformation plays a pivotal role in normal cell functioning and biological processes. However, protein folding itself is a complex mechanism, and in order for a protein to achieve its correct native structure, the protein must avoid various environmental challenges such as elevations in temperature and changes in pH\(^{(1)}\). In the event of protein misfolding, such as when cellular repair mechanisms fail\(^{(2)}\), a cascade of events can lead to mechanism-based toxicities, protein aggregation and disease.

Since the first reported case of brain amyloid plaque formation in 1854\(^{(3)}\), the accumulation of abnormally folded protein deposits has been recognized as a key histopathological feature in at least 25 debilitating human diseases (Table 1.1). Although most amyloid-derived diseases occur spontaneously, a small proportion are hereditary and some are transmissible\(^{(4)}\). Furthermore, the accumulation of amyloid can occur systemically or localized in a specific tissue type, and manifest as both intracellular and extracellular deposits. Although each amyloid forming protein arises from very different pre-cursors, primary structures and biochemical functionalities, their aggregated amyloid states share common discriminating characteristics such as ordered $\beta$-sheet structure, fibril dimensions and x-ray diffraction patterns (see section 1.3).
Table 1.1. Some proteins associated with disease and amyloid fibril formation.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Amyloid protein</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Alzheimer’s disease</td>
<td>Aβ peptides, tau</td>
<td>(5-11)</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>α-synuclein</td>
<td>(12-14)</td>
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<tr>
<td>Huntington’s disease</td>
<td>Huntingtin</td>
<td>(15, 16)</td>
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<tr>
<td>Creutzfeldt–Jakob disease &amp; Transmissible spongiform encephalopathies (Prion diseases)</td>
<td>Prion protein (PrP)</td>
<td>(17-19)</td>
</tr>
<tr>
<td>Adult-onset (Type II) diabetes</td>
<td>Islet amyloid polypeptide (IAPP) or Amylin</td>
<td>(20)</td>
</tr>
<tr>
<td>Atrial amyloidosis</td>
<td>Amyloid atrial natriuretic factor (ANF)</td>
<td>(21, 22)</td>
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<td>Aortic medial amyloidosis</td>
<td>Medin</td>
<td>(23)</td>
</tr>
<tr>
<td>Senile systemic amyloidosis &amp; Familial amyloid polyneuropathy</td>
<td>Transthyretin (TTR)</td>
<td>(24-26)</td>
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<tr>
<td>Primary systemic amyloidosis</td>
<td>Immunoglobulin light (AL) and heavy (HL) chains</td>
<td>(27, 28)</td>
</tr>
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<td>Secondary systemic amyloidosis</td>
<td>Serum amyloid A (SAA)</td>
<td>(24, 27)</td>
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<tr>
<td>Hereditary systemic amyloidosis</td>
<td>Lysozyme</td>
<td>(24, 25, 29)</td>
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<tr>
<td>Haemodialysis-related amyloidosis</td>
<td>β2-microglobulin</td>
<td>(30-33)</td>
</tr>
<tr>
<td>British familial dementia</td>
<td>ABri</td>
<td>(34-36)</td>
</tr>
<tr>
<td>Finnish hereditary systemic amyloidosis</td>
<td>Gelsolin</td>
<td>(37)</td>
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<tr>
<td>Cystic fibrosis</td>
<td>CFTR protein</td>
<td>(1, 38, 39)</td>
</tr>
<tr>
<td>Down’s syndrome</td>
<td>Aβ peptides</td>
<td>(9, 40-43)</td>
</tr>
</tbody>
</table>
This suggests that these proteins follow a common folding pathway that encompasses a conserved intermediate state or conformational transition prior to adopting their final β-sheet conformation\(^{(44)}\).

Currently, Alzheimer’s disease (AD) is the single highest cause of dementia worldwide, accounting for 60-80\% of all dementia cases\(^{(45)}\). Although dementia does not discriminate against race or gender, age is a major risk factor, with dementia affecting one in nine people aged over 65 and three in ten people aged 85 or older in Australia\(^{(46)}\). In 2011, an estimated 298,000 people were affected with dementia in Australia, and this value is expected to triple to over 900,000 by the year 2050. Furthermore, an estimate of $2.0 billion dollars was attributed towards the use and cost of health care facilities for dementia cases alone in Australia in 2009-10\(^{(46)}\). The enormous medical implications of amyloidosis in AD has motivated intense research efforts, leading to a number of reviews on the structure and growth of amyloid fibrils\(^{(4, 6, 47-56)}\).

### 1.2 Alzheimer’s disease and the amyloid-beta (Aβ) peptide

AD is a neurodegenerative disorder characterized by the intracellular formation and accumulation of neurofibrillar tangles and the deposition of insoluble extracellular amyloid plaques\(^{(57)}\). These aggregated protein deposits appear to cause disease through an increase in localized toxicity that progressively affects normal brain functions such as memory, judgement and cognition and result in the failure of important cellular processes\(^{(58, 59)}\). The amyloid cascade hypothesis proposed by Hardy \textit{et al.}\(^{(60, 61)}\)
suggests that the aggregation of Aβ protein induces neuronal dysfunction and the hyperphosphorylation of the microtubule associated τau protein, leading to neuronal death. This hypothesis was further supported by transgenic mice models where it was determined that Aβ accumulation precedes changes in the metabolism of τau protein\(^\text{(62, 63)}\). Glenner and Wong\(^\text{(10)}\) first discovered Aβ during the purification and sequencing of microvascular amyloid deposits from the meninges of AD brains. However, it was not until its isolation and characterization from plaque cores of post-mortem AD cortices\(^\text{(8)}\) when the link between Aβ protein aggregation and deposition into amyloid fibrils was recognized. Since then, Hardy’s amyloid cascade hypothesis has been revised to indicate the importance of soluble oligomeric species as being the most potent neurotoxins and the current paradigm in the field of AD\(^\text{(64, 65)}\) (see section 1.5).

Aβ is produced by two sequential proteolytic cleavages of the transmembrane amyloid precursor protein (APP) in the lipid-rich microdomains (lipid rafts) of endosomes and the plasma membrane\(^\text{(66)}\). Known as the amyloidogenic pathway, APP is initially cleaved by β-site APP cleaving enzyme 1 (BACE1) which releases a large N-terminal APP ectodomain fragment (APPsβ)\(^\text{(67)}\), followed by cleaving with γ-secretase, which releases APPsβ and leads to secretion of Aβ peptides\(^\text{(68)}\). This proteolytic Aβ product ranges between 29-43 amino acids in length depending on the β- and γ-secretase cleaving sites as illustrated in Figure 1.1.

Truncated and pyroglutamate forms of Aβ, which account for a portion of the Aβ peptides extracted from AD human brains, have also been heavily reported\(^\text{(69-71)}\) and
arise from the intramolecular dehydration of N-terminally exposed glutamate residues\(^{(72)}\).

Alternatively, the more dominant processing of APP occurs outside the domain of lipid rafts and does not result in the production of \(\text{A}\beta\)\(^{(66)}\). During this non-amyloidogenic pathway, sequential proteolytic cleavaging of APP occurs first by \(\alpha\)-secretase, a disintegrin and metalloprotease 10 (ADAM10)\(^{(75)}\), then \(\gamma\)-secretase to yield an N-terminal soluble APP (sAPP\(\alpha\)) fragment and a C-terminal fragment known as C83 transmembrane domain\(^{(76)}\).
Misprocessing of APP via the amyloidogenic pathway has been recognized as the key event in the pathogenesis of AD, since the accumulation of Aβ leads to amyloid fibril formation. Aβ fibrillogenesis studies have revealed that the carboxy-terminally extended variant Aβ1-42 forms insoluble amyloid fibrils more rapidly than Aβ1-40 and increased levels of longer C-terminal forms probably contribute to causation in both sporadic and familial AD (FAD)\(^{(77, 78)}\). In fact, this is evident in a number of studies where cultured cell systems expressing FAD-mutant forms of APP or presenilin overexpressed Aβ1-42\(^{(79-81)}\). Furthermore, increases in the ratio of Aβ1-42 production relative to Aβ1-40 were also measured in the plasma of patients diagnosed with FAD\(^{(82)}\) and similarly was observed in transgenic mice models\(^{(80, 81, 83)}\).

Comprehensive analysis of the early stage Aβ self-assembly reveal that Aβ1-42 forms transient pentamer/hexamer units that assemble into beaded superstructures resembling early protofibrils. Though not observed for Aβ1-40, this key structural transition could explain the rapid assembly of Aβ1-42 in AD\(^{(84)}\).

Additionally, there is well documented evidence suggesting that genetic mutations in APP significantly lower the onset age in FAD and increase cognitive impairment and plaque formation\(^{(85-91)}\). Some of these and other common mutations are illustrated in the red text in Figure 1.1. The involvement of lipoprotein receptors has previously been recognized as a key regulator for both the recycling of APP in endosomes and Aβ formation. Neuronal expression of the lipoprotein receptor LR11 or SorLA in brain regions vulnerable to AD neuropathology showed a dramatic reduction in LR11 expression and increased Aβ production and cell death\(^{(92, 93)}\). However, when LR11
was overexpressed in these brain regions, extracellular levels of Aβ were significantly reduced and cellular levels of APP were lowered.

Enhancing α-secretase cleavage has been considered as a possible therapeutic approach for tackling AD. However, the lack of knowledge surrounding the molecular mechanisms for regulating α-secretase cleavage has made this approach difficult\(^{(94)}\). Alternatively, the inhibition of BACE1 activity has also been extensively studied as a strategy to inhibit Aβ deposition in AD. Studies have shown that preventing β-secretase cleavage can result in the inhibition of Aβ secretion in cultured neurons and in brain cells\(^{(95-99)}\). However, controversial studies have suggested that partial deletion of BACE1 activity leads to mild deficits in cognitive functioning and behavioural changes in BACE1 knockout mice\(^{(99-101)}\). Aβ has been shown to play an important physiological role in normal cell functioning and modulates the activities of certain synapses\(^{(102)}\). Thus inhibiting BACE1 activity could have deleterious effects such as mechanism-based toxicities or interference with normal cellular function.

### 1.3 Amyloid structure and fibril morphology

#### 1.3.1 Characteristics of amyloid fibrils

The formation of α-helices, β-strands and β-sheets is a naturally occurring process mediated via intra- and intermolecular hydrogen bonding, providing proteins with their native structure and function. What is now viewed as a property inherent to all amyloid-forming proteins is the formation of ordered β-sheet structure which is critical in the initiation of self-assembly and fibril growth\(^{(103)}\). The β-sheets themselves are
created through intermolecular hydrogen bonds between individual peptide strands which align either in an anti-parallel or parallel configuration\textsuperscript{(104)}. Amyloid fibrils contain bundles of these β-sheets where the peptide backbone aligns orthogonal to the fibre axis in a cross-β-sheet configuration\textsuperscript{(6, 105, 106)} (Figure 1.2).

\textbf{Figure 1.2.} Schematic of the cross-β structure found in amyloid fibrils. (a) Hydrogen bonds form between β-strands (red dashed lines) either in an anti-parallel or parallel arrangement. (b) The common cross-β structure of an amyloid fibril with a meridional spacing of 4.7-4.8 Å and equatorial spacing of 9-10 Å. (c) TEM of long straight Aβ16-22 fibrils. Scale bar 200 nm.

These characteristic cross-β-sheet structures show two distinctive X-ray diffraction reflections; a meridional at 4.7-4.8 Å that arises from the spacing between hydrogen bonded β-strands, and an equatorial between 9-10 Å that occurs from the distance between the β-sheets\textsuperscript{(106-109)}. Furthermore, the arrangement of β-strands perpendicular to the fibre axis is critical in defining the macrostructure of amyloid fibrils, which is
typically monitored by electron microscopy. These amyloid fibrils are typically long and straight diameters of 7-15 nm\(^{(110)}\). Detection of the β-sheet component of amyloid fibrils can be also be achieved by using various histological dyes such as Congo red (CR) and Thioflavin T (ThT). Amyloid fibrils display an apple-green birefringence when stained with CR and viewed under cross-polarized light; a property that is only observed when CR binds to proteins with extensive β-sheet structure\(^{(111-115)}\). When bound to ThT, the dye exhibits increased fluorescence intensity as the result of binding to β-sheet-rich deposits, and has been extensively used as a tool to monitor the fibrillization kinetics of various amyloid-forming proteins\(^{(116-120)}\).

### 1.3.2 NMR studies of amyloid fibril structure

The first peptide synthesis and solution studies of full length Ab peptide was carried out by Barrow and Zagorski using a combination of CD and NMR spectroscopy\(^{(78, 121, 122)}\). These studies showed that Aβ is soluble but aggregates rapidly in the presence of extrinsic factors such as low pH. The insoluble and heterogeneous nature of large amyloid fibrils has made it difficult to study the high-resolution structures of full length aggregated species, and so structural analysis has been performed on smaller fragments of these peptides. During early solid-state NMR (ssNMR) investigations, Lansbury et al.\(^{(123)}\) were able to construct a model for Aβ34-42 amyloid fibrils. Later studies revealed that Aβ10-35 amyloid fibrils contain an in register cross-β structure arranged with parallel β-sheets\(^{(124, 125)}\). Other fragments including Aβ16-22 (Figure 1.3) and Aβ11-25 have since become attractive due to their ability to form amyloid fibrils in solution. Amyloid fibrils formed by Aβ16-22 show a
well-ordered β-strand conformation that extends across the entire hydrophobic segment from L17 through to A21\(^{(126)}\). The anti-parallel organization of these β-sheets suggests a high degree of structural order at the molecular level that approaches that of peptide and protein crystals\(^{(126)}\).

\[\text{Figure 1.3. Chemical structure of the Aβ16-22 heptapeptide.}\]

Reports on Aβ11-25 indicate that the central hydrophobic segment (LVFFA) adopts a β-strand conformation and participates in anti-parallel β-sheets at both pH 7.4 and pH 2.4, however the registry of intermolecular hydrogen bonds show a dependence to pH\(^{(127)}\). Advancements in technology and the combination of various discriminating techniques have provided insights into the substructure and packing arrangements of larger amyloid residues at the atomic level\(^{(128, 129)}\). Several ssNMR models have since been determined and describe the molecular organization of side chains within amyloid fibrils\(^{(130-132)}\). More recent investigations using a combination of hydrogen deuterium (HD) exchange NMR and ssNMR have detected specific residues within amyloid proteins that are involved in forming the characteristic cross-β structure observed in fibrils\(^{(133, 134)}\). In particular, 3D structural studies on Aβ1-42 and Aβ1-40
amyloid fibrils revealed the presence of two β-sheets between residues 18-26 and 31-42 joined by a single β-turn. The β-sheets in the model for Aβ1-42 are stabilized by side chain interactions between residues F19 and G38, A21 and V36, and a salt bridge between D23 and K28, which are thought to contribute the peptides greater core stability and aggregations propensity in comparison to Aβ1-40. A structural model for Aβ1-40, based on ssNMR experimental constraints, further reveals that through intermolecular hydrogen bonding, parallel β-sheets are created from the β-strands formed between residues 12-24 and 30-40 (Figure 1.4).

**Figure 1.4.** (a) 3D structure of the two β-sheets and β-turn formed in the Aβ1-40 fibril and (b) atomic representation of a single Aβ1-40 molecule viewed down the long axis of the fibril. Figure modified from Tycko et al. [128, 137].
Although the first 10 residues remain predominantly disordered, the two β-sheets align via sidechain-sidechain interactions, facilitated from a bend in the peptide backbone arising from residues 25-29\(^{(136, 137)}\). Further salt bridge formation occurs between the D23 and K28 residues\(^{(136, 137)}\). Disulfide cross-linking experiments identified specific inter- and intramolecular atomic interactions (referred to as internal and external quaternary contacts) between specific residues in Aβ1-40\(^{(138)}\). These residue contact points are critical in developing an understanding of the structural hierarchy of amyloid fibrils.

1.4 Mechanisms for amyloid fibril self-assembly

Despite many years of research, the progression of amyloid-related diseases remains poorly understood. Currently, there are no effective in vivo treatments that reduce or inhibit the aggregation of these amyloid-forming proteins, and in order to develop strategies to combat disease progression, a more thorough understanding of the intricate mechanisms involved in amyloid fibril self-assembly is required. Predicting the mechanisms of amyloid aggregation, however, has proven challenging despite numerous models being proposed for similar protein systems. A number of studies have identified proteins unrelated to disease state that aggregate into amyloid-like fibrils\(^{(139-145)}\). Researchers suggest, in fact, that virtually any protein has the potential, under appropriate conditions, to form an amyloid fibril\(^{(143, 144, 146)}\). Furthermore, in spite of structural differences between these proteins they all demonstrate the ability to form a common core cross-β-sheet structure\(^{(106)}\) suggesting that these proteins may also share a common pathway towards amyloid fibrilization\(^{(147, 148)}\).
1.4.1 Nucleation-dependent polymerization model

The nucleation-dependent polymerization (NP) model was the first model used to explain the results of in vitro amyloid kinetic experiments, and has since been found to appropriately describe the protein aggregation for a diverse assortment of structure forming processes including microtubule formation\(^{149}\), flagellum formation\(^{150}\) and amyloid fibril formation\(^{151, 152}\). This model describes the stabilization of a number of protein monomers (through a series of otherwise unfavourable protein-protein association equilibria), into an intermediate nucleus, the spatial confinement resulting in a significant local increase in concentrations allowing them to polymerize into the very stable higher ordered fibrillar structures\(^{153, 154}\) (Figure 1.5).

![Figure 1.5. Schematic outlining the nucleation-dependent polymerization model of amyloid self-assembly.](image)
The lag phase is initiated through a series of thermodynamically unfavourable steps involving the association of several monomers into a nucleus. This stage is commonly observed during fibril self-assembly and is particularly sensitive to factors such as temperature, pH, ionic concentration and oxidation potential\textsuperscript{[127, 155-158]}, but more importantly, protein concentration and nucleus size\textsuperscript{[159-161]}. The main consensus behind the formation of an ordered nucleus is that this is the rate-limiting step in fibrillogenesis and that polymerization will not occur until the monomer concentration exceeds the critical protein concentration\textsuperscript{[153, 160]}. In terms of fibrillogenesis, higher monomer concentrations lead to a greater probability of the nuclei formation required to spontaneously initiate fibril assembly\textsuperscript{[160]}.

In regards to AD, the Aβ1-42 peptide has a lower critical protein concentration than Aβ1-40, which was reflected in a four to fivefold reduction in the presence of soluble monomer in fibrilized solutions of Aβ1-42 in comparison to Aβ1-40\textsuperscript{[162]}. This finding is consistent with the aggregation behaviour of Aβ1-42 that forms fibrils more rapidly than Aβ1-40\textsuperscript{[163, 164]}. Only after the critical protein concentration is achieved can the equilibrium shift towards the favourable growth and elongation of fibrils via monomer addition. Through this seeded growth, pre-fibrillar aggregates and other oligomeric intermediates are created and recruited to assemble exponentially into higher ordered filaments, protofilaments and matured fibrils\textsuperscript{[165, 166]}.
1.4.1.1 Amyloid seeding

In the event of slow nucleation, the introduction of a seed or pre-formed fibril can drastically accelerate the fibrilization process\(^{(77, 152, 154, 160, 167, 168)}\). This seeding process significantly reduces, or in some cases eliminates the lag phase time, by providing a self-assembling template for fibrilization\(^{(160)}\). Upon reduction of this lag phase period, seeding experiments have been observed to immediately induce fibril formation via a first-order growth profile as described by Harper and Lansbury\(^{(160)}\) (Figure 1.6).

![Figure 1.6](image)

**Figure 1.6.** Addition of a fibril seed eliminates the lag phase associated with self-assembly and accelerates fibril formation (figure adapted from Ref.\(^{(160)}\)).

Seeding to induce fibril formation is not a chemically discriminating event, as described by Ono *et al.*\(^{(169)}\). In this recent work, strong evidence suggests fibrils and oligomers of Aβ1-40, Aβ1-42 and α-synuclein can act as seeds and affect the aggregation pathways within and among species *in vitro*. 

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1.4.2 Alternative models for amyloid self-assembly

Although amyloid self-assembly is often associated with the nucleation-dependent process, the addition of fibril seeds do not always result in fibril formation, suggesting an alternative pathway for some oligomeric species and other higher ordered amyloid assemblies\(^\text{170}\). Recent studies have suggested that amyloid fibril formation is a more dynamic and reversible process than initially anticipated\(^\text{133, 171}\) and that a simple nucleated-polymerisation model does not adequately describe all aspects of amyloid fibril formation\(^\text{172}\). One such model describes the self-assembly of transthyretin, under partially denatured conditions, to be consistent with a non-nucleated process, since fibrilization was not accelerated by seeding which is commonly observed in the nucleated polymerization model\(^\text{173}\). Additionally, the authors postulate that under these conditions, transthyretin self-assembly follows a non-nucleated polymerization model where the highest energy species is the native monomer and the nucleus is not the rate-limiting step. Comprehensive atomic force microscopy (AFM) analysis of \(\beta_2\)-microglobulin fibrils by Gosal \textit{et al.}\(^\text{174}\) demonstrate the formation of flexible worm-like amyloid fibrils which are thought to be driven via a rapid non-nucleated linear assembly mechanism. The nature of these fibrils were distinctly different to traditional long-straight fibrils associated with classical amyloid self-assembly and suggests the existence of two distinct competitive pathways for amyloid fibril assembly.

\textit{Serio et al.}\(^\text{175}\) proposed a new model for the transformation of soluble precursors into amyloid fibrils in the yeast prion Sup 35. This new model describing the formation of
an additional kinetic pathway \textit{via} the clustering of oligomeric structures was termed the nucleated conformational conversion (NCC) model, and has proven more relevant in describing fibrillogenesis in many protein systems\cite{159}. NCC requires a structural conversion of a protein monomer once added to the growing fibril, as the fundamental mechanism of fibril elongation. Esler \textit{et al.}\cite{176} also demonstrated this through a ‘dock’ and ‘lock’ mechanism, whereby after the formation of a nucleus, the addition of structurally converted monomers becomes irreversible and fibril elongation proceeds.

In addition to the NCC model, other models such as the monomer-derived conversion (MDC) model and template-assisted (TA) conversion model have been proposed\cite{159,177,178}. These models similarly describe the formation of a critical intermediate state, which, when bound to a molecular chaperone, is able to heterodimerize and act as a template to assist in further conversion and fibril growth\cite{179}. Cohen \textit{et al.}\cite{180} proposed that a fibril-catalyzed secondary nucleation mechanism was responsible for the proliferation of A\beta1-42 aggregates. This was facilitated by a positive feedback loop between the monomeric and fibrillar forms of the peptide. Additionally, a new reversible model for apolipoprotein (apoC-II) amyloid fibril formation was proposed by Binger \textit{et al.}\cite{181,182} which involves the reversible nucleation and elongation of fibrils coupled with fibril breaking and end-to-end joining. Clearly with such a diverse range of processes involved, it becomes challenging to identify a single unifying model which best describes amyloid fibrilization, and it may be, based on current understandings, that several models are needed to describe specific cases.
1.5 Early events in amyloidosis – Toxic amyloid species

Detecting the early events in amyloid fibril formation has proven to be a difficult task due to the transient nature of these species and the large number of possible mechanistic intermediates as described in reviews by Haass and Selkoe(183), Jan et al.(184) and Benilova et al.(185). However, recent advances in technology have identified more than a dozen various intermediate states, ranging from monomer to dodecamers, spheres to protofibrils and crystalline to amorphous assemblies, which have resulted from increased knowledge of sigmoidal kinetic models and growth profiles(184, 185).

There has long been debate on whether the fibrillar amyloid forms or soluble Aβ oligomers are responsible for initiating the toxicity that causes the loss of synaptic function and dementia in AD(48, 50, 186, 187). During early in vitro studies, it was believed that Aβ-induced neurotoxicity was the property of the amyloid fibril, and that neurotoxicity was only detected in Aβ peptides that adopted this fibrillar aggregated state(188-191). Pre-dissolved non-toxic Aβ monomers were converted to fibrillar aggregates after a few days incubation in buffered conditions; these aggregates then displayed various levels of toxicity, which was consistent with the toxic Aβ fibril hypothesis at that time(192). However, studies following this have argued that Aβ protofibrillar structures and oligomers, rather than the fibrils themselves, are the toxic species(193-196). This was highlighted in human and transgenic mouse models that displayed cognitive decline before the deposition of amyloid, correlating with changes in soluble Aβ oligomer levels rather than APP levels or fibrillar amyloid deposits(197).
1.5.1 Small oligomeric aggregates

Several pre-fibrillar aggregated states have been identified in amyloidogenic proteins. These so-called ‘soluble oligomers’ which precede Aβ aggregation and are believed by many to be the causative agents of Aβ toxicity\(^{198-202}\) include various low-molecular weight species ranging in length from dimers through to dodecamers, and high-molecular weight supramolecular structures such as protofibrils, ADDLs (Aβ-derived diffusible ligands), and spherical and annular species\(^{184}\). Like amyloid fibrils, Aβ oligomers also share a common anti-parallel β-sheet structure, which may relate to their toxicity in the pathogenesis of AD\(^{64, 65, 203}\). In vivo injections of Aβ oligomers, in the absence of monomers and fibrils, showed a marked inhibition in hippocampal long-term potentiation and disrupted synaptic plasticity in rat cerebella\(^{204}\). Remarkably, these toxic oligomers further demonstrated a 10-fold inhibition in neuronal viability when neuronal cells were treated with Aβ1-42 oligomers as opposed Aβ1-42 to fibrils\(^{205}\). Aβ dimers have displayed high levels of neurotoxicity; rat hippocampal neuron glia cells were destroyed after treatment with Aβ1-40 and Aβ1-42 dimers\(^{206}\). A further disruption in cognitive function was observed in male Sprague-Dawley rats after being treated with soluble amyloid oligomers. This study identified that both dimers and trimers were necessary and sufficient to rapidly impair learned behaviour without inducing permanent neurological deficits\(^{207}\).
1.5.2 Large protofibrillar assemblies

Aβ protofibrils were first described by Harper et al.\textsuperscript{(164,194)} and Walsh et al.\textsuperscript{(163)}. These metastable structures were proposed to be on-pathway to amyloid fibrilization due to their rich β-sheet content. Other proteins unrelated to Aβ can also form these protofibrillar aggregates and increase neurotoxicity in cell cultures\textsuperscript{(147)}, suggesting that the toxicity of protofibrils is related to their bulk structure rather than the precise chemical nature of their amino acid sequence\textsuperscript{(208)}. Protofibrils share similar structural characteristics to amyloid fibrils and have demonstrated adverse effects on the normal metabolism of cultured neurons\textsuperscript{(209)}. In fact, their presence has led to dramatic changes in the electrical activity of cortical neurons, causing sharp increases in excitatory postsynaptic potentials, action potentials and membrane depolarizations that resulted in progressive neuronal loss\textsuperscript{(194)}. A marked reduction in cell viability was observed in cells treated with Aβ protofibrils, rather than monomeric or polymerized Aβ\textsuperscript{(210)}. The Arctic mutant Aβ1-40 further showed variations in both the morphology and size distribution of Aβ protofibrils, forming short filaments, large spherical particles and annular pore-like protofibrils which have potential neurotoxic implications in AD\textsuperscript{(211)}.

Oligomer cytotoxicity may even be related to the formation of membrane pores and ion channels by amyloidogenic proteins\textsuperscript{(212-214)}. Under conditions that do not favour fibril production, nanomolar additions of small diffusible Aβ1-42 oligomers (ADDLs) actively bind to cell membranes and cause cytotoxicity\textsuperscript{(195)}. These ADDLs also displayed catastrophic effects on matured neurons in organotypic central nervous system cultures\textsuperscript{(195)}. ADDL-like oligomeric assemblies have been isolated from
post-mortem AD brains and their presence correlated strongly with memory loss. A non-fibrillar and neurotoxic form of Aβ was also identified when Aβ1-42 was incubated with clusterin, a multifunctional apolipoprotein, leading to inhibition of Aβ aggregation according to neurotoxicity assays. With the multitude of different types of oligomers, it becomes difficult to identify which species are pathologically relevant in human diseases since many have overlapping properties and may form from different mechanistic pathways that are yet to be understood. Indeed, a continuous dynamic exchange between these different species may be relevant to their toxic implications in disease pathogenesis.

1.5.3 Critical structural conversions

Under biological conditions, most native state proteins must unfold or partially unfold before fibrillization can occur. This often involves the conversion of a soluble protein monomer into insoluble amyloid through the production of an unfolded intermediate species. In many cases, these native state intermediates transition from a random coil or α-helical conformation into ordered β-sheet and β-strand conformations, which initiate fibril elongation. There is strong evidence to suggest that helix-to-strand transitions play a prominent role in fibril self-assembly and neurotoxicity. The helical transition of Aβ1-42 has been implicated in the formation of membrane channels that allow the penetration of certain metal ions causing neuronal death.
In Aβ, the helix-to-strand transition is thought to be initiated in the N-terminal region, according to NMR studies and molecular dynamic simulations of Aβ1-28\(^ {122, 225}\). The formation of transient helical-containing intermediates were initially identified by Harper et al.\(^ {164}\) and later by Walsh et al.\(^ {163, 209}\) who studied the conformational changes in Aβ protofibrils and fibrils. Comprehensive secondary structural analysis of 18 Aβ isoforms all demonstrated a common oligomeric helical intermediate\(^ {226}\). These partially folded intermediates have an increased propensity for fibril formation and have been accepted as critical species in the formation of many amyloid fibrils\(^ {221, 227-229}\). However, their detection has proven to be a difficult task due to their short-lived nature and relative speed at which the monomers unfold and aggregate.

Once these helical-intermediates became recognized in the pathogenesis of disease, researchers focused their attention on modifying the helical propensity of these amyloidogenic proteins through the use of co-solvents such as trifluoroethanol (TFE) and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). These fluorinated solvents can create apolar microenvironments that mimic the lipid phase of membranes\(^ {230}\). In the case of the Aβ peptide, Aβ1-28, Aβ1-39 and Aβ1-42 isoforms all showed a preferred α-helical conformation in the presence of >40% TFE concentrations\(^ {121}\). Fezoui and Teplow\(^ {220}\) further confirmed through comprehensive circular dichroism analysis, that a preferred helical structure was adopted for Aβ1-40 and Aβ1-42 at 40% and 60% TFE concentrations respectively. The authors described this helical conversion as a necessary step towards decreasing the rate of fibril elongation and completely
suppressing fibrilization. Other studies have also suggested the β-sheet to α-helix transition is reversible by simply changing the composition of water and HFIP\textsuperscript{(231)}.

Several solution state NMR experiments have also revealed that Aβ1-40 and Aβ1-42 adopt a high propensity of helical structure in the presence of fluorinated alcohols\textsuperscript{(232-234)}. The identification of two helical regions in Aβ1-42 encompassing residues 8-25 and 28-38\textsuperscript{(235)}, present a possible solution model for the analysis of conformational transitions in Aβ, since these residues have previously been shown to form β-strands in the fibrilized state\textsuperscript{(132)}.

1.6 Ionic liquids – Solvents of the future

Ionic liquids (ILs) are a particular class of solvent comprised entirely of ions. These liquids are generally described as ‘fused’ or ‘molten’ salts with melting points below 100°C\textsuperscript{(236)}. Although the first synthesis of an IL, ethanolammonium nitrate, was reported in 1888 by Gabriel and Weiner\textsuperscript{(237)}, it was not until the last three decades when the application of ILs became more than just an extension to the chemical solvent library. The enormous range of anion and cation combinations renders these solvents very unique and their properties can be fine-tuned to suit almost any application. Perhaps the most notable of these IL characteristics include their excellent chemical and thermal stability, non-flammability, non-volatility and high ionic conductivity\textsuperscript{(236, 238)}. ILs have found increasing utility in a vast range of applications from solvent systems for synthesis and heterogeneous and homogeneous catalysis reactions\textsuperscript{(238-241)} to electrochemistry\textsuperscript{(242-246)}. 
ILs are divided into two main categories: aprotic and protic ionic liquids (PILs). Aprotic ILs are formed from the ionization of the parent base by accepting any group other than a proton to its basic site\textsuperscript{(247)}. Alternatively, PILs are traditionally formed by the stoichiometric neutralization of a Brønsted acid (HA) with a Brønsted base (B) according to equation 1\textsuperscript{(247)}:

\[ \text{HA} + \text{B} \rightleftharpoons \text{BH}^+ + \text{A}^- \quad (1) \]

Due to the presence of this mobile proton, PILs generally display Brønsted acidity depending on the acidity of the precursor acid. The key property that sets PILs apart from other types of ILs is their degree of ionization; the proton transfer between acid and base, which can be controlled by careful selection of ion pairs. This property provides proton donor and acceptor sites within the PIL complex that can be used to alter the hydrogen bonding network of various systems. The degree of proton transfer in a PIL can be further estimated according to the difference between the $pK_{\text{a,aq}}$ values of acid and base, as discussed by Yoshizawa \textit{et al.}\textsuperscript{(247)} The authors here postulate that for complete proton transfer to occur, a $\Delta pK_{\text{a,aq}} > 10$ is imperative. Ideally, to achieve a strongly ionizable PIL (large $\Delta pK_{\text{a,aq}}$ value), combinations of a strong acid and base are required to shift the equilibrium in favour of ionic products; however this does not always occur. Alternatively, the combination of a weak acid and base pair (small $\Delta pK_{\text{a,aq}}$ value) results in incomplete proton transfer and can lead to the formation of molecular species, ion pairs or other higher ordered aggregated products\textsuperscript{(248-250)}. 


The first reported synthesis of a room-temperature PIL was by Walden\(^{(251)}\) in 1914. Ethylammonium nitrate (EAN), with a measured melting point of 12.5°C\(^{(252)}\), became the main focus in PIL studies with researchers demonstrating its unique water-like properties\(^{(253-255)}\) and its ability to form a three-dimensional hydrogen-bonded network with its surrounding environment\(^{(256)}\). These properties encourage the use of EAN and other inspired ILs as increasingly popular ‘green’ replacement solvents for various protein studies encompassing protein dissolution, stabilization, denaturation and refolding\(^{(257, 258)}\).

Furthermore, knowledge on protein structure and function relationships in the presence of ILs became essential in understanding the mechanisms behind protein folding when compared with traditional solvent systems such as urea or guanidine hydrochloride. One such example includes recent work by Baker and Heller\(^{(259)}\) who used a combination of small-angle scattering and spectroscopic techniques to measure protein denaturation in cytochrome c and human serum albumin (HSA) in the water-miscible IL, 1-butyl-3-methylimidazolium chloride (BMIMCl). Both proteins were found to denature in high concentrations of this IL (50 vol.%) and adopted a state that was consistent to that observed in the chaotropic agents urea and guanidine hydrochloride\(^{(260)}\).

**1.6.1 Ionic liquids as solvents for protein studies**

**1.6.1.1 Thermal stability and protein refolding**

One of the major issues contributing to a proteins’ inability to correctly refold after being exposed to high temperatures is disordered, irreversible aggregation as a result
of the exposure of surface hydrophobic residues\textsuperscript{(261)}. Therefore, stabilizing agents are generally employed to reduce or prevent aggregation and (partially) restore the proteins activity and native structure\textsuperscript{(262, 263)}. Many proteins dissolved in aqueous solutions become denatured at higher temperatures due to disruptions in hydrogen bonding and hydrophobic interactions, and thus attention has been shifted towards ionic liquids with little to no water content to avoid unwanted side reactions, hydrolysis and substrate solubility\textsuperscript{(261, 264)}.

Perhaps the highlight in ionic liquid-mediated protein studies was described by Summers and Flowers\textsuperscript{(265, 266)} who successfully used EAN as an additive for the refolding of hen egg white lysozyme (HEWL). The authors demonstrated that when HEWL was denatured and reduced in neat EAN (containing negligible water), more than 90\% of the active protein was recovered after dilution. Additionally, when HEWL was denatured and reduced according to conventional procedures, followed by renaturation in EAN, 75\% of the lysozyme activity was restored\textsuperscript{(265)}. It was believed that HEWL aggregation was prevented by electrostatic repulsion induced by the cationic groups adsorbed on the exposed hydrophobic domains of the protein.

The Angell group\textsuperscript{(267)} also observed similar trends in the reversible thermal unfolding/refolding of lysozyme in EAN. In this paper, differential scanning calorimetry (DSC) experiments showed that lysozyme was able to fold and unfold over several denaturation cycles up to 100\degree C. The PIL itself was found to protect against aggregation, with \textasciitilde97\% retention in refoldable protein sample per denaturation cycle, as opposed to aqueous buffered samples where a 70\% loss of protein was measured.
after just one cycle. The activity of the N-H proton, according to NMR spectroscopy, was confirmed as a principal factor in controlling protein unfolding\textsuperscript{(268)}. Selectively tuning the “effective acidity” of the PIL was found to induce maximal protein refolding in HEWL between the N-H chemical shifts of 7 and 9 ppm, with any N-H chemical shift outside this range leading to irreversible enzyme folding.

Protein thermal stability however, is not limited to EAN alone, with researchers showing that simple hydroxylation of the ionic liquid cation alkyl chain is also effective as a refolding additive\textsuperscript{(269)}. In a study by Lange \textit{et al.}\textsuperscript{(269)}, maximum refolding yields for HEWL were higher for hydroxylated methylimidazolium salts than those carrying longer hydrophobic alkyl chains. This was also noted by Atkin \textit{et al.}\textsuperscript{(270)} who found that alkyl hydroxylated ethanolammonium formate (EtAF) demonstrated substantial HEWL stabilization against unfolding at high temperatures. The authors speculated that this enhanced stability was the result of extra hydrogen bond donor/acceptor sites on the hydroxylated cation, which impede with the hydrophobic interactions on the protein. In terms of enzyme kinetics, HEWL showed a substantial 6-fold increase in protein activity with EtAF present in comparison to buffered conditions, further emphasizing the role of these hydrogen bond donor/acceptor sites in protein stability\textsuperscript{(270)}.

According to DSC experiments in recent work by Fujita \textit{et al.}\textsuperscript{(271)}, the thermal stability of cytochrome c was dramatically increased in the presence of the aprotic choline dihydrogen phosphate (choline dhp) due to the absence of a denaturing peak in the DSC trace. The structural stability of cytochrome c was further emphasized by
temperature-induced FTIR spectroscopy, where it was found that only solutions containing choline dhp showed no changes in protein structure during exposure to temperatures of 110°C. Furthermore, cytochrome c structure was maintained and undisrupted after returning to room temperature. The authors highlight the importance of the ion pairs in choline dhp, which they describe as an “excellent combination of chaotropic cation and kosmotropic anion”, as it provides additional hydrogen bonding and acceptor sites as well as a similar proton activity to water, which actively contribute to its successful long term structural stabilization of cytochrome c.

1.6.1.2 Dissolution and inhibition of amyloid formation

The complex nature of amyloid fibrilization and the formation of toxic intermediate species has led to a number of investigations surrounding the control of aggregation and prevention of amyloid fibril formation. Although most of these studies utilize conventional solvents such as TFE and HFIP, a few studies describe the use of ILs as media for the dissolution and inhibition of amyloid fibrils. Byrne and Angell described the first reported case of a PIL as a solvent for amyloid fibril dissolution. In one particular method, the authors used additions of ethanol to fibrilize HEWL and then redissolved the fibrils in various PILs, discovering that 72% of enzyme bioactivity was restored when redissolved in EAN. This was a remarkable finding since non-fibrilized HEWL solutions in the same solvent showed 80% enzymatic bioactivity. Shortly after this, Kalhor et al. published similar work describing the application of several tetramethylguanidinium-based PILs as PIL-mediated amyloid inhibitors. Among the library of PILs synthesized, the authors
found that additions of tetramethylguanidinium acetate (TMGAc) inhibited the formation of HEWL amyloid fibrils \textit{in vitro} by nearly 50\% by reducing the number of β-sheet rich protofibrils required for self-assembly. This was confirmed by ThT binding assays and TEM, revealing a significant decrease in ThT intensity correlated with the formation of amyloid fibrils. Furthermore, the presence of intermediate species observed by native gel electrophoresis suggested that the PIL was selectively interacting with HEWL intermediates and trapping them into more stable conformations via cation-π interactions\textsuperscript{(275)}.

1.6.1.3 Enhancement of amyloid fibril formation

The relatively slow process of amyloid fibrilization in some proteins is a major setback in terms of developing drugs to regulate amyloid formation, due to the difficulty in developing rapid high throughput screening assays. Although strategies have evolved to accelerate fibrilization including acidic pH, increased temperature or directly modifying the proteins primary structure, ionic liquids have also found application in this field. For example, the fibrilization of HEWL in hydrated ammonium bisulfate showed enhanced amyloid fibril formation with predominant β-sheet secondary structure according to CD analysis, when heated to \(\sim 60^\circ\text{C}\)\textsuperscript{(276)}; a process which would take several weeks to occur if held at room temperature. Kim and co-workers\textsuperscript{(277-279)} also described that several ILs containing the methylimidazolium cation, in particular 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide (BIMbF\textsubscript{3}Im), could accelerate amyloid formation in α-synuclein, as depicted by an increase in ThT fluorescence intensity. The authors propose that the exposure of the hydrophobic
protein core facilitated by the increased interactions between solvent side chains, lead to extensive hydrogen bonding networks within the peptide backbone forming various conformational states.$^{277}$
1.7 Project Aims

To date, there have been no reported cases on the self-assembly and aggregation of Aβ peptides in ionic liquids. The main scope of this thesis is therefore focused on how PILs can be used to control Aβ self-assembly.

The result chapters are organized as follows:

Chapter 3 discusses the controlled fibrillization of Aβ16-22, the central component of full-length Aβ, in anion-modified PILs. This chapter highlights the necessity of competitive hydrogen bonding between PIL anion and water, and its contribution to the rapid self-assembly of Aβ16-22 amyloid fibrils. Additionally, evidence of early oligomeric assemblies in a C-terminal modified Aβ16-22 analogue incubated in a PIL is described.

Chapter 4 further explores the self-assembly of Aβ16-22, but as a function of cation-modified PILs. This chapter describes how subtle changes to the chemistry of the cation can facilitate amyloid fibril formation in a manner similar to that observed in anions. The structure-inducing effects of these PILs are further investigated on various Aβ isoforms linked to AD pathogenesis, showing that specific peptide-PIL interactions are responsible for direct changes in peptide solution conformations.

On the basis of these observations, Chapter 5 discusses the helical-inducing properties of PILs on the Aβ1-40 peptide. The helical-inducing nature of PILs is confirmed as an essential property for inhibiting amyloid deposition and for dissolving synthetic
pre-formed Aβ1-40 amyloid fibrils. In order to determine the therapeutic relevance of this approach, some commonly studied ILs were tested as potential solvents for the dissolution of naturally occurring brain amyloid plaques.

Chapter 6 describes the impact of ethylammonium nitrate (EAN) on the formation of various polymorphs in a key Aβ fragment, NH2-FF-COOH. The direct changes in hydrophobic assembly as the result of increasing EAN concentration, and its implication on the final supramolecular structure of the peptide are discussed.

Finally, Chapter 7 represents a change in focus and explores the effect of the main PIL solvent parameters such as acidity, basicity, polarity and polarizability, on the solution conformation of a thermo-responsive polymer. This polymer is used to model the events that occur during protein folding in PILs and forms the foundation for future studies investigating the role that PILs play in amyloid self-assembly. This work illustrates that two factors; the relative acidity of the PIL anion and the solvent dipolarity/polarizability ratio, are critical in enabling the PIL to form extensive hydrogen bonds with the polymer. These results provide valuable information on PIL-solute interactions that strongly correlate to findings in Aβ self-assembly studies.
CHAPTER 2: Materials and Methodology

2.1 Materials

Fmoc-protected L-amino acids, 1-hydroxybenzotriazole (HOBt), \(O\)-(benzotriazol-1-yl)-\(N,N,N',N'\)-tetramethyluronium hexafluorophosphate (HBTU), piperidine and \(N,N\)-diisopropylethylamine (DIPEA) were purchased from Auspep (Melbourne, VIC, Australia). Commercially available Wang resin and Fmoc-Glu(OtBu)-Wang resin were purchased from GL Biochem (Shanghai, China). Triethylsilane (TES) trimethylsilane (TMS), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), phenol, ninhydrin, potassium cyanide, pyridine, Thioflavin T (ThT), dimethyl sulfoxide (DMSO), molecular sieves 3Å, sodium phosphate monobasic, sodium phosphate dibasic, Nile Red, 4-nitroaniline, Reichardt’s dye, 8-anilino-1-naphthalene-sulfonic acid and all precursor acids and bases were purchased from Sigma-Aldrich (Castle Hill, VIC, Australia). Dichloromethane (DCM), \(N,N\)-dimethylformamide (DMF) and diethyl ether were purchased from ChemSupply (Port Adelaide, Australia). Acetonitrile, hydrochloric acid and sodium hydroxide pellets were purchased from Ajax Finechem (VIC, Australia). Poly(\(N\)-isopropylacrylamide) (PNIPAM) was purchased from Scientific Polymer (Atlanta, GA, USA). \(N,N\)-diethyl-4-nitroaniline and deuterium oxide (HDO) were purchased from Novachem (South Yarra, VIC, Australia). Polypeptide molecular ladder and mini protean 18% Tris-precast gels were purchased from Bio-Rad Laboratories Pty. Ltd. (Gladesville, VIC, Australia). Protein desalting columns were purchased from Thermo Fisher Scientific (Scoresby, VIC, Australia). Water was
purified and deionised using an in-house Advantage A10 Milli-Q System (Millipore) equipped with a 0.2 \( \mu \)m filter (Millipak®).

Purified A\( \beta \)-1-40, A\( \beta \)-1-42, A\( \beta \)11pE-40, and A\( \beta \)3pE-40 peptides were provided by Professor Colin Barrow (Deakin University) and their syntheses are described elsewhere\(^{(280)}\). The aprotic ionic liquids BMIMAc, BMIMCl and EMIMSO\(_3\) were supplied by Dr Nolene Byrne (Deakin University).

### 2.2 Peptide synthesis

Peptides were synthesised on a 0.2 mmol scale by traditional Fmoc solid-phase chemistry\(^{(281, 282)}\). Solid phase support was either Rink-amide resin [loading 0.68 mmol/g] (0.29 g, 0.2 mmol) or Wang resin [loading 0.34 mmol/g] (0.59 g, 0.2 mmol). Amino acids were synthesized onto the solid-phase from the carboxyl terminus, in reverse sequence order. Freshly activated molecular sieves were added to DMF and allowed to dry for 4 hours. The resin beads (0.2 mmol) were pre-swelled in DMF (3 mL) for 15 min. After draining excess DMF, the resin was deprotected with 5 mL of deprotection solution (20% piperidine in DMF) for 5 min. Resin beads were washed with DMF (2 \( \times \) 3 mL) and DCM (2 \( \times \) 3 mL) before a small portion of beads were removed for Kaiser ninhydrin testing (see section 2.3).

The coupling reagents HBTU (0.360 g, 0.48 mmol) and HOBt (0.136 g, 0.5 mmol) and a 5-fold excess of Fmoc-protected amino acid (1.0 mmol) were mixed with DMF (4 mL) and DIPEA (0.4 mL, 1.2 mmol) for 2 min to pre-activate the amino acid. This solution was transferred to the reaction vessel containing the deprotected resin and
allowed to mix for 20-30 min. The resin beads were drained and washed with DMF (2 × 3 mL) and DCM (2 × 3 mL) and a small portion of beads were removed for Kaiser ninhydrin testing. The resin beads were continuously deprotected and coupled with pre-activated amino acids until the final peptide sequence was synthesized onto the solid support. A final wash with DCM (2 × 3 mL) was completed to remove excess DMF from the resin and the product was dried under vacuum for 2 days.

Cleaving of the peptides from the solid-phase support was achieved by suspending the resin beads in a 5 mL mixture of TFA (95%), TES (3%) and water (2%) (v/v/v) for 3 hours, with occasional stirring. The resin was filtered through a pipette containing glass wool and the supernatant was collected in a centrifuge tube. Additional trifluoroacetic acid (3 × 1 mL) was used to wash the resin. The combined filtrate was dried under a stream of nitrogen gas to remove most of the TFA residue. Icy cold diethyl ether (40 mL) was added to the filtrate and precipitation of the peptide occurred immediately. The aqueous layer was extracted five times after centrifugation at 2,500 rpm for 5 min. In the case of NH₂-FF-COOH, icy cold distilled water (40 mL) was added to the filtrate instead of diethyl ether. Washed peptides were placed in a -80 °C freezer overnight and lyophilized the following day.

White crude products were yielded as follows: NH₂-KLVFFAE-COOH (58 mg, 86.9%), Ms: m/z 853.48 [M+H]⁺, calc. 853.47. Ac-KLVFFAE-COOH (66 mg, 76.1%), Ms: m/z 895.62 [M+H]⁺, calc. 895.49. Ac-KLVFFAE-NH₂ (58 mg, 40.2%), Ms: m/z 894.63 [M+H]⁺, calc. 894.50. NH₂-KLVWFAE-COOH (49.7 mg, 42.1%),
Ms: \( m/z \) 891.10 \([\text{M}+\text{H}]^+\), calc. 891.07. \( \text{NH}_2\text{-FF-COOH} \) (32 mg, 71.9%), Ms: \( m/z \) 312.37 \([\text{M}+\text{H}]^+\), calc. 312.36.

2.3 Kaiser ninhydrin testing

The Kaiser ninhydrin test\(^{(283)}\) is a sensitive technique for the detection of primary amines in solution and is commonly utilized in solid-phase peptide synthesis to determine if coupling reactions are complete. The Kaiser ninhydrin test involves the preparation of three solutions: (1) ninhydrin (2.5 g) was dissolved in ethanol (40 mL), (2) liquefied phenol (40 g) was dissolved in ethanol (10 mL), and (3) KCN (0.001 M, 1 mL) was diluted with pyridine (49 mL) to a final volume of 50 mL. After the deprotection and coupling of each amino acid during peptide synthesis, a small portion of resin beads were transferred into a test tube. 1 mL of each solution described above was added and mixed. The test tube was heated to 80 °C for 15 sec. A positive test, indicating the presence of free amine was represented by a dark blue/violet colour change of the solution and/or resin beads. A negative test was represented by no colour change in the solution and/or resin beads.

2.4 Peptide self-assembly

Peptides were dissolved in HFIP as a pre-treatment to break down aggregated material in the peptide lyophilisates, and prepared to a total stock concentration of 10 mg/mL. After 5 minutes of sonication, peptides were completely dissolved and present in monomeric form. An aliquot of stock solution was transferred to a vial where \( \text{N}_2 \) was purged into the vessel to eliminate residual HFIP solvent until a dried film was
produced. Films were then re-dissolved in MilliQ water and diluted to the appropriate concentration before being transferred to PBS or PIL solution. Fresh stock solutions were made prior to each self-assembly to eliminate the formation of aggregates.

2.5 Preparation of pre-formed Aβ1-40 fibrils for dissolution studies

2.5.1 Method 1 – pre-formed TeaMs fibrils

Aβ1-40 lyophilisates were pre-treated in HFIP as described above before the addition of 1 mL aqueous 10 wt% TeaMs solution. Fibril self-assembly was initiated within 10 min and allowed to further self-assemble for 1 hour. After 1 hour, the peptide solution was centrifuged at 10,000 rpm for 10 min and the supernatant was extracted. Fibrils were washed with 3 x 1 mL MilliQ water to remove residual PIL solution before being lyophilized. Self-assembled lyophilisates were weighed and 0.2 mg was transferred to a vial containing neat TeaMs solution for analysis.

2.5.2 Method 2 – preformed PBS fibrils

Aβ1-40 lyophilisates were pre-treated in HFIP as described above before the addition of 1 mL aqueous PBS solution. Fibrils were allowed to slowly self-assemble over 2 weeks. The peptide solution was centrifuged at 10,000 rpm for 10 min and the supernatant was extracted before the fibrils were lyophilized. Self-assembled lyophilisates were weighed and 0.2 mg was transferred to a vial containing neat TeaMs solution for analysis.
2.6 Protic ionic liquid synthesis

**Butylammonium lactate (BaLa).** Butylamine (15.0 g, 0.2051 mol) was added dropwise to lactic acid (21.73 g, 0.2051 mol). The mixture was stirred together in an ice bath for 2 hours, after which a clear viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent (yield; 34.2 g, 93.1 %).

\[
1^1\text{H NMR (500 MHz; HDO)} \delta (ppm) 0.91-0.96 (t, J 7.3, 3H), 1.36-1.42 (sextet, J 6.8, 3H), 1.49-1.52 (t, J 7, 2H), 1.65-1.71 (quint., J 7.2, 2H), 2.94-2.98 (t, J 7.5, 2H), 4.07-4.11 (q, J 6.5, 1H), 8.04 (s, 3H). \]

ESI-TOF-MS: calculated for C_{7}H_{17}O_{3}N [M]^+: Ms: \textit{m/z} 163.23 [M+H]^+, calc. 163.25.

**Choline dihydrogen phosphate (choline dhp).**

Choline hydroxide (40.0 g, 0.1485 mol) dissolved in methanol was added dropwise to phosphoric acid (14.55 g, 0.1485 mol). The mixture was stirred together in an ice bath for 2 hours, after which a clear viscous liquid remained. The neutralized product was placed under rotary evaporation to remove excess methanol from the starting material. The product crystalized into a white solid when transferred to reduced pressure 1 hour (yield; 53.1 g, 97.3 %).

\[
1^1\text{H NMR (500 MHz; HDO)} \delta (ppm) 3.19 (s, 9H), 3.49-3.51 (t, J 5, 2H), 4.03-4.06 (t, J 5, 2H). \]

ESI-TOF-MS: calculated for C_{7}H_{17}O_{3}N [M]^+: Ms: \textit{m/z} 201.17 [M+H]^+, calc. 201.19.

**Choline lactate (ChoLa).** Choline hydroxide (40.00 g, 0.1485 mol) dissolved in methanol was added dropwise to lactic acid (15.74 g, 0.1485 mol). The mixture was stirred together in an ice bath for 2 hours, after which a clear viscous liquid remained. The neutralized
product was placed under rotary evaporation to remove excess methanol from the starting material, then placed under reduced pressure for 1 hour to remove residual solvent (yield; 53.6 g, 96.2 %). $^1$H NMR (500 MHz; HDO) δ (ppm) 1.30-1.32 (d, J 7, 3H), 3.19 (s, 9H), 3.49-3.51 (t, J 5, 2H), 4.03-4.11 (m, 3H). Ms: $m/z$ 193.22 [M+H]$^+$, calc. 193.20.

**Choline malate (ChoMa).** Choline hydroxide (44.16 g, 0.164 mol) was added dropwise to malic acid (21.95 g, 0.164 mol). The mixture was stirred together in an ice bath for 2 hours, after which a yellow liquid remained. The neutralized product was placed under rotary evaporation to remove excess methanol from the starting material, then placed under reduced pressure for 1 hour to remove residual solvent. The product crystalized into a yellow solid after 1 hour at room temperature (yield; 59.4 g, 89.9 %). $^1$H NMR (500 MHz; DMSO) δ (ppm) 2.29-2.32 (dd, J 15.5, 11, 1H), 2.48-2.53 (dd, J 16, 3.5, 1H), 3.12 (s, 9H), 3.40-3.42 (t, J 5, 2H), 3.83-3.86 (m, 3H). Ms: $m/z$ 197.25 [M+H]$^+$, calc. 197.20.

**Diethylammonium lactate (DeaLa).** Diethylamine (15.0 g, 0.2051 mol) was added dropwise to lactic acid (21.73 g, 0.2051 mol). The mixture was stirred together in an ice bath for 2 hours, after which a yellow viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent (yield; 34.8 g, 94.7 %). $^1$H NMR (500 MHz; HDO) δ (ppm) 1.24-1.27 (t, J 7.5, 6H), 1.31-1.33 (d, J 7, 3H), 3.03-3.07 (q, J 7.4, 4H), 4.09-4.14 (q, J 7, 1H). Ms: $m/z$ 163.19 [M+H]$^+$, calc. 163.18.
Diethylammonium mesylate (DeaMs). Diethylamine (15.0 g, 0.2051 mol) was added dropwise to methanesulfonic acid (19.72 g, 0.2051 mol). The mixture was stirred together in an ice bath for 2 hours, after which a yellow viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent. The product crystalized into a yellow solid after 1 hour at room temperature (yield; 31.8 g, 91.6 %).

\[ ^1H \text{ NMR (500 MHz; DMSO)} \delta \text{ (ppm) 1.16-1.9 (t, J 7.3, 6H), 2.42 (s, 3H), 2.89-2.93 (q, J 6.5, 4H), 8.38 (s, 2H). Ms: m/z 137.20 [M+H]^+, calc. 137.16.} \]

Ethanolammonium lactate (ETALa). Ethanolamine (15.0 g, 0.2456 mol) was added dropwise to lactic acid (26.03 g, 0.2456 mol). The mixture was stirred together in an ice bath for 2 hours, after which a yellow viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent (yield; 38.9 g, 94.8 %).

\[ ^1H \text{ NMR (500 MHz; HDO)} \delta \text{ (ppm) 1.30-1.31 (d, J 7, 3H), 3.11-3.13 (t, J 5.3, 2H), 3.79-3.81 (t, J 5.3, 2H), 4.07-4.11 (q, J 7, 1H), 8.15 (s, 3H). Ms: m/z 135.15 [M+H]^+, calc. 135.14.} \]

Ethanolammonium malate (ETAMa). Ethanolamine (10.0 g, 0.164 mol) was added dropwise to malic acid (21.95 g, 0.164 mol). The mixture was stirred together in an ice bath for 2 hours, after which a yellow liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent. The product crystalized into a yellow solid after 1 hour at room temperature (yield; 26.4 g, 82.6 %).

\[ ^1H \text{ NMR (500 MHz; DMSO)} \delta \text{ (ppm) 4.07-4.11 (q, J 7, 1H), 8.15 (s, 3H). Ms: m/z 127.14 [M+H]^+, calc. 127.13.} \]
2.27-2.31 (dd, J 15.4, 7.3, 1H), 2.53-2.58 (dd, J 15.4, 5.9, 1H), 2.85-2.87 (t, J 5, 2H), 3.57-3.59 (t, J 5.3, 2H), 3.96-3.99 (t, J 6.5, 1H). Ms: \( m/z \) 195.17 [M+H]+, calc. 195.14.

**Ethylammonium lactate (EaLa).** Ethylamine (15.0 g, 0.3377 mol) was added dropwise to lactic acid (35.26 g, 0.3377 mol). The mixture was stirred together in an ice bath for 2 hours, after which a clear viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent (yield; 44.3 g, 88.1 %). \(^1\)H NMR (500 MHz; HDO) \( \delta \) (ppm) 1.24-1.27 (t, J 7.5, 3H), 1.31-1.33 (d, J 7, 3H), 3.00-3.05 (q, J 7.3, 2H), 4.09-4.14 (q, J 7, 1H). Ms: \( m/z \) 123.13 [M+H]+, calc. 123.14.

**Ethylammonium mesylate (EaMs).** Ethylamine (15.0 g, 0.3327 mol) was added dropwise to methanesulfonic acid (31.65 g, 0.3327 mol). The mixture was stirred together in an ice bath for 2 hours, after which a clear viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent and immediately crystalized into a white solid (yield; 41.8 g, 89.6 %). \(^1\)H NMR (500 MHz; HDO) \( \delta \) (ppm) 1.20-1.23 (t, J 7.3, 3H), 2.75 (s, 3H), 2.97-3.01 (q, J 7.3, 2H). Ms: \( m/z \) 141.23 [M+H]+, calc. 141.22.

**Ethylammonium nitrate (EAN).** Ethylamine (15.0 g, 0.3327 mol) was added dropwise to nitric acid (20.96 g, 0.3327 mol) over a bath containing liquid nitrogen and isopropanol. The mixture was stirred together in an ice bath for 2 hours, after which a clear liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent
Triethylammonium dihydrogen phosphate (TeaH₂PO₄). Triethylamine (15.0 g, 0.1482 mol) was added dropwise to phosphoric acid (14.52 g, 0.1482 mol). The mixture was stirred together in an ice bath for 2 hours, after which a clear viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent and immediately crystalized into a white solid (yield; 24.5 g, 83.0 %). ^1H NMR (500 MHz; DMSO) δ (ppm) 1.16-1.19 (t, J 7.3, 9H), 2.95-3.00 (q, J 7.3, 6H). Ms: m/z 199.20 [M+H]^+, calc. 199.19.

Triethylammonium hydrogen sulfate (TeaHSO₄). Triethylamine (15.0 g, 0.1482 mol) was added dropwise to sulphuric acid (14.54 g, 0.1482 mol). The mixture was stirred together in an ice bath for 2 hours, after which a clear viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent and immediately crystalized into a white solid (yield; 25.1 g, 85.0 %). ^1H NMR (500 MHz; HDO) δ (ppm) 1.26-1.29 (t, J 7.3, 9H), 3.18-3.22 (q, J 7.3, 6H). Ms: m/z 199.29 [M+H]^+, calc. 199.27.

Triethylammonium lactate (TeaLa). Triethylamine (15.0 g, 0.1482 mol) was added dropwise to lactic acid (15.71 g, 0.1482 mol). The mixture was stirred together in an ice bath for 2 hours, after which a clear viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent (yield; 27.4 g, 89.2 %).
CHAPTER 2

1H NMR (500 MHz; HDO) δ (ppm) 1.25-1.29 (t, J 7.3, 9H), 1.35-1.37 (d, J 7, 3H), 3.17-3.21 (q, J 7.3, 6H), 4.19-4.24 (q, J 6.8, 1H). Ms: m/z 191.28 [M+H]+, calc. 191.22.

Triethylammonium mesylate (TeaMs).

Triethylamine (15.0 g, 0.1482 mol) was added dropwise to methanesulfonic acid (14.25 g, 0.1482 mol). The mixture was stirred together in an ice bath for 2 hours, after which a yellow liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent. The product crystallized into a yellow solid after 1 hour at room temperature (yield; 26.8 g, 91.6 %). 1H NMR (500 MHz; HDO) δ (ppm) 1.28-1.31 (t, J 7.3, 9H), 2.80 (s, 3H), 3.19-3.24 (q, J 7.3, 6H). Ms: m/z 183.32 [M+H]+, calc. 183.29.

Triethylammonium tetrafluoroborate (TeaBF₄).

Triethylamine (30.0 g, 0.2965 mol) was added dropwise to tetrafluoroboric acid (54.25 g, 0.2965 mol). The mixture was stirred together in an ice bath for 2 hours, after which a clear viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour where the product crystallized into a white solid after 1 hour at room temperature (yield; 77.3 g, 91.8 %). 1H NMR (500 MHz; DMSO) δ (ppm) 1.34-1.36 (t, J 4.5, 9H), 3.25-3.31 (q, J 9, 6H), 8.99 (s, 1H). Ms: m/z 188.96 [M+H]+, calc. 188.97.

Triethylammonium trifluoroacetate (TeaTfac).

Triethylamine (15.0 g, 0.1482 mol) was added dropwise to trifluoroacetic acid (16.90 g, 0.1482 mol) over a bath containing isopropanol and liquid nitrogen. The mixture was stirred together in an ice bath for 2 hours, after which a yellow liquid remained. The neutralized product was placed
under reduced pressure for 1 hour to remove residual solvent (yield; 24.6 g, 77.1 %).

$^1$H NMR (500 MHz; HDO) $\delta$ (ppm) 1.26-1.29 (t, J 7.5, 9H), 3.17-3.22 (q, J 7.3, 6H).

Ms: $m/z$ 215.18 [M+H]$^+$, calc. 215.17.

**Triethylammonium triflate (TeaTf).**

Triethylamine (15.0 g, 0.1482 mol) was added dropwise to triflic acid (22.70 g, 0.1482 mol) over a bath containing isopropanol and liquid nitrogen. The mixture was stirred together in an ice bath for an additional 2 hours, after which a yellow viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent (yield; 30.4 g, 80.6 %).

$^1$H NMR (500 MHz; HDO) $\delta$ (ppm) 1.27-1.30 (t, J 7.3, 9H), 3.18-3.23 (q, J 7.4, 6H). Ms: $m/z$ 218.15 [M+H]$^+$, calc. 218.16.

**Tetramethylguanidinium lactate (TeaLa).**

1,1,3,3-Tetramethyl-guanidine (15.0 g, 0.1302 mol) was added dropwise to lactic acid (13.8 g, 0.1302 mol). The mixture was stirred together in an ice bath for 2 hours, after which a clear viscous liquid remained. The neutralized product was placed under reduced pressure for 1 hour to remove residual solvent (yield; 26.0 g, 90.3 %).

$^1$H NMR (500 MHz; HDO) $\delta$ (ppm) 1.31-1.32 (d, J 7, 3H), 2.95 (s, 12H), 4.06-4.11 (q, J 6.8, 1H). Ms: $m/z$ 205.24 [M+H]$^+$, calc. 205.22.

### 2.7 Time-of-flight mass spectrometry (TOF-MS)

Mass spectra were collected on a 6210 Mass Spectral Detector TOF mass spectrometer (Agilent Technologies) in positive ion mode. The following instrumental conditions
were used: drying gas, nitrogen (7 L/min, 350 °C); nebuliser gas, nitrogen (16 psi); capillary voltage, 4.0 kV; vaporizer temperature, 350 °C; and cone voltage, 60 V. The instrument was coupled to a 1200 series liquid chromatograph equipped with a binary pump (Agilent Technologies). Chemstation software (Agilent Technologies) controlled the HPLC pump and data acquisition. Samples were prepared in HFIP at a total concentration of 2 mg/mL and directly injected into the mass spectrometer with an injection volume of 20 μL.

2.8 Reverse-phase high performance liquid chromatograph (HPLC)

Chromatographic runs were performed on an 1200 Series HPLC system consisting of a quaternary pump, solvent degasser and autosampler (Agilent Technologies) with separation on a packed column (Zorbax 300SB RP-8e, 150 x 2.1 mm internal diameter, Agilent Technologies), equipped with a pre-column filter (Supelco™ Analytical 1/16", peek, 0.5 μm frit). The mobile phase was composed of [A]: aqueous TFA (0.1% v/v) and [B]: acetonitrile with TFA (0.1% v/v). Sample were prepared in HFIP at a total concentration of 2 mg/mL and directly injected into the HPLC with an injection volume of 5 μL. A flow rate of 0.5 mL/min was used and the column temperature remained constant at 25 °C. A gradient of 10% to 100% solvent [B], from 0-20 min was applied and peak detection was achieved with a photodiode array detector set at 214 nm and 254 nm.
2.9 Thioflavin T (ThT) assay

ThT fluorescence assays were performed on a Varian Cary Eclipse fluorescence spectrophotometer operating in emission mode, using a spectroscopic rectangular quartz cuvette (10 x 10 mm internal diameter, PTFE stopper) (Starna Scientific Ltd.). ThT dye was excited at 440 nm and samples were measured between the wavelengths of 460-600 nm, over three accumulations and from three independent sample sets. Data points were collected at 482 nm at a slow scan speed, with an excitation and emission slit window of 5 mm. Assays were carried out at room temperature, using a peptide and ThT concentration of 2 mg/mL and 0.5 mg/mL respectively. Aqueous PILs with 0.5 mg/mL ThT were used as controls and samples were sealed with a teflon cap to prevent solvent evaporation during data collection.

2.10 Intrinsic tryptophan fluorescence

The fluorescence for a tryptophan-19 substituted Aβ16-22 peptide was measured on a Jasco J-850 CD Spectrophotometer (ATA Scientific) (Taren Point, NSW, Australia) using a rectangular quartz cuvette (10 x 10 mm internal diameter, PTFE stopper) (Starna Scientific Ltd.). The fluorescence excitation wavelength was set at 280 nm and the emission spectra were recorded from 300-400 nm. Measurements were carried out at room temperature, using a constant peptide concentration of 2 mg/mL and samples were prepared in triplicate. Background measurements of aqueous PILs were recorded prior to analysis and showed no increase in fluorescence intensity.
2.11 Congo red staining and birefringence monitoring

A 10 μL aliquot of 5mM peptide solution aged for 1 day was allowed to dry overnight on a glass microscope slide. Congo red staining was performed by the addition of a 10 μL suspension of saturated Congo red and NaCl in 80% ethanol (v/v) solution. Birefringence was determined on an optical microscope, equipped with an Olympus DP71 camera and cross polarizers.

2.12 $^1$H and $^{31}$P nuclear magnetic resonance (NMR) chemical shifts

NMR spectra were recorded on a commercial Bruker AVANCE 500 MHz FT-NMR spectrometer. PIL samples were prepared in HDO and measured at 298 K. Proton and phosphorus chemical shifts were referenced relative to TMS and triphenylphosphine respectively, in the presence of HDO through an internal capillary. Data was processed on Bruker software version 3.1.

2.13 Measurement of spin-lattice ($T_1$) and spin-spin ($T_2$) relaxation times

Spin-lattice or longitudinal ($T_1$) relaxation values were recorded on a commercial Bruker AVANCE 500 MHz FT-NMR spectrometer using the ‘inversion recovery’ pulse sequence described by Farrar and Becker\(^{(284)}\). The inversion recovery pulse sequence (Figure 2.1) and measurement of the $^1$H-$T_1$ signal for a 50 wt% TeaMs solution (Figure 2.2) is illustrated below.
Figure 2.1. Inversion-recovery pulse sequence for measuring $T_1$.

Figure 2.2. Inversion-recovery $^1$H-$T_1$ determination of 50 wt% TeaMs at 500 MHz.

The sequence was repeated at a range of increasing time ($\tau$) values to give the decay rate of the magnetization. A time delay of $5T_1$ was used between measurements to ensure the sample returned to equilibrium without signal saturation. Measurements were collected by cooling solutions from 340 K to 260 K, allowing 20 min for the sample to equilibrate at each temperature interval. The temperature was calibrated within an internal accuracy of ±0.1 K.
Since signal linewidth is inversely proportional to the spin-spin or transverse ($T_2$) relaxation time, linewidths were estimated by measuring the half-height full-width of each $^1$H signal between the temperatures of 340-260 K. These values were recorded in Hz and errors were less than 1 Hz.

### 2.14 Circular dichroism (CD)

Circular dichroism data was obtained on a Jasco J-815 CD Spectrophotometer (ATA Scientific) (Taren Point, NSW, Australia) using a rectangular quartz cuvette (1 x 10 mm internal diameter, PTFE stopper) (Starna Scientific Ltd.). Wavelength scans were collected using a 1.0 nm bandwidth, 2.0 sec averaging time, over an average of 5 accumulations. Samples were measured between the wavelengths of 190-250 nm at a peptide concentration of 100 $\mu$M. Measurements below 190 nm were disregarded due to signal noise and interferences caused by the ionic liquids. Control samples consisting of solvent only were measured prior to each peptide sample and no baseline corrections were attempted. Circular dichroism data was analysed on Spectra Manager Version II (Jasco Analytical Instruments) and corrected using the Savitzky-Golay smoothing function at a convolution width of 9 points. Peptide secondary structure was further quantified by K2D2 online software$^{285}$.

### 2.15 Fourier transform infrared (FTIR) spectroscopy

FTIR measurements were performed on an Alpha FTIR spectrophotometer (Bruker Optik GmbH, Germany) equipped with a deuterated triglycine sulfate (DTGS) detector and a single-reflection diamond attenuated total reflectance (ATR) sampling module
(Platinum ATR QuickSnap™) consisting of 45° angle of incidence and a 2x2 mm² active sensing surface. A background spectrum of a clean ATR surface was individually acquired prior to each sample measurement using the same acquisition parameters. Aqueous PILs prepared in HDO were used as controls. The ATR-FTIR acquisition parameters used in OPUS 6.0 software suite (Bruker) throughout the study included a 4 cm⁻¹ spectral resolution, 128 co-added scans, Blackman-Harris 3-Term apodization, Power Spectrum phase correction and zero-filling factor of 2. Spectral post-processing including smoothing and second derivatization was performed using a 9-point Savitzky-Golay algorithm within 1800-1500 cm⁻¹ spectral range.

2.16 Dynamic light scattering (DLS)

Lower critical solution temperature (LCST) measurements were collected on a Zetasizer Nanoseries (Malvern Instruments) equipped with a thermostated sample chamber. The LCST was measured within a temperature range of 1-70 °C. A 500 μL aliquot of aqueous 0.5% (w/w) PNIPAM was mixed with aqueous PIL and introduced into a disposable cuvette which was capped to eliminate solvent evaporation. Samples were stored on ice prior to analysis and were equilibrated at each temperature interval for 3 min over 64 accumulations per temperature point. The temperature was increased by increments of 0.3 °C.
2.17 Determination of Kamlet-Taft solvatochromic parameters

The Kamlet-Taft solvatochromic parameters\(^{(287-289)}\) were determined by freshly preparing three stock solutions of Reichardt’s dye (1) (20.0 mg, 1.2 mol/L), \(N,N\)-diethyl-4-nitroaniline (2) (7.0 mg, 1.2 mol/L) and 4-nitroaniline (3) (5.0 mg, 1.2 mol/L) in methanol, and sonicated prior to use (Figure 2.3). 10 μL of the appropriate stock solution was transferred to each PIL solution and allowed to evaporate under vacuum. The \(\lambda_{\text{max}}\) of each sample was measured at room temperature on a Cary 300 Bio UV-Visible spectrophotometer, in a quartz cuvette (1 x 10 mm internal diameter, PTFE stopper) (Starna Scientific Ltd.) over the wavelength range of 300-800 nm for Reichardt’s dye and 300-600 nm for 4-nitroaniline and \(N,N\)-diethyl-4-nitroaniline. Reference samples were carried out in water and methanol and compared to values in literature.

![Figure 2.3. Structures of the Kamlet-Taft dyes and Nile Red polarity probe.](image-url)
The dipolarity/polarizability parameter ($\pi^*$) was calculated according to equation 2;

$$\pi^* = \left( \frac{v_{\text{max}(2)}}{1000 \text{ cm}^{-1}} \right) - 27.52 - 3.182 \quad (2)$$

Where $v_{\text{max}(2)}$ is the wavenumber at maximum absorbance for $N,N$-diethyl-4-nitroaniline (2) in the PIL. The hydrogen bond acceptor (HBA) basicity ($\beta$) was determined according to equation 3;

$$\beta = \frac{1.035v_{\text{max}(2)}}{1000 \text{ cm}^{-1}} - \frac{v_{\text{max}(3)}}{1000 \text{ cm}^{-1}} + 2.64/2.8 \quad (3)$$

Where $v_{\text{max}(2)}$ and $v_{\text{max}(3)}$ is the wavenumber at maximum absorbance for $N,N$-diethyl-4-nitroaniline (2) and 4-nitroaniline (3) in the PIL respectively. The molar transition energy ($E_T(30) (290)$) of Reichardt’s dye (1) in various PILs was determined according to equation 4;

$$E_T(30)/(\text{kcal mol}^{-1}) = \frac{28591}{\lambda(1)_{\text{max}}/\text{nm}} \quad (4)$$

Where $\lambda(1)_{\text{max}}$ is the maximum $\pi-\pi^*$ absorption band of Reichardt’s dye (1) in a given solvent. The normalized polarity ($E_T^N(290)$) was calculated according to equation 5;
The hydrogen bond donator (HBD) acidity ($\alpha$) was determined according to equation 6;

$$\alpha = \frac{(E_T(30)/(\text{kcal mol}^{-1}) - 14.6(\pi^* - 0.23) - 30.31)}{16.5}$$  \hspace{1cm} (6)

Random samples were selected for reproducibility of UV-visible measurements and mean deviations for $\alpha$, $\beta$ and $\pi^*$ parameters were calculated to be $\pm 0.02$.

### 2.18 Polarity measurements

A fresh stock solution of Nile Red (4) (1.0 mg, 0.1 mol/L) was prepared in methanol and sonicated prior to use. 10 $\mu$L of the stock solution was transferred to each PIL solution and allowed to evaporate under vacuum for 2 hours. The $\lambda_{\text{max}}$ (absorbance at maximum wavelength) of each PIL solution was measured at room temperature in a quartz cuvette (1 x 10 mm internal diameter, PTFE stopper; Starna Scientific Ltd.) by UV-visible spectrophotometry over the wavelength range of 300-800 nm. The molar transition energy of Nile Red ($E_{NR}$) was calculated according to equation 7;
\[ E_{NR} = \frac{hcN_A}{\lambda_{\text{max}}} \times 10^6 \]  

(7)

Where \( h \) is Planck’s constant, \( c \) is the speed of light, \( N_A \) is Avogadro’s number and \( \lambda_{\text{max}} \) is the wavelength of maximum absorption of Nile Red in the PIL.

### 2.19 Preparation of sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE)

Prior to running SDS-PAGE, peptides were pre-dissolved in HFIP to reduce aggregate formation and then dried with nitrogen gas. Ionic liquid solutions were added to the dried peptide films and were incubated at room temperature for 1 hour. 50 \( \mu \)L of sample was diluted with 120 \( \mu \)L of PBS pH 7.4 and desalted using protein desalting columns (Thermo Fischer Scientific) which were buffer exchanged prior to the addition of each sample. Samples were heated to 70 °C for 10 min, then vortexed and spun at 10,000 rpm for 1 min prior to loading on 16.5% Tris-Tricine gel. Samples were electrophoresed at 90 V for 3 hours to enhance the separation of low molecular-weight components. The gel was washed with 3 x MilliQ water and then stained by Coomassie Brilliant Blue for 30 min. The gel was destained overnight using glacial acetic acid and methanol buffer and imaged the following day.

### 2.20 Preparation of human tissue samples

Human brain tissues were provided by the Florey Institute of Neuroscience & Mental Health (FINMH). Approximately 600 mg of tissue were homogenized by razor
dissection and 4 volumes of TBS (10 mM Tris, 100 mM NaCl) containing protease inhibitors (Complete, EDTA-free; Roche) added to the tissues. Brain tissues were vortexed, then spun 100,000g for 30 min at 4 °C. The supernatant was collected and the remaining pellets were resuspended in membrane buffer and spun at 100,000g at 4 °C for an additional 30 min. The pellets were then washed 5 × in ddH₂O and centrifuged at 100,000g for 15 min at 4 °C. Homogenates were then prepared into equal aliquots and 120 μL of PIL solution was added and incubated at room temperature overnight. The following day, homogenates were spun at 13,200g for 15 min to separate the supernatant from the pellets. Aliquots of the supernatant were then further used for western blotting analysis.

2.21 Western blotting

For brain dissolution studies, aliquots of the supernatant (described in section 2.20) were separated by SDS-PAGE using 4-20% BT gels (Criterion, Bio-rad) according to the manufacturer’s instructions. Briefly, 50 μL of each extraction was diluted up to 300 μL in PBS pH 7.4 and desalted using protein desalting columns (Thermo Fischer Scientific) which were buffer exchanged prior to the addition of each sample. Samples were heated to 70 °C for 10 min, then vortexed and spun at 10,000 rpm for 1 min prior to loading on 4-20% blot transfer gel. Samples were electrophoresed at 180 V for 45 min before being transferred onto pre-assembled PVDF membrane stacks using a Trans-Blot® semi-dry transfer apparatus (Bio-rad). The membrane was then boiled for 2 min at 50% microwave power in PBS, pH 7.4.
After removing PBS, the membrane was blocked in TBST (10 mM Tris-HCl pH 8.5, 50 mM NaCl, 0.1% Tween-20) containing 5% skim milk. Primary antibodies WO2 and 4G8 were incubated on the membrane overnight at 4 °C. Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako) were diluted 1:10,000 in TBST and incubated for 1 hour at room temperature. The membrane was washed with 5 × 5 TBST after each primary and secondary antibody binding step and enhanced chemiluminescence (Immobilon, Millipore) was used to detect the bands. Images were captured and analysed by a LAS3000 chemiluminescence detector and Science Lab 2005 software (Fujifilm). Aβ peptides were detected using monoclonal mouse antibodies WO2 (diluted 1/50 from culture medium) and/or 4G8 (diluted to 1 μg/mL).

2.22 Transmission electron microscopy (TEM)

Transmission electron micrographs were taken using a Jeol 2100 TEM microscope using an accelerating voltage of 100 kV. A 12 μL droplet of self-assembled peptide solution was placed on the grid and left for 5 min to adsorb before excess fluid was wicked off. Grids were then negatively stained by placing one droplet of freshly prepared and filtered 2% (w/v) uranyl acetate on the grids for 5 min and then blotted continuously against double folded Whatman 50 filter paper. Grids were stored in an air-tight vessel and dried overnight before imaging. Fibril dimensions were estimated using ImageJ software (version 1.48t)(291).
2.23 Scanning electron microscopy (SEM)

Scanning electron micrographs were taken using a Zeiss Supra 55VP SEM. A 10 μL droplet was placed onto carbon tape mounted on an SEM stub (Cambridge sample stubs, 12.7 mm diam. x 11.4 mm ht, Tousimis) and vacuum dried. Samples were gold sputter coated (Leica Microsystems, BAL-TEC SDC 050 sputter coater) and further dried under vacuum overnight. Samples were imaged at a working distance of 3.5 mm using an EHT of 4.00 kV.

2.24 X-ray diffraction (XRD)

X-ray scattering of self-assembled NH$_2$-FF-COOH dipeptides were performed on a diffractometer (X’Pert PRO from PANalytical). The normalized XRD patterns were obtained in symmetrical Bragg geometry with Cu-Kα radiation (40 kV, 20 mA), equipped with a liquid nitrogen cooled Ge solid-state detector. Approximately 20 mg of lyophilized sample was gently mounted onto a glass surface. Samples were scanned from 2° to 45° at a scanning rate of 0.02°/min.

2.25 Optical microscopy

Self-assembled peptides (5 wt%) in 0.1mM PBS and aqueous EAN solutions were imaged at magnifications of x40 on an optical microscope, equipped with an Olympus DP71 camera. Briefly, a 10 μL aliquot of each solution was placed on a microscope slide and vacuum dried over night to eliminate residual solvent.
2.26 ANS binding assay

8-Anilino-1-naphthalene-sulfonic acid (ANS) binding assays were performed on a Varian Cary Eclipse fluorescence spectrophotometer in emission mode. ANS dye was excited at 350 nm and samples were measured between the wavelengths of 400-600 nm, over three accumulations and from three independent sample sets. The fluorescence maximum was recorded for each sample at a slow scan speed, with an excitation and emission slit window of 2.5 mm. Assays were carried out at room temperature, using a constant ANS concentration of 20 μM. Where protein was required, a total concentration of 0.05 mg/mL protein was used.
CHAPTER 3: Investigating Aβ16-22 Fibrilization Kinetics in Protic Ionic Liquid Anions and the Hofmeister Series

3.1 Preface

Sections of this work, describing the controlled fibrilization of Aβ16-22 in protic ionic liquids using a reverse Hofmeister strategy, have been published:


3.2 Introduction

The self-assembly of soluble proteins into insoluble filamentous deposits is a pathological hallmark for many debilitating diseases. These filamentous deposits are known as amyloid fibrils and are often the result of protein misfolding\(^{(146)}\). In Alzheimer’s disease (AD), the accumulation of fibrillar aggregates of amyloid-β (Aβ) peptide, which are produced as a result of enzymatic proteolysis of amyloid precursor protein (APP), triggers a cascade of cellular and molecular events that lead to synaptic failure, neuronal loss and eventually dementia\(^{(61)}\). Thus, increased knowledge of the mechanism of Aβ aggregation and the identity of the toxic intermediate species is required to aid in the development of diagnostic tools for disease progression\(^{(51, 292)}\).
The pathogenesis of AD is an area of active research and recent focus has been centred towards the process of amyloid fibrillization and the isolation and characterization of oligomeric species\(^{(183, 293, 294)}\). The diverse number of these oligomeric intermediates presents a difficult task for researchers to identify the exact nature of the toxic species and mechanism by which fibril formation is achieved. Currently, the neurotoxic species in amyloid fibril formation is believed to be some form of soluble oligomer formed during the early stages of Aβ aggregation\(^{(183, 185)}\). These soluble oligomers, rich in β-sheet structure, exist momentarily before they assemble into higher ordered structures known as amyloid fibrils\(^{(153, 154)}\).

Amyloid fibrilization often follows a sigmoidal kinetic growth profile\(^{(172, 295)}\) which is characterized by a preliminary lag phase followed by monomer addition which initiates fibril growth and elongation. This sigmoidal fibril growth is strongly dependent on the solvent environment, including ionic strength, pH and variations in temperature, which are known to perturb specific protein-protein and hydrophobic interactions required for amyloid self-assembly. Hydrogen bonding is also important in amyloid fibrilization since these interactions are the main features in the formation of β-sheets\(^{(145)}\).

In this chapter, PILs are utilized as a novel solvent media to study the self-assembly of amyloid fibrils in Aβ16-22. This peptide fragment is one of the shortest fibril-forming β-amyloid fragments reported\(^{(126)}\) and is an ideal probe to study amyloid fibrilization in PILs. Since PILs have a tuneable feature of proton transfer, the hydrogen bonding nature of the solvent will be modified through variations in anion
design, to better understand the mechanisms of self-assembly. Additionally, we briefly discuss the formation of a critical amyloid intermediate during self-assembly and identify a possible mechanism for amyloid fibrilization in PILs.
3.3 Results and Discussion

3.3.1 Controlling Aβ16-22 fibrilization kinetics with PILs according to a reverse Hofmeister strategy

The effect of PIL anion on the fibrilization of Aβ16-22 was explored using seven PILs designed with various anions including mesylate (Ms), triflate (Tf), trifluoroacetate (Tfac), hydrogen sulfate (HSO₄), tetrafluoroborate (BF₄), dihydrogen phosphate (H₂PO₄) and lactate (La). The common cation selected was triethylamine (Tea). Additionally, we included the aprotic ionic liquid choline dihydrogen phosphate (choline dhp) since this IL has been extensively used in protein studies and provides a good comparison to all PIL systems and phosphate buffer saline (PBS) studied here.

Since TeaHSO₄, TeaH₂PO₄ and choline dhp have melting points above room temperature, preliminary ThT kinetic measurements for Aβ16-22 were recorded in aqueous TeaH₂PO₄ solutions between the concentrations of 97% to 5% (v/v) PIL. The ThT intensity after 1 minute revealed that solutions greater than 90% TeaH₂PO₄ content demonstrated the fastest kinetics, which was also supported by the rapid formation of fibrils in solution displayed graphically in Figure 3.1 (http://www.rsc.org/suppdata/cp/c1/c1cp22256b/c1cp22256b.mpg). Using the fixed concentration of 90% (v/v), the fibrilization of Aβ16-22 in the aforementioned PILs and aprotic choline dhp were measured as a function of time and the corresponding kinetic profiles are displayed in Figure 3.2. Measurements were stopped once the ThT fluorescence intensity reached a maximum signal.
Figure 3.1. The rapid formation of Aβ16-22 fibrils in 90% TeaH2PO4. t = 0 sec; a solution of monomeric Aβ16-22 is injected into the PIL media. t = 10-20 sec; gentle agitation of the solution shows a distribution of white precipitate (mature amyloid fibrils).

Figure 3.2. Relative ThT intensity as a function of time for Aβ16-22 in 90 wt% TeaH2PO4 (dark blue), TeaHSO4 (red), choline dhp (brown), TeaTfac (green), TeaLa (purple), PBS (black), TeaTf (pale blue), TeaMs (orange) and TeaBF4 (pale green).
The choice of anion has a significant influence on the fibrilization kinetics as illustrated, with both TeaH$_2$PO$_4$ and TeaHSO$_4$ demonstrating remarkably fast kinetics. This is supported by a maximum ThT signal measured at the 1 minute interval. In fact, the fibrilization of A$\beta$16-22 in these two PILs is so fast that there was no obvious lag phase, which is interesting since traditional self-assembly models usually follow a sigmoidal growth profile. TeaTfac and choline dhp illustrate a short lag phase before proceeding to a maximum ThT signal after 12 hours. The relative ThT intensity of A$\beta$16-22 in choline dhp is almost double the intensity observed for TeaTfac, suggesting choline dhp has a greater tendency to fibrilize A$\beta$16-22. The differences in fibrilization kinetic profiles between choline dhp and TeaH$_2$PO$_4$ are likely attributed to the IL cation, since both ILs have the same anion. TeaLa and TeaTf display longer lag phases before the ThT fluorescence signal plateaus after 2 days and 11 days respectively, and both TeaMs and TeaBF$_4$ show no obvious increase in ThT intensity even after 18 days. The fibrilization kinetics of A$\beta$16-22 was also measured in a 0.1mM PBS and a maximum ThT signal was observed after 9 days incubation.

The difference in ThT maximum intensity between each PIL sample also varies as a function of anion. In the case of TeaTfac, TeaLa and TeaTf where the relative ThT maximum is less than 1, different aggregated states or amyloid structures may be present in solution. More importantly, the lack of fibril growth determined by no increase in ThT intensity over time in TeaMs and TeaBF$_4$, suggests these PILs are inhibiting fibrilization and possibly stabilizing pre-fibrillar oligomeric states which are
critical pre-cursors in amyloid self-assembly. Examination of the self-assembled fibrils reveal changes in the superstructure according to the PIL anion (Figure 3.3).

**Figure 3.3.** TEM of negatively stained Aβ16-22 nanostructures in 90 wt% ILs and PBS. Samples were imaged immediately after reaching their maximum ThT fluorescence. No structures were not observed for TeaMs and TeaBF₄ systems.
In the Tea\textsubscript{H$_2$PO$_4$} and Tea\textsubscript{HSO$_4$} systems, a dense network of fibrils is observed within several minutes of self-assembly. The rapid formation of these fibrils suggests that the starting product (monomer) quickly transforms into mature fibrils. This was supported in the ThT binding assay where both systems achieved a maximum ThT intensity within seconds. The fibrils formed in Tea\textsubscript{H$_2$PO$_4$} are long and branched, as opposed to the shorter fibrils formed in Tea\textsubscript{HSO$_4$}. No non-fibrillar aggregates were observed in the electron micrographs of both systems. The fibrils formed in choline dhp and TeaTf acc are homogeneous and uniform in length, with fibril widths of 13.9 ± 1.3 and 34.6 ± 2.4 nm respectively. In 0.1 mM PBS, the fibrils are longer in length and twist periodically along the fibre axis with widths of 35.5 ± 2.8 nm. Similar fibril morphology has previously been reported by Balbach \textit{et al.}\cite{126} in the same solvent system.

Interestingly, both TeaLa and TeaTf solutions produced sheets of different morphologies. The sheets formed in TeaLa are 163 ± 5.8 nm in width and no striations are observed on the surface, which would otherwise indicate the aggregation of short fibrils. These are distinctively different to the TeaTf system where narrow and twisted sheets are formed with widths of 31.8 ± 3.2 nm. It is possible that charge screening effects are contributing to the twisting of Aβ16-22 fibrils formed in high concentrations of TeaTf. This type of fibril morphology has previously been observed in the self-assembly of the AAKLVFF peptide in 300 mM NaCl solution\cite{158}. There was no evidence of nanostructures in the TeaMs and TeaBF\textsubscript{4} systems that was supported by the results in ThT assay. Even after 4 months incubation, both solutions remained
transparent suggesting that the fibrilization of Aβ16-22 is inhibited and that the monomeric state or some form of soluble oligomer is stabilized in solution. The changes observed in fibril superstructure when assembled in PILs may be the result of direct changes to the secondary structure of the peptide. This chiral ordering was further investigated by CD (Figure 3.4).

Figure 3.4. CD spectra of Aβ16-22 in 90 wt% TeaH₂PO₄ (dark blue), TeaHSO₄ (red), TeaMs (orange), TeaBF₄ (green), TeaTf (pale blue) and PBS (black).

The preliminary structure of Aβ16-22 in PBS solution displays a negative maximum at 217 nm and 225 nm, and a positive band at 202 nm, which is consistent with α-helical structure. Alternatively, in the PIL solutions Aβ16-22 predominantly forms a random coil structure with a slight α-helical character. This is represented by a positive π-π* absorption band near 195 nm, a negative band near 207 nm and a positive band near 222 nm (296). Since the preliminary conformation of the peptide is similar in
all PIL solutions, we propose that the changes in fibril ultrastructure and difference observed in the ThT binding assays, are the result of specific anion-water interactions. This is particularly evident in the comparison of Aβ16-22 in TeaHSO₄ and TeaMs where both PILs induce a random coil conformation, but only TeaHSO₄ leads to amyloid fibril production. Additionally, FTIR analysis of the amide I band reveals features in the secondary structure of Aβ16-22 after self-assembly (Figure 3.5).

**Figure 3.5.** FTIR spectra of the Aβ16-22 amide I region in (a) 90 wt% TeaH₂PO₄, (b) 90 wt% TeaMs and (c) 0.1 mM PBS (pH 7.0). Main amide I features are identified by black dashed lines. The blue spectra represent solution + Aβ16-22 and the orange spectra represent the solution on its own.
The FTIR spectra of the fibrils isolated from 90 wt% TeaH$_2$PO$_4$ show two dominant features of amide I bands at 1678 and 1624 cm$^{-1}$, which are indicative of β-turn and parallel β-sheet structures, respectively$^{(297)}$. Quantitative analysis of the amide I components suggests that Aβ16-22 preferably adopts β-type conformations since they yield more than 67% of the total structure (Table 3.1).

**Table 3.1.** FTIR amide I features and calculated percentage secondary structure of Aβ16-22.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>β-sheet</th>
<th>α-helical</th>
<th>Unordered</th>
<th>β-turn</th>
</tr>
</thead>
<tbody>
<tr>
<td>TeaH$_2$PO$_4$</td>
<td>1624 (24.1)</td>
<td>1646 (9.5)</td>
<td>1638 (22.5)</td>
<td>1678 (43.9)</td>
</tr>
<tr>
<td>TeaMs</td>
<td>1612 (16.3)</td>
<td>1646 (31.1)</td>
<td>1637 (34.4)</td>
<td>1674 (18.2)</td>
</tr>
<tr>
<td>PBS</td>
<td>1628 (52.1)</td>
<td>-</td>
<td>1638 (25.8)</td>
<td>1676 (7.1)</td>
</tr>
<tr>
<td></td>
<td>*1693 (15.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values indicate location of amide I band (cm$^{-1}$) with percentage structure reported in parentheses. *The presence of this higher energy band denotes anti-parallel β-sheet structure.

To confirm that this was not the result of the PIL solution, a control measurement was performed using 90 wt% TeaH$_2$PO$_4$ in HDO, and no presence of these spectral features was observed in the FTIR spectrum. Since no fibrils were formed in TeaMs, an aliquot was subsequently analyzed and the collected FTIR spectrum showed a distinctive broad single band at 1651 cm$^{-1}$ indicating the presence of α-helical structure, different from those observed in the control solution. After spectral deconvolution, it was found that less than 35% of the amide I bands contributed to β-type conformations. More importantly, there was an increase in α-helical structure, which could explain why
fibrilization was not observed in the ThT binding assay, since ThT is sensitive to β-sheet structure.

The fibrils collected from the PBS sample show typical anti-parallel β-sheet features through the presence of the strong band at 1628 cm\(^{-1}\) and a weaker band at 1693 cm\(^{-1}\)\(^{(298)}\). The relatively narrower width of the major peak at 1628 cm\(^{-1}\) has previously indicated the presence of stable and/or long β-strands and strong hydrogen bonds, which are commonly found in amyloid fibrils\(^{(299)}\). Although Aβ16-22 in 90 wt% TeaH\(_2\)PO\(_4\) does not display these sharp anti-parallel β-sheet features, the formation of parallel β-sheet structure provides the essential molecular transition for the peptide to rapidly convert from monomer to amyloid fibril.

3.3.1.1 Aβ16-22 fibrilization follows a reverse Hofmeister trend

To describe how these anions are influencing fibrilization, we considered the possibility of Hofmeister anion effects\(^{(300)}\). The empirical Hofmeister series relates to the minimal concentrations of various salts required to precipitate a given protein from aqueous solution, where the effectiveness of these salts are dominated by specific properties relating to the anion. These effects are typically pronounced at low salt concentrations (0.01 M to 0.1 M), where salts can dissociate into individual ions in water. Ranking the anions from fibril forming to fibril inhibiting behaviour, the following series is obtained:

\[
\text{H}_2\text{PO}_4^- > \text{HSO}_4^- > \text{Tf}$\text{ac}^- > \text{La}^- > \text{Tf} \parallel \text{Ms}^- > \text{BF}_4^- 
\]
The double bar (ǁ) indicates the crossover from destabilizing to stabilizing PILs. In terms of monomer stabilization, the dihydrogen phosphate and hydrogen sulfate anions are the most destabilizing, whereas the mesylate and tetrafluoroborate anions are the most stabilizing. Salts which enhance the stability of native proteins usually drive ‘salting-out’ of the protein\(^{(300, 301)}\), that is, they promote aggregates, or in this case amyloid fibrils. However, we find that the PILs enhancing A\(\beta\)16-22 stability are in fact those that are ‘salting in’ the peptide; the mesylate and tetrafluoroborate anions.

It is difficult to explain these reverse Hofmeister effects since common arguments reflecting ion surface charge density, relative ion size and hydrophobicity are not interrelated by this series\(^{(302-305)}\). In fact, some researchers have shown that proteins do not follow the typical Hofmeister behaviour\(^{(306)}\) and question whether it is actually a suitable explanation for the stability of proteins in ionic liquids\(^{(307)}\). Others have shown that proteins exhibit opposite Hofmeister effects, which may contribute to the protein’s net charge and specific interactions\(^{(308)}\). In some studies involving ‘neat’ ionic liquids as solvents, the relative ion effects on enzyme activity and stability become more complicated than a ranking of ions, as described in a review by Zhao\(^{(309)}\).

Since the Hofmeister series best describe salt effects at low concentration, we propose that the accelerated fibrilization observed is likely the result of competitive hydrogen bonding between the anion of the PIL and water. In this scenario, the hydrogen bonds between A\(\beta\)16-22 monomers and water molecules are broken (destabilized) due to the strong water-attracting nature of the kosmotropic anions. These kosmotropic anions then enhance amyloid fibrilization through a ‘salting out’ scenario by lowering the
peptides solubility in solution. Alternatively, weak interactions between chaotropic anions and water facilitate the ‘salting in’ process and render long-term solubility and stability of the peptide in solution. Alternative explanations including ion solvation could suggest that the PIL ions may not completely dissociate into individual ions and instead co-exist as ion pairs, higher order aggregates, or a microsegregated ionic liquid phase.

3.3.2 Amyloid self-assembly is facilitated through competitive PIL anion-water hydrogen bonds

NMR spectroscopy offers valuable information about the phase changes, structural character and reorientational dynamics of molecules under a magnetized field. In particular, solvent relaxation constants, which are used to evaluate the dynamics and molecular interactions of a substance under various thermal conditions, can provide sufficient detail about competing effects between components in solution, particularly when slow dynamics dominate in ionic liquids. Therefore, to gain insight into the hydrogen bonding environment between water and PIL anions, we first investigated the proton chemical shifts of each PIL as a function of concentration by $^1$H NMR spectroscopy.

Evidently, all PILs containing anions with at least one available hydroxyl group ($\text{H}_2\text{PO}_4^-$, $\text{HSO}_4^-$ and $\text{La}^-$) showed an increased proton shift of water, reflecting the formation of hydrogen bonds. For comparative measures, the proton stack plots of Tea$\text{H}_2\text{PO}_4$ and TeaMs are illustrated in Figure 3.6, since these two systems
demonstrated extreme time scale differences in Aβ16-22 fibrilization kinetics and are expected to form different hydrogen bond networks with water.

**Figure 3.6.** $^1$H NMR chemical shift of water as a function of (a) TeaH$_2$PO$_4$ and (b) TeaMs concentration. The small sharp peak at ~4.8 ppm and 0 ppm correspond to HDO and TMS, respectively.
In aqueous TeaH$_2$PO$_4$, there is a large downfield chemical shift of water from 4.90 ppm at 10 wt% to 8.02 ppm at 90 wt% concentration. The smaller peak at ~4.8 ppm corresponds to HDO in the internal capillary. This downfield shift suggests that the proton nuclei of water are becoming less electron dense$^{(315)}$ as the concentration of the PIL is increased. In effect, this causes the nuclei to become less shielded and shift downfield in the spectrum as the result of increased hydrogen bonding with the hydrated hydroxyl groups. Additionally, since the formation of hydrogen bonds causes a resonance shift towards the lower field, the gradual broadening of this peak further implies it is undergoing chemical exchange with the surrounding environment.

The change in the chemical shift of water is negligible in TeaMs, which is anticipated due to the PIL’s chaotropic nature. However, some chemical exchange is still occurring, as evidenced by broadening of the water signal at ~4.8 ppm. Interestingly, the protons corresponding to the methyl group on the mesylate anion (~2.8 ppm) progressively shift upfield at higher PIL concentration and suggest that the anion is weakly interacting with water. The TeaH$_2$PO$_4$ anion has no measurable protons and so the chemical shift of phosphorus was investigated using $^{31}$P NMR (Figure 3.7). A difference of almost 3 ppm is measured for the chemical shift of phosphorus as a function of TeaH$_2$PO$_4$ concentration. This contrasts the shift observed for the mesylate anion in TeaMs and further suggests the prevalence of strong anion-water interactions in TeaH$_2$PO$_4$. 
Figure 3.7. Difference in chemical shift of phosphorus as a function of TeaH$_2$PO$_4$ concentration, probed by $^{31}$P NMR.

To further probe the dynamics of the protons in each PIL system, the spin-lattice relaxation times ($T_1$) of TeaMs and TeaH$_2$PO$_4$ were measured as a function of temperature (Figure 3.8). Measurements were performed at a concentration of 90 wt% PIL since these conditions were previously described in the fibrilization kinetic assay. The most useful information gained from $T_1$ values is the relaxation time and location of the $T_1$ minimum during changes in temperature. Values below 263 K for TeaMs and 268 K for TeaH$_2$PO$_4$ could not be measured due to significant signal broadening owing to the slow motion of the PILs.
Figure 3.8. $^1$H relaxation times ($T_1$) for (a) 90 wt% TeaMs and (b) 90 wt% TeaH$_2$PO$_4$ as a function of temperature (K). Note the difference in y-axes values.
The CH$_3$ protons located on the mesylate anion did not reach a minimum within the temperature range investigated and this indicates that the $^1$H nuclei are continuing to undergo relatively unrestricted rotation. The triethylammonium cation contains three different sets of protons; the N-H proton reaches a $T_1$ minimum of 0.412 sec at 298 K, the CH$_2$ protons reach $T_1$ of 0.448 sec at a lower temperature of 292 K and the terminal CH$_3$ protons reach $T_1$ of 0.555 sec at an even lower temperature of 273 K. As the protons move further out from the nitrogen, their relative mobility increases at a given temperature. This increase in proton mobility suggests a longer time is required to achieve relaxation, which is consistent with our findings here. The slower motion of the water protons in solution results in faster relaxation of the nuclei, measuring the lowest $T_1$ value of 0.357 sec at 292 K in this system.

Alternatively, the $^1$H-$T_1$ values in TeaH$_2$PO$_4$ differ substantially from those observed in TeaMs. As mentioned earlier, the mobility of the proton nuclei increase as one moves from the CH$_2$ to CH$_3$ protons on the triethylammonium cation, however their $T_1$ values and minima occur at higher temperatures; 0.552 sec at 320 K for CH$_2$ and 0.684 sec at 298 K for CH$_3$. The water protons reach a $T_1$ minimum of 0.673 sec at 304 K. This also coincides with the relaxation of the N-H proton. Whilst we would have expected the N-H proton to reach a $T_1$ minimum at higher temperatures than the CH$_2$ protons on the same cation, this was not the case. This trend may be facilitated by chemical exchange between the N-H and water protons as illustrated earlier. Rapid intermolecular exchange between these groups often prevents their coupling with adjacent C-H groups, which is demonstrated by the faster relaxation of the
CH$_2$ protons$^{(316)}$. Furthermore, these variations in proton dynamics, which lead to differences measured in $T_1$ values, are unlikely the result of intramolecular segmental motion as suggested for other ionic liquid systems bearing aromatic protons$^{(314)}$. This was confirmed by measuring the linewidths of each proton signal for 90 wt% TeaMs (Figure 3.9 and Table 3.2) and 90 wt% TeaH$_2$PO$_4$ (Figure 3.10 and Table 3.3).

![Figure 3.9: $^1$H NMR spectra of 90 wt% TeaMs during cooling. Protons are designated according to the molecular structure above.](image)

Figure 3.9: $^1$H NMR spectra of 90 wt% TeaMs during cooling. Protons are designated according to the molecular structure above.
Figure 3.10: 1H NMR spectra of 90 wt% TeaH₂PO₄ during cooling. Protons are designated according to the molecular structure above.
Table 3.2: The linewidths (Hz) of $^1$H signals in 90 wt% TeaMs as a function of temperature.

<table>
<thead>
<tr>
<th>Temperature (K)</th>
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Table 3.3: The linewidths (Hz) of $^1$H signals in 90 wt% TeaH$_2$PO$_4$ as a function of temperature.

<table>
<thead>
<tr>
<th>Temperature (K)</th>
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In TeaMs, the estimated linewidths decrease as the temperature is cooled from 330 K to 268 K. When cooled to 263 K, the linewidths of all proton nuclei begin to broaden due to slower proton dynamics in solution\textsuperscript{(314)}. In the supercooled region, the mesylate CH\textsubscript{3} protons show narrower linewidths due to greater mobility, which is consistent with the $T_1$ values measured earlier. With the exception of the mesylate CH\textsubscript{3} protons, there is no significant difference between the linewidths of the remaining protons to confirm that segmental motion is contributing to changes in $T_1$.

This is also observed for the TeaH\textsubscript{2}PO\textsubscript{4} system, where the linewidths between different proton groups are very similar. The broad linewidth measured for the N-H proton at 340 K is likely due to chemical exchange as described earlier, while the narrowing of water protons between the temperature range of 330 K and 286 K suggests that these protons are very mobile. The greater mobility of these water protons implies their susceptibility to hydrogen bonding with the surrounding environment, which is in agreement with the longer $T_1$ values observed earlier.

Additionally, the total relaxation time of each proton nuclei in the PIL systems provides useful information. TeaMs exhibits a slightly lower, but longer $T_1$ range at room temperature (0.36-0.72 sec), in comparison with TeaH\textsubscript{2}PO\textsubscript{4} (0.58-0.68 sec). We have established that strong hydrogen bonds between the hydroxyl, water and N-H species exists in solution, and as such the relatively fast dynamics of these protons require a longer time for relaxation to occur. Increases in van der Waals and hydrogen bond interactions are known to affect the viscosity of PIL solutions\textsuperscript{(317-319)} and can also contribute to slower proton dynamics and longer $T_1$ relaxation times. Therefore, it
cannot be ruled out that viscosity could impact the $T_1$ values measured. The strong
evidence of chemical shifting and exchange between proton groups in TeaH$_2$PO$_4$,
which are absent in TeaMs, further confirm why rapid amyloid self-assembly in
TeaH$_2$PO$_4$ is achieved.

3.3.2.1 Mechanism for PIL-water-mediated amyloid fibrilization

We have identified that hydrogen bond formation between specific PIL anions and
water molecules is essential in driving the self-assembly of A$\beta$16-22. This
PIL-mediated mechanism of amyloid fibril formation is illustrated in Figure 3.11.

**Figure 3.11.** Schematic of the proposed PIL-mediated self-assembly pathway. (a) The
enhancement of A$\beta$16-22 amyloid fibrils by the addition of a kosmotropic anion, and (b) the
inhibition of amyloid fibrils by the addition of a chaotropic anion. Straight blue arrows
indicate the formation of ordered hydrogen bonds and distorted blue arrows represent the
formation of unordered hydrogen bonds.
In the presence of a kosmotropic anion at high PIL concentration, competitive hydrogen bonding between the PIL anion and surrounding water molecules causes the hydrogen bonds between water and peptide monomers to be broken. As a consequence, new hydrogen bonds are formed between water molecules and PIL anions, which create a more ordered microenvironment around the peptide monomers. These peptide monomers then rapidly form on-pathway nuclei that further assemble into amyloid fibrils.

In the presence of a chaotropic anion, unfavourable interactions between water and anion cause disordering of the bulk water-anion complex. This unordered environment slows down or inhibits the folding and self-assembly of the amyloid monomer, such that it remains stabilized in a hydrated soluble state. Although it is unclear the nature of this species, we anticipate this species to be some form of pre-fibrillar assembly such as an oligomer or a lower molecular weight species. Additionally, the ability to inhibit fibrilization holds promise in terms of developing therapeutic drugs for the treatment of Alzheimer’s disease and will be expanded upon in Chapter 5.

### 3.3.3 Formation of transient Aβ16-22 oligomeric spheres in TeaHSO₄

In addition to the native Aβ16-22 peptide, an N-terminal variant was synthesized to determine the impact of the N-terminal charge on the self-assembly process. Previously, the N-terminal acetylated Aβ16-22 variant was shown to assembling into either nanotubes or fibrils depending on solvent pH\(^{(320, 321)}\). In the presence of PILs where it is expected that charge screening effects predominate\(^{(322)}\), changes in protein
self-assembly are likely attributed to increased ion-water interactions through the formation of hydrogen bonds.

The fibrilization kinetics of the acetylated Aβ16-22 peptide was measured in a solution of 90 wt% TeaHSO₄ since TeaHSO₄ is very kosmotropic and the ThT fluorescence assay is displayed in Figure 3.12.

![ThT fluorescence assay of Ac-Aβ16-22 in 90 wt% TeaHSO₄. Electron micrographs of negatively stained Ac-Aβ16-22 nanostructures sampled from the 1 minute and 24 hour interval of fibrilization.](image)

**Figure 3.12.** ThT fluorescence assay of Ac-Aβ16-22 in 90 wt% TeaHSO₄. Electron micrographs of negatively stained Ac-Aβ16-22 nanostructures sampled from the 1 minute and 24 hour interval of fibrilization.

After 1 day incubation, a maximum ThT intensity was achieved before the fluorescence signal plateaued. Investigation through TEM analysis revealed no fibrils during the initial stages of fibrilization, but rather small spherical nanostructures, which were densely populated across the grid. These structures have previously been
termed spherical oligomers\(^{84, 184, 323}\) and are known to be critical precursors in amyloid self-assembly that are rich in β-sheet structure. This supports the abnormally high ThT intensity measured during the first hour of self-assembly, since ThT is sensitive to β-sheet structure. The formation of these oligomeric nanostructures is an important finding as it has been suggested that oligomers play a key role in the cytotoxicity associated with the formation of amyloid fibrils and consequently, amyloidosis in neurodegenerative disorders such as Alzheimer’s disease\(^{55, 148, 183, 215, 323, 324}\).

After 24 hours incubation, another micrograph was taken and this showed the formation of short amyloid fibrils that appeared to elongate outward from a common centre. The small spherical structures are still observed after 24 hours, and were stabilized in aqueous TeaHSO\(_4\). This is an interesting finding since β-sheet-rich oligomeric aggregates generally disappear upon fibril formation due to their transient and unstable nature. Furthermore, a noticeable inflection point occurs approximately 2 hours into self-assembly. Although we did not sample this time interval, we postulate that this is due to a sudden structural conversion from spherical oligomers to early stage fibrillar structures, which would account for the observed increase in β-sheet formation and greater ThT fluorescence intensity.

Subtle changes to the chemistry of the peptide monomer do not appear to affect the final morphology of the amyloid fibril, since similar structures were observed in native Aβ16-22 in the same solvent. This implies that both native and acetylated Aβ16-22 follow a similar self-assembly mechanism. However, preliminary stages of self-assembly differ for these peptides. Native Aβ16-22 showed no lag phase and
proceeded to form mature amyloid fibrils within minutes of incubation in 90 wt% TeaHSO₄, whereas Ac-Aβ16-22 has a distinctive 1 day lag phase and forms spherical oligomers before proceeding to mature fibrils. By simply replacing the N-terminal amino group with an uncharged acetyl group, the peptide loses a key hydrogen bonding site and the total net charge of the peptide is reduced. Although further analysis of this system is required, the co-existence of these oligomeric spheres and amyloid fibrils is consistent with the complexity of the amyloid fibrilization process\textsuperscript{(163, 168, 325)}.  


3.4 Conclusion

Our findings with the PIL anion series are consistent with previous reports that indicate that anions have a significant impact on biomolecules\(^{326,327}\). The choice of PIL anion is critical in the fibrilization of Aβ16-22. Kosmotropic anions such as H\(_2\)PO\(_4\)- and HSO\(_4\)- significantly enhance fibrilization via a ‘salting out’ scenario, whereas chaotropic anions such at Ms- and BF\(_4\)- completely inhibit fibrilization via a ‘salting in’ scenario. The chaotropic PILs were more likely to stabilize the monomeric form of Aβ16-22 while kosmotropic PILs were better for accelerating amyloid fibrilization, at a shorter time scale in comparison to conventional solvent systems. NMR chemical shift analysis revealed that the fibrilization kinetics in Aβ16-22 were driven by competitive hydrogen bonding between the anion of the PIL and water.

In a modified Aβ16-22 analogue, both amyloid fibrils and oligomeric spheres co-exist in solutions of 90 wt% TeaHSO\(_4\). These oligomeric spheres are believed to be a critical toxic intermediate in the pathogenesis of Alzheimer’s disease. The accelerated fibrilization of Aβ16-22 fibrils observed in 90 wt% TeaH\(_2\)PO\(_4\) holds considerable promise for biotechnological applications\(^{328}\) since amyloid fibrils are exceptionally strong materials.
CHAPTER 4: Enhanced Aβ Fibrilization in Protic Ionic Liquid Cations

4.1 Preface

Sections of this work, describing the enhanced conversion of amyloid fibrils in protic ionic liquids with primary amines, have been published:


4.2 Introduction

Although there is still ongoing debate, the interactions between biomolecules and cations are lower than with anions of the same charge density\(^{(326, 327)}\). This is thought to be caused by factors such as polarizability and hydration, which are lower in cationic species\(^{(326, 327)}\). Others have proposed that cations exhibit Hofmeister activity and, depending on the kosmotropic or chaotropic character of the cation in question, are more likely to display their effects through ion pairing with the conjugate anion species\(^{(329)}\). In fact, this indirect approach exhibited by cations can likely change the behaviour of the anion and consequently the PIL system\(^{(329)}\).

Since anion interactions are more readily understood, most protein folding and self-assembly studies have focussed on the effect of anions in bulk solutions. To date,
only a handful of studies have actually explored changes in PIL cations and their impact on biomolecules\textsuperscript{(265, 270)}. Most recently, Byrne \textit{et al.}\textsuperscript{(330)} described a simple modification of the cationic PIL group that could significantly enhance the stability of the native structure of \textit{tobacco mosaic virus} in the presence of ethylammonium mesylate (EaMs), in contrast to aqueous buffered conditions where a loss of structure was observed over the same experimental time scale.

In an expansion of the approach demonstrated by the PIL anion series discussed in Chapter 3, this chapter aims to investigate the impact of cation-modified PILs on the self-assembly of A\textbeta{}16-22. We describe how PILs designed with primary amines can facilitate amyloid fibril formation and use this information to determine structural changes in pathologically related isoforms of the A\textbeta{} peptide.
4.3 Results and Discussion

4.3.1 Fibrilization kinetics of Aβ16-22 in cation-modified PILs

The effect of PIL cation on the self-assembly of Aβ16-22 was explored using six PILs designed with the lactate anion and various cations including ethanolammonium (ETA), ethylammonium (Ea), diethylammonium (Dea), triethylammonium (Tea), butylammonium (Ba) and tetramethylguanidinium (TMG). Additionally, the aprotic choline lactate (ChoLa) was also synthesized as a comparison to the protic cations. The self-assembly of Aβ16-22 in 90 wt% ILs was monitored over time by ThT fluorescence assay and is illustrated in Figure 4.1.

![Figure 4.1](image-url)

**Figure 4.1.** Relative ThT intensity as a function of time for Aβ16-22 in 90 wt% ILs. ETA Lα (black), TeaLα (purple), ChoLα (pale blue), BaLα (blue), DeaLα (red), EaLα (green) and TMG Lα (orange). Error bars are within 5%.
When Aβ16-22 is self-assembled in ETALa, a maximum ThT intensity is observed after 4 days incubation, with very little changes in ThT intensity thereafter. In comparison to the other PILs, the relative ThT intensity is highest in ETALa and suggests that the Aβ16-22 monomers are almost completely converted to mature fibrils after 4 days. Despite the similarities in molecular structure between the ETALa and EaLa PILs, the absence of the hydroxyl group on the ethylammonium cation shows a significant impact on the fibrilization of Aβ16-22, with a relative ThT intensity almost 10-fold greater in ETA+. This additional hydrogen bond donor/acceptor site in ETA+ allows the PIL to co-ordinate more efficiently with the assembling peptide. Similar observations have been made by Atkin et al., where protein secondary structure was stabilized by cations with multiple hydroxyl groups. Additionally, a 7-fold enhancement in ThT intensity is observed when the cation alkyl chain length is increased from ethylamine to butylamine. Although we assume the strength of electrostatic interactions between the ethylammonium cation-protein and butylammonium cation-protein to be similar, hydrophobic interactions are in fact more significant in the butylammonium cation due to its longer alkyl chain length. This effectively increases the PILs interaction with Aβ16-22 as it assembles.

The effect of hydrogen bonding through the amine protons is demonstrated by EaLa, DeaLa and TeaLa. In the presence of TeaLa, a maximum ThT intensity is measured after 2 days incubation, with a significantly reduced lag phase in comparison to DeaLa and EaLa. This outcome was not expected since the addition of hydrogen bonding sites are thought to enhance protein folding and self-assembly. A possible reason for
this could be explained by the PIL anion and cation combination. Eggers and Valentine\(^{(329)}\) proposed that kosmotropic cations, as opposed to chaotropic ones, have a higher tendency of ion pairing with the kosmotropic anions in solution and results in a reduction of free anions in solution.

### 4.3.2 Primary PIL amines enhance the conversion of Aβ16-22 monomers to amyloid fibrils

To further explore the effects of hydrogen bonding in primary, secondary and tertiary PIL cations, new PILs were synthesized containing the mesylate anion, since this anion displayed chaotropic properties (Chapter 3), and ThT fluorescence assays were performed at various PIL concentrations (Figure 4.2).

It is immediately evident that in most mesylate-containing PILs at various concentrations, the self-assembly of Aβ16-22 does not follow typical sigmoidal growth as observed previously in the lactate anion PIL series. At 10 wt% PIL concentration, both EaMs and TeaMs show a maximum ThT intensity at 1 minute before the fluorescence intensity decays, while DeaMs displays a maximum after 2 hours incubation. Blank measurements were recorded for each PIL concentration at each time interval and this fluorescence decay was not observed. The interesting feature of this figure is the difference in absolute intensity where EaMs demonstrates a five-fold increase in ThT intensity when compared to TeaMs, suggesting that amyloid fibrils are five times more likely to form in the presence of EaMs.
Figure 4.2. ThT intensity of Aβ16-22 in various PIL concentrations. EaMs (black), DeaMs (red) and TeaMs (blue).

When the PIL concentration is increased to 30 wt%, the ThT intensity in EaMs is increased to almost double that observed at 10 wt% concentration. TeaMs shows a four-fold increase in ThT intensity in comparison to the 10 wt% solution, with a maximum intensity again measured at 1 minute. The ThT maximum in 30 wt% DeaMs is measured at 2 hours, which is slightly quicker than that observed in the 10 wt% solution. At 50 wt% concentration, EaMs again demonstrates rapid fibrilization with a maximum ThT intensity proceeding at 1 minute incubation, and shows a similar
fibrilization kinetic profile to that measured at 30 wt%. DeaMs illustrates a substantial increase in the kinetics of fibrilization with a maximum intensity measured at 5 minutes whereas TeaMs significantly decreases to 1 hour.

At higher PIL concentrations (70 wt%), the kinetics of fibrilization slows down significantly in EaMs and DeaMs, where a maximum ThT intensity was observed at 45 minutes. More importantly, TeaMs displays no increase in ThT intensity and there were no signs of fibrillar aggregates in solution according to TEM analysis. This inhibition of fibrilization may be driven by the structure-inducing capabilities of the mesylate anion, which will be discussed further in Chapter 5. This is a contrast to lower PIL concentrations where no evidence of structural-inducing effects were observed. For all PIL concentrations of EaMs and TeaMs, the relative ThT intensity is significantly higher for EaMs than for TeaMs, owing to the availability of amine hydrogen bonds in EaMs. The formation of ion pairs between the ethylammonium cation and the mesylate anion is also significantly reduced due to the chaotropic nature of the anion, allowing the cation to freely interact with the assembling peptide. Since the proton activity in EaMs, DeaMs and TeaMs is very similar ($pK_a = 11.2, 11.3, 11.3$ respectively)\(^{(331)}\), the solution pH is not a critical factor for enhancing fibrilization.

To further investigate the changes in amyloid fibrilization, intrinsic fluorescence of a tryptophan-19 substituted Aβ16-22 peptide was examined in both PILs, containing the primary and tertiary amines. Tryptophan fluorescence is widely used as a tool to monitor protein folding in regards to changes in local structure and dynamics\(^{(332)}\). The substitution of the tryptophan group in position 19 has previously been shown not to
impact amyloid fibrilization\(^{(333)}\) and thus, provides a more sensitive measurement of the local hydrophobic environment since tryptophan has a stronger fluorescence intensity and higher quantum yield than the phenylalanine residue. The fluorescence emission spectra of the tryptophan substituted Aβ16-22 peptide as a function of EaMs and TeaMs concentration is shown in Figure 4.3.

The emission spectrum was also recorded in PBS and is illustrated in both PIL systems. A gradual blue shift is observed in the emission spectrum of EaMs as a function of PIL concentration. At 10 wt\% EaMs, the \(\lambda_{\text{max}}\) occurs at 350 nm and shifts to 342 nm in 70 wt\% EaMs. Blue shifts are often attributed to an increase in hydrophobicity around the tryptophan residue\(^{(332)}\) and imply direct changes to the local environment of the residue. Control measurements of the aqueous PIL solution without the tryptophan substituted peptide showed no \(\lambda_{\text{max}}\) confirming that the signal obtained is the result of changes surrounding the tryptophan residue.

In comparison to TeaMs, no blue shift was observed in the tryptophan emission spectra upon increasing the PIL concentration, and all TeaMs concentrations measured a \(\lambda_{\text{max}}\) of 364 nm. Interestingly, the signal measured at 10 wt\% TeaMs superimposes that of the PBS which also has a \(\lambda_{\text{max}}\) of 364 nm. The fluorescence intensity of the tryptophan residue also increased as a function of TeaMs concentration, with the exception of 30 wt\%, and in comparison to EaMs, the fluorescence intensity is almost three times larger at the same concentration.
Figure 4.3. Fluorescence emission of the tryptophan residue as a function of (a) EaMs concentration and (b) TeaMs concentration. PBS (black), 10 wt% (blue), 30 wt% (red), 50 wt% (green) and 70 wt% (orange). Excitation wavelength 280 nm.

Glutamic acid is known to quench the tryptophan signal when protonated\(^{(334)}\). Therefore, the tryptophan residues may be shielded from glutamic acid by TeaMs, resulting in the enhanced tryptophan emission. In terms of amyloid self-assembly, these hydrophobic tryptophan residues are buried deep within the hydrophobic core of
assembling peptide. The blue shift observed in EaMs suggests a tighter packing density of tryptophan, which may affect the final packing of individual β-sheets and the formation of amyloid fibrils. Furthermore, the absence of a blue shift in TeaMs could imply that this PIL is altering the fibrilization pathway of Aβ16-22 or potentially inhibiting amyloid fibril formation completely. Electron micrographs were taken to illustrate the degree of fibrilization in EaMs and TeaMs and are illustrated in Figure 4.4.

![Figure 4.4](Image)

**Figure 4.4.** Overview of a TEM grid section outlining the degree of Aβ16-22 fibrils in 10 wt% EaMs and TeaMs.

The coverage of fibrillar material across the grid section clearly shows EaMs ability to induce Aβ16-22 fibrils. This is in contrast to TeaMs where only trace amounts of fibrils are observed. These micrographs validate the differences measured in the fibrilization kinetic assays for both systems, where EaMs induces up to a five-fold enhancement in ThT fluorescence intensity, and this result emphasizes the impact of hydrogen bonding through the N-H proton(s) of the cation on amyloid formation. These protons behave similarly to the hydroxyl protons previously described for
TeaH$_2$PO$_4$ and other hydroxyl-containing ionic liquids$^{333}$, and demonstrates that subtle modifications to the chemistry of the cation can be just as effective in enhancing peptide amyloid fibrilization as anion species. The direct peptide-PIL interactions measured by differences in the tryptophan environment likely accounts for the differences observed in the ability to convert Aβ16-22 monomers into amyloid fibrils.

### 4.3.3 Peptide-PIL interactions influence the solution conformational changes in Aβ isoforms

The consequence of these peptide–PIL interactions likely causes a change in peptide secondary structure. In this section, the impact of varying EaMs and TeaMs concentrations are explored on other key Aβ variants that have direct implications in AD. These isoforms include Aβ1-40 and Aβ1-42, naturally occurring components in amyloid deposits, and Aβ3pE-40 and Aβ11pE-40 which are endogenous N-terminally truncated pyroglutamate fragments suspected to be important post-translational products found in amyloid plaques$^{280, 336-338}$. Changes in the secondary structure of Aβ1-40, Aβ1-42, Aβ11pE-40 and Aβ3pE-40 were examined using circular dichroism in 10 wt%, 30 wt%, 50 wt% and 70 wt% concentrations of TeaMs and EaMs. Each spectrum is depicted graphically in Figure 4.5 with their corresponding percentage secondary structures outlined in Table 4.1. These values were determined using K2D2 deconvolution software$^{285}$ in addition to percentage error estimates, which were less than 1.7 % in all cases.
Figure 4.5. CD spectra of Aβ peptides as a function of EaMs and TeaMs concentration. PIL concentrations are 10 wt% (blue), 30 wt% (red), 50 wt% (green) and 70 wt% (black).
Table 4.1. Estimates of Aβ percentage secondary structure using K2D2 online software. Values are rounded off to the nearest whole number.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Solvent Composition</th>
<th>α-helix (%)</th>
<th>β-sheet (%)</th>
<th>Random Coil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ1-42</td>
<td>10 wt% EaMs</td>
<td>8</td>
<td>44</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>30 wt% EaMs</td>
<td>9</td>
<td>43</td>
<td>48</td>
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<tr>
<td></td>
<td>50 wt% EaMs</td>
<td>23</td>
<td>27</td>
<td>50</td>
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<td></td>
<td>70 wt% EaMs</td>
<td>31</td>
<td>15</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>10 wt% TeaMs</td>
<td>9</td>
<td>40</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>30 wt% TeaMs</td>
<td>13</td>
<td>32</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>50 wt% TeaMs</td>
<td>20</td>
<td>25</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>70 wt% TeaMs</td>
<td>25</td>
<td>19</td>
<td>56</td>
</tr>
<tr>
<td>Aβ1-40</td>
<td>10 wt% EaMs</td>
<td>12</td>
<td>49</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>30 wt% EaMs</td>
<td>19</td>
<td>36</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>50 wt% EaMs</td>
<td>22</td>
<td>31</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>70 wt% EaMs</td>
<td>30</td>
<td>28</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>10 wt% TeaMs</td>
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<td>30 wt% TeaMs</td>
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<td>50 wt% TeaMs</td>
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<tr>
<td></td>
<td>70 wt% TeaMs</td>
<td>47</td>
<td>15</td>
<td>38</td>
</tr>
<tr>
<td>Aβ3pE-40</td>
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<td>48</td>
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<tr>
<td></td>
<td>30 wt% EaMs</td>
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<td></td>
<td>50 wt% EaMs</td>
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<td></td>
<td>70 wt% EaMs</td>
<td>37</td>
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<tr>
<td></td>
<td>10 wt% TeaMs</td>
<td>20</td>
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<td>53</td>
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<td></td>
<td>30 wt% TeaMs</td>
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<td>29</td>
<td>51</td>
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<tr>
<td></td>
<td>50 wt% TeaMs</td>
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<td>52</td>
</tr>
<tr>
<td></td>
<td>70 wt% TeaMs</td>
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<td>15</td>
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</tr>
<tr>
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<td>2</td>
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<td>47</td>
</tr>
<tr>
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<td>30 wt% EaMs</td>
<td>5</td>
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<td>70 wt% EaMs</td>
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<td>49</td>
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<tr>
<td></td>
<td>70 wt% TeaMs</td>
<td>24</td>
<td>29</td>
<td>47</td>
</tr>
</tbody>
</table>
As a general observation, the structure inducing effect of each PIL becomes more apparent at higher PIL concentrations with increasing in CD ellipticity with concentration. Higher PIL concentrations induce folding due to specific PIL-peptide interactions, which are less noticeable at high water content due to ion solvation. The presence of isodichroic points at 202-204 nm in various CD spectra implies there is a direct structural conversion between the β-sheet and α-helix conformational states. The α-helix content may be overestimated, since a shift to longer wavelength β-structure occurs on aggregation. This can result in an overestimation of α-helical content, particularly for the more rapidly aggregating species.

Analysis of the estimated secondary structural components reveals distinct differences between the α-helical and β-sheet components of each Aβ isoform. When Aβ1-40 is compared with full-length Aβ1-42 in various EaMs concentrations, both peptides show a gradual loss in β-sheet structure and a gain in α-helical structure, with little change in the total amount of random coil. However, at least part of this apparent gain in α-helical structure may be a shift to more soluble β-sheet species with an observed increase in the single minima intensity around 216 to 222 nm, with artificial calculates as an increase in α-helical content (refer to shape of curves in Figure 4.5). At high EaMs concentrations, Aβ1-40 has almost double the estimated amount of β-sheet content than Aβ1-42. This is particularly interesting since it is well known that Aβ1-42 forms amyloid fibrils more rapidly through the formation of β-sheets that are contributed by the C-terminal hydrophobic residues(77, 79, 164).
Aβ3pE-40 also shares a similar ratio of secondary structural components in comparison with Aβ1-42 at 70 wt% EaMs, however below this concentration, Aβ3pE-40 exhibits equal ratios of β-sheet and random coil structure and demonstrates very little helical content. These results suggest that β-sheet structure for Aβ3pE-40 is more stable in aqueous solutions of EaMs than the β-sheet structure for any other Aβ isoform in this study.

In Aβ11pE-40, there is a gradual increase in helical structure that coincides with the loss of β-sheet structure as the concentration of EaMs is increased to 50 wt%. During this transition, there is no significant variation in random coil content. However at 70 wt% concentration, the percentage of helical structure remains unchanged, while an abrupt increase in β-sheet structure is measured overlapping with the loss of random coil structure. Electron micrographs were taken for Aβ11pE-40 at various EaMs concentrations to determine the impact of this structural transition on the aggregate superstructure (Figure 4.6).

**Figure 4.6.** TEM of Aβ11pE-40 aggregates in 30 wt%, 50 wt% and 70 wt% EaMs solutions.
As expected, Aβ11pE-40 fibrils were clearly observed at low EaMs concentrations, which supports the high percentage of β-sheet secondary structure. At 50 wt% EaMs concentrations, where helical content is at its maximum, fibrillar aggregates are in co-existence with larger amorphous structures. More interesting are the structures formed at 70 wt% EaMs concentration. These globular aggregates range in size from 22 to 96 nm in diameter and appeared in large clusters across the grid surface. Recently, similarly sized structures termed spherical aggregates, were found in submicellar concentrations of SDS according to atomic force microscopic imaging\(^{339}\). These aggregated structures were thought to act as nucleation sites to form larger aggregated species. In this context, the high β-sheet content measured during assembly in high EaMs loadings is equivalent to larger pre-fibrillar oligomeric species described by Cerf \textit{et al.}\(^{203}\) that are also rich in β-sheet structure.

The ability to promote β-sheet structure in high EaMs concentrations emphasizes that intermolecular hydrogen bonding between the PIL and water are preferred over intramolecular hydrogen bonding required to promote α-helix conversion. This varies from the behaviour of α-helical inducing solvents such as TFE and HFIP, since these solvents are weaker proton donors than water and preferentially induce α-helical structure by allowing intramolecular hydrogen bond formation to occur. Barrow \textit{et al.}\(^{121}\) determined a higher propensity of α-helical structure in the Aβ1-39 isoform than in the larger Aβ1-42 isoform, in various concentrations of TFE and HFIP. In this case, Aβ1-40 adopted a higher percentage of helical structure at 70 wt% TeaMs concentration in comparison to Aβ1-42. This result was expected since Aβ1-42 forms
more stable β-sheet structures than Aβ1-40, and suggests similarities between the helical-inducing behaviour of TeaMs and the conventional solvent systems TFE and HFIP. Furthermore, the difference in α-helix and β-sheet ratios between 70 wt% EaMs and 70 wt% TeaMs also supports the helical-inducing structure of TeaMs, which is likely attributed to increased intramolecular hydrogen bond formation. Aβ3pE-40 again illustrates similar secondary structural ratios to that observed in Aβ1-42. Aβ11pE-40 is least affected by the helical-inducing nature of TeaMs out of all Aβ isoforms investigated, which is evident by a higher percentage ratio of β-sheet in relation to α-helical content up to 70 wt% TeaMs. This is also indicated by the absence of any noticeable absorption at 208 nm according to the CD spectra, suggesting that little α-helical structure exists in solution. This may be explained by the hydrophobicity of the Aβ11pE-40 peptide, since the absence of the N-terminal hydrophilic residues renders it the most hydrophobic peptide investigated in this study.

Previous reports on the thermal unfolding of C-peptide, the terminal peptide fragment of RNase A, have suggested that the formation of α-helices are an enthalpy-driven process in which unfolding of the helix is triggered by an increase in temperature\(^{340}\). This study indicated that peptide hydrophobic interactions were not as important in the stabilization of helices, and instead may be more important in the formation and stabilization of β-sheet conformations, as suggest by Barrow et al.\(^{121}\). Since hydrophobic interactions are more prevalent in Aβ11pE-40, it is likely that these interactions are supporting the stabilization of β-sheet structure, even in the presence of helical-inducing TeaMs.
4.4 Conclusion

We have demonstrated that PILs designed with a primary amine can significantly enhance the conversion of Aβ16-22 monomers to amyloid fibrils. Solutions of EaMs resulted in a five-fold enhancement in ThT fluorescence in comparison to TeaMs. Through intrinsic fluorescence measurements of tryptophan-substituted Aβ16-22, we revealed that the aqueous PIL directly interacts with the peptide. This was confirmed by a significant blue shift in the fluorescence spectrum in the presence of EaMs, which suggested that the tryptophan residues become exposed to a more hydrophobic environment as the concentration of the PIL is increased. The additional hydrogen bonding sites on the primary amine could cause a tighter β-sheet packing density within the peptide, resulting in the enhanced production of fibrils rather than monomers. This was clearly illustrated in electron micrographs highlighting the dense network of fibrils assembled in EaMs.

The PIL-peptide interactions of EaMs and TeaMs were further explored in a series of Aβ isoforms and it was determined that each PIL demonstrated greater structural inducing effects at higher concentrations. The ratio of secondary structural constituents vary between different Aβ isoforms and suggests the importance of N- and C-terminal residues and peptide hydrophobic interactions when exposed to structure-inducing PILs.
CHAPTER 5: Structural Induction and Fibrils Dissolution of Aβ1-40 in Ionic Liquids through the α-Helical Intermediate

5.1 Preface

Sections of this work, describing the inhibition and enhancement of amyloid fibril self-assembly, have been published:


5.2 Introduction

In amyloidogenic proteins, the underlying mechanism of fibril formation involves a partial destabilization of the protein’s native conformation, either through changes in the local environment or through point mutations\(^{(24, 34)}\). The amyloid-β protein (Aβ) found in Alzheimer’s disease self-assembles into β-sheet rich fibrils from its irregularly structured monomeric precursor. Although large fibrillar aggregates are important in clinical determination, it is aggregation to smaller oligomeric species that appears to result in a neurotoxic species that play a role in disease progression\(^{(198, 200, 204)}\). Studying the formation of oligomers is complex since these early stage aggregates are highly unstable and usually very difficult to isolate.
The ability to manipulate a protein’s secondary structure and study the interactions and mechanisms responsible for conformational change has attracted significant attention in the field of protein folding. Solvent additives such as urea and guanidine hydrochloride (GdnHCl) have been long known as protein denaturants, and to this day are still extensively used in protein folding studies\(^{(342-345)}\). Whether these denaturants are directly interacting with the protein through van der Waals forces or hydrogen bonding, or indirectly by altering the structure of water through the solvation of hydrophobic groups, is still debateable\(^{(346, 347)}\). In addition to this, other additives such as hexafluoroisopropanol (HFIP), and trifluoroethanol (TFE)\(^{(121, 220, 348-351)}\) have been used as protein stabilizing agents. Both solvents are known to induce or stabilize \(\alpha\)-helical conformations in a diverse range of proteins and peptides including \(\beta\)-lactoglobulin and various A\(\beta\) isoforms\(^{(220, 349)}\).

In aqueous solutions of TFE and HFIP, both A\(\beta\)1-40 and A\(\beta\)1-42 isoforms predominantly exhibit \(\alpha\)-helical structure without fibrilization occurring\(^{(121)}\). The formation of \(\alpha\)-helical intermediates was described as a critical structural transition in the fibrillogenesis of proteins and was further confirmed through studying the fibrilization of 18 different A\(\beta\) peptide fragments, all of which contained the helical intermediate\(^{(209, 226)}\). Furthermore, it was proposed by Kallberg \textit{et al.}\(^{(221)}\) that the stabilization of an \(\alpha\)-helical conformation could supress the formation of amyloid fibrils. A series of proteins including A\(\beta\) were studied and showed an inhibition in fibril formation when residues were removed or modified to a non-discordant sequence.
Following on from the preliminary structural induction studies outlined in chapter 4, the research described in this chapter aimed to investigate the effect of aqueous TeaMs PIL on the \( \alpha \)-helical content of A\( \beta \)1-40 and its consequences on amyloid fibril self-assembly. Additionally, we discuss how synthetic amyloid fibrils, pre-formed in PBS and aqueous TeaMs solutions, can be dissolved in neat PIL media. Finally, we briefly investigate a series of aprotic and protic ionic liquids as potential solvents for the dissolution of naturally occurring brain amyloid plaques.
5.3 Results and Discussion

5.3.1 Structure inducing ionic liquids – Enhancing α-helical structure in the Aβ1-40 peptide from Alzheimer’s disease

Due to its role in the pathogenesis of Alzheimer’s disease, the Aβ1-40 peptide was investigated in TeaMs to determine if any changes in secondary structure and fibril formation could be induced by an increase in PIL concentration. Previously, low concentrations of TFE have been shown to convert predominantly unstructured Aβ monomers into partially ordered conformers which exhibit significantly reduced lag phases and greater propensities for fibril elongation\(^{(220)}\). To determine if TeaMs had a similar effect, the secondary structure of Aβ1-40 was investigated in aqueous solutions of TeaMs (Figure 5.1).

At TeaMs concentrations of 10-50 wt%, Aβ1-40 adopts a classical β-sheet secondary structure identified by the negative ellipticity at 218 nm. Furthermore, this β-sheet content increases in intensity as the concentration of TeaMs is increased from 10 wt% to 50 wt%. At 60 wt% concentration, there is no further increase in β-sheet structure, however a shoulder at 209 nm equal to the 218 nm minimum can be seen and suggests the formation of some α-helical structure. The overlayed signals between 10-60 wt% TeaMs reveals an isodichroic point at 203 nm, which is the point where the elliptical intensities are equal. This isodichroic point is indicative of the presence of only two conformational states that are populating the TeaMs concentration-dependent assembly pathway; the transition from β-sheet to α-helix.
Visual observations of these solutions revealed the presence of aggregated precipitates that redissolved upon the addition of >60 wt% TeaMs, coinciding with the diminishing β-sheet content in Aβ1-40.
When TeaMs concentration is increased to 70-80 wt%, the intensity of the 209 nm minimum also increases, suggesting that Aβ1-40 predominantly adopts an α-helical conformation since there is no longer a clear minimum at 218 nm. Furthermore, at the concentrations of 90-100 wt% TeaMs, Aβ1-40 is predominantly disordered (Figure 5.2) and no longer possesses an isodichroic point.

![CD spectrum of Aβ1-40 in 90 wt% (red) and 100 wt% TeaMs (black).](image)

**Figure 5.2.** CD spectrum of Aβ1-40 in 90 wt% (red) and 100 wt% TeaMs (black).

This indicates that electrostatic screening abilities of concentrated TeaMs at higher concentrations prevents the formation of sufficient intermolecular and intramolecular hydrogen bonds that are required for folding. The induction of α-helical structure by TeaMs and its relative effect on Aβ1-40 fibrilization was examined by TEM (Figure 5.3).
In 10 wt% and 50 wt% TeaMs solutions, $\alpha\beta$1-40 shows a dense population of fibrillar aggregates across the entire grid section. In both aqueous TeaMs solutions, long homogeneous fibrils are observed with similar morphologies that are consistent with the enhanced $\beta$-sheet secondary structure observed using CD spectroscopy. In addition to this, both TeaMs solutions in the presence of $\alpha\beta$1-40 showed a positive ThT fluorescence intensity, further confirming the presence of $\beta$-sheet structure.

In contrast, the micrograph of $\alpha\beta$1-40 in 100 wt% TeaMs shows no sign of fibrillar aggregates and ThT binding assays also revealed no fluorescence intensity, indicating the absence of fibril formation and no $\beta$-sheet structure. At TeaMs concentrations above 60 wt% there were no fibrils observed in solution, indicating that the inhibition of fibrils is likely due to TeaMs stabilizing the monomeric state of $\alpha\beta$1-40 through $\alpha$-helical induction. To screen the presence of these monomers in high concentrations of TeaMs, SDS-PAGE was performed on $\alpha\beta$1-40 incubated in various concentrations of TeaMs (Figure 5.4).
It is observed that Aβ1-40 monomers (~4.3 kDa) are present in all TeaMs concentrations, however the intensity of the bands increased upon the addition of more PIL. This is interesting since the initial amount of Aβ1-40 monomers incubated were the same in all samples. The low concentration of monomers formed in 10 and 30 wt% TeaMs implies that the equilibrium shifts in favour of fibril formation. Earlier we observed predominant β-sheet structure by CD when Aβ1-40 assembled at these TeaMs concentrations, leading to the formation of amyloid fibrils. The presence of monomers indicates that at low wt% TeaMs, a small percentage of Aβ monomers do not completely convert into fibrils, perhaps due to the peptide interacting with the hydrated TeaMs molecules. This equilibrium shifts in favour of monomer formation, particularly in the presence of 70 wt% and 90 wt% TeaMs concentrations, which
complements the absence of β-sheet structure and fibril formation determined earlier. Although it is understood that SDS induces oligomer dissociation and alternative techniques such as CD spectroscopy are more conclusive at the secondary level of peptide assembly, it is likely that this enhancement in α-helical structure by TeaMs could replicate the structural inducing effects exhibited by TFE.

Indeed, the ability to induce α-helical structure in Aβ, a predominantly α-helical peptide in its native state, is a critical step towards understanding the folding mechanisms associated with the peptides self-assembly. Induction of α-helical structure in high concentrations of TeaMs shows similarities to the conformations induced in high concentrations of TFE (>40 vol%)\(^{(352)}\). The exact mechanism of how TFE induces α-helical structure is still debateable. Some explanations incorporate the role of solvent polarity, where low polarity solvents are thought to weaken the hydrophobic interactions that stabilize native protein structure and simultaneously strengthen local hydrogen bonds\(^{(349, 350)}\). Hong et al.\(^{(230)}\) discuss that relative dielectric constants and hydrogen bond strengths are important factors and suggests that the formation of dynamic clusters of the alcohol side groups via hydrophobic interactions are responsible for the enhanced structural stability observed in protein and peptide systems. Molecular dynamic studies have previously shown that TFE molecules can aggregate around peptides, displacing water molecules and consequently remove alternative hydrogen bonding neighbours, in addition to providing a low dielectric environment that is favourable in the formation of intra-peptide hydrogen bonds\(^{(353)}\). It is generally accepted that the balance between nonpolar and polar groups in TFE
disrupt the intramolecular hydrogen bonds which are essential for β-sheet formation, while simultaneously promoting hydrogen bonding in the monomeric helical species.

We propose that the mechanism by which TeaMs can alter the hydrogen bonding nature of Aβ1-40, such that the α-helical intramolecular bonds are favoured over the β-sheet forming intermolecular bonds, may be the consequence of the unique hydrogen bonded network system of the PIL. Other conventional solvent systems including TFE, HFIP and SDS have shown to induce α-helical structure in different amyloid-forming peptides by creating a membrane-like environment\(^{(230, 339, 354)}\). It is therefore also possible that the presence of aggregated species within the ionic liquid could be creating a bilayer effect. There have been reports which suggest that ionic liquids in aqueous solution are not completely dissociated into ions, and these molecules can form clusters in aqueous solution\(^{(355)}\). Recent studies have demonstrated that aggregated species do indeed exist in PILs, and ‘poorly ionic liquids’ which describe many PILs have a tendency to form aggregates in aqueous solution, while still retaining their nanostructured features\(^{(250, 356)}\).

The structural induction of TeaMs is not limited to Aβ1-40. The α-helical inducing effects of TeaMs was examined on a modified Aβ fragment, Ac-KLVFFAE-NH\(_2\) which has previously shown to form fibrils and nanotubes in various solvent conditions\(^{(320, 321, 357)}\) and is identified as a key sequence involved in the β-sheet fibril formation of full-length Aβ1-40. This fragment is based on the Aβ16-22 sequence, but contains a C-terminal and an acetylated N-terminus. The structure-inducing effect of TeaMs as a function of concentration is illustrated in Figure 5.5.
Upon increasing TeaMs concentration from 10 wt% to 50 wt%, an increase in α-helical structure is indicated by the increased signal at 206 nm. The presence of an isodichroic point at 201 nm further suggests that there is a mixture of only two conformations; α-helical and random coil, which is observed at higher TeaMs concentration.
loadings. Although pH is an important factor in protein folding and aggregation, the changes induced by TeaMs are not the consequence of pH variation in solution. To confirm this, the secondary structure of the modified Aβ16-22 peptide was measured in various phosphate buffer solutions of different pH and is shown in Figure 5.5. In all ten solutions ranging from pH 2 to 11.5, there was no change in peptide secondary structure which indicates that the structural induction observed for these Aβ peptides is not driven by pH.

The relative magnitude of ellipticity in both spectra is also noteworthy. In TeaMs a considerably higher molar ellipticity was observed, in comparison to that observed in aqueous phosphate buffer solutions of different pH values. This may be due to reduced chiral ordering as a result of increased intramolecular hydrogen bonding in the presence of the PIL. Ionic liquids have previously been shown to increase the solubility of difficult to solubilize molecules due to the electrostatic nature of the ionic liquid\(^{(358)}\).
5.3.2 Dissolution of pre-formed Aβ1-40 fibrils in TeaMs

The ability to reverse the formation of amyloid fibrils is currently an area of active research. Several approaches have been identified to inhibit amyloid fibril formation including the use of aromatic dyes such as Congo red, curcumin, and macrocyclic β-sheet mimics to disrupt fibril self-assembly\(^\text{359, 360}\). Since we have identified that a number of mesylate-containing PILs could inhibit the formation of Aβ1-40 fibrils through the formation of an alternative structure via the induction of disruptive intramolecular hydrogen bonds, we next investigated whether TeaMs had the ability to dissolve pre-formed Aβ1-40 amyloid fibrils.

In this study, Aβ1-40 amyloid fibrils were prepared according to the two methods outlined in Chapter 2, section 2.5. Briefly, \textit{method 1} involved the rapid self-assembly of Aβ1-40 fibrils in an aqueous solution containing 10 wt% TeaMs and \textit{method 2} involved the slow self-assembly of Aβ1-40 fibrils in PBS solution. Each sample was lyophilized and 0.2 mg of fibrils from each method were transferred to 1 mL solutions of neat TeaMs for analysis. The change in chiral ordering during the dissolution of Aβ1-40 fibrils formed according to \textit{method 1} is illustrated in Figure 5.6.
Figure 5.6. Time-dependent CD spectra of (a) Aβ1-40 fibrils generated according to method 1, during dissolution in neat TeaMs and (b) the CD signal at 216 nm. The line is added as a visual aid.

During analysis, solutions were gently agitated to ensure sedimentation did not result in the loss of CD signal by removing the structures from the measurement light path. A marked reduction in ellipticity at 216 nm represents the progressive loss of β-sheet
structure in the fibrils as they dissolve in the presence of neat TeaMs. This is clearly illustrated above where the β-sheet content rapidly decreased and plateaus after 12 hours. During this time an increase in α-helical content is observed, as indicated by the increase in ellipticity at 206 nm. To probe the solubilization of Aβ1-40 fibrils during dissolution, an aliquot of sample was removed during the early, middle and late stages of dissolution and these sample were imaged using TEM (Figure 5.7).

![TEM of method 1 pre-formed Aβ1-40 fibrils dissolved in neat TeaMs at different time intervals.](image)

**Figure 5.7.** TEM of method 1 pre-formed Aβ1-40 fibrils dissolved in neat TeaMs at different time intervals.

The degree of fibrillized material decreased as a function of incubation time in TeaMs. During the early stages of dissolution, Aβ1-40 fibrils appear dispersed across the grid section. These fibrils are homogenous and very short or ‘fragmented’ in comparison to those described earlier in section 5.3.1. After 12 hours incubation, a reduced number of fibrils were observed, albeit with conserved morphologies. This reduction in number of fibril is consistent with the gradual loss in β-sheet structure indicated by CD analysis. The fibril dissolving property of neat TeaMs becomes apparent during the late stages of dissolution, where after 24 hours incubation there is very little to no
evidence of fibrillar aggregates in the micrograph. This observation in addition to the gain in helical structure implies that the fibrils have dissolved in neat TeaMs. The conformational conversion from β-sheet to α-helix suggests that this dissolution proceeds via the formation of an α-helical intermediate in Aβ1-40, the stability of which is likely facilitated by increased TeaMs-peptide interactions.

Conversely, analysis of the dissolution of method 2 pre-formed Aβ1-40 fibrils by CD reveals the formation of a random coil conformation with a partial β-structure\(^{(36)}\) over a 48 hour incubation period (Figure 5.8).

![Figure 5.8](image)

**Figure 5.8.** Time-dependent CD spectra of Aβ1-40 fibrils generated according to method 2, during dissolution in neat TeaMs.

This structural transition is significantly different to the sheet-helix transition observed for the method 1 fibrils. A decrease in the 196 nm and 220 nm minima imply greater ordering into a possible β-structure as incubation time is increased. It is not clear if this
is related to increased solubility or actual structural change over time. Analysis of the electron micrographs taken show the presence of Aβ1-40 fibrils at both time points, with shorter fibrils observed after 48 hours (Figure 5.9).

![Figure 5.9. TEM of method 2 pre-formed Aβ1-40 fibrils dissolved in neat TeaMs at different time intervals.](image)

Although the fibrils at longer incubation appear shorter, their abundance on the grid does not change in comparison to shorter incubation periods in TeaMs. Additionally, there are differences in morphology between these fibers and those generated according to method 1. The rapid formation of fibrils in 10 wt% TeaMs leads to shorter fragmented fibrils whereas forming these structures slowly in PBS produces longer homogenous fibrils.

By allowing Aβ1-40 monomers to assemble in an environment that is not disrupted by the structure breaking properties of TeaMs, monomer polymerization and fibril elongation is favoured. This has implications on the packing density of individual
β-sheets, since well-developed amyloid fibrils have a greater propensity to form tightly packed intermolecular β-structures. This packing density can be monitored by FTIR analysis of the amide I region, where greater inter and intramolecular ordering is usually indicated by a lower energy shift of the secondary structure. The pre-formed Aβ1-40 fibrils formed by both methods were analysed by FTIR and the results are depicted in Figure 5.10.

![FTIR spectra of the amide I region for Aβ1-40 fibrils pre-formed according to method 1 (blue) and method 2 (red).](image)

**Figure 5.10.** FTIR spectra of the amide I region for Aβ1-40 fibrils pre-formed according to *method 1* (blue) and *method 2* (red).

The Aβ1-40 fibrils formed according to *method 1* show two prominent FTIR spectral features including a sharp peak at 1628 cm⁻¹ and a shoulder band centred at ~1654 cm⁻¹, which represents β-sheet and α-helical structures, respectively. This shoulder is not observed for the spectra of the fibrils generated from *method 2*. Instead the main band is observed at 1623 cm⁻¹ indicating the presence of β-sheet structure.
Analysis based on second derivative spectra within the amide I region further identified an additional small peak at 1694 cm\(^{-1}\) assignable to anti-parallel β-sheet structure. In particular, the main absorbance band centred at 1623 cm\(^{-1}\) is significantly shifted to lower wavelength when compared to that observed in the pre-formed TeaMs fibrils, indicating that there is a tighter packing density of β-sheets within the fibril network. Furthermore, the absence of a band at 1654 cm\(^{-1}\) indicates that PBS formed Aβ1-40 fibrils preferably adopt β-sheet structure, which is consistent with the longer fibrils observed by TEM.

The tighter packing density of these β-sheets in the PBS formed Aβ1-40 fibrils implies that these fibrils exist in a higher ordered state. In terms of dissolution, it may be difficult for neat TeaMs to disrupt this network of organized intermolecular hydrogen bonds and that would explain why fibril degeneration was not observed. On the other hand, dissolution is enhanced for the pre-formed TeaMs fibrils since the intermolecular arrangement of hydrogen bonds are more loosely packed. The presence of a band at 1654 cm\(^{-1}\) indicates that TeaMs does induce α-helical structure, even at low PIL concentrations. It is also possible that Aβ1-40 peptide oligomers are trapped in TeaMs during self-assembly, leading to restricted fibril growth. In fact, this has been previously reported by Kalhor et al.\(^{(275)}\) where it was determined that HEWL protein oligomers were not converted into protofilaments and fibrils because they were trapped by the ionic liquid in a more stable conformation. The ability for TeaMs to fibrilize and subsequently dissolve Aβ1-40 fibrils confirms the multifunctional properties of this PIL system, which can be modified according to water content.
5.3.2.1 Brain amyloid dissolution in ionic liquids

In collaboration with researchers from the Mental Health Research Institute, Melbourne University, a selection of ionic liquids, both aprotic and protic in nature, were tested on their ability to dissolve brain amyloid plaques. Aprotic ionic liquids were synthesized with the 1-butyl-3-methylimidazolium (BMIM) and 1-ethyl-3-methylimidazolium (EMIM) cations since these have shown increased solubility and dissolution of biomaterials including lysozyme, native silk fibroin and cellulose\(^{(269, 362, 363)}\). Additionally, the PIL cation 1,1,3,3-tetramethylguanidine (TMG) was selected, as this cation was previously used in the dissolution of HEWL\(^{(275)}\). It is difficult to dissolve amyloid plaques from brain and separate Aβ components since other components present and post-translational modifications contribute to the amyloid structure. Masters et al.\(^{(8)}\) previously described the solubility of plaques consisting of various proportions of amyloid cores in >70% formic acid (FA) solution, and for this reason we have tested the dissolution ability of our ionic liquid systems against FA. Western blots of the Aβ1-40 peptide derived from dissolving brain amyloid plaques in neat ionic liquids and FA are shown in Figure 5.11.
Figure 5.11. Western blot profiles of Aβ derived from brain amyloid plaques after 24 hours incubation at room temperature in ionic liquids and formic acid. Plaques were pre-treated (a) with urea buffer or (b) without urea buffer prior to blotting. Samples were separated on 4-12% tris-tricine polyacrylamide gels and probed with 4G8 and WO2 antibodies. 15s and 30s correspond to the enhanced chemiluminescence imaging time.

The profiles of ionic liquid-dissolved Aβ1-40 showed a marked increase in low molecular weight oligomers ranging from monomers to tetramers, after overnight incubation at room temperature, and are clearly observed after 30 seconds of enhanced chemiluminescence (ECL). Although all ionic liquid samples show a distribution of low molecular weight oligomers, BMIMAc shows a greater formation of tetramers due to the intensity of the 16 kDa band. Interestingly, TMGLa showed the largest distribution of low molecular weight aggregates around the 4 kDa mark, suggesting that the dissolution of Aβ1-40 monomeric species is favoured. TMGLa is the only
protic ionic liquid investigated here and results indicate that the proton transfer property of this PIL enhances the formation of Aβ1-40 monomers. The lactate anion has previously shown enhanced dissolution of *Candida Antarctica* lipase B, thought to be contributed to preferential hydrogen bond formation between the PIL anion with the polypeptide backbone\(^{(306)}\). Despite the presence of these low molecular weight bands in each ionic liquid system, the intensity of these bands is significantly greater in 80% FA. In terms of proton availability between these solutions, the acid protons in the PIL systems have all been neutralized, whereas in FA there is no neutralized acid proton. This indicates that FA can readily donate/accept protons in solution, allowing it to actively dissolve various Aβ components as illustrated. Additionally, an extremely high molecular mass Aβ (~250 kDa) remained in the stacking gel of the samples pre-treated in urea buffer. This species is particularly stronger in the BMIMCl, EMIMSO₃ and FA samples as illustrated by the darker band. This high molecular mass Aβ corresponds to fibrils\(^{(203)}\).

However, the influence of SDS on the aggregation and migration behaviour of proteins has also been reported\(^{(364)}\), and these unwanted solvent interactions are usually overcome by the addition of small volumes of urea to the sample matrix. Therefore, to determine whether the presence of urea in solution is affecting the dissolution of the Aβ plaques in ionic liquids, another blot was prepared in the absence of urea. As depicted in Figure 5.11, the majority of low molecular weight bands have disappeared from the blot, leaving only trace amounts of Aβ1-40 monomers (4 kDa) detectable in each ionic liquid sample after 30 seconds of ECL. Furthermore, there are no high
molecular weight species in the stacking gel as previously noticed for the samples treated with urea buffer. In FA, the monomeric band is maintained and only trace amounts of oligomers between 8-16 kDa are seen. The absence of urea in the preparation of each sample appears to largely affect the ability of the ionic liquid samples to dissolve the Aβ components. Without urea present, Aβ1-40 likely forms unordered aggregates that are harder to dissolve by the aprotic ionic liquids since their proton availability is negligible. This does not affect the dissolution in FA since the monomeric species was easily formed. Quantification of these species is necessary, but may be difficult in the urea-treated samples since fibrils remaining in the stacking gel could be underestimated\textsuperscript{(203)}. 
5.4 Conclusion

In this chapter, the structural inducing effect of TeaMs was explored for the Aβ1-40 peptide. Aβ1-40 amyloid fibrils were formed in aqueous TeaMs solutions (<50 wt%) and showed distinctive β-sheet structure by CD analysis. When incubated at higher TeaMs concentrations (>60 wt%), α-helical structure was preferentially formed which resulted in the inhibition of fibrilization. The helical-inducing effects of TeaMs, which are also characteristic of other mesylate containing ionic liquids, are driven by the preferred formation of intramolecular hydrogen bonds, and behaves similarly to other solvent systems such as TFE and HFIP which are commonly used to induce helical structure in proteins.

Synthetic Aβ1-40 pre-formed fibrils that were self-assembled rapidly in aqueous TeaMs conditions can also be dissolved in neat TeaMs through the structural conversion of β-sheets to α-helices. However, when these fibrils are slowly assembled in normal aqueous conditions in the absence of a chaotropic PIL, intermolecular hydrogen bonding in the amyloid fibril becomes more organized and the packing density of β-sheets is greatly increased. Evidently, this tighter β-sheet network makes dissolution more difficult for TeaMs. Furthermore, although our selected ionic liquid series showed only a partial dissolution of naturally occurring brain amyloid in comparison to formic acid, the presence of urea during the preparation of the plaques is likely influencing the formation of these oligomeric species in the ionic liquid solvents and requires further investigation.
CHAPTER 6: Crystalline to Amorphous Transition of the Central Alzheimer’s Aβ Dipeptide in Aqueous PIL Solution

6.1 Introduction

It is well established that amyloid fibrils result from the unfolding or denaturation of the native state protein. Once unfolded, protein monomers self-assemble via various on-pathway intermediates leading to amyloid fibril formation\(^{(153, 154)}\). However, there have been documented cases of various stable supramolecular structures, or polymorphs, that aggregate as the result of protein unfolding under different conditions\(^{(104, 365, 366)}\). Some of these assemblies include the formation of nano-crystalline fibrils\(^{(158, 367)}\) and spherulites\(^{(368)}\) which have previously been identified in post-mortem tissue of diseased brains, and may suggest their relevance in the disease state\(^{(369)}\). In particular, a recent study identified the formation of small transient nano-crystalline Aβ assemblies which preceded amyloid fibril formation\(^{(370)}\). These ordered structures were viewed as critical intermediates in the early stages of the fibrilization process.

Polymorphism in amyloid proteins has been known for some time\(^{(371, 372)}\), however there is ongoing debate as to whether variations in the supramolecular assembly or changes in the internal structure of protein monomers during assembly are the contributing cause\(^{(128, 138, 373, 374)}\). Proteins rely heavily on the formation of several weak non-covalent bonds including ionic, van der Waals, hydrogen bonding, hydrophobic and \(\pi-\pi\) interactions\(^{(375-379)}\). For smaller peptides containing aromatic
moieties, the contribution of hydrophobic and $\pi$-$\pi$ interactions are particularly important\(^{(380)}\) since geometrical restrictions of the aromatic residues can promote directional and orientational growth into fibrillar structures that are essential for amyloid fibril formation\(^{(380, 381)}\).

One of the most versatile synthetic peptides to date is the structural recognition motif for Alzheimer’s Aβ peptide. NH$_2$-FF-COOH (Figure 6.1), which consists of two consecutive phenylalanine residues, plays a decisive role in fibril formation, attributed to the $\pi$-stacking interactions of the phenylalanine rings\(^{(382)}\). Numerous reports describe the formation of various polymorphic structures including nanowires\(^{(383, 384)}\), nanotubes\(^{(385-387)}\) and nanospheres\(^{(388-390)}\), which carry their own unique chemical and physical properties.

![Figure 6.1. Chemical structure of the NH$_2$-FF-COOH dipeptide.](image)

Recently, peptide nanotubes designed from the NH$_2$-FF-COOH dipeptide showed a thermally induced phase transition from a linear assembly exhibiting hexagonal nanocrystalline structure, to a cyclic assembly displaying orthorhombic nanocrystalline structure\(^{(291)}\). This thermally induced polymorphism causes profound changes at the molecular level that influence the final morphology of the assembled
structures. The same peptide sequence capped with an N-terminal tert-butoxycarbonyl (Boc) group could form nanotubes or nanospheres depending on the solvent composition\(^{(389)}\). Since short aromatic peptides contain all the necessary molecular information required for amyloid formation, they are viewed as excellent models to study the mechanistic issues of polymorphism in amyloid formation\(^{(392, 393)}\).

This chapter aims to investigate the various structural transitions of NH\(_2\)-FF-COOH when self-assembled in aqueous ethylammonium nitrate (EAN) solutions. EAN was selected as a peptide precipitating agent due to its hydrophobic and ionic character, as well as its ability to hydrogen bond with water, and has previously been identified as a successful medium for lysozyme crystallization in both neat and aqueous state\(^{(266, 394)}\). Additionally, the impact of aqueous EAN solutions on NH\(_2\)-FF-COOH hydrophobicity during self-assembly will also be discussed.
6.2 Results & Discussion

6.2.1 Characterization of NH$_2$-FF-COOH crystalline assemblies

Pre-dissolved monomeric NH$_2$-FF-COOH dipeptide was self-assembled over one week in PBS and various concentrations of EAN. In PBS, 0.1 M and 0.5 M concentrations of EAN, NH$_2$-FF-COOH assembled into visible microstructures within the first few hours, while no visible structures were observed for solutions containing 1.0 M and 2.0 M EAN after one week. Aliquots of each solution were imaged via optical microscopy and SEM to determine the morphology of the assembled structures (Figure 6.2).

In aqueous PBS conditions, there is a clear formation of long thin crystal-like NH$_2$-FF-COOH fibrils. The corresponding SEM image highlights the surface uniformity, which closely resembles that observed in amyloid fibrils. Additions of EAN to the solution matrix cause the dipeptide to form various polymorphic assemblies. At 0.1 M EAN concentration, similar crystal-like fibrils are observed by SEM. Upon increasing the PIL concentration to 0.5 M, another clear morphological change to short flat fibrils is observed during self-assembly. SEM analysis shows the alignment of several short fibrils along the longitudinal axis that create clusters of microsheets.
Figure 6.2. Optical microscopic images (top) and SEM images (bottom) of the self-assembled NH$_2$-FF-COOH dipeptide in PBS and aqueous 0.1 M, 0.5 M, 1.0 M and 2.0 M EAN solutions.
At 1.0 M EAN concentration, there was almost no visible precipitate in solution, and optical microscopy revealed the formation of small circular microspheres. A closer examination of these structures by SEM revealed aggregates of thin flaky sheets. At 2.0 M EAN concentration, only unstructured aggregates were observed in solution, confirmed by the absence of fibrillar material by SEM.

Previous studies have identified that this dipeptide is capable of forming amyloid-like structures\(^{(395)}\). To confirm whether the PIL-assembled structures exhibit amyloid characteristics, samples were stained with Congo red (CR) dye and viewed under cross polarizers on an optical microscope (Figure 6.3).

![Figure 6.3: Optical microscopic images of CR stained NH\(_2\)-FF-COOH assemblies in aqueous 0.1 M and 0.5 M EAN solutions under cross-polarizers.](image)

NH\(_2\)-FF-COOH assembled in 0.1 M and 0.5 M EAN showed a clear gold-green birefringence, suggesting a high molecular level order that is commonly observed in
the amyloid state. No birefringence was evident in the assemblies at 1.0 M and 2.0 M EAN solutions, which indicates very little structural order at the molecular level.

To confirm the crystalline or amorphous state of these structures, the normalized x-ray diffraction patterns of each lyophilized sample were obtained and these are depicted in Figure 6.4. The presence of sharp diffractive peaks at low angle 2θ correlate to a high degree of molecular crystallinity, whereas no diffraction peaks and broad spanning regions indicate amorphous structure(396).

![XRD patterns of the self-assembled NH$_2$-FF-COOH dipeptide in PBS (blue) and aqueous 0.1 M (green), 0.5 M (orange), 1.0 M (red) and 2.0 M (black) EAN solutions. Miller indices (hkl) assignments correspond to the hexagonal unit cell, $P6_1$.](image)

**Figure 6.4.** XRD patterns of the self-assembled NH$_2$-FF-COOH dipeptide in PBS (blue) and aqueous 0.1 M (green), 0.5 M (orange), 1.0 M (red) and 2.0 M (black) EAN solutions. Miller indices (hkl) assignments correspond to the hexagonal unit cell, $P6_1$. 
The diffraction array for NH$_2$-FF-COOH assembled in PBS has previously been identified by Gorbitz et al.\textsuperscript{(397, 398)}. The authors correlated the NH$_2$-FF-COOH diffraction peaks to a $P_6_1$ space grouping which is indicative of a hexagonal crystal system. Analysis of the Miller indices (hkl) indicate similar 2\(\theta\) diffraction peaks, which agree with the hexagonal symmetry identified for the single crystal structure of NH$_2$-FF-COOH\textsuperscript{(399)}. This is further supported by the presence of crystals according to SEM and optical microscopy discussed earlier. The molecular and morphological transitions in NH$_2$-FF-COOH as a function of EAN concentration are accompanied by distinctive changes in the XRD crystal structure of the assemblies.

When self-assembled in 0.1 M EAN concentration, NH$_2$-FF-COOH exhibits similar Bragg reflections to those observed in PBS despite the reduction of several peak intensities, and suggests that the dipeptide remains predominantly crystalline. At 0.5 M EAN concentration, most of the low angle 2\(\theta\) diffraction peaks have disappeared although crystalline structures were still observed by optical microscopy. A noticeable broad band spanning across angle 2\(\theta\) of 8° to 25° is observed at 1.0 M and 2.0 M EAN concentrations, which indicates the structures are predominantly amorphous. The impact of EAN also illustrates significant changes to NH$_2$-FF-COOH secondary structure as shown by CD analysis (Figure 6.5).
In PBS, there is evidence of β-turn structure that is characterized by a strong positive band at 198 nm and a second positive band at 218 nm. These bands correspond to the π-π* and n-π* transition, respectively, in the phenylalanine residues and are common features in β-turn peptides containing aromatic residues\(^{400-402}\). Upon the addition of EAN, there is a gradual increase in ellipticity which implies an increase in chiral ordering as the result of increased solubility in solution. Furthermore, the ratio of the π-π* band relative to n-π* increases as a function of EAN concentration and indicates a direct change in the rotation of the phenylalanine residues during assembly. The intermolecular arrangement of the NH\(_2\)-FF-COOH phenylalanine residues, driven by hydrophobic π-π stacking interactions, is a critical feature for the formation of
nanotubes and crystals, as discussed by Gazit et al.\textsuperscript{(380, 383)}. Thus, during crystal growth, molecules of EAN may interfere with the assembling peptide monomers by altering the orientation of the aromatic residues. This causes changes in the intermolecular $\pi$-$\pi^*$ associations between neighbouring phenylalanine residues, as illustrated by the band ratio change above, and ultimately disrupt the potential for self-assembly and crystal growth.

### 6.2.2 EAN facilitates changes in NH$_2$-FF-COOH hydrophobic packing

Interactions between particular hydrophobic side chain residues are thought to play a key role in protein aggregation and stability. However, the non-specific nature of hydrophobic interactions can facilitate polymorphism within amyloid fibrils\textsuperscript{(138, 403)}. To further investigate how EAN influence the morphological transitions in NH$_2$-FF-COOH, changes in the hydrophobic environment were probed by 8-anilino-1-naphthalene-sulfonic acid (ANS) binding assays. ANS is commonly employed as a fluorescence probe for the determination of surface hydrophobicity and actively binds to hydrophobic regions in protein\textsuperscript{(404-407)}. Hydrophobic changes on protein surfaces are indicated by two factors; a distinctive blue-shift in the ANS emission maximum, and an enhancement in the ANS fluorescence intensity signal. Figure 6.6 illustrates the main emission band for ANS when bound to NH$_2$-FF-COOH in each solvent condition, corrected by subtracting the PIL signal.
Figure 6.6. ANS fluorescence spectra for NH$_2$-FF-COOH after 30 min incubation in PBS (blue) and aqueous 0.1 M (green), 0.5 M (orange), 1.0 M (red) and 2.0 M (black) EAN solutions. Black arrow indicates a hypsochromic shift in the ANS fluorescence spectra. Excitation wavelength 350 nm.

In PBS, a maximum ANS emission is recorded at the wavelength of 502 nm, which is in close agreement with the reported 505 nm maximum for protein-bound ANS molecules in aqueous conditions$^{(404)}$. Increasing the concentration of EAN causes a significant blue-shift in the emission maximum of peptide-bound ANS molecules, measuring a wavelength shift to 483 nm at 2.0 M concentration. The fluorescence intensity of ANS also shows a noticeable increase from 543 a.u to 588 a.u upon increasing EAN concentration. Since the PIL signal was subtracted, changes in the fluorescence intensity are directly correlated to changes in the ANS binding to
NH₂-FF-COOH. Variations in ANS fluorescence intensity were further examined over a 5 hour incubation period in each solution and are displayed in Figure 6.7.

![Figure 6.7](image)

**Figure 6.7.** ANS binding assay for NH₂-FF-COOH during self-assembly in PBS (blue) and aqueous 0.1 M (green), 0.5 M (orange), 1.0 M (red) and 2.0 M (black) EAN solutions. Excitation wavelength 350 nm.

In both PBS and 0.1 M EAN solutions, ANS demonstrates a strong binding affinity for NH₂-FF-COOH, which is marked by an increase in the fluorescence intensity over the first 30 minutes. This suggests that during the initial stages of self-assembly, NH₂-FF-COOH is predominantly unfolded such that hydrophobic residues on the peptide surface are exposed to binding with surrounding ANS molecules. After 30-45 minutes incubation, there is a sudden decay in fluorescence intensity that coincides with the formation of crystalline fibrils in solution. This observation, which
can be viewed as the start of monomer polymerization, indicates that ANS molecules are becoming unbound as the result of peptide folding into a higher organized state. As NH$_2$-FF-COOH self-assembles, the phenylalanine residues assemble via strong intermolecular hydrophobic $\pi$-$\pi$ interactions, causing ANS molecules to become displaced. NH$_2$-FF-COOH shows a similar trend during self-assembly in 0.5 M EAN, where a maximum fluorescence signal is measured after 45 minutes, before gradual signal decay is measured.

Interestingly, at 1.0 M and 2.0 M concentrations, there is no evidence of signal decay even after 5 hours incubation. Instead, the fluorescence intensity gradually plateaus, suggesting that the hydrophobic residues on the peptide surface are predominantly exposed. Furthermore, at higher EAN concentrations, the relative fluorescence intensity measured for the 2.0 M EAN solution is significantly greater than that observed for PBS and 0.1 M EAN solutions. This suggests that at higher EAN concentrations, hydrophobic and hydrogen bond interactions between the PIL and peptide are increased, which may help stabilize the unfolded state of the peptide. In fact, Summers and Flowers\(^{(265)}\) previously described that during lysozyme unfolding in EAN, the exposed hydrophobic core of the protein favourably interacts with the hydrophobic alkyl side chain of the ethylammonium cation; an interaction that becomes more dominant as the concentration of EAN is increased. Thus, the stabilization of these hydrophobic peptide residues with molecules of EAN prevents their self-assembly into higher order structures.
Structural polymorphism in amyloid fibrils has important implications in disease progression. For example, prion proteins have been found to give rise to multiple disease strains in both mammalian and yeast studies\(^ {408, 409} \) as the result of different structural forms of the prion protein during aggregation. Indeed, the formation of structurally and morphologically distinct forms of NH\(_2\)-FF-COOH may help explain why polymorphism exists in A\(_\beta\)1-40. Although a correlation between AD cognitive impairment and total amyloid deposition has yet to be observed\(^ {55} \), some studies postulate that certain A\(_\beta\) polymorphs could be more toxic than others\(^ {132} \).
6.3 Conclusion

The work described in this chapter relates to polymorphism in the Aβ peptide related NH$_2$-FF-COOH dipeptide. In aqueous solution, NH$_2$-FF-COOH forms large amyloid fibril-like microstructures with a highly ordered hexagonal crystal structure. Increasing the concentration of EAN in solution leads to the formation of various polymorphs including amyloid crystals, flat sheets and porous spherical assemblies, with noticeable changes in the chiral arrangement of phenylalanine residues.

Analysis of the hydrophobic environment during self-assembly showed that higher concentrations of EAN inhibited the formation of crystalline fibril growth by stabilizing the unfolded state of NH$_2$-FF-COOH. This supported by the high ANS fluorescence intensity measured during self-assembly. Alternatively, in diluted EAN solutions, strong hydrophobic and π-π interactions between peptide monomers predominate, causing NH$_2$-FF-COOH to unfold and self-assemble into ordered crystalline structures.
CHAPTER 7: Evaluation of PIL Solvent Parameters on the Phase Behaviour of Poly(N-isopropylacrylamide) (PNIPAM)

7.1 Preface

Sections of this work, describing the key interactions between water and PIL and their impact on polymer phase behaviour, have been published:


7.2 Introduction

Ionic liquids (ILs) have found application in a diverse range of fields from electrolytes\(^{(410, 411)}\) to solvents for biomolecule stabilization\(^{(271, 412)}\). Protic ionic liquids (PILs) are a subclass of the IL\(^{(236)}\) family that have an additional tuneable feature as a result of the proton transfer, the proton activity\(^{(413)}\). The proton activity can be linked to the pH of the PIL and has been used as a relative scale to select appropriate PILs for organic transformation reactions\(^{(414, 415)}\) and the solvation of biomolecules\(^{(268)}\). However, the proton activity is not the only parameter that determines protein stability and solubility.
The empirical Hofmeister series, which describes the ability of ions to ‘salt in’ or ‘salt out’ proteins\(^\text{258, 416}\), has recently been used to describe the influence of ionic liquids on biomolecule stabilization, in which major attention has been centred on imidazolium-based cations in the aprotic ionic liquid family. These ions are regarded as kosmotropic or chaotropic depending on their relative abilities to induce the structuring of water\(^\text{300, 417, 418}\). Kosmotropes (structure makers) are thought to enhance the solubility of proteins whereas chaotropes (structure breakers) reduce the solubility of proteins\(^\text{302, 419-421}\). The Hofmeister effect does not always explain these observation, and other factors such as specific ion binding and relative polarizabilities have been implicated\(^\text{327, 422}\).

Poly(N-isopropylacrylamide) [PNIPAM] is a thermo-responsive amphiphilic polymer which has often been used as a model system for the cold denaturation of peptides and proteins\(^\text{423, 424}\), as it contains both hydrophilic (amide) and hydrophobic (isopropyl) groups. PNIPAM has shown to follow the Hofmeister trend in a number of studies\(^\text{425-428}\), including ionic liquids\(^\text{429, 430}\). It is well-known that PNIPAM develops a lower critical solution temperature (LCST) at 32 °C in aqueous solution\(^\text{431}\), which is the result of the polymer’s transition from a soluble hydrophilic random coil at lower temperatures, to an insoluble hydrophobic globule at high temperatures. The hydrophobic collapse of the polymer chain is thought to be directly related to the arrangement of intramolecular and intermolecular hydrogen bonds within PNIPAM\(^\text{432}\).
In the work previously described in Chapter 3, the stabilization of Aβ16-22 monomers was observed to follow a reverse Hofmeister trend in which kosmotropic anions enhanced the fibrilization process. Since fibrilization is directly linked to protein destabilization, and in the case of Aβ16-22, the formation of amyloid fibrils, it therefore becomes important to understand which solvent parameters are contributing to this observation and how this can be used to correctly select appropriate PILs for biomolecule stabilization. Since the diversity of ionic liquids is endless, choosing the appropriate cation and anion combination permits fine-tuning the solvent properties for a particular purpose. The polarity of ionic liquids is sensitive to the nature of anions and cations, and given the complexity of interactions that arise from a solvent’s polarity, most single parameter approaches cannot describe the system accurately.

Kamlet and Taft developed a multi-parameter system that probes particular solvent properties based on the interaction between a set of closely related dyes by UV/visible spectrophotometry. These properties include hydrogen bond acceptor (HBA) basicity ($\beta$)$^{(287)}$, hydrogen bond donor (HBD) acidity ($\alpha$)$^{(289)}$ and dipolarity/polarizability effects ($\pi^*$)$^{(288)}$. Numerous studies have investigated these parameters in ionic liquid systems$^{(429, 433-437)}$. For instance, Lee et al.$^{(436)}$ describe how distinct variations in the HBA basicity and HBD acidity arise from modifying the methyl group on pyridinium-based ionic liquids. Furthermore, Nayak et al.$^{(429)}$ were able to measure the degree of hydrogen bonding and ion pairing within their ionic liquid systems in relation to how ionic liquids interact with polymers.
In PILs, the hydrogen bond donating and accepting abilities become particularly important, particularly when studying the effects of protein self-assembly, since protein self-assembly relies heavily on the extended hydrogen bond network between individual peptide strands. Solvent polarity also has a profound influence on the outcome of many chemical reactions and is known to play an important role in influencing the phase behaviour of polymers\cite{438, 439}. The polarity of the microenvironment surrounding the main chain of PNIPAM changes significantly at phase transition and results in changes in polymer structure\cite{440, 441}. Parameters such as dielectric constant, dipole moment and refractive index are available to help select the right ionic liquid for a given purpose. However, these parameters do not take into account specific solvent-solute interactions which exist in determining the polarity of solvents. This led to the development of solvent polarity empirical scales, which are described by Deye et al.\cite{442}. There are various methods for measuring the polarity of solvents, particularly in ionic liquid systems\cite{443, 444}, including the solvatochromic dyes pyridinium N-phenolate betaine\cite{445} (Reichardt’s dye), which was made popular by Reichardt and co-workers\cite{290, 446-448}, and Nile Red\cite{443-445}.

In this chapter, the phase behaviour of PNIPAM is investigated in a series of aqueous PIL solutions. In addition, other key solvent parameters including the HBA basicity, HBD acidity, dipolarity/polarizability effects and polarity will be discussed in order to identify the critical interactions between water and PIL complexes, and compare these relative to observations found with respect to biomolecule stabilization.
7.3 Results & Discussion

7.3.1 Preliminary determination of PNIPAM LCST in a PIL

Numerous studies discuss the phase behaviour of PNIPAM in aqueous salt solutions\(^{426, 427, 449}\) and solvents\(^{450-452}\). However, very few actually describe these changes in ionic liquids, particularly PILs. Since we are interested in how PILs affect polymer phase behaviour, we initially measured the phase transition in a 1.48 M aqueous solution of DeaMs as a function of temperature to determine the LCST (Figure 7.1).

![Figure 7.1. Typical LCST transition of PNIPAM in 1.48 M DeaMs. The insert illustrates the LCST. Pictures illustrate the transition from soluble coil (left) to insoluble globular (right) state.](image)
The LCST is clearly seen at 19.3 °C in the insert. Leading up to this temperature interval, the polymer remains in a hydrophilic coil structure and is completely miscible in solution. However, beyond this temperature point, the polymer becomes immiscible and restructures into a hydrophobic globular state that can clearly be observed by eye.

### 7.3.2 PNIPAM phase behaviour in PIL anions

Using the results from DeaMs described in the previous section, we next explored a library of PILs designed with various anions and cations using the aprotic choline dhp as a comparison. Starting with selectively modifying the PIL anion, the impact of PIL concentration on the LCST of PNIPAM is depicted in Figure 7.2.

![Figure 7.2.](image)

**Figure 7.2.** LCST of 0.5% (w/w) aqueous PNIPAM solution as a function of IL anion; TeaBF₄ (pale blue), TeaTf (orange), TeaTfac (green), TeaMs (black), TeaHSO₄ (red), TeaLa (purple), TeaH₂PO₄ (dark blue) and choline dhp (white).
At high water content there is almost no difference observed in the LCST between various PILs, however as the PIL concentration is increased the LCST points spread out depending on the type of anion. In fact, the impact of choice of anion on the LCST is dramatic, with a 20 °C difference observed between TeaH$_2$PO$_4$ and TeaTfac at a fixed PIL concentration of 2 mol/L. The trend of most to least LCST inducing PIL anion is as follows;

$$\text{H}_2\text{PO}_4^->\text{HSO}_4^->\text{La}^->\text{Ms}^->\text{Tfac}^->\text{Tf}^->\text{BF}_4^-$$

Similarly with Aβ16-22 fibrilization as discussed in Chapter 3, this series identifies the kosmotropes as the most LCST-inducing anions and the chaotropes as the least LCST-inducing anions, although some changes in the ordering of the chaotropic anions are observed. From this observation, it is highly likely that Hofmeister anion affects are enforced and that the hydrogen bonding environment of these anions may be influencing PNIPAM phase behaviour. Interestingly, choline dhp exhibits the most LCST-inducing effect on PNIPAM, requiring only 1.62 M of the IL to reach an LCST of 1 °C. This suggests that the composition of anion and cation has a greater ability to convert the polymer from a hydrophilic coil to a hydrophobic globule under thermal conditions.

Interestingly, there are two distinct trends occurring. Firstly, PILs containing nonfluorinated anions such as H$_2$PO$_4^-$, HSO$_4^-$, La$^-$ and Ms$^-$ show a constant reduction in LCST as a function of concentration. Secondly, the fluorinated anions Tfac$^-$, TF and BF$_4^-$ gradually increase the LCST. Although it is not illustrated in Figure 7.2, TeaTfac
requires a concentration of $>10.8$ mol/L before the LCST starts to increase. Both TeaTf and TeaBF$_4$ raise the LCST above that of PNIPAM in water, with TeaBF$_4$ demonstrating the greatest enhancement. Previous studies using aprotic ILs with the BF$_4^-$ anion have reported an increase in the LCST and the presence of a UCST$^{(429)}$. However, in this study there was no observation of a UCST within the temperature range studied, or across the entire PIL-water phase diagram for any of the fluorinated PILs investigated. Wang et al.$^{(453)}$ previously reported that the LCST of PNIPAM in the aprotic IL BMIMBF$_4$ disappears at higher IL concentrations. This finding was attributed to the increase in intermolecular hydrogen bonds between the IL-water and polymer complex, which enhances the polymers stability at higher temperatures. Since a similar increase in PNIPAM LCST was observed in the TeaBF$_4$ and TeaTf systems, it is likely that the stability exhibited by PNIPAM at higher temperature is caused by the increased hydrophobicity of the PIL, in which surrounding water molecules are being pushed away from the polymer. Costa et al.$^{(451)}$ noticed a similar reversal in LCST behaviour at different mole fractions of cononsolvents. Solvent mixtures comprising of methanol-water and in particular acetone-water showed a sudden increase in PNIPAM solubility, which the authors describe are contributed to the stronger hydrophobic hydration effects of the cononsolvent mixtures.

Since we suspect hydrogen bonding interactions to play a critical role here, the proton chemical shift of water was probed by $^1$H NMR at various PIL concentrations (Figure 7.3).


Figure 7.3. Proton chemical shift of water as a function of IL anion concentration. TeaH$_2$PO$_4$ (dark blue), choline dhp (white), TeaHSO$_4$ (red), TeaLa (purple), TeaMs (black), TeaTf-ac (green), TeaTf (orange) and TeaBF$_4$ (pale blue).

Evidently, the shift in the HDO peak is highly dependent on the PIL anion. The kosmotropic anions H$_2$PO$_4^-$ and HSO$_4^-$ demonstrate a significant downfield shift in comparison to other PILs in this study, with TeaH$_2$PO$_4$ exhibiting the greatest downfield shift. In comparison with TeaMs, little to no chemical shift was measured at various concentrations. Furthermore, all fluorinated PILs display an upfield shift, with TeaBF$_4$ exhibiting the most upfield shifted water protons.

These observations reinforce that the water-PIL hydrogen bond network is dependent on the choice of PIL, with this difference being reflected in the LCST of PNIPAM. The PILs with the greatest impact on water (kosmotropic) lower the LCST of PNIPAM more efficiently. An interesting comparison can be made between the two ILs
containing the dihydrogen phosphate anion, the protic TeaH₂PO₄ and the aprotic choline dhp. Although choline dhp reduces the LCST of PNIPAM at lower concentrations, it is TeaH₂PO₄ that has the strongest interaction with water according to ³¹P NMR chemical shift analysis (Figure 7.4).

![Figure 7.4. Difference in chemical shift of phosphorus as a function of IL concentration, probed by ³¹P NMR. TeaH₂PO₄ (dark blue) and choline dhp (white).](image)

The reduced LCST of PNIPAM in the presence of choline dhp can be best explained by the combined influence of the kosmotropic H₂PO₄⁻ anion and the hydroxyl group on the cation. Since both H₂PO₄⁻ anion and hydroxyl group are competing for water molecules, PNIPAM can reach lower phase transition temperatures in choline dhp than in TeaH₂PO₄. Additionally, the marked trend in the proton chemical shift of water, in relation to the hydrophobic character of the fluorinated anions, is further depicted in the LCST of PNIPAM, where anions of a stronger hydrophobic nature
(BF$_4^-$ > Tf > Tfac$^-$) cause an enhancement in polymer solubility. From these results, these fluorinated anions show a greater repulsion towards water molecules, as depicted by the upfield proton chemical shifts, and suggest they interact weakly with surrounding water molecules. Recently, Reddy et al.$^{(454)}$ determined that the BF$_4^-$ anion in BMIMBF$_4$ strongly attracts the protons of water molecules surrounding PNIPAM. This interaction was responsible for the polymer’s collapse in solution. Our results disagree with this study since we have clearly shown that the BF$_4^-$ anion in the TeaBF$_4$ PIL is not attracted to the protons on water. A comparison between the proton chemical shifts of water for the PILs against their precursor acids (Figure 7.5) also emphasizes the unique chemical environment of the PIL systems.

Figure 7.5. Proton chemical shift of water as a function of acid concentration. Ms$^-$ (black), HSO$_4^-$ (red), Tf (orange), BF$_4^-$ (pale blue), Tfac$^-$ (green) and H$_2$PO$_4^-$ (dark blue).
In all cases, the precursor acid causes a downfield shift in the water peak. The largest downfield shift was observed for methanesulfonic acid and the least downfield shift was observed for phosphoric acid. The PIL-water trend is remarkably different to the acid-water trend and this is most likely attributed to the presence of freely available protons. In the PIL state, the base has neutralized the acid proton, whereas in the acid state, this proton is still available to interact with water molecules.

7.3.3 PNIPAM phase behaviour in PIL cations

Following the anion series, we next investigated a series of ILs designed with different cations and their impact on PNIPAM LCST (Figure 7.6).

![Figure 7.6. LCST of 0.5% (w/w) aqueous PNIPAM solution as a function of IL cation; TMGLa (black), DeaLa (red), BaLa (dark blue), ETALa (pale blue), EaLa (orange) and ChoLa (green).]
The trend of most to least LCST inducing IL cation is as follows;

\[
\text{Choline}^+ > \text{Ea}^+ > \text{ETA}^+ > \text{Ba}^+ > \text{Dea}^+ > \text{TMG}^+
\]

Similarly to the anions investigated earlier, differences in LCST are more prominent as the concentration of PIL is increased, with the aprotic ChoLa and protic TMGLa illustrating the strongest and weakest effects on LCST, respectively. Interestingly, the phase behaviour of PNIPAM in this series of PILs never increases above that of water, indicating that the effect of cations on the LCST behaviour is less important than that of the anions. In fact, cations in aqueous salt mixtures are known to have a minimal effect on the LCST of PNIPAM\(^{(426, 455)}\). Their less dominant nature in comparison to anions of the same charge density, are believed to be the result of their reduced polarizable nature and lowered hydration effects\(^{(326, 327)}\). The corresponding proton chemical shift analysis also indicates that the degree of chemical shift is much less than that observed for the anion series (Figure 7.7). However, it is interesting to note the impact of the cation hydroxyl groups in ETALa and ChoLa. Although both cations carry a single hydroxyl group, ETA\(^+\) results in the largest chemical shift of water, whereas choline\(^+\) results in the smallest shift. Both ILs are structurally similar, however the addition of three methyl groups to the central nitrogen atom in the aprotic choline cation causes the PIL to weakly interact with water.
Nevertheless, the choline cation is a known chaotrope and is expected to have a weak association with water due to its ‘structure breaking’ nature. Alternatively, ETA$^+$ is a primary amine containing up to three N-H hydrogen bonds which are freely available to interact with water molecules. Since ChoLa has the greatest influence on lowering the phase transition of PNIPAM, it is likely that strong ion pairing between the anion and cation occurs in this aprotic IL, which may be responsible for the polymer’s state changes at lower ChoLa concentrations.

7.3.4 Kamlet-Taft solvatochromic parameters in PILs

Differences in PIL design suggest that there are specific properties of PILs that strongly influence the phase behaviour of PNIPAM. Therefore, to understand these
properties, solvatochromic analysis of the hydrogen bond donor (HBD) acidity $\alpha$, hydrogen bond acceptor (HBA) basicity $\beta$ and dipolarity/polarizability effects $\pi^*$ were measured using the spectroscopic Kamlet-Taft dyes 4-nitroaniline, $N,N$-diethyl-4-nitroaniline and Reichardt’s dye. This technique utilises UV-visible spectroscopy to measure differences in the wavelength shift of certain probe dyes that are sensitive to solvent polarity and hydrogen bond acidity and basicity.

Preliminary UV-visible measurements were performed in methanol and water and the intramolecular charge transfer band (ICT) correlating to the longest wavelength absorption maxima of each dye was measured in nanometres and converted to wavenumbers where necessary, according to equations 2-6 in Chapter 2. The $E_{T}(30)$ and $E_{T}^{N}$ parameters, defining the molar transition energy of Reichardt’s dye and the normalized polarity, respectively, are also included (Table 7.1). Since the measured values are in close agreement to those in literature, the experimental parameters could be confidently applied to PIL samples.

**Table 7.1.** Kamlet-Taft parameters of water and methanol.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$E_{T}(30)$</th>
<th>$E_{T}^{N}$</th>
<th>$\pi^*$</th>
<th>$\alpha$</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>55.45</td>
<td>0.76</td>
<td>0.731</td>
<td>1.080</td>
<td>0.605</td>
</tr>
<tr>
<td></td>
<td>(55.6)$^{(433)}$</td>
<td>(0.762)$^{(456)}$</td>
<td>(0.73)$^{(436)}$</td>
<td>(1.05)$^{(457)}$</td>
<td>(0.61)$^{(437)}$</td>
</tr>
<tr>
<td>Water</td>
<td>62.92</td>
<td>0.99</td>
<td>1.332</td>
<td>1.010</td>
<td>0.137</td>
</tr>
<tr>
<td></td>
<td>(1.000)$^{(426)}$</td>
<td>(1.33)$^{(456)}$</td>
<td>(1.12)$^{(458)}$</td>
<td>(0.14)$^{(456)}$</td>
<td></td>
</tr>
</tbody>
</table>

Reported values are given in parentheses.
7.3.4.1 $\pi^*$ values

Solvent dipolarity/polarizability ($\pi^*$) is a measure of a solvent’s ability to stabilize a neighbouring charge or dipole by means of nonspecific dielectric interactions. The dipolarity/polarizability of each PIL solution was measured using the $N,N$-diethyl-4-nitroaniline dye according to equation 2. Although $\pi^*$ provides valuable information about the dipolarity and polarizability of the solvent system, it is the property of the solute that is actually being measured, that is, the differential stabilization of the more polar excited state of the solvatochromic dye. The interaction between the probe dye and the ions directly depend on the extent of coulombic interactions between them. For example, it has been reported that when the charge surrounding the anion of an ionic liquid becomes delocalized over more atoms, the polarizability of the solvent decreases due to stronger coulombic forces between the ions\textsuperscript{(433, 437)}. This weakens the interaction between the ions and probe dye, reducing the polarity on the solvatochromic scale. The $\pi^*$ values for each PIL as a function of concentration are illustrated in Figure 7.8.

The non-fluorinated and fluorinated PILs have been separated for ease of interpretation. At low water content, all non-fluorinated and fluorinated PILs measured a $\pi^*$ value similar to that observed for nonaqueous molecular solvents and aprotic ionic liquids\textsuperscript{(433, 436, 437, 459)}. It has been reported that both hydrophobic character and size of the anion can impact the $\pi^*$ values\textsuperscript{(435)}. For example, ionic liquids designed with smaller and more compact anions, such as halogens or those in the tetrahedral geometry, lead to higher $\pi^*$ values.
Figure 7.8. Kamlet-Taft $\pi^*$ parameter for (a) nonfluorinated ILs TeaLa (purple), TeaMs (black), TeaHSO$_4$ (red), TeaH$_2$PO$_4$ (dark blue) and choline dhp (white) and (b) fluorinated PILs TeaBF$_4$ (pale blue), TeaTf (orange) and TeaTfac (green).

In this case, the BF$_4^-$, H$_2$PO$_4^-$, Ms$^-$ and HSO$_4^-$ anions, all of which are tetrahedral in geometry, have a $\pi^*$ value above 1.1 at low water content. Also, as the hydrophobic
character of the fluorinated PILs increases from Tfac\(^-\) to BF\(_4^-\), the \(\pi^*\) value increases, which is in agreement with previous trends\(^{(425)}\). As the water content is increased, the \(\pi^*\) value changes significantly, depending on the PIL anion. Both TeaH\(_2\)PO\(_4\) and TeaHSO\(_4\) have \(\pi^*\) values similar to water (1.332) at a volume fraction of water \(\phi_{\text{water}}\) \(>0.3\), suggesting a water-like environment, even in the concentrated PIL state. Interestingly, Cammarata \textit{et al.}\(^{(460)}\) suggest that depending on the type of anion, water can either interact strongly with the anion or exist largely as bulk water. Since water involves ice-like structured pools and disordered zones, the addition of a highly ordered anion such H\(_2\)PO\(_4^-\) or HSO\(_4^-\) may cause the disordered zones to become more ordered. This being the result of hydrogen bonding between the anion and water molecules, could indicate why a waterlike behaviour is seen in the kosmotropic PILs, which becomes more evident at higher PIL concentrations.

TeaMs shows a sudden drop in the \(\pi^*\) value at \(\phi_{\text{water}} = 0.8\). Since this PIL has a lower \(\pi^*\) value than TeaH\(_2\)PO\(_4\) and TeaHSO\(_4\) at the same volume fraction of water, it is considered to be less waterlike. The fluorinated PILs show lower dipolarity/polarizability values at low water content in comparison with other PILs in this study. TeaTfac has the lowest \(\pi^*\) value in the series suggesting that strong coulombic interactions exist between the ions of the PIL, which lead to weaker interactions with the probe dye. Interestingly, choline dhp shows the highest \(\pi^*\) value, and as this IL concentration is increased, the dipolarity/polarizability effect becomes greater than water. Only one other ionic liquid has been reported to have a \(\pi^*\) value greater than water at room temperature; 1-methylimidazolium propionate.
[HmIm][CH$_3$CH$_2$COO] (1.50)$^{433}$. This large $\pi^*$ value for choline dhp implies strong interactions between the IL and probe dye, which may indicate why this IL interacts strongly with PNIPAM, as reflected in the LCST.

Although the $\pi^*$ value seems to be greatly influenced by the basicity of the anion in an ionic liquid, it is reported that the cation has a very small effect on the polarity of a PIL$^{433}$. The dipolarity/polarizability for a series of ILs designed with different cations and the common lactate anion, were examined through the same principles and are illustrated in Figure 7.9.

**Figure 7.9.** Kamlet-Taft parameter $\pi^*$ for ILs with various cations. ChoLa (green), EaLa (orange), TMGLa (black), DeaLa (red), ETALa (pale blue) and BaLa (dark blue).
Although none of these PILs share a similar $\pi^*$ value to that of water in the neat state, there is a significant difference between the dipolarity/polarizability in each IL sample. The aprotic ChoLa and protic BaLa registered the largest and smallest $\pi^*$ values in the series, respectively. In context with the LCST of PNIPAM, the large $\pi^*$ value obtained for ChoLa suggests that increased polarizability effects around the isopropyl side chains of PNIPAM effectively lowers the LCST.

If EaLa and BaLa are compared, a significantly smaller $\pi^*$ value is obtained for BaLa, suggesting that increasing the alkyl chain length of the cation in the PIL leads to a lower polarity value. Similar results were obtained in a study by Bini et al.\(^\text{(461)}\) who measured smaller $\pi^*$ values for methylimidazolium cations containing longer alkyl side chains. However, these are contrary to results reported in a recent study by Lee et al.\(^\text{(436)}\), who showed that an increase in the alkyl chain length of pyrrolidinium-based ionic liquids leads to higher polarity values.

To further understand the implications of hydrogen bonding sites on the cation, with respect to the effect on the $\pi^*$ value, ETALa provides an exceptional example when compared with EaLa. The presence of the terminal hydroxyl group on ETALa leads to a decrease in the $\pi^*$ value as a function of water. Conversely, Zhang et al.\(^\text{(462)}\) showed that the polarity of hydroxyl substituted ionic liquids, in comparison to their corresponding non-hydroxyl ionic liquids, causes an increase in $\pi^*$ value. These findings suggest that aprotic ionic liquids and PILs are very different in terms of their polarity and dipolarity/polarizability effects. Finally, with the exception of EaLa, cations consisting of primary amines tend to register lower $\pi^*$ values than those of...
secondary, tertiary and quaternary amines. Since primary amines carry additional hydrogen bonding sites, this enables them to interact more strongly with the probe dye in terms of coulombic interactions, which reduces the polarity of the PIL.

7.3.4.2 $\beta$ values

The hydrogen bond acceptor (HBA) basicity of a solvent can be measured by the Kamlet-Taft parameter, $\beta$. The $\beta$ parameter describes the basicity of the cation with the solvatochromic probe dye and is said to be largely determined by the nature of the anion\textsuperscript{(435, 463)}, with the cation having a secondary effect. For this study, the $\beta$ values of each PIL as a function of water were measured using $N,N$-diethyl-4-nitroaniline and 4-nitroaniline dyes according to equation 3. It is generally accepted that large $\beta$ values are associated with a strong basic character of the cation towards the probe dyes. Since this method relies on the comparative analysis in the UV-visible spectrum of two closely related dyes; one that is capable of hydrogen bond donation and the other that is not, differences in solvent HBA character can be determined. Again, it is important to note that the values being measured are the result of PIL-dye interactions. The HBA basicity of PILs bearing different anions, as a function of water, is shown in Figure 7.10.
Figure 7.10. Kamlet-Taft $\beta$ parameter for (a) nonfluorinated ILs TeaLa (purple), TeaMs (black), TeaHSO$_4$ (red), TeaH$_2$PO$_4$ (dark blue) and choline dhp (white) and (b) fluorinated PILs TeaBF$_4$ (pale blue), TeaTf (orange) and TeaTfac (green).
The β values for TeaH2PO4, TeaHSO4, TeaMs and TeaLa show very little variation as a function of water, and in all cases the β value increases linearly as the volume fraction of water is reduced. Since TeaLa has a slightly larger β value at ϕ\text{water} = 0.1, this implies that the Tea+ cation has a greater tendency to accept protons from the probe dye when coupled with the La\textsuperscript- anion than with H2PO\textsubscript{4}\textsuperscript{-} and HSO\textsubscript{4}\textsuperscript{-} anions. Additionally, since the ΔpK\texttext{a} is lower for TeaH2PO4 than TeaMs, we find the larger β value in TeaMs very interesting. Choline dhp had the lowest β value out of all ILs investigated here, which is consistent with aprotic ionic liquid values\textsuperscript{(435, 459, 461)}. Shukla et al.\textsuperscript{(433)} proposed that for an ionic liquid to exhibit large β values, the precursor acid must undergo complete proton transfer with the base.

The presence of ionic species (from strong precursor acids) favours large β values and a combination of neutral and ionic species (for weak precursor acids) favours low β values. Since choline dhp has a low β value, it is assumed that the H2PO4\textsuperscript{-} anion is acting as a weak proton donor to its corresponding base pair. The β values for the fluorinated PILs TeaTfac, TeaTf and TeaBF\textsubscript{4} show a much greater spread at ϕ\text{water} >0.5. The largest β value for the PILs in this study was measured for TeaTfac, indicating that in this complex, the Tea\textsuperscript{+} cation strongly accepts protons from the probe dye. Since the β value is large, TeaTfac exists mainly in an ionic state, which indicates that it is the least protonated in the series. It is also observed that as the hydrophobic character of the PIL is increased from Tfac\textsuperscript{-} to BF\textsubscript{4}\textsuperscript{-}, the Tea\textsuperscript{+} cation becomes a weaker hydrogen bond acceptor. Additionally, we measured the HBA for the primary and secondary amines in the PILs EaMs and DeaMs (Figure 7.11).
Here we determined that the primary amine has a lower $\beta$ value across the dilution series. This implies that EaMs is more protonated than the tertiary TeaMs, which is in good agreement with findings from previous work completed by Stoimenovski et al.\(^{(464)}\). In context with PNIPAM, Nayak et al.\(^{(429)}\) discuss that low $\beta$ values are associated with weak anion interactions with the amide of PNIPAM.

TeaMs showed a larger $\beta$ value than TeaH$_2$PO$_4$ and TeaHSO$_4$, which supports the LCST trend observed earlier. Since hydrophobic effects play a secondary role in polymer phase behaviour, the less hydrophobic Tfac$^-$ anion is more likely to interact with PNIPAM because its conjugate acid is interacting strongly with the probe dye. There have been several instances in which ionic liquids designed with the BF$_4^-$ anion have demonstrated low $\beta$ values\(^{(429, 437, 465)}\). The interaction between the BF$_4^-$ anion and
PNIPAM is considered weak because of competitive hydrogen bonding between the cation and anion for the polymer. The low \( \beta \) values measured for \( \text{TeaBF}_4 \) at low water content is evidence of weak anion-PNIPAM interaction. The \( \beta \) values for a series of PILs designed with various cations and the common anion, lactate, is shown in Figure 7.12.

![Figure 7.12. Kamlet-Taft parameter \( \beta \) for ILs with various cations. BaLa (dark blue), TMGLa (black), EaLa (orange), DeaLa (red), ChoLa (green) and ETALa (pale blue).](image)

In all PILs, the \( \beta \) values increased as the volume fraction of water was reduced and the range of these \( \beta \) values at high PIL content was in the relatively narrow range of 0.6-0.9. In comparison to the anions discussed earlier, the cations in this PIL series have a greater tendency to accept protons from the probe dye, since larger \( \beta \) values were measured. When the cation alkyl chain length is increased from EaLa to BaLa, there is a significant increase in \( \beta \) value. BaLa, which measured the lowest \( \pi^* \) value,
has the largest β value. Since the proton NMR analysis of water in the presence of 
BaLa showed a smaller chemical shift in comparison to EaLa, this suggested that 
increasing the alkyl chain length of the cation in the PIL weakened the interaction with 
water and increased the interaction with the probe dye. ChoLa, which measured the 
highest π* value, has one of the lowest β values in this series. This suggests that the 
choline cation has a weaker interaction with the probe dye across the dilution series. 
Also, ETALa drastically lowered the β value in comparison to EaLa, indicating that 
the addition of a hydroxyl group caused ETALa to become a weaker hydrogen bond 
acceptor with the probe dye. ETALa has a very similar β value to PILs bearing 
kosmotropic anions (TeaH2PO4 and TeaHSO4) at φwater = 0.1, and since water showed 
a larger downfield shift in proton NMR, ETALa had a stronger interaction with water 
molecules than the probe dye. This explains why ETALa showed one of the lowest 
phase transition temperatures for PNIPAM.

7.3.4.3 α values

The last Kamlet-Taft parameter describes the hydrogen bond donating (HBD) acidity 
α of the anion. This parameter is largely determined by the nature of the cation, with 
the anion playing a secondary role. The α values of each PIL as a function of water 
were measured using N,N-diethyl-4-nitroaniline and Reichardt’s dye according to 
equation 6. While $E_{r30}$ values are unique for any given solvent, the α value is strongly 
influenced by the value of π* used. For example, the higher the π* value used, the 
smaller the α parameter measured. Due to the nature of the cations in the PILs
investigated, $\alpha$ values could only be measured for four PILs, and these are displayed in Figure 7.13.

![Figure 7.13. Kamlet-Taft $\alpha$ parameter for BaLa (dark blue), ETALa (pale blue), TMGLa (black) and ChoLa (green).](image)

No intramolecular charge transfer band was detected for Reichardt’s dye at $\phi_{\text{water}} > 0.5$ in each PIL. The $\alpha$ values for each IL at $\phi_{\text{water}} = 0.0$ shows a direct relationship between the $\pi^*$ values determined earlier. BaLa, which measured the lowest $\pi^*$ value, has the largest $\alpha$ value, and vice versa for the aprotic ChoLa. Generally, cations that contain additional hydrogen bonding substituents such as hydroxyl groups, tend to have higher $\alpha$ values\(^{(434)}\), which is consistent with the findings for ETALa in this study. The dilution profile of each IL system is unique. The aprotic ChoLa is the only IL in this study that measures a lower $\alpha$ value upon decreasing water content, suggesting that ion pairing between the cation and anion in ChoLa is strong. Nayak \textit{et al.}\(^{(429)}\) also
determined strong ion pairing in BMIMOAc, which was reflected by low $\alpha$ values at high ionic liquid concentration, which may be consistent to aprotic ionic liquids only. TMGLa displays low $\alpha$ values across the dilution series and also suggests strong ion interactions within the ionic liquid. Alternatively, both BaLa and ETALa are expected to have weak ion pairing between anion and cation, since their $\alpha$ values are larger at low water content. The Welton research group\(^{434, 437}\) propose that there are two modes of competition occurring simultaneously between the anion, the cation and the probe dye. They show that as the anion becomes a better hydrogen bond acceptor, the concentration of free non-hydrogen bonded base decreases, which in turn reduces the amount of available base to interact with the probe dye. In the context of PILs in this study, where the acid is common in all systems, it is the cation hydrogen bond donor strength that is the determining factor.

7.3.5 Polarity study of aqueous PIL solutions

From preliminary experiments, Reichardt’s dye was determined to be unsuitable for polarity measurements in the triethylammonium-based PILs, since the intramolecular charge transfer band was not visible due to the formation of the phenoxide moiety of Reichardt’s dye\(^{444, 466}\). Instead, Nile Red was used as the polarity probe since it is soluble over a wide range of solvents and ionic liquids. When dissolved in highly polar solvents, the wavelength of its visible absorption maximum ($\lambda_{\text{max}}$) shifts to longer wavelengths. Table 2 illustrates the molar transition energies, $E_{\text{NR}}$ (kJ mol\(^{-1}\)) of Nile Red in triethylammonium-based PILs, calculated according to equation 7.
Table 7.2. Spectroscopic data of the Nile Red polarity parameter calculated for a series of triethylammonium-based PILs as a function of water at 25 °C.

<table>
<thead>
<tr>
<th>PIL</th>
<th>$\phi_{\text{water}}$</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$E_{\text{NR}}$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TeaHSO$_4$</td>
<td>0.1</td>
<td>595.2</td>
<td>200.9</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>596.5</td>
<td>200.5</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>597.5</td>
<td>200.2</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>598.7</td>
<td>199.8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>599.2</td>
<td>199.6</td>
</tr>
<tr>
<td>TeaH$_2$PO$_4$</td>
<td>0.1</td>
<td>593.2</td>
<td>201.6</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>594.8</td>
<td>201.0</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>595.8</td>
<td>200.7</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>597.2</td>
<td>200.3</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>598.3</td>
<td>199.8</td>
</tr>
<tr>
<td>TeaLa</td>
<td>0.1</td>
<td>563.5</td>
<td>212.4</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>571.1</td>
<td>209.0</td>
</tr>
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The $E_{NR}$ values measured for TeaHSO$_4$ and TeaH$_2$PO$_4$ show similar polarity to that of water (201.7 kJ mol$^{-1}$)$^{(443)}$. When water is added to these two PILs, the variation in polarity is small, which further supports the kosmotropic nature of these anions and the strong water structuring effects, which occur for these PILs. In fact, oxyanions such as H$_2$PO$_4^-$ and HSO$_4^-$ lead to highly polar PILs due to the extensive hydrogen bonding network in the anions$^{(433)}$. The lower polarity values for these two PILs arise from the increasing polarizability of the anions, which is consistent with the $\pi^*$ values determined earlier. The shorter wavelength shift of TeaTfac at $\phi_{\text{water}} = 0.1$ implies that TeaTfac is the least polar PIL in this series. TeaTfac has a polarity which is comparable to low molecular weight alcohols such as ethanol at 218.2 kJ mol$^{-1}$$(443)$.

The polarity of TeaBF$_4$ could not be numerically determined because the main absorption band split into two maxima (Figure 7.14). Because of the long wavelength shift of Nile Red in TeaBF$_4$, this PIL is considered more polar than TeaHSO$_4$, however it has been reported that band splitting of the main absorption band is associated with low solvent polarity, such as is the case with cyclohexane$^{(467)}$. 
Figure 7.14. The UV-Visible absorbance spectra of Nile Red in 90 wt% PIL solutions. TeaTf (green), TeaLa (purple), TeaMs (black), TeaTf (orange), TeaHSO₄ (red), TeaH₂PO₄ (dark blue) and TeaBF₄ (pale blue). Note the band splitting on the TeaBF₄ peak.

The $E_{NR}$ values for the lactate-based PIL series are summarised in Table 7.3. The polarity ($E_T(30)$) and normalized polarity ($E_T^N$) values of Reichardt’s dye in four lactate-based PILs are also included. These were calculated according to equations 4 and 5.

In comparison with the two spectroscopic dyes (Nile Red and Reichardt’s dye), pure ETALa exhibited the lowest $E_{NR}$ value and the highest $E_T^N$ value in the series, which was anticipated due to its strong interaction with water as determined using proton chemical shift information.
Table 7.3. Spectroscopic data of Nile Red and Reichardt’s dye polarity parameters calculated for a series of lactate-based ILs as a function of water at 25 °C.

<table>
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<tr>
<th>PIL</th>
<th>( \phi_{\text{water}} )</th>
<th>( \lambda_{\text{max}} ) (nm)</th>
<th>( E_{\text{NR}} ) (kJ mol(^{-1}))</th>
<th>( E_{T(30)} )</th>
<th>( E_{T}^{N} )</th>
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</table>
Since water has the largest $E_{TN}$ value (1.000)\(^{(436)}\) and the lowest $E_{NR}$ value (201.7 kJ mol\(^{-1}\))\(^{(443)}\) of all solvent systems, ETALa can be considered as a fairly polar PIL, although TeaH\(_2\)PO\(_4\) and TeaHSO\(_4\) are still considerably more polar since their $E_{NR}$ values were equivalent to that of water.

The influence of cation chain length is also noticeable, where an increase in chain length from EaLa to BaLa causes the PIL to be less polar. The longer chain length increases the hydrophobic character of the cation, resulting in a lowering of net polarization and polarity of the PIL, which is in agreement with previous observed trends\(^{(433, 443)}\). In general, the polarity of the cation series is lower than that of the anions, and so the polarity effects in PILs are mainly driven by the anion, since anions are more polarizable and hydrate more strongly with water\(^{(327)}\).
7.4 Conclusion

In this chapter, the LCST of PNIPAM was investigated in a series of PILs designed with various anions and cations. It was found that the triethylammonium PILs, consisting of the kosmotropic anions H$_2$PO$_4^-$ and HSO$_4^-$, reduced the LCST of PNIPAM more effectively than the chaotropic anions. The aprotic IL choline dhp was shown to stabilize the globular form of PNIPAM the greatest. The fluorinated anions enhanced the chaotropic nature of the PILs, with TeaTf and TeaBF$_4$ increasing the LCST of PNIPAM above that of water, as a function of concentration. This increase in LCST using fluorinated PILs is likely due to the presence of an enhanced hydration layer around the polymer, caused by unfavourable water-PIL interactions. The lactate-based PILs showed a less pronounced effect on LCST in comparison to the anion series. However, ETALa and the aprotic ChoLa enhanced the LCST through additional hydrogen bonding sites on the PILs.

The solvent properties of the hydrated PIL solutions were explored through Kamlet-Taft solvatochromic analysis and results indicate that the water-PIL interactions, mainly the hydrogen bond accepting $\beta$ and dipolarity/polarizability $\pi^*$ parameters, are responsible for the observed differences in the PNIPAM LCST trend. Furthermore, the $^1$H NMR chemical shifts of water showed that the kosmotropic anions interact strongly with water, whereas no interaction was observed for the chaotropic anions. The strength of coulombic interactions between anion and cation pairs strongly influences the interaction strength between the ions and the probe dye, where ions that have a greater tendency to hydrogen bond lead to weaker interactions.
with the solute. Overall, the hydrogen network of the hydrated PIL solution is likely the most important solvent property for protein stabilization.
8.1 **General**

In context to the work described in this thesis, protic ionic liquids have provided insight into understanding the complex interactions that comprise amyloid assembly in the context of the Aβ peptide. Ideally, to further the scope of this work, other amyloidogenic proteins such as the prion protein, TTR and α-synuclein, all which have their own unique aggregation mechanism, are worth investigating using ionic liquids.

8.2 **Investigating Aβ16-22 Fibrilization Kinetics in Protic Ionic Liquid Anions and the Hofmeister Series**

Investigations into the self-assembly of Aβ16-22 in PILs showed the importance of anion selection in peptide fibrilization. Kosmotropic anions such as H₂PO₄⁻ and HSO₄⁻ significantly enhanced Aβ16-22 fibrilization *via* a ‘salting out’ scenario whereas chaotropic anions such as Ms⁻ and BF₄⁻ inhibited fibrilization *via* a ‘salting in’ scenario. When analyzed by NMR spectroscopy, critical differences were observed between the proton chemical shift of water molecules between kosmotropic and chaotropic anions, suggesting that Aβ16-22 fibrilization kinetics are driven by competitive hydrogen bonding between the PIL anion and water. Additionally, an N-terminal acetylated analogue of the Aβ16-22 peptide revealed the co-existence of amyloid fibrils and oligomeric spheres in solution.
The accelerated fibrilization of Aβ16-22 holds considerable promise for biotechnological applications since amyloid fibrils are exceptionally strong materials. Furthermore, the ability to rapidly form amyloid fibres should facilitate higher-throughput amyloid inhibitor assay development, including screens for pyroglutamate Aβ fragments and the pathological form of Aβ in AD. This work could be further applied to other amyloid-based diseases by tailoring the ionic liquid to suit the aggregation mechanism of these proteins. The controlled formation of amyloid over the short time scales demonstrated in this study may enable the exploration of fundamental mechanisms of amyloid formation, resulting in an improved understanding of amyloid formation in neurodegeneration.

8.3 Enhanced Aβ Fibrilization in Protic Ionic Liquid Cations

In addition to PIL anions, cations can be further tailored to accelerate or inhibit the fibrilization of Aβ16-22. PIL cations designed with a primary amine enhanced the conversion of Aβ16-22 monomers to amyloid fibrils, almost five-times greater than cations bearing tertiary amines. By substituting the phenylalanine residue located at position 19 with tryptophan, intrinsic fluorescence measurements showed that the five-fold enhancement in fibrilization was due to a direct interaction between the PIL cation and self-assembling peptide. The impact of these primary and tertiary amines were further explored in full-length Aβ, demonstrating greater structural inducing effects at higher PIL concentrations. However, further investigations need to be carried out on the spectroscopic analysis of full-length Aβ and its isoforms, particularly in the presence of higher PIL concentrations where signal interferences are probable.
8.4 Structural Induction and Fibrils Dissolution of Aβ1-40 in Ionic Liquids through the α-Helical Intermediate

In exploring the structure inducing effects in full-length Aβ, low concentrations (<50 wt%) of the chaotropic PIL TeaMs led to the formation of amyloid fibrils with distinctive β-sheet structures. At higher TeaMs concentrations (>60 wt%), α-helical structure was preferred and fibrilization was inhibited. This fibril inhibition occurred with PILs containing the mesylate anion, which promoted solubilization of Aβ1-40 via the formation of intermolecular hydrogen bonds between the solvent and peptide, in a manner similar to helical-inducing solvents such as TFE and HFIP.

Additionally, synthetic Aβ1-40 fibrils which were self-assembled in aqueous TeaMs solution could be dissolved in neat TeaMs, with the solubilization occurring through a β-sheet to α-helix conversion. However, neat TeaMs could not dissolve Aβ1-40 fibrils that were pre-assembled in 100% aqueous conditions, indicating the importance of intermolecular hydrogen bonding in the initial fibril state. Although x-ray scattering techniques were pursued to monitor this change in fibril structure, the results were inconclusive and suggested that further analysis of the microstructure within the ionic liquid region was necessary. Furthermore, the work outlined in chapter 5 was also attempted on the Aβ1-42 peptide, however given the greater aggregation propensity of this peptide and the hydrophobic nature of the ionic liquid conditions used, preliminary data collection was unsuccessful and requires further investigation in the future.
8.5 Crystalline to Amorphous Transition of the Central Alzheimer’s Aβ Dipeptide in Aqueous PIL Solution

In addition to hydrogen bonding, the impact of $\pi-\pi$ interactions were investigated in NH$_2$-FF-COOH, a dipeptide representing the central hydrophobic domain in full-length Aβ. At low EAN concentrations (<0.5 M), NH$_2$-FF-COOH formed large amyloid fibril-like microstructures with a highly ordered hexagonal crystal structure according to x-ray diffraction analysis. At higher EAN concentrations (>1.0 M), NH$_2$-FF-COOH assembled into flat sheets and spheres with no definite crystalline structure. In diluted EAN conditions, ANS binding assays revealed that hydrophobic and $\pi-\pi$ interactions, between peptide monomers dominate, initiating peptide unfolding and assembly into ordered crystalline structures. Although further studies are required to characterize these polymorphic structures and their mechanisms of self-assembly, these results provide further impetus for examining Aβ polymorphic models and their role in disease progression, and also for exploring the application of these materials as novel biomaterials.

8.6 Evaluation of PIL Solvent Parameters on the Phase Behaviour of Poly(N-isopropylacrylamide) (PNIPAM)

Using PNIPAM as a model for peptide folding, the impact of other solvent parameters including acidity, basicity, polarity and polarizability, which are also associated with protein folding in PILs, were measured by exploring the Kamlet-Taft solvatochromic parameters. Similarly to the trend observed for Aβ16-22 fibrilization, PNIPAM was able to achieve lower critical solution temperatures when incubated in kosmotropic
PILs, as opposed to chaotropic PILs. The basicity of the PIL (reflected through the anion), and the polarizability of the anion (reflected through its kosmotropicity), were identified as the key properties for stabilizing the globular state of PNIPAM in PILs and impact protein stabilization and amyloid fibril formation.
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