The copper-amyloid-beta-peptide complex of Alzheimer's disease: affinity, structure, fibril formation and toxicity
Sarell, Claire Jessica

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The Copper-Amyloid-beta-Peptide Complex of Alzheimer’s Disease: Affinity, Structure, Fibril Formation and Toxicity.

Claire Jessica Sarell

A thesis submitted to
THE UNIVERSITY OF LONDON
For the degree of
DOCTOR OF PHILOSOPHY

School of Biological and Chemical Sciences
Queen Mary, University of London
September 2010
I declare that the work presented in this thesis is my own.

Claire Jessica Sarell
Abstract

Senile plaques of Alzheimer’s disease (AD) patients are composed primarily of the amyloid-β-peptide peptide (Aβ), and within these plaques Cu²⁺ ions are found concentrated and directly bound to Aβ. Cu²⁺ homeostasis is severely impaired in AD patients and recent in vivo studies implicate Cu²⁺ in the etiology of AD. However the role of Cu²⁺ ions in AD is currently highly disputed due to the low reported affinity of Aβ for Cu²⁺ (nM and µM), and Cu²⁺ binding to Aβ is thought to result in amorphous aggregation rather than fibril formation. These two aspects, along with the coordination geometry, stoichiometry and toxicity of the Cu²⁺-Aβ complex were investigated in this thesis. In Chapter 3, circular dichroism and fluorescence spectroscopy alongside competitive metal capture show a surprisingly high picomolar affinity for both monomeric and fibrillar Aβ. In Chapter 4 electron paramagnetic resonance was used to study the structure and stoichiometry of the copper-Aβ complex in both monomeric and fibrillar Aβ. Both Aβ forms were able to bind a full stoichiometric complement of Cu²⁺ ions, with identical square planar coordination geometry. Importantly Cu²⁺ ion binding did not disrupt fibril structure. In Chapter 5 of this thesis it is shown that, in contrast to the predominant belief in the AD field, stoichiometric and sub-stoichiometric amounts of Cu²⁺ actually accelerate the rate of fibril formation. Finally, the toxic effects of Aβ and Cu²⁺ were studied in a PC12 clonal cell line. The presence of Cu²⁺ ions were found to enhance Aβ cell toxicity, and substoichiometric concentrations of Cu²⁺ were found to be the most toxic, suggesting that Cu²⁺ induced fibril formation and Cu²⁺ induced toxicity may be linked. Therefore this study finds considerable support for an altered amyloid hypothesis where Cu²⁺ dyshomeostasis has a central role in AD.
This thesis is dedicated to my family
Acknowledgements

The past four years have been predominantly happy ones, and John Viles has played a leading role in them. His support, generosity, patience and knowledge, both as a scientist and as a supervisor, has set the highest of benchmarks for me to judge myself against, and to try my best to emulate. I will miss his sense of humour and anecdotes as much as his advice and ideas.

I’m lucky enough to have parents who see the best in me, and are excited by all my achievements, however minor and insignificant. I can hear their voices in my head, even when they are miles away, encouraging me when I feel like giving up, reminding me to take a break, and always offering help. My sister’s kindness and generosity is something I am incredibly grateful for, and if I am half as successful in both my private and personal life as the three of them then I will be happy. Henry, thank you for asking how my research is going, and then listening and challenging me on it. Your patience, honesty, hugs and sense of humour has made writing up much more enjoyable than it would otherwise have been. You’re kind of a big deal.

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### Abbreviations

The three-letter and single-letter standard amino acid codes are used to describe amino acid residues.

- **1D**: One dimensional
- **2D**: Two dimensional
- **3N10**: 3 Nitrogen 1 Oxygen
- **4N**: 4 Nitrogen
- **8-OHG**: 8-hydroxyguanosine
- **ADDLs**: Aβ-derived diffusible ligands
- **Ac**: Acetylation
- **AD**: Alzheimer’s disease
- **Aβ**: Amyloid-β peptide
- **APP**: Amyloid precursor protein
- **Å**: Angstrom
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<tr>
<td>AICD</td>
<td>APP intracellular domain</td>
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<td>AFU</td>
<td>Arbitrary fluorescence units</td>
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<td>Atomic force microscopy</td>
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<tr>
<td>cm</td>
<td>Centimetre</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>c</td>
<td>Concentration</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>D₂O</td>
<td>Deuterated water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>et al.</td>
<td>et alia</td>
</tr>
<tr>
<td>fAD</td>
<td>Familial Alzheimer’s disease</td>
</tr>
<tr>
<td>fM</td>
<td>Femtomolar</td>
</tr>
<tr>
<td>F-moc</td>
<td>N-(9-fluorenyl)methoxycarbonyl</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HEPES</td>
<td>(N-[2-hydroxyethyl] piperazine-N’-[2 ethanesulfonic acid])</td>
</tr>
<tr>
<td>·OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>L-</td>
<td>Left-</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mK</td>
<td>Millikaysers</td>
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<td>Millilitre</td>
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<td>Minute</td>
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<td>Molar</td>
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<td>Nanometre</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl D-aspartate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl D-aspartate receptor</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>l</td>
<td>Path length (in cm)</td>
</tr>
<tr>
<td>pM</td>
<td>Picomolar</td>
</tr>
<tr>
<td>PS</td>
<td>Presenilin</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin-1</td>
</tr>
<tr>
<td>PS2</td>
<td>Presenilin-2</td>
</tr>
<tr>
<td>R-</td>
<td>Right-</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>O$_2$•⁻</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total correlation spectroscopy</td>
</tr>
<tr>
<td>TFE</td>
<td>Trifluoroethanol</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>Ultraviolet/Visible</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight to volume</td>
</tr>
<tr>
<td>δ</td>
<td>Chemical shift</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
</tbody>
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1. Introduction
1.1. **General Introduction to Alzheimer’s Disease**

Alzheimer’s disease (AD) is a form of senile dementia, and is thought to affect 42% of the UK population, through either having the disease or knowing a close friend or family member with the condition (Alzheimer's Research Trust / YouGov 2008). Furthermore a new case of dementia occurs in England and Wales every 3.2 minutes (Matthews, et al. 2005). AD is currently incurable, which puts a financial and emotional strain on those caring for the patient. In people aged 60 or over, dementia contributed 11.2% of all years lived with disability, more than stroke (9.5%), cardiovascular disease (5.0%) or all cancers (2.4%) (World Health Organization 2003). AD is ultimately fatal, characterised by increasing destruction of the neuronal tissues in the brain. Symptoms include jealousy, memory lapses, paranoia, loss of reasoning powers, incomprehension and stupor.

In Figure 1.1 a time line of some selected developments in AD is outlined. There are many important findings that have been omitted, but the key developments considered to be relevant to this thesis are shown. The first event is the observation in 1906 by Alois Alzheimer of the neurofibrillary tangles and amyloid plaques in the brain of a patient who had displayed the symptoms of dementia (Alzheimer 1907). Strikingly, isolation of the major protein in the AD plaques, amyloid-beta-peptide (Aβ), did not occur until 1984 (Glenner, et al. 1984). Another major event is the development of the amyloid cascade hypothesis in 1992 (Hardy, et al. 1992), which hypothesises that formation of fibres and plaques by Aβ is the key event in AD (discussed in more detail later in this chapter). Since 1985 there have been large strides forward in understanding the disease, but a therapeutic that halts or reverses progression of the disease remains elusive.
## 1.2. Amyloid-beta-Peptide

The two fundamental characteristics of the AD brain are extracellular amyloid plaques and intracellular neurofibrillary tangles. ~90% of the amyloid plaques consist of the fibrillar form of Aβ (Masters, et al. 1985). The remaining 10% is a variety of other components including membrane lipids from degenerated axons, metal ions, and traces of other components from the interstitium (Atwood, et al. 2002). *In vivo* Aβ can be between 39 and 43 residues long, with Aβ(1-40) and Aβ(1-...
42) being the two most biologically important forms. Aβ(1-40) is also more common, with Aβ(1-42) only ~10% as abundant as Aβ(1-40).

The sequence of Aβ(1-43) is shown below:

```
  5  10  15  20  25  30  35  40
DAEFR   HDSGY   EVHHQ   KLVFF   AEDVG   SNKGA   IIGLM   VGGVV   IAT
```

The first 15 residues are mainly hydrophilic, and contain His6, His13 and His14 (in bold) which are important for Cu$^{2+}$ chelation. The propensity of the amino acids in Aβ(1-43) to form fibrils can be predicted by the TANGO algorithm (Figure 1.2) (Fernandez-Escamilla, et al. 2004). It is evident that residues 17-21 (the central hydrophobic cluster) and residues 30-42 in the C-terminal tail have a high propensity for fibril formation.

Figure 1.2 also shows that Aβ(1-42) has two more amino acids with a propensity to form β-fibrils than Aβ(1-40), illustrating why Aβ(1-40) is more soluble and forms fibrils slower than Aβ(1-42). Aβ(1-42) is the more amyloidogenic form, and is the main form in amyloid plaques (Suzuki, et al. 1994). Consequently, an increase in the ratio of Aβ(1-42):Aβ(1-40) is thought to be a key event in AD development. The 43rd residue of Aβ(1-43) is threonine, which has a low aggregation score, and Aβ(1-43) is not thought to be a predominant species in vivo, thus it is not considered further in this thesis.

### 1.2.1. Location and Function of Aβ

Aβ is constitutively anabolised and catabolised in the brain throughout life in all humans. It is present in the cerebrospinal fluid (CSF) and plasma in humans with a typical concentration of 5 nM (Vigo-Pelfrey, et al. 1993). Aβ is predominately generated in the golgi bodies through the amyloidogenic processing of the amyloid precursor protein (APP), see Section 1.2.3, then trafficked to the cell membrane (Gasparini, et al. 2001).
Chapter 1. Introduction

Figure 1.2. TANGO output for the propensity of residues within Aβ to form β-fibrils. Input parameters were the sequence of Aβ(1-43), pH 7.4, 37 °C, and an ionic strength of 160 mM. The algorithm assumes that in β-fibrils the nucleating regions will be fully buried and tend to satisfy their hydrogen-bonding potential. Three energy contributions are assessed by the algorithm: a residue-specific cost in conformational entropy for fixing that residue in a beta-strand conformation and side chain-side chain interactions of residue i with residues at positions i+1 and i+2. (Fernandez-Escamilla et al. 2004).

The normal physiological role of Aβ is not clear. It seems unlikely that Aβ only has detrimental functions as Aβ production occurs throughout the cell. Interestingly, the application of Aβ to cortical neurons promoted endocytosis of NMDA (N-methyl-D-aspartic) receptors (Snyder, et al. 2005). These receptors occur at the post-synaptic membrane at glutamatergic synapses, and are vulnerable to excitotoxicity in AD. Excitotoxicity can occur when NMDA receptors are overactivated, such as by a pathological excess of the neurotransmitter glutamate. Over-activation results in high levels of Ca\(^{2+}\) ions entering the cell which activates a number of enzymes that go on to damage cell structures. Thus Aβ’s normal function may be to depress synaptic function, protecting against excitotoxicity.
Aβ is also part of a range of signalling cascades (Lee, et al. 2002) and able to induce pro-inflammatory activities (Paris, et al. 1999). It has also been suggested to have a role as a cellular antioxidant or sacrificial quencher of reactive oxygen species (Nadal, et al. 2008, Teng, et al. 2005). Very recently Aβ has also been proposed to be an anti-microbial peptide, which may normally function in the innate immune system protecting against a range of microorganisms. AD brain homogenates had significantly higher antimicrobial activity than aged matched non-AD samples (Soscia, et al. 2010).

1.2.2. Amyloid Precursor Protein

Amyloid Precursor Protein (APP) is a transmembrane protein encoded on chromosome 21 in humans (Kang, et al. 1987). APP$_{695}$ is the principal neuronal form, two other forms (APP$_{751}$ and APP$_{770}$) are more common in other cell types (Kang, et al. 1987, Kitaguchi, et al. 1988, Ponte, et al. 1988, Tanzi, et al. 1988). APP has a short cytoplasmic region and a large extracellular domain. The Aβ sequence is within residues 672 to 711 (Aβ(1-40)) or 713 (Aβ(1-42)) of APP$_{770}$. This section of APP spans the 28 residues of the extracellular domain and the 12-15 residues of the transmembrane domains. APP contains two copper binding sites, one within the Aβ domain which is covered in more detail in Chapter 4, and a second (the APP copper binding domain (CuBD)) between residues 124-189 of APP. The CuBD binds Cu$^{2+}$ with a nanomolar affinity (Hesse, et al. 1994).

Multiple roles for APP have been suggested, broadly falling into three categories:

- acting as a contact receptor in its transmembrane form, with a role in forming functional synapses through cell adhesion
- a trophic role in its secreted form after cleavage, promoting cell proliferation and motility
- a role in metal ion homeostasis. APP shares structural homology to copper chaperones and may function as a Cu$^{+}$ binding neuronal metallochaperone (Bayer 2006) or as a cell surface metalloreductase, as APP is capable of reducing Cu$^{2+}$ to Cu$^{+}$ (Barnham, et al. 2003). APP knock out mice show an
increase in copper levels in the brain (White, et al. 1999) whereas overexpression of APP was reported to result in significantly reduced copper levels in transgenic mice brains (Maynard, et al. 2002).

1.2.3. APP Trafficking and Processing

APP trafficking is shown in Figure 1.3. APP matures through the constitutive secretory pathway, during which it undergoes N-glycosylation (glycans attached to a nitrogen in asparagine or arginine side chains) in the endoplasmic reticulum and O-glycosylation (glycans attached to the hydroxy oxygen of serine, threonine and tyrosine side-chains) in the golgi bodies. Upon exiting the golgi body, APP is trafficked to the plasma membrane. Once APP reaches the cell surface, it is internalised and either trafficked via the endocytic/recycling pathway back to the
cell surface, or degraded in the lysosome (Thinakaran, et al. 2008). APP trafficking is likely to vary depending on the cell type, and the intracellular organelles/transport vesicles involved in APP trafficking in neurons require further characterisation.

During trafficking APP can undergo one of two processing events. The non-amyloidogenic pathway, without the production of Aβ, is the sequential processing of APP by α and γ-secretases and occurs mainly at the cell surface (Sisodia 1992). The amyloidogenic pathway is the cleavage of APP by β-secretase then γ-secretase, resulting in Aβ secretion and occurs during trafficking through the endocytic organelles (Koo, et al. 1994).

**Non-amyloidogenic pathway**

In the non-amyloidogenic pathway APP is cleaved at the cell membrane by α-secretase (Sisodia 1992). The identity of α-secretase is most likely to be a member of the ADAM (*a d*isintegrin *a nd* *m*etalloprotease) family, probably ADAM10 and/or ADAM17 (Parvathy, et al. 1998). The α-secretase site in APP is between residues 687 and 688 of APP (Lys16 and Leu17 of the Aβ sequence), 12 amino acids upstream from the extracellular side of the membrane (see Figure 1.4).

![Figure 1.4. The region of APP primary structure region which includes the sequence of amyloid-beta-peptide (Aβ). Residues in Aβ are enlarged and in bold, and residues residing in the transmembrane region of the neuronal cell are in italics. Secretase cleavage sites are in red for α-secretase, blue for β-secretase and green for γ-secretase. Major cleavage sites are shown with unbroken arrows, and minor cleavage sites are shown with broken arrows (adapted from (Barrow 1999)).](image-url)
\(\alpha\)-secretase cleavage results in the release of an extracellular domain (sAPP\(\alpha\)) (Parvathy, et al. 1999) and leaves a membrane tethered \(\alpha\)-C-terminal fragment (CTF\(\alpha\)) (Allinson, et al. 2003). sAPP\(\alpha\) may be involved in cell differentiation and proliferation (Caille, et al. 2004, Kwak, et al. 2006) CTF\(\alpha\) is then cleaved within the transmembrane domain by \(\gamma\)-secretase to release the soluble 3kDa N-terminal fragment p3 extracellularly and APP intracellular domain (AICD) into the cytoplasm. AICD may be involved in nuclear signalling via transcriptional regulation (Cao, et al. 2001) while p3’s function is yet to be elucidated, but it has been shown to be non-toxic to neuronal cells (Minogue, et al. 2009). The non-amyloidogenic pathway is shown in Figure 1.5a.

\(\gamma\)-secretase is a multimeric membrane-bound protease complex composed of four essential subunits: 1) presenilin-1 or –2 (PS1 and PS2) (De Strooper, et al. 1998, Thinakaran, et al. 1996, Wolfe, et al. 1999), 2) nicastrin (Yu, et al. 2000), 3) APH-1 and 4) PEN-2 (Francis, et al. 2002). The presenilin subunits are aspartyl proteases which provide the active core of the \(\gamma\)-secretase complex. The \(\gamma\)-secretase complex is found in multiple cellular locations including the ER, golgi, endosomes and plasma membrane and has a role in both APP processing pathways.

**Amyloidogenic pathway**

In the amyloidogenic pathway (Figure 1.5b) APP is cleaved by \(\beta\)-secretase between residues 671 and 672 of APP to leave the C-terminal fragment (CTF\(\beta\)) tethered to the membrane and release a soluble N-terminal fragment (sAPP\(\beta\)). CTF\(\beta\) is cleaved by the \(\gamma\)-secretase complex to release A\(\beta\). Importantly \(\gamma\)-secretase cleaves at multiple sites within the transmembrane domain of APP, resulting in A\(\beta\) being between 38 to 43 residues in length. The position of the \(\gamma\)-secretase cleavage site is of key importance in determining the ratio of A\(\beta\)(1-40) to A\(\beta\)(1-42). The \(\gamma\)-secretase is also a therapeutic target, discussed further in Section 1.9.

The major \(\beta\)-secretase in neurons is BACE1 (beta-site APP-cleaving enzyme). BACE1 is a 501 amino acid, transmembrane aspartyl protease (Hussain, et al. 1999, Vassar, et al. 1999) which acts on APP intracellularly during the secretory/endocytic steps of APP trafficking (Koo and Squazzo 1994) (see Figure 1.3). BACE1 contains
a dileucine motif (LL) which causes BACE1 to be internalised to the endosomes where it can co-localise with APP (Pastorino, et al. 2002). Deletion of the β-secretase gene in mice resulted in only minor behavioural changes and the mice remained viable and fertile (Harrison, et al. 2003, Roberds, et al. 2001). Absence of BACE1 will prevent production of Aβ (Luo, et al. 2001), making it a highly attractive therapeutic target, which is discussed in Section 1.9.

**Figure 1.5. Processing of APP.** (a) The non-amyloidogenic pathway with sequential processing of APP by membrane bound α and γ-secretases, does not release Aβ. (b) The amyloidogenic pathway of APP is the β and γ-secretase cleavage of APP, generating Aβ. Adapted from McDowall 2010.

### 1.2.4. Influences on the APP Processing Pathways in Sporadic AD

Membrane properties could be key in affecting APP processing. Aβ generation in a neuronal cell line was shown to be dependent on lipid rafts (Cordy, et al. 2003). Lipid rafts are important in membrane signalling and trafficking and are identified by their high levels of sphingolipids, cholesterol and saturated fatty acids. The key proteins in the amyloidogenic pathway, APP, BACE and the presenilins, all co-localise to the rafts, (Riddell, et al. 2001) whereas the likely candidate for the α-secretase, ADAM10, does not (Kojro, et al. 2001).
Decreased intracellular copper levels occur in AD, and this decrease also promotes amyloidogenic processing of APP (Cater, et al. 2008). BACE1 binds a single Cu\(^+\) ion with high affinity, coordinating the ion with cysteine residues from the C-terminal (Angeletti, et al. 2005). Thus APP processing could be altered through disrupted BACE1-Cu\(^+\) binding, potentially increasing BACE1 activity or BACE1 association with APP, resulting in the amyloidogenic pathway being favoured. Copper deficiency also leads to decreased activity of the antioxidant protein SOD1, resulting in oxidative stress (Paynter, et al. 1979). Oxidative stress appears to favour the amyloidogenic pathway by increasing the expression, protein levels and activity of BACE1 and γ-secretase (Tabaton, et al. 2007, Tamagno, et al. 2002).

1.2.5. Aβ Catabolism
Factors that affect APP processing and Aβ anabolism have been intensely studied, however in sporadic AD, Aβ anabolism does not appear to change, yet Aβ(1-42) levels accumulate in both normal and AD brains from the age of 40 (Funato, et al. 1998). Thus attention has focused on changes in Aβ clearance from the brain. Aβ has a relatively short half life in the brain, ~2 hrs in young mice. However in mice with amyloid deposits Aβ’s half-life is doubled, suggesting deterioration of Aβ catabolism could have an important role in AD (Cirrito, et al. 2003).

Neprilysin is a key enzyme in Aβ catabolism, degrading Aβ at or near the synapses, and also inside vesicles during axonal transport (Huang, et al. 2006). Importantly there is a 20-40 % reduction in neprilysin levels in the brains of aged mice, and disruption of the neprilysin gene causes an accumulation of Aβ in the mouse brain (Iwata, et al. 2005). Thus a decrease in neprilysin levels may be partly responsible for Aβ accumulation and onset of AD.

1.2.6. Genetics of Familial AD
Familial Alzheimer’s disease (fAD) results in an early onset of AD, usually in patients only aged 45-60 years old. FAD only accounts for a small number of total AD cases (~5 %). However, it has proved instrumental in understanding the risk factors in AD and in giving Aβ a central role in the pathogenesis of AD.
A summary of some of the key mutations that cause fAD are shown in Table 1.1. Down’s syndrome arises from trisomy of chromosome 21, which is the chromosome the APP gene is located on. The extra copy results in an increase in Aβ and the early onset of AD. Mutations within APP that lead to fAD are predominantly located at, or near, the α, β and γ cleavage sites, resulting in the amyloidogenic processing pathway being favoured, and leading to the production of more Aβ. The arctic mutation does not affect APP processing but results in much more aggressive amyloidosis (Cheng, et al. 2004). Additionally the Flemish, and Arctic mutations of APP also make Aβ more resistant to neprilysin degradation (Tsubuki, et al. 2003).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Biochemical cause</th>
<th>Molecular effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down’s syndrome (trisomy of chromosome 21)</td>
<td>More APP production</td>
<td>More Aβ</td>
</tr>
<tr>
<td>APP K670N/ M671L (Swedish)</td>
<td>Potentiation of β-secretase cleavage</td>
<td>More Aβ</td>
</tr>
<tr>
<td>APP A692G (Flemish)</td>
<td>Inhibition of α-secretase cleavage</td>
<td>More Aβ</td>
</tr>
<tr>
<td>APP E693G (Arctic)</td>
<td>More amyloidogenic</td>
<td>Forms fibrils faster</td>
</tr>
<tr>
<td>APP I716V (Florida)</td>
<td>Alteration of site of γ-secretase cleavage</td>
<td>More Aβ(1-42)</td>
</tr>
<tr>
<td>APP V717I (London)</td>
<td>Alteration of site of γ-secretase cleavage</td>
<td>More Aβ(1-42)</td>
</tr>
<tr>
<td>PS1 and PS2 mutations</td>
<td>Alteration of APP processing</td>
<td>More Aβ(1-42)</td>
</tr>
</tbody>
</table>

Table 1.1. Summary of mutations which result in early onset Alzheimer’s disease.
The site of the mutation in APP770 is followed by the commonly used name for the mutation in parentheses. Adapted from (Hardy 1997).

To date, no cases of familial AD caused by mutations or dysfunction of β-secretase, or lack of α-secretase have been found. Instead mutations in presenilin 1 (PS1) and presenilin 2 (PS2) genes (part of the γ-secretase complex) account for the majority of fAD cases. More than 150 mutations in PS1 and ~10 mutations in PS2 have been
identified (Tabaton and Tamagno 2007). Mutations of PS shift the preferred site of \( \gamma \)-secretase cleavage from residue 40 to 42 of A\( \beta \) (Scheuner, et al. 1996, Thinakaran, et al. 1996) thus increasing the A\( \beta \)(1-42):A\( \beta \)(1-40) ratio.

**1.2.7. Genetics of Sporadic AD**

Although ageing is still the only known definite risk factor in sporadic AD, there are so-called AD “susceptibility” genes which, in conjunction with environmental factors, increase susceptibility to AD. Indeed after advanced age, having a parent with sporadic AD (especially if it is the mother) is the most significant risk factor for developing AD (Mosconi, et al. 2010).

The apolipoprotein E (ApoE) gene on chromosome 19 has been consistently implicated in increasing susceptibility to sporadic AD. ApoE is a major apolipoprotein and a cholesterol carrier in the brain. In humans, the ApoE gene exists as three different polymorphic alleles: \( \varepsilon 2 \), \( \varepsilon 3 \) and \( \varepsilon 4 \). The \( \varepsilon 3 \) allele is the most common allele, prevalent in ~ 60 % in the population (Mahley 1988). Interestingly, the \( \varepsilon 4 \) allele is only present in ~ 15 % of the general population, but is present in ~ 40 % in AD patients. The \( \varepsilon 4 \) allele results in a 2.3-3 fold increased risk of AD compared to the \( \varepsilon 3 \) allele, whereas the \( \varepsilon 2 \) allele actually decreases the risk of AD (Farrer, et al. 1997). The \( \varepsilon 4 \) allele is thought to increase the risk of AD through higher plasma cholesterol levels, resulting in enhanced A\( \beta \) deposition and formation of plaques (Shobab, et al. 2005). However as only ~ 40 % of AD sufferers carry the \( \varepsilon 4 \) allele its presence can not be necessary to cause the disease, with multiple other genes increasing susceptibility for AD.

Finally there are also environmental factors which are thought to increase the likelihood of developing AD, including traumatic brain injury (Roberts, et al. 1994), inflammation (Akiyama, et al. 2000) and occupational exposure to pesticides (Baldi, et al. 2003) and high blood pressure and high cholesterol (Kivipelto, et al. 2001). Alternatively the consumption of unsaturated fatty acids and omega-3 polyunsaturated fatty acids might help to reduce the risk of AD (Morris, et al. 2003).
1.3. The Amyloid Cascade Hypothesis

Genetic evidence strongly supports Aβ as the causative agent of Alzheimer’s disease but the role of Aβ in the progression of AD is still unclear. The best hypothesis for the key events in the development of AD is the amyloid cascade hypothesis (Hardy and Higgins 1992), shown in Figure 1.6. In this hypothesis genetic and environmental factors result in an increase in the ratio of Aβ(1-42):Aβ(1-40) resulting in Aβ fibril formation and synaptic injury. Downstream of Aβ deposition altered kinase/phosphatase activities occur, resulting in hyperphosphorylation of the protein tau. Finally oxidative injury and neuronal dysfunction results in widespread cell death and dementia (Hardy 1997).

There are some holes in the amyloid cascade hypothesis. Formation of Aβ plaques is a key event in the hypothesis but the number and presence of amyloid deposits do not correlate with AD severity, and neurodegeneration sometimes precedes plaque formation. Likewise, immunisation of AD patients with Aβ(1-42) was found to clear amyloid plaques from the brain but not prevent progression of neurodegeneration (Holmes, et al. 2008). However, this can be explained by the “toxic oligomer” hypothesis (see Section 1.7) where it is small, diffusible, oligomers of Aβ which cause toxicity, not fully formed plaques. A second problem with the hypothesis, which is still unexplained, is that APP and Aβ are present and ubiquitous in all humans throughout their lives, but amyloid deposits are focal (mainly near synapses and the cerebrovascular lamina media) and temporal (mainly occurring in people over 65). Thus it is still a mystery as to why fibril formation occurs, and why some people develop sporadic Alzheimer’s disease and others do not. One alternative to the amyloid cascade hypothesis is that the damage observed in AD brains is caused by intracellular neurofibrillary tangles (NFTs) composed of tau, not Aβ.
Chapter 1. Introduction

Missense mutations in \textit{APP}, \textit{PS1}, or \textit{PS2} genes (fAD)
Ageing, plus genetic and environmental risk factors (sporadic)

\begin{itemize}
\item Increased $A\beta(1-42)$ production and accumulation
\item $A\beta(1-42)$ oligomerisation and deposition as diffuse plaques
\item Subtle effects of $A\beta$ oligomers on synapses
\item Microglial and astrocytic activation (complement factors, cytokines etc.)
\item Progressive synaptic and neuritic injury
\item Altered neuronal ionic homeostasis; oxidative injury
\item Altered kinase/phosphatase activities $\rightarrow$ tangles
\item Widespread neuronal/neuritic dysfunction and cell death with transmitter deficits
\end{itemize}

\textbf{DEMENTIA}

\textit{Figure 1.6. Amyloid cascade hypothesis} (Hardy \textit{et al.} 1992).

1.4. \textbf{Tau}

Tau is a microtubule binding protein whose primary function is to stabilise microtubules. Tau is not the focus of this thesis and so will only be mentioned briefly. For a more in-depth review please see (Avila 2006) or (Ballatore, et al.
The microtubule-binding ability of tau is post-translationally regulated, primarily by serine/threonine-directed phosphorylation. This phosphorylation occurs under normal, non-disease conditions at a small number of potential phosphate-acceptor amino acid residues and effectively modulates the binding affinity of tau for the microtubules.

However during neurodegeneration nearly all these residues become phosphorylated— the “hyperphosphorylation” of tau. This results in an abnormal loss of binding of tau to the microtubules and a break-down in the equilibrium between free tau and microtubule associated tau. The increased cytosolic concentrations of free tau leads to aggregation and fibrillisation resulting in the formation of NFTs, composed of paired helical filaments. NFTs can sequester normal tau and become physical obstacles to the transport of vesicles and other cargo. Thus tau has both a loss of function (no longer stabilising microtubules) and also a toxic gain of function (formations of NFTs). Because of the prominent accumulation of NFTs, AD is considered a tauopathy, along with a range of other frontotemporal dementias.

1.4.1. Tau vs Aβ

Initially there was a debate as to whether Aβ or tau was responsible for AD onset and progression. “Tauists” point out that neuron loss and neurodegeneration could be caused by the NFTs, as the tau-mediated collapse of the microtubules causes substantial damage and numerous toxic effects, including loss of signalling and the death of the cell. However, mutations in the gene encoding tau cause frontotemporal dementia with parkinsonism, and NFTs but no amyloid plaques (Spillantini, et al. 1998). In contrast, Aβ(1-42) accumulation subsequently induces tau aggregation, phosphorylation, neuronal death and dementia, suggesting tau aggregation is downstream of Aβ fibril formation (Price, et al. 1999). These findings, in addition to the genetic evidence in Section 1.2.6, are strongly in favour of Aβ as the disease-causing agent, and the debate is essentially settled, with a win for the “baptists” (β-amyloid proteins supporters). However it is still not clear how Aβ induces neurodegeneration, and it is still possible that a common factor might induce both plaques and tangles.
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1.5. Aβ Structure

1.5.1. Monomeric Aβ Structure

The starting population of freshly solubilised Aβ in vitro is a rapidly exchanging pool of Aβ monomers and low order oligomers, with transiently different secondary structure (Bitan, et al. 2003). The protein backbone undergoes inherent fluctuations, searching through possible conformations available to the polypeptide chain (Dinner, et al. 2000). There are different possibilities for the conformational changes that Aβ undergoes during fibril formation, such as random coil (RC) → α-helix, RC → β-strand and α-helix → β strand transitions. Whether fibril formation begins with Aβ as predominantly unstructured (random coil) or with α-helical structure in vitro depends on experimental conditions. Solution NMR performed in the absence of organic solvents such as trifluoroethanol or sodium dodecyl sulfate have shown that monomers of both Aβ(1-40) and Aβ(1-42) are devoid of α-helical or β sheet structure (Zhang, et al. 2000). However in the presence of ‘membrane-mimics’ such as organic solvents, both forms of Aβ are predominantly α-helical (Barrow, et al. 1992, Soto, et al. 1995). In vivo it is possible that the protein undergoes a number of changes in secondary structure during fibril formation. In fact differences in the structure of low molecular weight Aβ species in freshly solubilised Aβ have been suggested as an explanation for the observed differences in fibril morphology (Goldsbury, et al. 2005), described in Section 1.5.3.

1.5.2. Fibrillar Aβ Structure

The secondary structure of Aβ fibrils, like all amyloid proteins, is composed of β-sheet. This is due to β-sheets being the most thermodynamically stable secondary structure for oligomers (Perczel, et al. 2007). X-ray fibre diffraction patterns can be used to detect the structure of fibrils and significantly, they indicate that β-strands align perpendicularly to the fibril axis, and this ‘cross-β motif’ is a defining feature of all fibres (Serpell 1999, Sunde, et al. 1997). Figure 1.7a shows an X-ray diffraction pattern of fibrils.
Figure 1.7. X-ray diffraction of fibrils. (a) Characteristic X-ray diffraction pattern of fibrils. (b) Diagram of characteristic meridional signals at 4.7-4.8 Å and the equatorial signal at 10.7 Å. The diagram in (c) shows the ~4.8 Å signal arises from the spacing between hydrogen bonded β-strands within a β-sheet and the equatorial signal at 10.7 Å arises from the intersheet spacing. Taken from (Rambaran et al. 2008) and (Serpell 2000).

The diffraction pattern includes a strong meridional signal at 4.8 Å, arising from the spacing between hydrogen bonded β-strands within a β-sheet. The equatorial signal at 10.7 Å arises from the intersheet spacing, emphasised in Figure 1.7b and c (Makin, et al. 2005). The cross-β nature of the fibres is important as it greatly restricts the possible individual states of the individual β-strands.

Due to the large size, heterogeneous morphology, and poor solubility of protofibrils and amyloid fibrils, it is very difficult to obtain high-resolution structure determination of these species. Solid state NMR has proved to be vital in understanding the tertiary molecular structures of fibrils. In the Tycko model shown in Figure 1.8a (Tycko 2006), the structure of a protofilament is shown. Protofilaments are the underlying subunits of fibres, which also have a fibrillar morphology and laterally associate to form fibres (Serpell, et al. 2000).
Chapter 1. Introduction

Figure 1.8. Structural model for the formation of a protofilament. (a) Two peptide molecules, with residues 1-8 not shown due to their lack of structure. Residues 10-22 and 30-40 have β-strand conformation and form two separate in-register parallel β-sheets. Blue arrows show side-chain-side-chain interactions and side-chain-backbone interactions (b) Cartoon representation of the four layered β-sheet fibril. Each cross-β unit is a double-layered structure, with parallel β-sheets formed by residues 12–21 (red arrows) and 30–40 (blue arrows) (Tycko 2006).

In Tycko’s model residues 1-8 are disordered, 10-22 and 30-40 have β-strand conformations and form two in-register parallel β-sheets, and these two β-strand segments are connected by a turn involving residues 23-29. Intersheet contacts occur between odd-numbered side chains in the N-terminal β-strand and even numbered side chains in the C-terminal β-strand. A salt bridge between Asp23 and Lys28 stabilises the two β-strands. The amino acid side chains extend perpendicularly to
the fibril axis and determine important properties of the fibrils including packing distance between sheets and contact surfaces of the protofilaments (Meinhardt, et al. 2009).

There are some small differences between Tycko’s model in Figure 1.8 and other models of Aβ fibrils, for example Antzutkin et al’s study on Aβ(10-35) resulted in a model where residues 1-9 are structurally disordered but 10-22 and 30-35 form β-strands, with residues 23-29 forming a loop between the sheets (Antzutkin, et al. 2002). However small differences in experimentally derived structures of fibres are not surprising in light of findings that the molecular structure of fibrils can be affected by the fibril growth conditions (see Section 1.5.3).

Hydrogen/deuterium (H/D) exchange techniques can provide important information about structural rearrangements that occur during fibril formation. H/D exchange shows the pattern and extent of hydrogen bonding within the fibril and suggests which of the backbone amide protons are engaged in H-bonds and the proportion of total backbone amides that are engaged. Core portions of β-sheet are more highly protected than loop and random coil regions. Kheterpal et al found that all of the backbone amide hydrogens of monomeric Aβ undergo rapid H/D exchange, indicating the absence of protective structure. In contrast, in Aβ fibrils at least 50 % of the backbone residues were resistant to exchange, suggesting a highly protected, rigid core structure (Kheterpal, et al. 2000). Additionally H/D exchange has shown relatively free exchange within the fibril of the amide protons from residues 1-14 suggesting that this portion of the peptide is not involved in the hydrogen-bonded core. Residues 15-23 and residues 28-35 showed protective structure, consistent with the Aβ peptide folding back on itself to form an enclosed structure (Whittemore, et al. 2005). This is similar to the structure in Figure 1.8 suggested by Tycko, although with slightly different stretches of amino acids proposed to take part in the β-strand parts of the fibril and the β-turn.

Overall morphology of the fibrils is commonly visualised using atomic force microscopy (AFM) and electron microscopy (EM). Cryo-EM has been particularly successful at determining fibril structure at high resolution. Figure 1.9 shows the structure of one morphology of an Aβ(1-40) fibre.
Figure. 1.9. A 3D reconstruction of an Aβ(1-40) fibril imaged by cryo-EM. (A) Ice-embedded Aβ(1-40) fibril cropped from a raw electron micrograph, displaying the distance of one helical pitch. (B) Projection of the 3D fibril reconstruction. (C) Side-view surface rendering of the fibril reconstruction (Sachse et al. 2008).

The averaged 3D fibril structure shows a width of 19 nm and a helical pitch (twist) of 285 nm. The fibril consists of two protofilaments and a local twofold symmetry within each region suggests that pairs of β-sheets are formed from equivalent parts of two Aβ(1-40) peptides contained in each protofilament (Sachse, et al. 2008). The pairing between the β-sheets occurs via tightly packed interfaces, and this supports the possibility of a dry steric zipper within the fibrils. The presence of a dry steric zipper was shown by an x-ray microcrystallography study performed on fragments of fibril forming peptides (including Aβ). It is formed by two closely interdigitated β-sheets, with the side chains intermeshing with close complementarity (Sawaya, et al. 2007). The presence of a steric zipper was found to be common to 30 other segments from fibril-forming proteins that form amyloid-like fibrils, adding further evidence to the idea that amyloid fibrils share a common architecture.
Once protofilaments have formed, they can laterally associate to form more mature amyloid fibrils. It is not clear how many protofibrils form a fibre. An X-ray fibre diffraction study suggested that thin sections of amyloid fibrils formed from fragments of Aβ show cross-sections containing five or six protofilaments (Kirschner, et al. 1987). However an alternative study using cryo-EM suggests that only one or two protofilaments make up a fibre (Schmidt, et al. 2009). It is likely that the variation is due to different fibril growth conditions, as discussed in Section 1.5.3. A summary of the formation of fibrils is shown in Figure 1.10.

Figure 1.10. Aβ fibril assembly. Different structures which form along the pathways of fibril formation in Alzheimer’s Disease, beginning with the Aβ peptide, which oligomerises to form β sheets, then protofilaments, and finally fibrils (Serpell 2000).

Once formed, the fibrils can then associate further to form the plaques observed in AD brains (Figure 1.11a). It is not clear whether there is an underlying structure to the plaques, however a promising suggestion is that they form spherulites. These micrometre-sized, roughly spherical structures are composed of ordered arrays of amyloid fibrils in radial arrangements which characteristically show a typical Maltese cross pattern of light extinction under the polarizing microscope (Figure
1.11b). Importantly it has been shown that spherulites formed in vitro with Aβ are nearly identical to the plaques observed in vivo in AD brain tissue, suggesting that spherulites and amyloid plaques may be the same species (Exley, et al. 2010).

![Microscopic examination of fibrillar deposits in Alzheimer's disease.](image)

**Figure 1.11.** Microscopic examination of fibrillar deposits in Alzheimer's disease. (a) Immunohistochemical detection of amyloid plaques (arrows). (b) Typical Maltese cross aspect of plaques stained with Congo red and visualized by polarized microscopy (Rostagno et al 2009).

1.5.3. Polymorphism in Aβ Fibrils

The cross-β and parallel nature of Aβ fibrils is generally agreed on. However a large degree of polymorphism in the size and degree of twisting of the fibrils is observed by techniques such as transmission electron microscopy and atomic force microscopy, not only between different labs and sample preparation techniques but even within the same sample pool (Hortschansky, et al. 2005). The different structures can also affect the toxicity of the fibrils. Electron microscopy and solid state NMR showed that fibrils grown under quiescent conditions had a maximum diameter of $9 \pm 1$ nm, a periodic twist and were toxic to neuronal cell cultures. Conversely, fibrils grown under agitated conditions had a diameter of $5 \pm 1$ nm, no twisting and were not toxic to cell cultures (Petkova, et al. 2005). Interestingly the parent fibrils, which had been grown under these two different conditions, could seed fresh solutions of Aβ. The fresh solutions of Aβ were grown under identical conditions, yet the parent morphology was ‘passed on’ to the daughter and granddaughter fibrils (Figure 1.12). Solid state NMR shows that the differences are at a molecular level, for example β-strand segments in quiescent Aβ(1-40) fibrils included residues 10 to 14, 16 to 22, 30 to 32, and 34 to 36, whereas those in agitated Aβ(1-40) fibrils include residues 10 to 22, 30 to 32, and 34 to 36. This study suggests that, if different fibril growth conditions do lead to fibril conformations
with varying toxicities, then this may explain the conflicting reports as to whether fibrils are or are not toxic (discussed in Section 1.7).

1.6. Fibril Formation
Fibril formation is predominantly associated with disease, however it is also important in nature. *E. coli* contains the protein curlin, which forms fibrils to colonise inert surfaces and mediates binding to host proteins (Chapman, et al. 2002). Furthermore, in humans melanosomes (lysosome-related organelles) contain functional amyloid which allows melanin granules to form (Berson, et al. 2003).

However there are a large number of systems *in vivo* designed to prevent unwanted aggregation and fibril formation from occurring (Meriin, et al. 2005), and the majority of amyloids seem to not be ‘selected for’, but are accidental and result in disease, including Huntington’s disease, Parkinson’s disease, prion diseases and Alzheimer’s disease.

![Figure 1.12. TEM images of Aβ(1–40) fibrils formed by different incubation conditions.](image)

Figure 1.12. TEM images of Aβ(1–40) fibrils formed by different incubation conditions. Parent fibrils were prepared by incubation of Aβ(1–40) solutions either under quiescent conditions or with gentle agitation. Daughter and granddaughter fibrils were grown under identical conditions but were seeded with sonicated fragments of parent and daughter fibrils, respectively (Petkova *et al* 2005).
1.6.1. Fibril Detection

A range of techniques can detect the formation of fibrils. The presence of amyloid fibrils has been defined by scoring 4 points using the scoring system shown in Table 13 (Nilsson 2004).

Thioflavin T (ThT) is a benzothiazole dye that binds rapidly and with a high specificity to amyloid fibrils. The free dye has an excitation maxima at 385 nm and an emission maxima at 445 nm. Upon binding a new excitation maximum appears at 450 nm and enhanced emission occurs at 482 nm (LeVine 1993). This dye is used throughout this thesis to monitor fibril formation and is discussed in more detail in Chapter 2.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Score for a positive test</th>
</tr>
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<tbody>
<tr>
<td>ThT binding</td>
<td>2</td>
</tr>
<tr>
<td>Congo Red binding</td>
<td>2</td>
</tr>
<tr>
<td>β-sheet secondary structure</td>
<td>2</td>
</tr>
<tr>
<td>Protofibril intermediate or seeded kinetics</td>
<td>1</td>
</tr>
<tr>
<td>Low solubility in denaturant or protease resistance</td>
<td>1</td>
</tr>
<tr>
<td>Gel formation</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 1.2. Characteristics of fibril formation (Nilsson 2004)

Congo Red (CR) is a histologic dye that will bind to amyloid. In normal light the amyloid appears red, but between crossed polarizers the colour changes to a yellow/green, i.e. the dye exhibits birefringence. The mode of interaction of CR with fibrils is not completely understood, although the general consensus is that binding is dependent on the presence of cross β-pleated sheets (Klunk, et al. 1989). Additionally it has been suggested that binding could occur between two negatively charged sulfate groups of CR and two positively charged amino acid residues of two separate protofilaments (Frid, et al. 2007). A potential problem with CR is that it has recently been shown to disturb the processes of protein misfolding and aggregation by stabilising native protein monomers or partially folded intermediates, and
decreasing the concentration of toxic oligomers (Frid, et al. 2007). Thus its use could itself alter the formation of fibrils.

TEM imaging of the fibres is also an excellent method for determining their formation. As described previously, amyloid fibrils viewed by TEM are straight, unbranched, ~6-12 nm in width and of indeterminate length ((Makin and Serpell 2005)). However upon a grid viewed in TEM the fibril sample can contain a multitude of morphologies of aggregates, including spherical, amorphous, and sheet like/rope-like assemblies of many fibrils wrapped together (Goldsbury, et al. 1997). Thus it is not always clear cut which species are fibrils and which are not, or likewise what percentage of fibrils is needed to reliably state that a protein sample has formed fibrils.

Fourier transform infra-red (FTIR) and circular dichroism (CD) spectroscopy can both be used to compare the protein sample before and after fibril formation, an increase in β sheet secondary structure is observed upon fibril formation. In FTIR a peak near 1645 cm⁻¹ is indicative of random coil, and after fibril formation has occurred a peak between 1620 and 1640 cm⁻¹ suggests that β sheet formation has occurred. Likewise in CD a transition from a band at 197 nm to a band at 217 nm reflects the random coil to β-sheet transition. However β sheet secondary structure is not entirely indicative of the presence of amyloids as it can also be displayed by other kinds of aggregates.

Seeding also indicates the formation of fibrils. Unseeded Aβ should have a lag phase before undergoing fibril formation (for further details please see Chapter 5). To determine whether a preparation of Aβ is in the fibrillar form, a seed from a potentially fibrillar sample (usually 10 % of the total volume) is added to a fresh sample of monomeric Aβ, e.g. 100 µl of seed to 900 µl of monomeric Aβ. The lag phase should now be significantly reduced or removed altogether. If however the seed is actually only aggregate or monomeric Aβ, the addition will have no effect on the lag phase of the monomeric sample.

The final three determinants of fibril formation according to Nilsson’s table are a change in solubility, protease resistance and gel formation. When Aβ undergoes a
change in structure from random coil to β-strand and subsequently forms β sheet, it results in key changes that are measurable \textit{in vitro}. The first is that the peptide becomes highly insoluble, even in denaturant. The second is that the fibrillar form of Aβ is no longer vulnerable to protease treatment. Likewise the formation of fibrils may result in some protein samples visibly showing an increase in viscosity and gel formation. Sometimes precipitates will be observed. This is likely to be dependent on the molecular weight of the protein being studied. As Aβ is a relatively short peptide, it would have to be present at a high concentration to have this effect.

1.6.2. Protein Misfolding Pathways
Aβ undergoes a misfolding pathway in AD to form amyloid fibrils. It is generally agreed that this pathway is based on a nucleation event followed by elongation. Considering that fibrils are much more stable thermodynamically than the monomeric form of Aβ, it is surprising that we do not all develop Aβ fibrils and AD at a much younger age. Fortunately the overall rate of amyloid formation is stunted by the slow generation of nuclei (nucleation phase), which once formed, is followed by a much more rapid elongation phase. The slow formation of the nucleus can be observed in growth curves of fibril formation, where the characteristic lag phase can be removed if a preformed fibril is added to the system.

1.6.3. Identification of the Nucleus
The nucleating event that triggers fibril formation is still unclear. Four proposed nucleating events are described below:

\textbf{α-helix formation (Soto, et al. 1995)}
Soluble Aβ has been proposed to be in equilibrium between α-helix and β sheet secondary structure. The transition to β-sheet may be the nucleating factor, with the ability to form fibrils only occurring once β-sheet structure has been obtained. Increasing the propensity for α-helical secondary structure results in a reduction in fibril formation ability and likewise amino acid substitutions that disfavour α-helical formation accelerate formation of fibrils.
Folding nucleus formation (Lazo, et al. 2005)

An intramolecular nucleation event in both Aβ(1-40) and Aβ(1-42) has been suggested, where Val24-Lys28 forms a “folding nucleus” by forming a turn. The turn brings the central hydrophobic cluster (CHC) of Leu17-Ala21 and the hydrophobic C-terminus in contact, allowing hydrophobic side chain interactions to form, a pre-requisite of fibril formation. However it has been shown that residues 14-23 are the minimal fibril forming fragment of Aβ (Tjernberg, et al. 1999), thus casting some doubt as to whether residues 24-28 are the nucleating factor in Aβ fibril formation.

Micelle formation (Lomakin, et al. 1996)

The formation of micelles as the nucleus has also been suggested. In this model if a critical Aβ(1-40) concentration is reached or exceeded, then nucleation occurs through the formation of micelles, with a hydrodynamic radii ($R_h$) of 7 nm. It is assumed that these micelles act as sites of nucleation. At Aβ(1-40) concentrations less than the critical protein concentrations, micelles do not form and subsequently, nucleation is heterogeneous, with multiple nucleation pathways, possibly through seeding on impurities.

Paranuclei formation (Bitan, et al. 2003)

The formation of paranuclei for Aβ(1-42) has also been observed. Solubilised Aβ(1-42) can form paranuclei, composed of pentamers and hexamers. Paranuclei are not observed for Aβ(1-40) as their formation is mediated by Ile41 and Ala42. Once formed, the paranuclei then assemble further to form early protofibrils However preventing the formation of the paranuclei did not prevent fibril formation, although it did influence the abundance and size of early Aβ oligomers.

Thus in conclusion it is apparent that the identification of the nucleus is still unclear, and whether secondary structure changes (formation of a turn or transition to β-sheet) is the crucial stage, or if formation of a specific species (micelle or paranuclei) is more important is still to be established.
1.6.4. Elongation
Once formed, the nucleus can either decay back to the ground state (reduce in size or return to being unstructured/α-helical) or advance by a series of elongation steps to low molecular weight oligomers which then increase in size to oligomers and fibres. A typical fibril formation pathway is shown in Figure 1.13, however elongation of fibrils is not necessarily a linear process, as oligomers of Aβ can be depicted as “on-pathway” precursors to fibre formation, as in Figure 1.13, or alternatively off-pathway and in competition with fibre formation. Wu et al. used conformational specific antibodies to identify two different species, fibrillar oligomers (smaller aggregates 5-15 nm diameter) and fibrils (straight 10-20 nm diameter, 500–900 nm length). Despite being structurally and immunologically related to fibrils, fibrillar oligomers would not seed fibrils, only more fibrillar oligomers, suggesting they are an off-pathway stage of fibril formation (Wu, et al. 2010). Individual species in the fibril formation pathway, and their associated toxicity, is described in the next section.

![Figure 1.13. Aβ fibril formation. Typical fibril formation pathway, assuming that all species formed are on-pathway. Aβ forms a β-strand structure and low molecular weight (LMW) oligomers, then further association results in oligomers, protofibrils and fibrils, before finally forming plaques.](image)

1.7. Nature of the Toxic Species
The mechanism by which Aβ is toxic is hotly debated. Plaques are one of the most obvious features of the Alzheimer’s disease brain, and initially were considered the toxic species, however there are several pieces of evidence that cast doubt as to their
responsibility for the neurodegeneration observed. Firstly, plaque formation is not unique to the disease state, and studies have shown that there is not always a link between the presence of amyloid plaques and cognitive decline (Yankner, et al. 2008). Secondly, it is the soluble low molecular weight species of Aβ that seem to correlate best with AD severity, including the patient’s age at death (McLean, et al. 1999). The different species that have been isolated from the fibril formation pathway and suggested to be toxic are discussed below.

**Dimers**

Dimers are one of the smallest species thought to be toxic. Aβ aliquots were removed at a range of time points during fibril formation and the species stabilised by photo-induced chemical cross-linking. Of all the low molecular weight species, spanning monomers to hexamers, it was dimers that correlated best with inhibition of cytochrome c oxidase (COX) in human mitochondria (Crouch, et al. 2005).

**Aβ-derived diffusible ligands**

Aβ-derived diffusible ligands (ADDLs) are a group of Aβ oligomers whose molecular mass ranges between 17-42 kDa (Lambert, et al. 1998). ADDLs are composed of ~4-10 monomers and are toxic to neuron cultures and also able to inhibit long term potentiation (Walsh, et al. 2002). Their culpability is also strengthened by a study showing that ADDL concentration is ~70 fold higher in AD patients than non-sufferers (Gong, et al. 2003).

**Aβ*56**

The only species of a definite size that has been isolated so far is Aβ*56, a 56 kDa oligomer which was isolated from transgenic mice brains. This soluble oligomer is larger than the size range of the ADDLs and had the strongest inverse correlation with mouse performance in a Morris water maze test, which assesses spatial memory. Even more convincingly, injecting Aβ*56 into the brains of four month old rats correspondingly decreased their spatial memory (Lesne, et al. 2006).

**Oligomers/Protofibrils**

Oligomers and protofibrils are high molecular weight species that form before fibrils. Aβ oligomers are small globular structures that measure ~ 2–5 nm.
Oligomers were ~40x more toxic to neuronal cells in vitro compared to monomeric preparations and ~10x more toxic than fibrils (Dahlgren, et al. 2002). Injection of oligomers into rat brains has been shown to disrupt cognitive function including inhibiting hippocampal long-term potentiation (LTP) in vivo (Cleary, et al. 2005, Walsh, et al. 2002).

Protofibrils are thought to be composed of beaded chains of 5 nm diameter beads with a length of greater than 150 nm. They are sometimes described as flexible and curved and have been shown to be toxic in a number of studies (Dahlgren, et al. 2002, Hartley, et al. 1999, Walsh, et al. 1999). However there is a great deal of variation in different groups definition of oligomers and protofibrils, and this complicates gaining a true understanding of whether these groups constitute a discrete toxic species or not. This is discussed further in Section 1.7.1.

**Fibrils/Plaques**

Despite the data in favour of oligomers as the toxic species, there remains some strong support for mature fibrils and plaques as a toxic species in fibril formation. Perhaps the most convincing evidence for plaques being the toxic species comes from transgenic mice studies. In vivo multiphoton microscopy showed that plaque formation was followed by neurodegeneration, not the other way round, implicating fibres rather than toxic oligomers. This study established a direct causal relationship between plaques and neurodegeneration, with neuronal morphological changes evident in the days after plaque formation (Meyer-Luehmann, et al. 2008). Additionally mapping of neuronal cell density showed that neuronal loss only occurred in the immediate vicinity of amyloid plaques (Urbanc, et al. 2002). Finally monomeric and oligomeric Aβ were both found to have no effect on membrane conductance, whereas fibrils caused a large increase in electrical conductivity, indicative of a toxic effect on the membrane (Schauerte, et al. 2010). Thus it remains to be seen whether plaques can be written off as “the tombstones” of Alzheimer’s disease, rather than the toxic agent.

### 1.7.1. Problems with Studies on the Toxic Form of Aβ

A major problem with studies that aim to characterise a particular species or group that forms during fibril formation is that under experimental conditions (usually
conducted at much higher Aβ concentrations than would occur in vivo) Aβ will aggregate rapidly. This difficulty in confidently identifying one stable intermediate which can be reliably isolated explains why some of the “toxic species” include a large range of oligomeric sizes, such as the ADDLs. Thus unless methods are used to stabilise each species (though this itself will change the nature of the sample), it is probable that aggregation will continue throughout the experiment and thus monomers will rapidly dimerise, small oligomers will become large oligomers and so on. To highlight one study, Stine et al used atomic force microscopy (AFM) on Aβ(1-42) to elucidate conditions for fibril formation and oligomerisation (Stine, et al. 2003). Throughout the paper they define the following species:

Unaggregated peptide: 1.1 nm
Small diameter fibrils: 2 nm
Globular aggregates: 2 - 5 nm
Oligomers: 2 – 4nm
Intermediate diameter fibrils: 4- 6 nm
Amyloid fibrils: 4 nm
Protofibrils: < 200 nm
Short fibrils: < 1 μm
Mature fibrils: > 1 μm

A problem with studies such as these, where size is used as a species determinant, is that the Aβ monomeric population does not fold and assemble synchronously, a distribution of conformational/assembly states exists before fibril formation is completed. Thus an experimental sample that exists as homogenously one species, for example protofibrils of a certain diameter is highly unlikely. Attempts at specificity have been made, such as Kayed et al who developed an antibody which will recognise fibril forming intermediates, however even this antibody will recognise both small spherical species and larger elongated species highlighting the difficulty in defining clearly and reproducibly an intermediate in fibril forming pathways (Kayed, et al. 2003).

The methods used to characterise the sizes of Aβ species are also subject to scrutiny. Hepler et al found significant discrepancies in the apparent molecular weight of Aβ
species depending on whether gel electrophoresis, atomic force microscopy (AFM) or size exclusion chromatography coupled to multiangle laser light scattering detection (SEC/MALLS) were used (Hepler, et al. 2006). Thus caution must be used when interpreting results and defining species as the toxic agents of AD.

1.8. Mechanism of Aβ Toxicity

The mechanism of Aβ toxicity, as well as the toxic species, is still unclear. Membrane alterations, intracellular interactions and oxidative stress are three major areas where Aβ is thought to promote toxicity.

1.8.1. Membrane Alterations

Within APP, Aβ resides partially in the membrane. Consequently Aβ has a predominantly polar N terminus, and largely hydrophobic C terminus. This hydrophobic tail has led to the hypothesis that the toxicity is due to Aβ interactions with cell membranes (Simakova, et al. 2007). There are several ways this could lead to toxicity:


2. The conductance of lipid bilayers and cell membranes will increase in the presence of Aβ oligomers, through Aβ indirectly thinning the membrane, or causing localised structural defects (Sokolov, et al. 2006).

3. Aβ forms ion channels, resulting in increased conductance of Ca$^{2+}$ and other cations. This results in cell death via triggering Ca$^{2+}$ sensitive apoptosis signalling pathways (Arispe, et al. 2007).

The formation of ion channels is yet to be convincingly proven. However it is easy to imagine the damage that a fibril could cause by forming near or on the cell membrane. In fact a study using human islet amyloid polypeptide found that the degree of fibril growth at the membrane and membrane damage were closely related, suggesting that it is the process of fibril formation, rather than a particular toxic species, that distorts and damages cell membranes (Engel, et al. 2008).
1.8.2. Intracellular Interactions

An emerging role for intracellular Aβ is that it exerts toxicity at very early stages in AD dysfunction. Theoretically the intracellular environment should favour fibril formation, due to the lower pH of certain intracellular organelles and crowding effects. Increases in intraneuronal Aβ deposits in transgenic mice occurred at the same time as abnormalities in behaviour and physiology (Billings, et al. 2005), but before plaque or tangle formation. Additionally in transgenic mice Aβ(1-42) accumulates in presynaptic and especially postsynaptic compartments at the synapse. This accumulation was associated with abnormal synapse morphology, and also occurred before amyloid plaques formed (Takahashi, et al. 2002). In a very recent study in clonal cell lines intracellular intermediates preceded extracellular Aβ plaques and cell death, and it was suggested that fibrils grow out of cells, rather than associate with them extracellularly. It is not until the cell ultimately dies due to intracellular fibril formation that the fibrils (and all intracellular structures) are released into the extracellular milieu (Friedrich, et al. 2010).

An intracellular protein, endoplasmic reticulum amyloid-β-peptide binding protein (ERAB) has been identified that can mediate the cellular toxicity of Aβ (Yan, et al. 1997). Expression of ERAB is higher in the brains of AD patients compared to age-matched controls. ERAB is predominantly localised within neurons in the brain and Aβ toxicity was markedly increased in the presence of ERAB as measured by both the MTT reduction assay and monitoring induction of apoptosis. ERAB has since been shown to be an L-3-hydroxyacyl-coenzyme A dehydrogenase type II (NADH2) (He, et al. 1998). It is also now more commonly referred to as Aβ-binding alcohol dehydrogenase (ABAD) and emphasis has shifted to its role in the mitochondria, with the discovery that ERAB/ABAD binds to Aβ within mitochondria (Lustbader, et al. 2004). Prevention of ERAB/ABAD binding to Aβ within mitochondria resulted in protection from Aβ induced cytochrome C release from mitochondria, a marker for cellular stress and apoptosis. Additionally protection from Aβ-ABAD toxicity coincided with a reduction in ROS generation, suggesting that the interaction between ABAD/ERAB and Aβ results in mitochondrial dysfunction due to the production of ROS and ultimately cell death (Lustbader, et al. 2004).
The theory that intracellular changes begin the cascade of neurodegeneration is an attractive one. However studies of the Aβ secretory pathway show that most Aβ produced is secreted from the cell. Indeed intraneuronal Aβ(1-42) levels actually decrease with increasing development of AD (Gouras, et al. 2000). Thus extracellular Aβ would have to be re-internalised, and it is debatable whether intracellular Aβ would reach concentrations high enough to cause the significant toxicity that would be expected from the trauma observed in AD brains.

1.8.3. Oxidative Stress

Increased levels of oxidative damage are characteristic of AD brains. Oxidative stress has also been observed to occur before the formation of amyloid plaques (Nunomura, et al. 2006). In early studies, Aβ was suggested to generate ROS (reactive oxygen species) spontaneously (Hensley, et al. 1994). However further studies using electron paramagnetic resonance spin-trapping, found no evidence of spontaneous formation of peptide-derived free radicals during the incubation of Aβ(1-40) (Turnbull, et al. 2001), and it now seems highly unlikely that Aβ is capable of generating ROS alone.

It was then suggested that Aβ could produce ROS in the presence of transition metals through Fenton/Haber-Weiss reactions (Huang, et al. 1999). The ability of Cu²⁺ to undergo Fentons/Haber-Weiss reactions and generate ROS is described in Section 1.10.3 of this chapter. Aβ has a methionine at position 35 and in Aβ isolated from plaques the methionine is oxidised (Dong, et al. 2003). Potentially, Met35 could donate electrons for metal ion reduction, and in doing so initiate a redox cycle. Additionally it has been suggested that the methionine is central to Aβ toxicity (Butterfield, et al. 2002). However redox potentials suggest that Aβ(1-42) itself cannot be oxidised by Cu²⁺ and instead requires other cellular species (Jiang, et al. 2007). This is supported with results from ROS detection assays where Aβ(1-40) and Aβ(1-42), either as monomers or as fibrils could not generate ROS in the presence of Cu²⁺ alone but required physiological reductants such as ascorbate (Nadal, et al. 2008).

Alternatively, Aβ has been proposed to have a protective role as an antioxidant. 8-hydroxyguanosine (8-OHG) is an indicator of oxidative damage. Neurons containing
extensive Aβ deposits, contained the lowest levels of 8-OHG (Nunomura, et al. 1999). This inverse relationship between amyloid deposits and 8-OHG levels suggests that Aβ might actually be produced in response to oxidative stress. Additionally hydroxyl radicals generated by Fenton/Haber-Weiss reactions of Cu²⁺ and ascorbate are quenched by Aβ, suggesting Aβ has a role as a sacrificial quencher of ROS (Nadal, et al. 2008). An explanation for these two contrasting roles of Aβ, pro-oxidant and antioxidant, may be the ability of Aβ not to catalytically generate ROS, but instead to chelate Cu²⁺ in a redox active form, perhaps resulting in an accumulation of redox-active Cu²⁺ at the cell membrane.

In summary the exact toxic mechanism is still unclear, but it is possible the theories are closely interconnected. The exact temporal and spatial events that lead to AD brain neurodegeneration are likely to be linked to both energetics (production of ROS) and mechanics (membrane damage).

1.9. Current Therapeutics

Studies of familial AD patients who have mutations within the APP or presenilin genes show that Aβ(1-42) levels are elevated by as little as 30 % (Scheuner, et al. 1996) yet this is enough to markedly accelerate Aβ deposition. Thus therapeutics that can reduce Aβ(1-42) levels by as little as 30 % might slow AD development enough to increase the asymptomatic stage of the disease and consequently decrease the number of years that patients live with AD.

Currently four drugs are approved by regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) to aid management of AD. Three of the drugs (Donepezil, Galantamine and Rivastigmine) are acetylcholinesterase inhibitors. Acetylcholinesterase inhibitors have been developed in response to post-mortem and biopsy studies which found decreases in acetylcholine synthesis and release in AD brains (Bowen, et al. 1977, Davies, et al. 1976). By inhibiting acetylcholinesterase activity, more acetylcholine is available at the synapse and subsequently cognitive function is improved.

Memantine, the fourth drug, is an N-methyl-D-aspartic acid receptor (NMDAR) antagonist. It was developed because NMDAR-mediated glutamate excitotoxicity is
associated with the neuronal death characteristic of AD. Glutamate is the most abundant neurotransmitter in the body, and is bound at chemical synapses by the NMDAR, on the membranes of post-synaptic neurons. However excitotoxicity can occur when receptors for glutamate such as the NMDAR are overactivated. Overactivation from excess glutamate allows high levels of calcium ions to enter the cell, activating a number of enzymes, which damage cell structures, resulting in cell death. The extent of AD symptoms correlates well with the neuronal death caused by excess glutamate. Thus using an NMDA receptor antagonist such as Memantine should prevent excess glutamate binding and subsequent excitotoxicity.

In the UK the National Institute for Clinical Excellence (NICE) only recommends treatment by three of the four drugs. Donepezil, Galantamine and/or Rivastigmine are recommended for treatment in moderate Alzheimer's disease only. These drugs show small improvements in cognition and/or behaviour (Rogers, et al. 1998). Controversially NICE has not recommended the use of Memantine. It was intended to be used for patients with moderate to severe AD as it showed reduced clinical deterioration in patients with AD (Reisberg, et al. 2003). However NICE ruled that its cost outweighed its benefits. Unfortunately all four drugs show modest improvement only, and there are no indications that treatment delays or halts progression of the disease.

As of 2010, more than 800 clinical trials are in the process of being carried out for identification of a possible treatment for AD (http://www.clinicaltrials.gov). Table 1.3 outlines some of the current research areas that are being developed into therapeutics.

Aβ antibodies are also being investigated. The seminal study in this area used transgenic mice which over-express mutant human APP (Val717Phe)(Schenk, et al. 1999). Synthetic human Aβ(1-42) was used as an immunogen (active immunisation). Treatment began before plaque formation was observed. Eight of the nine transgenic mice immunised with Aβ(1-42) developed serum antibody titres against Aβ(1-42) of greater than 1:10000. The result of this was that immunisation with Aβ(1-42) almost completely prevented Aβ deposition, with seven of the nine mice having no detectable Aβ deposits in their brains. Additionally there was a near-
total reduction in the Aβ burden in the hippocampus. In contrast, control animals that had been immunised with phosphate buffered saline only contained numerous Aβ deposits and had a significantly greater Aβ burden ($P = 0.0005$). The Aβ(1-42) immunised mice lacked all the hallmarks of neurodegeneration characteristic of AD, including an absence of dystrophic neurites, and dramatically reduced astrocytosis. Presumably immunisation either prevents deposition and/or promotes clearance of Aβ in the brain. The same study also observed the effect of immunisation after plaque formation had occurred, and found immunisation with Aβ significantly slowed the development of existing pathologies in the animals.

Passive immunisation has also been closely studied. The ability of Aβ binding proteins to act as an Aβ “sink” was assessed. The antibody m266 showed a low picomolar affinity and thus could bind Aβ in the presence of physiological buffers and endogenous Aβ binding proteins. The same transgenic mouse model as used in the Schenk study above was used to assess the effects of m266 in vivo. Unbound Aβ plasma levels were reduced from 140 pg/ml in control mice to essentially undetectable levels in mice treated with m266, suggesting all plasma Aβ was bound to m266. Additionally the amount of Aβ bound to m266 increased over several days to ~ 1000 fold above endogenous plasma Aβ levels. This strongly implies that circulating m266 facilitates the transfer of Aβ into the plasma from the CNS (DeMattos, et al. 2001). The same group produced a follow-up study where they determined the effect of m266 on learning and memory. The group administered m266 to transgenic mice and found that it formed a complex with Aβ in both the plasma and CSF of the mice, and resulted in rapid reversal of memory impairment in learning and memory tasks (Dodart, et al. 2002).

However a further study using passive immunization in transgenic mice found that although administration of Aβ immunisation significantly reduced amyloid, it also induced a two-fold increase in cerebral microhaemorrhages and a significant increase in haemorrhage severity over controls. This may results from passive immunization compounding the weakening of cerebral blood vessels which occurs during amyloid deposition (Pfeifer, et al. 2002). Furthermore a phase II clinical study using a serum Aβ antibody (AN1792) was halted when 6 % of the patients developed meningoencephalitis (Orgogozo, et al. 2003).
A further study was carried out with the antibody An1792, and found that immunisation of patients with An1792 was associated with a long term reduction in Aβ load and a variable degree of plaque removal. Unfortunately the study also found no support for immunisation improving cognitive function. Two individuals with the highest levels of Aβ antibodies and almost complete elimination of plaques still had clear end stage dementia before death, and there was no evidence of immunisation having an effect on long-term survival (Holmes, et al. 2008). However the authors themselves concede that the small numbers of participants in the study may have masked potential smaller benefits of the treatment, thus it may be too early to write off immunisation as a possible AD therapeutic.

Another approach is decreasing Aβ production by targeting the secretases. γ-secretase inhibitors can be characterised into two generations of therapeutics. Firstly, administration of a γ-secretase cleavage site peptidomimetic resulted in a lowering of Aβ production (Wolfe, et al. 1998). This substrate-based difluoro ketone was designed based on the APP γ-secretase cleavage site for Aβ(1-42) and was demonstrated to be a specific inhibitor of Aβ biosynthesis in APP-transfected cells. However the peptidic and bulky properties of these mimics make them unlikely therapeutics for further development in in vivo studies, although they have provided valuable information on characterising γ-secretase’s catalytic core (Lundkvist, et al. 2007).

The second wave of therapeutics targeting γ-secretase are mostly structurally related through containing either sulphonamide/sulfone or benzodiazepine/benzolactam moieties (Harrison, et al. 2004). Early therapeutics had unfortunate side-effects with profound changes in gastrointestinal tract tissue morphology and altered lymphocyte development, probably due to impaired Notch signalling (Wong, et al. 2004). The Notch signalling pathway is essential during embryogenesis (Artavanis-Tsakonas, et al. 1995) and in adult life (Radtke, et al. 2004) for controlling cellular differentiation. The Notch receptor undergoes proteolytic cleavage at two sites, S2 and S3 before translocating to the nucleus and regulating transcription (Lundkvist and Naslund 2007). However it has now been found that cleavage at S3 is carried out by γ-secretase (De Strooper, et al. 1999), and in fact there are more than 30
substrates of \( \gamma \)-secretase (Nyborg, et al. 2006). However developing \( \gamma \)-secretase therapeutics that avoid Notch associated toxicity is possible. Indeed a recent benzodiazepine-derived \( \gamma \)-secretase inhibitor named LY-450137, developed in a collaboration between Washington University, St Louis and Eli Lilly has avoided these side-effects. The group measured central nervous system A\( \beta \) production and clearance rates through stable isotope labelling kinetics (SILK) (Bateman, et al. 2009). They found that LY-450137 significantly decreased production of A\( \beta \) in human central nervous system, with up to 84% inhibition at a 280 mg dosage.

A different strategy to using \( \gamma \)-secretase inhibitors is the development of \( \gamma \)-secretase modulators. Tarenflurbil is an enantiomer of a non-steroidal anti-inflammatory drug (NSAID). NSAIDs include drugs such as ibuprofen and aspirin. NSAIDs interact directly with the \( \gamma \)-secretase complex. The mechanism is unclear but the proteolytic cleavage site is altered, resulting in less A\( \beta \)(1-42) and more A\( \beta \)(1-38). NSAIDs are attractive as therapeutics as they already have well-characterised toxicity profiles. However a large 18 month phase III trial of an NSAID showed no benefit, perhaps because taking the drug via oral administration resulted in insufficient brain concentrations (Eriksen, et al. 2003). There are also the same concerns with modulating \( \gamma \)-secretase activity as there is for inhibiting it, due to the presenilin component of \( \gamma \)-secretase’s role in the Notch signalling pathway.

In contrast, deletion of the \( \beta \)-secretase gene does not result in any serious toxicity and \( \beta \)-secretase is essential in generating A\( \beta \), thus \( \beta \)-secretase inhibitors are considered to be one of the most promising targets for an AD therapeutic (Harrison, et al. 2003, Roberds, et al. 2001). There are several encouraging studies using \( \beta \)-secretase inhibitors. GSK188909 is a BACE-1 inhibitor which was the first therapeutic shown to reduce brain amyloid levels in transgenic mice following oral administration (Hussain, et al. 2007). Inhibitors of \( \beta \)-secretase have mainly been designed to block the active site of the enzyme, for example there is a crystal structure of the protease domain of BACE complexed to an eight-residue inhibitor at 1.9 angstrom resolution (Hong, et al. 2000). However despite having high activity against the enzyme many have failed in cellular studies, and one suggestion is that inhibitors need to be designed to be able to target organelles within the cell where BACE is active, for example endosomes (Rajendran, et al. 2008). In this study,
membrane anchoring of the inhibitor resulted in the inhibitor gaining access to endosomal \( \beta \)-secretase as well as enhancing the interaction between the inhibitor and the enzyme, both of which had a dramatic effect in amplifying the potency of a \( \beta \)-secretase inhibitor (Rajendran, et al. 2008). Additionally conjugating a \( \beta \)-secretase inhibitor to a carrier peptide to aid blood brain barrier penetration reduces A\( \beta \) in the brain (Chang, et al. 2004). Indeed targeting BACE still seems to be the most viable approach for a single therapeutic in AD (Hunt, et al. 2009).

<table>
<thead>
<tr>
<th>Mechanism of Action of drug</th>
<th>Example of Drug</th>
<th>Stage of Development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholinesterase inhibitors</td>
<td>Donepezil (Aricept)/ Rivastigmine, Galantamine</td>
<td>Recommended by NICE for use in patients with moderate AD</td>
</tr>
<tr>
<td>NMDAR antagonist</td>
<td>Memantine</td>
<td>Not recommended by NICE</td>
</tr>
<tr>
<td>Directly bind A( \beta ) and inhibit aggregation or enhance clearance of plaques</td>
<td>Tramiprosate and ACC-001</td>
<td>At phase II and III in clinical trials</td>
</tr>
<tr>
<td>Secretase modulator</td>
<td>NSAIDs, GSK188909, statins</td>
<td>At various stages in clinical trials.</td>
</tr>
<tr>
<td>Metal chelation</td>
<td>Clioquinol (PBT2)</td>
<td>Entering Phase III</td>
</tr>
<tr>
<td>Tau aggregation inhibitor</td>
<td>Rember</td>
<td>Entering Phase III</td>
</tr>
</tbody>
</table>

**Table 1.3. Selected AD drug development programs.** Each of the categories is discussed in more detail in the text, adapted from Rafii et al. 2009.

As well as developing drugs that directly inhibit BACE1 activity, statins, a class of drug which lower cholesterol, have also been proposed as a therapeutic in AD. Interestingly a study of individuals of 50 years and older who were prescribed statins, a cholesterol lowering drug, showed that they had a substantially lowered risk of developing dementia (Jick, et al. 2000). Statins are competitive inhibitors of the enzyme that regulates the synthesis of cholesterol. High cholesterol has been suggested to affect APP processing through increased formation of intracellular lipid.
rafts, inside of which APP and BACE1 processing occurs (Cordy, et al. 2003, Sidera, et al. 2005, Won, et al. 2008). Statins have also been shown to decrease the risk of dementia through up-regulating the expression of the α-secretase ADAM10 (Kojro, et al. 2001). However, it is not clear still whether disturbed cholesterol metabolism may be a consequence of AD rather than a cause, and thus whether statins are a potential treatment target.

Metal chelators have been shown to solubilise amyloid plaques through metal depletion (Cherny, et al. 1999) and treatment with a copper-zinc chelator inhibited Aβ accumulation in transgenic mice (Cherny, et al. 2001). The Bush/Tanzi group have been particular champions of metal chelators based on 8-hydroxyquinoline derivatives such as clioquinol and PBT2. PBT2 showed a time and dose-dependent improvement in AD patients in a preliminary clinical trial compared to patients on a placebo (Lannfelt, et al. 2008).

However clioquinol was linked to an outbreak of subacute myelo-optic neuropathy in the 1960s in Japan, thus the proposed long-term administration of clioquinol to AD patients has some risks associated with it. Despite this two more phase 3 trials being planned with PBT2. Additionally metal chelators may remove Cu\(^{2+}\) from Aβ when administered, but once Cu\(^{2+}\) is bound to the chelator, there would be nothing to prevent further Cu\(^{2+}\) binding to Aβ, thus metal re-distribution, for example through induction of metallothionein expression, rather than chelation may be a more effective strategy.

Finally there are also drugs being developed that are not focused on preventing Aβ production, but act on tau instead. Both methylene blue (tradename Rember), a histological dye and a small peptide called NAP, have shown promising results due to their ability to interfere with tau aggregation and dissolve the neurofibrillary tangles (Gozes, et al. 2005, Gura 2008). Targeting both tau and amyloid-β formation/aggregation seems wise, as it is likely that there will not be one “superdrug” to cure AD, but instead a synergistic combination of drugs which will need to target both Aβ and tau pathology. Indeed a recent study using a cocktail of four drugs, including NSAIDS, statins, β and γ inhibitors found that the cocktail
resulted in the drugs actually having an additive effect, and importantly producing no detrimental effects in respect to the notch pathway (Asai, et al. 2010).

1.10. The Chemistry of Copper

The interaction between Aβ and Cu\(^{2+}\) is the major theme of this thesis, thus this section of the introduction discusses copper in the body, its redox chemistry and aspects of metal-ligand binding. Copper is a crucial cofactor in enzymes such as cytochrome c and superoxide dismutase, and it can drive a range of processes in the body that are essential for life. It can exist in biological systems in two states: Cu\(^+\) (reduced) and Cu\(^{2+}\) (oxidised). Defects in copper homeostasis are directly responsible for human disease, for example mutations in the \(ATP7a\) or \(ATP7b\) genes, which encode Cu\(^+\) transporting ATPase pumps, result in Menkes and Wilson’s disease respectively. Menkes disease is a lethal disorder of intestinal copper hyperaccumulation with severe copper deficiency in peripheral tissues. Wilson’s disease results in hepatic and neuronal copper overload, which requires chronic therapy to enhance copper excretion or reduce copper absorption.

1.10.1. Copper Life Cycle

We ingest \(\sim\) 1 mg of copper daily (Linder, et al. 1998). Based on studies in rats, a brief summary of copper’s fate upon leaving the intestine is as follows. Once copper crosses the intestinal membrane it enters the blood. Almost all the copper then rapidly leaves the blood and enters the liver and kidney before re-emerging into the blood plasma for the second time. The copper is then taken up by other tissues and organs, such as the brain. After copper has left the kidneys/liver it can also enter the bile for excretion. The concentration of ionic copper and amino acid chelates in plasma is low (\(\sim\) 11 pmol/L and 0.55 µmol/L respectively (Linder, et al. 1998)). Instead copper is mainly bound to proteins such as albumin, transcuprein and ceruloplasmin (Linder, et al. 1998).

1.10.2. Copper in the Brain

Interestingly, in comparison to Cu\(^{2+}\) in the blood, studies comparing radiolabelled free copper, copper-albumin, and copper-ceruloplasmin showed that free copper was the major species entering the brain (Choi, et al. 2009). The blood brain barrier (BBB) is the major entrance for copper to the brain, whereas the blood -
cerebrospinal fluid (BSF) barrier regulates copper homeostasis in the cerebrospinal fluid (CSF).

Once copper has crossed the BBB/BSF barrier the high affinity copper transporter Ctr1 mediates Cu\(^{+}\) transport into neurons. The mammalian mechanism of reducing extracellular Cu\(^{2+}\) to Cu\(^{+}\) is still unclear. Interestingly, APP possesses a copper binding domain capable of reducing Cu\(^{2+}\) to Cu\(^{+}\) and has been suggested as the metalloreductase responsible (Multhaup, et al. 1996). This is supported by a study using \textit{S. cerevisiae} where the endogenous cell surface metalloreductase Fre1 was replaced with APP, resulting in restored Cu\(^{2+}\) reductase activity and copper uptake (Suazo, et al. 2009).

Although free Cu\(^{2+}\) may be the major species entering the brain, once inside the cell redox active metals such as Cu\(^{+}\) are usually tightly chaperoned – there are no free Cu\(^{+}\) ions in the cell (Rae, et al. 1999). However the concentration of copper in the synaptic cleft of rat cortical synaptosomes (isolated terminals of neurons) was calculated to reach local concentrations of 100- 250 micromolar (Kardos, et al. 1989). Both A\(\beta\) and Cu\(^{2+}\) are released at the glutamatergic synapse, and subsequently this has implications which are explored further in Chapter 7.

\subsection*{1.10.3. Redox Chemistry of Copper}
Copper is a redox active metal as it can change its valence state under biological conditions. The one electron difference between Cu\(^{+}\) and Cu\(^{2+}\) allows copper to promote radical reactions. Dissolved molecular oxygen in the body is liable to react with redox active metals such as copper to produce ROS. Superoxides (O\(\text{2}^{-}\)) are one type of ROS that are produced in the mitochondrial transport chain, and dealt with by superoxide dismutase (SOD). SOD is a copper-containing enzyme which catalyses dismutation of O\(\text{2}^{-}\) to hydrogen peroxide (H\(\text{2}O\text{2}\)) by the copper ions in SOD undergoing oxidation and reduction:

\begin{equation}
\text{Enzyme–Cu}^{2+} + \text{O}_2^{-} \rightarrow \text{Enzyme–Cu}^{+} + \text{O}_2
\end{equation}

\begin{equation}
\text{Enzyme–Cu}^{+} + \text{O}_2^{-} + 2\text{H}^{+} \rightarrow \text{Enzyme–Cu}^{2+} + \text{H}_2\text{O}_2
\end{equation}

Hydrogen peroxide is widespread in the cell and is only a weak oxidising or reducing agent, and generally poorly reactive. However it can react with iron and
copper to form damaging species, such as the hydroxyl radical (OH\(^{•}\)), via Fenton’s cycling and the Haber-Weiss reaction (below).

\[
\text{Cu}^{+} + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{OH}^{•} + \text{OH}^{-} \quad \text{(Fenton reaction)}
\]

\[
\text{Metal catalyst} \quad \text{O}_2^{••} + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^{•} + \text{OH}^{-} \quad \text{(metal-catalysed Haber Weiss-reaction)}
\]

Thus, through redox cycling, copper has the potential to produce a range of harmful species which can go on to generate a variety of oxidative damage in the cell (Figure 1.14). Accumulation of ROS can result in the modification of proteins, lipids and DNA leading ultimately to cell death.

**Figure 1.14. Diagram of Cu\(^{2+}\) induced oxidative stress.** Dissolved molecular oxygen is liable to react with redox active metals (M\(^x\)) such as Cu and Fe. In the reduced state M\(^{x-1}\) will reduce O\(_2\) to superoxide O\(_2^{••}\) which is then dismutated or disproportionated to H\(_2\)O\(_2\), for example by superoxide dismutase. If H\(_2\)O\(_2\) is not cleared by scavenging mechanisms (e.g. catalase or glutathione peroxidase) it can generate the highly reactive hydroxyl radical (OH\(^{•}\)) upon reaction with an encountered reduced metal M\(^{x-1}\) (adapted from Strozyk 2006).

**Metal-Ligand Binding**

The potential complexes that may form between A\(_{\beta}\) and Cu\(^{2+}\) is covered in some detail in Chapter 4. Below is a brief summary of the principles behind metal-ligand binding, including different metal-ligand geometries, why Cu\(^{2+}\) preferentially binds
to nitrogen atoms, and why certain amino acids are more likely to take part in the binding complex.

1.10.4. Crystal Field Theory-Copper-Ligand Complex Geometry

The interaction between a transition metal, such as Cu$^{2+}$, with ligands can be described using crystal field theory. Cu$^{2+}$ is a d-block element with five degenerate d-orbitals (all five d-orbitals have the same energy level), see Figure 1.15. Hypothetically, if a spherically symmetrical field of negative charges was placed around Cu$^{2+}$, all five d-orbitals would be raised equally in energy as a result of the repulsion between the applied negative field and the negative electrons in the orbital, and the five d-orbitals would remain degenerate.

![Figure 1.15. The d-orbitals of copper. Spatial arrangements of the 5 d-orbitals.](image)

However this degeneracy is lost when a real ligand approaches the metal ion. For example, six ligands approaching to form an octahedral complex along the axes of the coordinate system: z, -z, x, -x, y and -y. Under these conditions the ligand electrons will interact strongly with the electron orbitals lying along the x, y and z axes, the $d_{z^2}$ and $d_{x^2-y^2}$ orbitals. These two orbitals will be raised in energy to the same extent. Less repulsion will be felt by the three remaining orbitals which do not lie exactly along the x, y and z axis ($d_{xy}$, $d_{xz}$, $d_{yz}$). Thus the d-electrons closer to the
ligands will have a higher energy than those further away, resulting in the d-orbitals splitting in energy.

**Figure 1.16. The splittings of the d-orbitals of copper.** Splitting of d-orbitals in (a) an octahedral field, (b) a tetrahedral field and (c) a square planar field.

Depending on how the energy levels split, different complexes result (Figure 1.16). The splitting is affected by the following factors:

- the nature of the metal ion.
- the metal's oxidation state. A higher oxidation state leads to a larger splitting.
• the arrangement of the ligands around the metal ion.
• the nature of the ligands surrounding the metal ion.

The most common type of complex is octahedral. In octahedral symmetry the d-orbitals split into two sets with an energy difference, Δoct (the crystal-field splitting parameter). An example of an octahedral coordination geometry is in Cu(H₂O)₆. Tetrahedral complexes are the second most common type of complex. Four ligands form a tetrahedron around the metal ion. The d-orbitals again split into two groups, with an energy difference of Δtet where the lower energy orbitals will be dz² and dx² - y², and the higher energy orbitals will be dxy, dxz and dyz - opposite to the octahedral case. Furthermore, since the ligand electrons in tetrahedral symmetry are not oriented directly towards the d-orbitals, the energy splitting will be lower than in the octahedral case. These tetrahedral complexes are an intense blue, due to strong absorption ~ 600 nm and are described as type I copper complexes. Tetragonal complexes can be square planar or square pyramidal and are often referred to as type II copper complexes. In square planar conformations four ligands are distributed close to Cu²⁺ in the equatorial plane. Two other ligands are further away and perpendicular to the equatorial plane and are more weakly bonded to the Cu²⁺ and subsequently are less important when considering their role in the complex. Human serum albumin and the prion protein both coordinate Cu²⁺ in this conformation. The complex formed by Aβ-Cu²⁺ is investigated in Chapter 4.

1.10.5. Hard and Soft Metal Ligands- Cu²⁺'s “Preference” for Certain Ligands

Metal ions will bind preferentially to certain ligands, and this can be predicted through the hard and soft metal ligand theory, which classes ligands and metals as “hard” or “soft” (Pearson 1963). Hard metals include group 1 and 2 of the periodic table, and lighter transition metals in higher oxidation states e.g. Fe³⁺. They are small, often have a high oxidation state and are weakly polarisable. Soft metal ions include those of the heavier transition metals and those in lower oxidation states e.g. Cu⁺. They are large and have low oxidation states and are highly polarisable. Likewise ligands can be classified as hard or soft, depending on their preference for hard or soft metal ions. Hard metals react faster and form stronger bonds with hard ligands, and likewise for soft metals and ligands, all other factors being equal.
Cu$^{2+}$ and Zn$^{2+}$ are both classified as borderline metals as they tend to have lower oxidation state and larger size than hard metals but a lower oxidation state and smaller size than soft metals. Corresponding borderline ligand donor atoms are N or Cl. Ca$^{2+}$ on the other hand is a hard metal and binds preferentially to hard bases such as H$_2$O, OH$, \text{ROH}$, and RO$. Thus Cu$^{2+}$ will “prefer” to bind to nitrogen ligands and Ca$^{2+}$ will “prefer” to bind to oxygen ligands.

### 1.10.6. Potential Binding Ligands

In addition to Cu$^{2+}$ ‘preferring’ certain atoms, the protonation state of an amino acid will influence the propensity of Cu$^{2+}$ to bind. Cu$^{2+}$ will bind preferentially to amino acids that are deprotonated and thus have a low pK$_a$, for example histidine’s pK$_a$ is ~ 6.5 and the N- terminus pK$_a$ is ~ 8. Thus at around neutral pH both will mainly be deprotonated, compared to say, arginine whose pK$_a$ is ~12.5.

### 1.11. Evidence for a Role for Copper in AD

Some interactions between APP, Aβ and Cu$^{2+}$ have been mentioned already, but a summary of the key pieces of evidence for a role for Cu$^{2+}$ in AD are discussed below.

A notable characteristic of both AD and Down’s syndrome patients is altered metal ion concentrations in the brain (Bayer 2006). Copper levels are significantly elevated in the brains of AD patients, from 4.4 ±1.5 μg/g in the neuropil of control patients to 19.3 ±6.3 μg/g in AD neuropil and up to 30.1±11.0 μg/g in the senile plaque core (Lovell, et al. 1998). Raman microscopy of senile plaque cores isolated from AD brains displayed vibrational frequencies arising from copper and zinc ions directly bound to the histidine imidazole rings of Aβ in the plaques (Dong, et al. 2003). In addition, synchrotron X-ray fluorescence has identified hot spots in which Cu$^{2+}$ and Zn$^{2+}$ are concentrated within Aβ associated amyloid plaques (Miller, et al. 2006). Finally copper levels in serum are 54 % higher in patients with AD than controls (Squitti, et al. 2002). This all indicates that disrupted metal ion homeostasis occurs in AD (Bush 2003, Cuajungco, et al. 1997).
Normal levels of $\text{Cu}^{2+}$ in the brain are more than sufficient to be neurotoxic if $\text{Cu}^{2+}$ homeostasis and compartmentalisation of $\text{Cu}^{2+}$ is impaired (Gaggelli, et al. 2006, Opazo, et al. 2002). Oxidative damage is a key feature of the pathogenesis of AD (Barnham, et al. 2004, Behl, et al. 1992, Butterfield, et al. 2007) and $\text{Cu}^{2+}$ Fentons cycling is the probable source of ROS. A recent key study shows transfer of copper from $\alpha\beta$ to a redox inactive form bound to metallothionein negates $\alpha\beta$’s neurotoxic properties (Meloni, et al. 2008). Likewise neurons with lower antioxidants are more susceptible to $\alpha\beta$-Cu toxicity (White, et al. 1999) and cells can be rescued from $\alpha\beta$-Cu toxicity by the presence of catalase, an enzyme that catalyses the decomposition of hydrogen peroxide into water and oxygen (Behl, et al. 1994, Loew 1900, Opazo, et al. 2002).

Soluble $\alpha\beta$(1-40) and $\alpha\beta$(1-42) are present in the cerebrospinal fluid (CSF) and blood plasma from birth, (Lambert, et al. 1998, Vigo-Pelfrey, et al. 1993), but the trigger that promotes oligomerisation and fibril formation is a subject of debate. Physiological levels of $\text{Cu}^{2+}$ and $\text{Zn}^{2+}$ will cause marked aggregation of $\alpha\beta$ (Atwood, et al. 1998, Atwood, et al. 2004, Bush, et al. 1994) and while some studies suggest $\text{Zn}^{2+}$ and $\text{Cu}^{2+}$ promote amorphous aggregation of $\alpha\beta$ (Bush, et al. 1994) but inhibit fibril formation (Raman, et al. 2005, Yoshiike, et al. 2001), others have suggested fibril assembly only occurs in the presence of $\text{Cu}^{2+}$ ions (Huang, et al. 2004) and this is investigated further in Chapter 5. Specific $\text{Cu}^{2+}$ chelators can reverse the aggregation that occurs in AD, and cause solubilisation of amyloid deposits from post-mortem AD brain tissue (Cherny, et al. 1999) and thus $\text{Cu}^{2+}$ chelators represent a possible therapy for Alzheimer’s disease (Bush 2002, Cherny, et al. 2001, Ritchie, et al. 2003).

Recent studies in a Drosophila model of Alzheimer's disease show that the iron-binding protein ferritin and also catalase are potent suppressors of the $\alpha\beta$ toxicity. Likewise, treatment with the iron-binding compound clioquinol increased the lifespan of flies expressing $\alpha\beta$(1-42). These studies suggest that oxidative stress, probably mediated by the hydroxyl radical and generated by the Fenton reaction, is essential for $\alpha\beta$(1-42) toxicity in vivo and provide strong support for a role for metal ions in Alzheimer's disease (Rival, et al. 2009). Additionally it has been shown that trace amounts of copper in a cholesterol high diet induces amyloid plaques and

However, despite these findings there are two commonly expressed objections to the role of copper in AD. The first objection is that AD is not associated with elevated exposure to environmental copper. It is important to clarify this misconception. The total concentration of copper within the brain is potentially more than sufficient to be neurotoxic. As a consequence, the brain has efficient homeostatic mechanisms in place to maintain compartmentalisation of metal ions, which when compromised cause neurodegenerative diseases such as Wilson's and Menkes' disease. However as outlined above there is evidence to suggest that homeostatic mechanisms for metal ions are impaired in AD patients, and AD is characterised by altered metal ion-dependent processes and metal ion concentrations in the brain.

The second objection is that the affinity of Aβ for Cu$^{2+}$ is too low to bind these metals at their extracellular concentrations. The affinity of Cu$^{2+}$ for Aβ is key to its physiological significance and there are large discrepancies between the affinities reported for monomeric Aβ, and no reports of Cu$^{2+}$ affinities for fibrillar Aβ. Dissociation constants for monomeric Aβ range from µM to nM (Garzon-Rodriguez, et al. 1999, Hatcher, et al. 2008, Syme, et al. 2004, Tougu, et al. 2008), and in one report up to atto-molar $K_d \sim 10^{-18}$ M (Atwood, et al. 2000). This is investigated further in this thesis in Chapter 3.

1.12. Aims of this Thesis

Thus from this chapter it is clear that, although a great deal of progress has been made since 1985 when Aβ was first isolated, many important details are missing, and a great deal of controversy as to the role of Cu$^{2+}$ remains. The four main aims of this thesis are outline below, but more detailed aims are within each of the four results chapters.

1. Determine the affinity of both monomeric and fibrillar Aβ to ascertain if the Aβ-Cu$^{2+}$ complex has a high enough affinity to make it physiologically relevant.
2. Investigate if the Cu\(^{2+}\) binding complex and stoichiometry differ between monomeric and fibrillar A\(\beta\) to see if the process of fibril formation results in a different binding complex.

3. Compare the fibril formation rates of A\(\beta\) in the presence and absence of Cu\(^{2+}\), to establish the role of Cu\(^{2+}\) ions in fibril formation.

4. Study the toxic effects of A\(\beta\) monomeric and fibrillar A\(\beta\) in the presence of Cu\(^{2+}\) to see if Cu\(^{2+}\) ions enhance or reduce A\(\beta\) toxicity.
2. Materials, Methods and Method Theory
2.1. Materials

2.1.1. Peptide Synthesis and Purification
Peptides representing various fragments of Aβ were synthesised by employing solid phase F-moc chemistry and produced by either the ABC facility at Imperial College, London or Zinisser Analytic, Maidenhead. After removal from the resin and deprotection, the samples were purified using reverse phase HPLC and characterised using mass spectrometry and $^1$H NMR. Synthesised peptides were supplied lyophilised and stored in the freezer at –18 °C or –70 °C.

2.1.2. Design of Peptides and Peptide Sequences
Peptides were synthesised of the human sequence of Aβ(1-40) and Aβ(1-42) in which the N-terminus and C-terminus were left as the native amino and carboxyl groups respectively. In the Aβ fragments used, the truncated C-terminus was blocked as the corresponding ethyl ester while at the N-terminus Asp1 retains a free amino group. Together with the wild-type Aβ(1-28) fragments, four analogs of Aβ(1-28) were synthesised in which His6, His13 and His14 residues are replaced by alanine and one in which the N-terminus was acetylated - Aβ(Ac1-28). Two shorter fragments were also synthesised: Aβ(1-11) and Aβ(Ac10-16). Sequences are given below:

Aβ(1-42): DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIIGLMVGGVVI
Aβ(H6A): DAEFRADSGYEVHHQKLVFAEDVGSNK
Aβ(H13A): DAEFRHDSYEVHAQKLVFAEDVGSNK
Aβ(H14A): DAEFRHDSYEHQAQLVFAEDVGSNK
Aβ(Ac1-28): Ac-DAEFRHDSYEVHQQKLVFAEDVGSNK
Aβ(Ac10-16): Ac-YEVHHQK

2.1.3. General Chemicals and Suppliers

Chemicals
Alamar blue (Invitrogen); Copper(II) Chloride dihydrate (99.9%) (Aldrich); Deuterium Oxide (99.9% atom D min, D$_2$O) (Goss Scientific); N-ethylmorpholine (C$_6$H$_{13}$NO) (Sigma); Glycine (H$_2$NCH$_2$COOH) (BDH); HEPES (N-[2-hydroxyethyl] piperazine-N’-[2 ethanesulfonic acid]) (Sigma); L-Histidine (99 %, L-α-Amino-β-[4-imidazolyl] propionic acid) (Sigma); Horse Serum (Sigma); Hydrochloric acid (SpectrosoL, 11.6 M, HCl) (BDH); Methanol (99.93%, HPLC grade, CH$_3$OH) (Aldrich); MTT ((3-(4,5-
Chapter 2. Materials and Methods

Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma); Nitrilotriacetic acid (C₆H₉NO₆) (Sigma); Opti-MEM Reduced Serum Media (Invitrogen); Potassium Chloride (saturated solution, KCl) (Sigma-Aldrich); Potassium phosphate dibasic (1.0 M, HK₂O₄P) (Sigma); Potassium phosphate monobasic (1.0 M, H₂KO₄P) (Sigma); RPMI-1640 + glutamine no phenol red (Sigma); DSS (sodium 2,2 dimethyl-2-silapentane -5-sulphonate) (Goss Scientific); Sodium Hydroxide (NaOH) (Sigma); T (C₁₇H₁₉ClN₂S) (Aldrich); Tris(hydroxymethyl)methylamine (H₂NC(CH₂OH)₃ (BDH); Trypan Blue (Sigma), Uranyl acetate (obtained with kind permission from Norbert Krauss); Zinc Chloride (ZnCl₂) (Sigma). All chemicals were of analar grade or better.

Other
Carbon coated 300 mesh copper grids (Agar Scientific)
PC12 cells (Health Protection Agency Culture Collection)
EPR tubes 178 mm in length, 4 mm diameter (Fluorochem Limited)

2.2. General Experimental Methods

General experimental methods used throughout this thesis are described here. Additionally the theory of the major techniques used, circular dichroism, fluorescence and electron paramagnetic resonance are also discussed in this chapter. Methods specific to each chapter can be found at the start of each individual chapter.

2.2.1. Peptide Solubilisation

Theoretical Background

There are several different methods currently used for the solubilisation of Aβ. Chaotropic agents such as dimethylsulfoxide (DMSO) (Stine, et al. 2003), organic acids such as trifluoroacetic acid (TFA) (Zagorski, et al. 1999), organic cosolvents, usually trifluoroethanol (TFE) and hexafluoroisopropanol (HFIP) (Chen, et al. 2001, Zagorski, et al. 1992) are common in the literature. Alternatively basic conditions, such as using sodium hydroxide (NaOH) to obtain a pH of ~10.5 have also been shown to effectively solubilise Aβ (Fezoui, et al. 2000).

A problem with using strong acids or organic solvents such as HFIP is that they can cause conformational changes that may influence fibrillogenesis. This adds to the variability that is already a major problem in fibril growth studies (Shen, et al. 1995). In
addition the presence of these organic solvents or acids can be directly cytotoxic and thus interfere with toxicity studies, such as in Chapter 6 of this thesis. Using strongly basic conditions for solubilisation avoids these problems. An additional advantage of using high pH solubilisation conditions is that it avoids Aβ being near its isoelectric point. Synthetically produced Aβ contains bound trifluoroacetate or fluoride ions (depending on whether F-moc or t-BOC peptide synthesis is used), which, when Aβ is solubilised, will create an acidic environment. When Aβ is subsequently added to buffer at a neutral pH the solution passes through the isoelectric point of Aβ (5.3), where the potential for Aβ aggregation and precipitation is maximised. This can result in highly variable kinetic behaviour and fibril morphologies (Wood, et al. 1996). However, by using strongly basic conditions Aβ avoids the transition from low pH via its pI to physiological pH and thus escapes the problems that can result from Aβ being at its pI (Fezoui, et al. 2000, Teplow 2006).

The NaOH method has been shown to be as effective as two other commonly used methods, the DMSO/water/Tris method and the NaCl/PO₄ method (Lashuel, et al. 2003) and also HFIP (Hortschansky, et al. 2005). Furthermore, the Teplow lab has found that NaOH pretreatments results in fewer aggregates and diminishes variability (Fezoui, et al. 2000). Because of this, NaOH was used to solubilise lyophilised Aβ in the studies in this thesis.

Other methods that are used to try to achieve a homogenous population of monomers in Aβ samples are centrifugation, sonication and filtration. Centrifugation was used on one solubilised batch of Aβ(1-40) to see if the light scatter was reduced, however there was no improvement. Sonication was not used, as any form of agitation such as pipetting or shaking was found to accelerate fibril formation. Filtration, such as through 0.2 µM filters, has been used by groups to remove large aggregates from Aβ stocks, however this is insufficiently exclusive as assemblies of 200 nm are generally already fibrillar. There are filters with smaller pores such as a 10 kDa exclusion limit which are more effective. Although these filters will initially create populations of only monomers and dimers, the stock solution will still rapidly (within seconds (Teplow 2006)) reach an equilibrium comprised of a population of low order assemblies. Thus filtration was not used in the solubilisation method in this thesis.
Experimental Specifics

For experiments described in this thesis, lyophilised full length Aβ(1-40) or Aβ(1-42) was solubilised at 0.7 mg/mL in NaOH in water (ultra high quality, >18 Ω cm resistivity) at pH ~10.5 and gently rocked at 5 °C for a minimum of 48 hours. The pH was checked during this period and kept at pH 10.5. Peptide concentrations were determined using the extinction coefficient of 1280 M⁻¹ cm⁻¹ (due to the single tyrosine residue) at 280 nm (Gill, et al. 1989). Typically, the freeze-dried peptides contained ~10% moisture by weight. UV/visible electronic absorption spectra were obtained with a Hitachi U-3010 double beam spectrophotometer, using a 1 cm path length quartz cuvette. The peptides were then aliquoted and frozen at -20 °C.

Evidence that the method used in this thesis to solubilise Aβ was successful in creating seed free Aβ is:

- The minimal light scatter as observed by UV-Vis absorbance spectroscopy.
- The lack of an absorbance band at 217 nm which is indicative of β-sheet secondary structure, and a strong band at 198 nm, indicative of irregular conformations, as measured by circular dichroism.
- The baseline levels of thioflavin T (ThT) fluorescence of the solubilised batches, indicative of a lack of fibrils present. The readings in the lag phase are comparable to the readings of buffer with ThT.
- The long lag time for both Aβ(1-40) and Aβ(1-42) (~75 hours) at pH 7.4 which is in contrast to the effect of deliberate seeding which removes the lag phase.
- Size exclusion chromatography of solubilised batches shows the population is predominantly dimeric and trimeric (courtesy of Hannah Wright/Nadine Younan).

After Aβ is solubilised it is referred to in this thesis as either soluble or monomeric Aβ. However it is likely that freshly solubilised Aβ exists as a mixture of monomers and low-order oligomers, and that these species are in equilibrium. The presence of low order oligomers is not as much of a concern as the ability to prepare peptide stock solutions which all resemble each other in their distribution of peptide assembly states and thus give reproducible experimental results.
2.2.2. Cu$^{2+}$ and Competing Ligand Addition

A Cu$^{2+}$ stock was made to a concentration of 25 mM in water as CuCl$_2$ (ultra high quality, >18 $\Omega$. cm resistivity) and then subsequently diluted down to a concentration that allowed a small volume (10 $\mu$l – 50 $\mu$l) to be added to the peptide sample to allow limited dilution of the peptide sample. The addition of competing ligands to the A$\beta$ peptides was also performed using small aliquots from stock aqueous solutions (10 – 50 mM) of glycine, L-histidine or nitrilotriacetic acid (NTA).

2.2.3. Buffers

The spectroscopic techniques used in this thesis have some influence on the choice of buffer. The affinity studies in Chapter 3 were typically performed in the absence of buffer and performed in ultra high quality (>18 $\Omega$. cm resistivity) water with 160 mM NaCl, pH 7.4. 10 mM phosphate buffer, 10 mM N-Ethylmorpholine (EM) buffer and 10 mM N-(2-Hydroxyethyl) piperazine-N$'$[2-ethanesulfonic acid]) (HEPES) were used in the EPR pH dependence study in Chapter 4. The fibril growth studies in Chapter 5 and 6 used 50 mM HEPES buffer with 160 mM NaCl, pH 7.4. Buffers were made to their respective concentration in water (ultra high quality, >18 $\Omega$. cm resistivity). The structures of the buffers used in this thesis, their pK$_a$, and their affinity for Cu$^{2+}$ are shown in Figure 2.1 and Table 2.1.

![Figure 2.1. Structure of four buffers commonly used in this thesis. N-2-hydroxyethylpiperazine-N$'$-ethanesulphonic acid (HEPES), N-ethylmorpholine buffer (EM), Tris-HCl and phosphate buffer.]

<table>
<thead>
<tr>
<th>Buffer name</th>
<th>Molecular Formula a</th>
<th>pH range a at 21 °C</th>
<th>Log K Cu b</th>
<th>pKₐ at (25°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-2-hydroxyethylpiperazin e-N’-ethanesulphonic acid buffer (HEPES)</td>
<td>C₈H₁₈N₂O₄S</td>
<td>7.2–8.2</td>
<td>3.22</td>
<td>7.48 e</td>
</tr>
<tr>
<td>Phosphate Buffer (mono and dibasic potassium phosphate combination)</td>
<td>K₂HPO₄/KH₂PO₄</td>
<td>5.8–8.0</td>
<td>3.20</td>
<td>7.20 d</td>
</tr>
<tr>
<td>Tris-HCl buffer</td>
<td>NH₂C(CH₂OH)₃</td>
<td>7.1–8.9</td>
<td>4.05</td>
<td>8.10 e</td>
</tr>
<tr>
<td>N-Ethylmorpholine buffer (EM)</td>
<td>C₆H₁₃NO</td>
<td>7.0–8.2</td>
<td>Not available</td>
<td>7.70 e</td>
</tr>
</tbody>
</table>

2.2.4. pH Measurements and Adjustments
The pH was adjusted using small amounts of 10-100 mM NaOH or HCl and measured using a Thermo Electron Orion 3 Star pH benchtop with an electrode with a 3 mm stem diameter and 55 mm in length (catalogue number KCMAW11) to allow the pH of samples to be read whilst in 1 cm path length quartz cuvettes. For the EPR and multi-well plate fluorescence experiments, the pH of the samples was determined before and after the experiment was carried out. The pH of most experiments was pH 7.4, unless stated otherwise, to recreate physiological conditions as much as possible.

2.2.5. Fibril Growth Conditions
The solubilised peptide (described in Section 2.2.1) was unfrozen, diluted to the correct concentration and used immediately. A range of buffers were used, depending on the spectroscopic technique.

For experiments in Chapters 3 and 4, the solubilised peptide was diluted to 50 μM in the appropriate buffer, with 160 mM NaCl at pH 7.4. The peptide was then left for 72 hours at 37 °C with agitation. Cu²⁺ was added at the end of fibril formation. Fibril growth was monitored using a Hitachi F-2500 fluorescence spectrophotometer and confirmed by the
sigmoidal Thioflavin T fibril growth curve. An excitation wavelength of 444 nm was used and data collected over the range of 455 – 520 nm with an average of 3 scans and gentle magnetic stirring.

In fibril growth experiments in Chapters 5 and 6 fibrils were grown in the presence of Cu$^{2+}$ and lower concentrations (typically 5 μM) of Aβ were necessary. The fibrils were typically grown at 30 °C, as this was found to be the optimum temperature to allow the growth curve to have both a lag phase and still form within a week. Additionally 30 seconds agitation was performed every 30 minutes, before a reading was taken. Fibril growth was typically monitored using 10 μM ThT and were grown in a BMG fluostar galaxy fluorescence spectometer. An excitation filter wavelength of 440 nm and an emission filter wavelength of 490 nm were used. For further information on the theory of fibril formation please refer to Chapter 5.

### 2.3. Spectroscopy Introduction

Multiple spectroscopies were used in this thesis to study the relationship between Aβ and Cu$^{2+}$, see Figure 2.2. The basis of the spectroscopic techniques is well covered in a number of books and reviews, see individual sections for references. However each of the main techniques used in this thesis has been outlined in brief.

Electromagnetic radiation is composed of coupled oscillating electric and magnetic fields. In optical spectroscopy, such as UV-Vis (Section 2.3.1), circular dichroism (Section 2.4) and fluorescence (Section 2.5) the radiation is at shorter wavelengths (the UV and visible) and will typically excite valence electrons to a higher energy orbital. Alternatively in EPR (Section 2.6) longer wavelength are used (microwaves) and are of a low enough energy that they can only ‘flip’ the spin orientations of electrons (EPR) between two energy levels.

Populations of the various energy levels in spectroscopy will adopt a Boltzmann distribution, so that at temperature T (kelvins), the number of molecules in an upper state ($n_{\text{upp}}$) relative to the number in a lower state ($n_{\text{low}}$) is given by equation 1 below:

\[
\frac{n_{\text{upp}}}{n_{\text{low}}} = \exp\left(-\frac{\Delta E}{kT}\right). \quad (\text{eq 1})
\]
Chapter 2. Materials and Methods

Where \( k \) is the Boltzmann constant and \( \Delta E \) is the energy difference between the states. Each of the spectroscopic techniques monitors the absorption and excitation of molecules at certain wavelengths, to the upper state, followed by the molecule’s return to the lower state.

![Electromagnetic spectrum with corresponding spectroscopic techniques.](image)

**Figure 2.2.** Electromagnetic spectrum with corresponding spectroscopic techniques.

### 2.3.1. Ultraviolet-Visible Spectroscopy

Ultraviolet-visible (UV/Vis) electronic absorption spectra were obtained with a Hitachi U-3010 double beam spectrophotometer. Peptide concentration determinations were carried out using a 1 mL volume, 1 cm path length quartz cuvette (Hellma). Subtraction of a baseline using a blank sample, containing water, was performed. Spectra were recorded at room temperature, at a scan rate of 300 nm min\(^{-1}\). For wavelength scans (800 nm to 200 nm) a 1 nm sampling interval was used. Aβ has an extinction coefficient of 1280 M\(^{-1}\) cm\(^{-1}\) at 1280 nm (Gill and von Hippel 1989) and the concentration determined using the Beer-Lambert law.
2.4. Circular Dichroism (CD) spectroscopy

A brief overview of the theory of CD is provided below. For further information Alex Drake is an excellent reviewer of this technique (Drake 1994).

Theoretical Background

2.4.1. Circularly Polarised Light

![Diagram of circular dichroism](https://example.com/diagram.png)

Figure 2.3. Principles of Circular Dichroism. (a) Left and (b) right circularly polarised light. (c) The basic components of a circular dichroism spectrometer. Adapted from Drake 1994.

Plane polarised light can be divided into two forms of circularly polarised light of equal intensity (Figure 2.3a and b). The differential absorbance of left (L) and right (R) circularly polarised light by a chromophore is the fundamental principle behind circular dichroism spectroscopy. Left-handed circularly polarised light rotates in a counter-clockwise direction, and right-handed (R) circularly polarised light rotates in a clockwise direction. For an optically inactive chromophore the difference between
absorption of R and L circularly polarised light will be zero. The degree of optical activity is determined by a molecules asymmetry. A simple diagram of how a CD spectrometer works is shown in Figure 2.3c.

2.4.2. Monitoring Secondary Structure Changes Using CD in the UV region.
CD spectroscopy of the secondary structure of peptides arises from the amide group of the peptide bond within the polypeptide backbone. The peptide bond is slightly shorter than a standard single bond, reflecting the partial delocalization of $\pi$ electrons from the carbonyl group into orbitals shared with the lone pair electrons of the amide nitrogen. This partial double bond character inhibits rotation around the peptide bond and provides the $\pi$ electrons required for the transitions studied in CD.

Two electronic absorptions occur, with electrons in $n$ or $\pi$ orbitals transitioning to the higher energy $\pi^*$ orbital. $\pi \rightarrow \pi^*$ transitions give a strong absorption (molar absorptivities between 1000 – 10,000 mol$^{-1}$ cm$^{-1}$) around 190 nm. Molar absorptions from $n \rightarrow \pi^*$ transitions are relatively low (10-100 mol$^{-1}$ cm$^{-1}$) and absorb ~ 210 nm, (often masked by the $\pi - \pi^*$ transition). These transitions define the spectral range 250-170 nm for secondary structure analysis.

2.4.3. Circular Dichroism Secondary Structure Determination
CD in the far-UV region represents the super-position of a variety of secondary structure elements. The different proportions of the various secondary structures that compose the spectra can be defined using various computer packages if needed (e.g. Dichroweb). There are distinct CD spectra bands for both $\alpha$-helix and $\beta$-sheet structure (see Figure 2.4). Two negative bands of similar magnitude with maxima at 222 nm and 208 nm, and a positive band at 192 nm, are characteristic of an $\alpha$-helix CD spectrum. $\beta$-sheet CD spectra are much weaker than $\alpha$-helix CD and have a negative band at 217 nm ± 5 nm and a positive band near 195 nm. Irregular conformations give a negative band at ~ 198 nm and a very weak positive band at 218 nm.
Figure 2.4. Typical CD bands of \(\alpha\)-helix and \(\beta\)-sheet secondary structure and random coil (unstructured). Adapted from Brahms et al. 1980.

2.4.4. Secondary Structure Studies Experimental Specifics
CD spectra were recorded on a Chirascan (Applied Photophysics) CD spectrometer at 25 °C. A cell with a 0.1 cm path length was predominantly used for spectra recorded between 185 and 270 nm with sampling points every 0.5 nm. A minimum of 3 scans were recorded for all spectra including baseline spectra which were subtracted from each spectra. Applied Photophysics Chirascan Viewer was used to apply minimal smoothing to the data if required. Direct CD measurements (\(\theta\), in millidegrees) were converted to molar ellipticity, \(\Delta\varepsilon\) (M\(^{-1}\) cm\(^{-1}\)), using the relationship \(\Delta\varepsilon = \theta/(33000cl)\), where \(c\) is the concentration and \(l\) the path length.

2.4.5. Calculating A\(\beta\)-Cu\(^{2+}\) Affinity Using Ligand-Metal Charge Transfer Studies
In addition to secondary structure, CD is also useful at studying the ligand coordination geometry around a metal ion. Ligand-metal charge transfer transitions and d-d transitions occur in the near-UV and visible region (270-800 nm). Ligand metal charge transitions, in A\(\beta\)-Cu\(^{2+}\) for example, occur when the electron in Cu\(^{2+}\)’s d-orbital absorbs
light at ~ 300 nm resulting in a transition to the $\pi$ orbital of a histidine in A\textbeta. d-d transitions occur when the electrons in the lower d-orbitals absorb light (~ 600 nm) and are excited to a higher energy d-orbital. It has been shown that although Cu$^{2+}$-A\textbeta does not give rise to CD bands from d-d electronic transitions, the positive CD band at 320 nm can be assigned to A\textbeta-Cu$^{2+}$-imidazole charge-transfer transitions (Syme, et al. 2004).

There are three main sources of optical activity that allow CD spectra of the transition metal ions, such as Cu$^{2+}$, in the visible region (Klewpatinond, et al. 2007, Martin, et al. 1966):

a) The configurational effect is usually responsible for the majority of the optical activity. It occurs when the distribution of donor atoms coordinating the metal ion are not symmetrical, and explains why free Cu$^{2+}$ is CD silent.

b) The conformational effect is due to ring puckering. However this effect is less applicable to tetragonal peptide complexes such as A\textbeta-Cu$^{2+}$ where coordination tends to be planar (see Chapter 4- Structure of the A\textbeta-Cu$^{2+}$ complex).

c) The vicinal effect. This effect gives the strongest optical activity when a chiral carbon atom is in the backbone of a ring between two chelated donor atoms, typically main chain amides. In peptide complexes such as A\textbeta-Cu$^{2+}$ which are fairly planar, the main determinant of optical activity is the vicinal affect of chiral substituents.

An advantage of circular dichroism over absorption spectroscopy is that CD spectra in the visible region are only produced when the metal ion is in a chiral environment. Thus free Cu$^{2+}$ in water or Cu$^{2+}$ bound to non-chiral molecules such as glycine does not have chirality and thus is CD silent, while Cu$^{2+}$ bound to L-histidine will give a very different CD spectrum, with a weak band at 320 nm and a stronger band at 700 nm. This was exploited in calculating an affinity for the A\textbeta-Cu$^{2+}$ complex, see Chapter 3.

2.5. **Fluorescence Spectroscopy**

The theory of fluorescence is briefly described below. For further details on the technique there are a number of good textbooks (e.g. (Varley 1994)) and a good review
specifically on fluorescence quenching (Section 2.5.6) is pages 238 – 265 of (Lakowicz 1999).

**Theoretical Background**

### 2.5.1. Fluorescence Signal

Substances that contain delocalised electrons, in the form of conjugated double bonds, typically exhibit fluorescence, e.g. a fluorescent dye such as Thioflavin T – see Section 2.5.4 or a tyrosine residue – see Section 2.5.5. Generally, the molecule of interest has a ground electronic state (a low energy state), and an excited electronic state of higher energy. Within each of these electronic states are various vibrational levels. When the molecule absorbs light, its orbital electrons are excited to one of the various vibrational states in the excited electronic state. The electrons will then lose energy, partly through collisions but mainly as heat (vibrations) until it reaches the lowest vibrational state of the excited electronic state. The electron will then drop down to one of the various vibrational levels of the ground electronic state again, emitting a photon in the process. Because the emitted radiation has less energy than the original absorbed light (e.g. due to heat loss) it will be of a longer wavelength (see Figure 2.5).

![Figure 2.5. State Diagram of Fluorescence.](image)

**Figure 2.5. State Diagram of Fluorescence.** Electrons have two excited states composed of vibrational levels. Upon absorption of a photon the electron is raised to the excited state. Through collisions and heat loss energy is lost and re-emitted at a longer wavelength. Adapted from Lakowicz 1999.

### 2.5.2. Fluorescence Units

Unlike in other types of spectroscopy, in fluorescence it is difficult to gain machine-independent values and thus to compare exact values between fluorescence spectrophotometers. For example, the percentage of the fluorescence picked up by the
detector is dependent upon the system, it will vary between different detectors, and with wavelength and time, as the detector inevitably deteriorates. Thus although the concentration of the fluorophore will generally be related to the intensity of the fluorescence signal, the units used in fluorescence are usually described as arbitrary fluorescence units, or AFU.

2.5.3. Instrumentation for Fluorescence Spectroscopy
Two different fluorescence spectrophotometers were used in this thesis:

Single Cell Fluorescence
For single cell experiments, a light beam from the xenon lamp is converged onto the entrance slit of the excitation monochromator through two lenses (see Figure 2.6). After monochromation the excitation beam is converged on to the sample cell. The beam is then directed to the computer resulting in a fluorescence spectrum.

![Figure 2.6. Simplified scheme for a single sample holder spectrofluorometer (adapted from Lakowicz 1999).](image)

Multiwell Fluorescence
For fibril growth studies, a BMG Fluostar galaxy was used. This fluorometer allows multiwell plates to be used. An outline of the set up of a multiwell plate reader is shown in Figure 2.7. It is essentially the same as a single cell fluorescence spectrometer, except that filters are used and the emission from the sample is detected above the plate, rather than at 90° as in single cell readers. The BMG Fluostar galaxy allows both agitation and temperature of the sample to be controlled, this is described further in Chapter 5.
2.5.4. Thioflavin T Assay Theory

Fluorescence was used in this thesis for two major purposes. Firstly, the use of the fluorescent dye Thioflavin T (ThT) will be discussed.

ThT is a positively charged dye at pH’s between 5 and 9 and interacts with the cross-β sheet structure common to amyloids (Sunde, et al. 1997). The structure and size of ThT is shown in Figure 2.8. ThT exhibits enhanced fluorescence emission at ~ 482 nm upon binding to amyloid when excited by light of a wavelength of 455 nm. Binding of ThT to the peptide occurs with apparent K_d’s of 0.54 and 2 μM for aggregated Aβ(1-28) and Aβ(1-40) respectively (LeVine 1993). Salts such as NaCl have no effect on ThT binding (LeVine 1993).
2.5.5. Mechanism of ThT Fluorescence

Several different models have been proposed to explain why ThT only fluoresces when bound to β-sheet fibrils and not in the presence of monomers or amorphous aggregates.

**Monomeric Model** (Krebs, et al. 2005)

ThT binding to amyloids has been studied by confocal microscopy and has been shown to bind in a specific and regular fashion, with the short axis of ThT (6.1 Å) perpendicular to the fibril axis closely surrounded by side chains. In this model the ThT binds in a monomeric form in the grooves formed by the residues n and n+2 of the β-strands on the surface of the fibril. Krebs et al suggest that it is the steric interactions between the dye and the side chains of the fibrils that result in fluorescent enhancement.

**Excimer Model** {Groenning, 2007 #925}

The binding of ThT was observed using anisotropy methods. Significant increases in the anisotropy are observed using between bound and unbound ThT, suggesting that ThT has less rotational motion in the presence of the binding compounds compared to free ThT. In this model it is the ability of ThT to bind as a dimer and form an “excimer” that results in the characteristic fluorescence.

**Micelle Model** (Khurana, et al. 2005)

At the ThT concentrations commonly used in experiments (10 – 20 μM) ThT will form micelles, with a critical micelle concentration of 4 μM and a micelle diameter of 3 nm. Atomic force microscopy (AFM) was used to visualise the micelles along the fibril length, and in this model fluorescent enhancement occurs due to hydrogen bond
formation between the charged nitrogen in the thiazole group of the ThT micelle and the amyloid fibrils.

**Lateral Stacking Model** (Wu *et al.* 2008)
The binding of ThT to model fibrils of Aβ(16-22) was studied via molecular dynamics. ThT was found to bind preferentially in the grooves of the β sheets rather than at the ends of the sheets, and bind to hydrophobic and aromatic residues rather than to negatively charged residues. Hydrophobic and ring-ring interactions were the main mode of binding between ThT and the fibrils. ThT was found to bind with the positively charged nitrogen parallel to the β sheet surface and exposed to the solvent. Fluorescent enhancement results from ThT binding firstly as a monomer then additional ThT molecules laterally stacking onto the bound ThT molecule to form an oligomer of up to seven monomers.

Of the four models, the lateral stacking model is attractive as it incorporates some of the other mechanisms outlined above, including reduction of the internal motion of monomeric ThT by binding to the grooves of fibrils, excimer formation and hydrogen bonding of a ThT micelle.

### 2.5.6. Tyrosine Fluorescence Quenching by Cu$^{2+}$ Ions
The second application for fluorescence was establishing the affinity of Aβ for Cu$^{2+}$ by monitoring the intrinsic fluorescence of the tyrosine residue. There are three aromatic amino acid residues (tryptophan, tyrosine, and to a lesser extent phenylalanine) which can contribute to the intrinsic fluorescence of peptides. These three residues have distinct absorption and emission wavelengths. Aβ does not contain tryptophan residues but does contains a tyrosine at position 10, which, when solvent exposed in H$_2$O, will absorb at around 280 nm and emit fluorescence at around 308 nm.

The intrinsic fluorescence of a peptide can be quenched. Cu$^{2+}$ is a well known quencher of fluorescence. The probable method of quenching is by the donation of an electron from the fluorophore (in this case Tyr10) to the quencher (Cu$^{2+}$). The quenching results from the formation of a nonfluorescent complex between the tyrosine and Cu$^{2+}$. When this complex absorbs light, it immediately returns to the ground state without emission of a photon.
2.6. Electron Paramagnetic Resonance (EPR) Spectroscopy

Below is a description of the main principles of EPR that are relevant to this thesis. However for further detail, two excellent books on EPR are (Bersohn 1966) and (Weil 2007).

Theoretical Background

2.6.1. What is EPR?

EPR (also known as Electron Spin Resonance, ESR) studies the resonant transitions between energy levels of electron magnetic dipole moments. An external magnetic field is applied to align the magnetic dipole moments of the electrons. The magnetic field also increases the gap between the two electron spin energy states until the difference between them matches the energy of the applied electromagnetic radiation (a fixed microwave source), thus allowing the electrons to move between their two energy states. Since there are normally more electrons in the lower energy state there is a net absorption of energy which is converted into an EPR spectrum.

2.6.2. What Can be Studied in EPR?

Each electron possesses an intrinsic magnetic dipole moment that arises from its spin. In most systems electrons occur in pairs and thus the net magnetic dipole moment equals zero. Only species which contain unpaired electrons (e.g. Cu$^{2+}$ which has one unpaired electron in its outer shell) possess the net spin moment necessary for suitable interaction with an electromagnetic field. Although electrons of paramagnetic materials have permanent magnetic moments even in the absence of an applied field, they are randomly orientated due to thermal agitation resulting in zero net magnetic moment. But when a magnetic field is applied the dipoles will align.

2.6.3. The Magnetic Dipole Moment and Spin of the Electron

The magnetic dipole moment is an important vector quantity in EPR. Magnetic dipole moments placed in a magnetic field tend to be aligned so that they point in the same direction as the field, a “parallel” orientation. This is the minimum energy orientation for a magnetic dipole moment. The intrinsic angular momentum of an electron is another vector, and is also known as the spin “S”. All elementary particles of a given kind have the same spin quantum number which is always unitless. For example, an electron has spin $\frac{1}{2}$, whereas the copper nucleus has a spin of 3/2.
2.6.4. Energy levels and Why Microwave Electromagnetic Radiation Is Used

A simple energy level diagram for an electron with spin \( \frac{1}{2} \) is shown in Figure 2.9. By varying the applied magnetic field \( B \), the energy level separation can be changed. Resonant absorption occurs if the frequency is adjusted so that \( \Delta E = h \nu \).

This ties into the basic phenomena of EPR, shown in equation 2, where the application of the correct electromagnetic resonance radiation \((h \nu)\), whose quanta have energy equal to the difference in energy levels \((\Delta E)\) between the two electron orientations \((+ g \mu_B B_z \) and \((- g \mu_B B_z)\), will result in transitions between these two states. The two states are described by the \( g \)-factor \((g)\), the Bohr Magneton \((\mu_B)\) and the strength of the experimental magnetic field \((B_z)\) in gauss.

\[
\Delta E = h \nu = g \mu_B B_z \quad \text{(eq 2)}
\]

For example, if \( B_z \) is about 3400 gauss, and \( g \) has the value for an unbound electron 2.002319, and the Bohr magneton is \(0.92732 \times 10^{-20} \text{ erg/gauss}\), all divided by Planck’s constant of \(6.63 \times 10^{-27} \text{ erg} \cdot \text{sec}\) then \( \nu \) is \(9.5 \times 10^9 \text{ cycles per second}\). This frequency is in the microwave region and explains why microwave radiation is used in EPR. Specifically in this thesis microwave radiation is used in the X-band region of 8 to 12 GHz.

The generation of microwaves is such that, experimentally, it is easier to vary the magnetic field by small increments than vary the frequency of the radiation. The position of the line in an EPR spectrum is indicated by the value of the magnetic field at which absorption of energy by the sample has occurred. Due to differences in EPR spectrometers and experimental conditions neither field or frequency is used to denote line position, instead line positions are stated in \( g \)-factors (see Section below). To minimise the noise the magnetic field is usually modulated, and as a result the detected signal appears as a first derivative.
Figure 2.9. Energy level diagram for a free electron with spin $\frac{1}{2}$. An unpaired electron can move between the two energy levels by either absorbing ($+\frac{1}{2}g\mu_B B_z$) or emitting ($-\frac{1}{2}g\mu_B B_z$) electromagnetic radiation such that the resonance condition, $hv = \Delta E$, is obeyed. The two states are described by the g-factor ($g$), the Bohr Magneton ($\mu_B$) and the strength of the experimental magnetic field ($B_z$) in gauss. Adapted from Weil 2007.

2.6.5. The g-factor

The g-factor ($g$) is a dimensionless quantity which can be thought of as a unique fingerprint for identification of a compound. Comparisons between the g-factor for a free electron in a vacuum ($g_e = 2.002319$) and the g-factor for an electron in an experimental system (calculated using equation 2) can be used to obtain information on the nature of the atomic or molecular orbital containing the unpaired electron. This is particularly relevant to studies with transition metal ions, such as in this thesis, where interactions between the electron spin and orbitals can drastically affect the g-factors.

The magnetic interactions involving the orbital angular momentum of the unpaired electron are the sole cause for the deviations of g-factors from 2.002319. Since the orbital angular momentum of the unpaired electron depends on its chemical environment in an atom, molecule or crystal the g-factor also depends upon these factors. Thus the g-factor deviations have some similarity to the property of chemical shifts in NMR spectroscopy. The two magnetic interactions involving the orbital angular momentum are spin-orbit coupling and also the interaction of the orbital angular momentum with the external magnetic field. The spin-orbit interaction arises from the nucleus “orbiting” around the electron (from the electron’s point of view) and the motion of this positive charge generates a magnetic field at the electron. Additionally
the orbital motion of the electron produces a magnetic moment which interacts with the external magnetic field.

2.6.6. Anisotropy of g

When the electron is orbiting close to nuclei, the orbital motion of the electron is quenched because of the electrostatic effect between the negatively charged electron and the lattice of nuclei. The orbital motion of the electron will be affected differently depending on whether the orbital motion is about the x, y, or z axes of the molecular system, i.e. whether the electrostatic effect is equal along all the axes or whether anisotropy occurs. Some examples of potential molecular geometries for Aβ-Cu\(^{2+}\) are shown in Figure 2.10. Octahedral geometry is isotropic (Figure 2.10a), whereas axial geometry has only linear rotational symmetry and anisotropy is observable except when the applied magnetic field is in the plane perpendicular to the axis of symmetry (Figure 2.10b). Tetrahedral geometry is anisotropic for all rotations (Figure 2.10c).

![Diagram](image)

**Figure 2.10. Anisotropy of g.** (a), (b), and (c) are potential coordination geometries for the Aβ-Cu\(^{2+}\) complex and their resulting EPR spectra.

The EPR spectra in Figures 2.10 a, b and c illustrate how the anisotropy of g affects the EPR spectra. Cu\(^{2+}\)-EPR is usually of the axial type (Figure 2.10b). Two components of
the EPR spectrum shown are the $g_\parallel$ (the parallel $g$ factor) and $g_\perp$ (the perpendicular $g$-factor). These are the $g$-factors appropriate to the magnitudes $B_\parallel$ and $B_\perp$ of the applied magnetic field, when it is parallel or perpendicular to the molecular system’s symmetry axis respectively. Figure 2.10b shows a major absorption at $g_\perp$ (high field) and a lesser absorption at $g_\parallel$ (low field). The $g_\parallel$ and $g_\perp$ components of the spectra are further complicated by the anisotropy of the hyperfine splittings ($A$), described in the next section.

2.6.7. Splitting and Hyperfine Splitting

After unpaired electrons are placed in a magnetic field the number of energy levels are increased from one to two ($E = + \frac{1}{2} g \mu_B B_z$ and $E = - \frac{1}{2} g \mu_B B_z$) (Figure 2.11). Increasing the number of energy levels through exposing the electrons to a magnetic field is called splitting.

\begin{align*}
M_s = +1/2 & \quad M_I = +3/2 \quad 1 \\
M_s = +1/2 & \quad M_I = +1/2 \quad 2 \\
M_s = +1/2 & \quad M_I = -1/2 \quad 3 \\
M_s = +1/2 & \quad M_I = -3/2 \quad 4 \\
M_s = -1/2 & \quad M_I = -3/2 \quad 5 \\
M_s = -1/2 & \quad M_I = -1/2 \quad 6 \\
M_s = -1/2 & \quad M_I = +1/2 \quad 7 \\
M_s = -1/2 & \quad M_I = +3/2 \quad 8
\end{align*}

**Figure 2.11. Hyperfine splitting of the Cu$^{2+}$ electron.** $M_s$ = angular momentum vector of electron, $M_I$ = angular momentum vector of nucleus. Only certain transitions from the ground state ($- \frac{1}{2} g \mu_B B_z$) to the excited state ($+ \frac{1}{2} g \mu_B B_z$) are allowed and/or detected by EPR. Allowed transitions are $8 \rightarrow 1$, $7 \rightarrow 2$, $6 \rightarrow 3$ and $5 \rightarrow 4$. This results in four splittings which, if the magnetic field is kept constant, results in the smaller difference between energy states (e.g. $5 \rightarrow 4$) occurring at a lower frequency and the largest energy difference (e.g. $8 \rightarrow 1$) occurring at a higher frequency.
In addition to the interaction between an electron spin and the external magnetic field is the further interaction between the electron spin and internal magnetic fields, e.g. the magnetism of nearby nuclei. The magnetic moment of the nucleus is restricted to a few particular orientations relative to the external magnetic field e.g. the copper nucleus has a spin of 3/2, and thus has nuclear spin orientations of +3/2, +1/2, -1/2 and -3/2. The magnetic energy of the electron will subsequently be affected by the nuclear magnetic moment orientation, and rather than one resonance absorption line obtained for a given value, there will be absorption lines obtained over a range of values of magnetic field. This causes the EPR spectrum to consist of a number of lines rather than just one (see Figure 2.12). The further splitting in energy levels from the interaction between the nucleus and electrons is called hyperfine splitting, analogous to J-coupling in NMR.

As well as the anisotropy in $g$ there may be an anisotropy in the hyperfine splittings ($A$) as well. Typically for Cu$^{2+}$-EPR, the hyperfine splitting $A_\perp$ (the nuclear hyperfine splitting at $g_\perp$) is poorly resolved, while the $A_\parallel$ (the nuclear hyperfine splitting at $g_\parallel$), is typically much larger, around 10 – 25 mK, with the four features at $g_\parallel$ resolved.

An idealised Cu$^{2+}$-Aβ EPR spectra is shown in Figure 2.12 with its two component parts arising from the axial coordination of Cu$^{2+}$, and the anisotropy of the $g$ and $A$ factor resulting in parallel and perpendicular components, with the parallel components at a lower field to the perpendicular ones. The hyperfine splitting ($A$) results in four splittings within both components of the spectra.
Figure 2.12. An idealised EPR spectrum for Aβ-Cu$^{2+}$. Axial geometry gives $g_{\|}$ and $g_{\perp}$ which are then split into four by the orientation of the copper nucleus with its 3/2 spin.

2.6.8. Using EPR to Indicate Coordination Ligands

The magnitude of $A_{\|}$ and $g_{\|}$ values can be used to infer the binding ligands in the complex. Peisach and Blumberg published an influential paper describing how EPR spectra of Cu$^{2+}$ tetragonal complexes could be used to infer the identity of the ligands in the equatorial plane (Peisach, et al. 1974). They noted that two components of the EPR spectra, the $A_{\|}$ and $g_{\|}$ could be related to the ligand type. They studied the relationship between the $A_{\|}$ and $g_{\|}$ values for different classes of model compounds and produced a guide that can be used to suggest binding ligands in the complex, see Figure 2.13. For example all nitrogen ligands (4N) might typically give $A_{\|}$ values of between 20 and 24 mK and $g_{\|}$ of between 2.1 and 2.2, whereas all oxygen ligands (4O) would have $A_{\|}$ values in the range 12–14 mK and $g_{\|}$ values between 2.35 and 2.45.
Figure 2.13. Relationship between $A_{II}$ and $g_{II}$ for Type 2 axial complexes of copper proteins (Figure adapted from (Peisach et al 1974)).

### 2.6.9. Line Widths and Relaxation Times

When studying transition metals with EPR, a problem arises due to the very short relaxation time $\tau_1$. This is the characteristic lifetime of the electron in its excited state and represents reorientation of the electron spin. The process of exciting the electrons starts with the spin system being irradiated by microwave radiation and the electrons being excited to their higher energy state. The absorbed radiation is then lost by energy diffusion which allows continuing absorption of photons to occur. However the short $\tau_1$ that occurs with transition metals can result in line broadening beyond detection. Because of this EPR experiments as described here were taken at low temperatures, (~15-20 kelvin), since relaxation times tends to increase with decreasing temperatures.
2.7. Transmission Electron Microscopy Experimental Specifics

Following incubation and fibril formation (as described in 2.2.5) aliquots were taken from test samples and frozen at −80 °C. Samples were then freeze-dried and resuspended to a concentration of approximately 0.5 mg/mL. A 10 µL aliquot of each sample was then spotted onto a carbon coated copper grid (Agar Scientific) and negatively stained using the single droplet procedure with 2 % (w/v) aqueous uranyl acetate. TEM images were recorded on a JEOL JEM 2010 transmission electron microscope.

2.8. Software packages

The Kaleidagraph spreadsheet/graph package was used to present data. Origin and Microsoft Excel were both used for data manipulations. See individual experimental sections for equipment software.
3. Fibrillar and Monomeric Aβ have a Picomolar Affinity for Cu$^{2+}$
Chapter 3. Fibrillar and Monomeric Aβ have a Picomolar Affinity for Cu$^{2+}$

Abstract

The affinity of Cu$^{2+}$ for monomeric Aβ is strongly disputed and there have been no reports of Cu$^{2+}$ affinity for fibrillar Aβ. Determining the affinity of Aβ for Cu$^{2+}$ is highly important in evaluating if there is a role for Cu$^{2+}$ in AD. Additionally there are currently metal chelators being developed as therapeutics in AD, thus determining the correct affinity is extremely important to ensure their efficacy.

In this chapter the affinity of Cu$^{2+}$ for both monomeric and fibrillar Aβ(1-42) was measured using two independent methods: fluorescence quenching and circular dichroism. Interestingly the binding curves were almost identical for both fibrillar and monomeric forms. Competition studies with free glycine, L-histidine and nitrilotriacetic acid (NTA) indicate an apparent dissociation constant of 10$^{-11}$ M, at pH 7.4 for both forms of Aβ. There was excellent agreement between the two techniques. Previous studies of Cu-Aβ have typically placed the affinity two or more orders of magnitude weaker, largely because the affinity of competing ligands or buffers has been under-estimated.

A picomolar Cu$^{2+}$ dissociation constant for Aβ makes Aβ a physiologically relevant Cu$^{2+}$ chelator, with this dissociation constant much lower than Cu$^{2+}$ concentrations at the synapse. Thus the work in this chapter supports a modified amyloid cascade hypothesis in which Cu$^{2+}$ is central to Aβ neurotoxicity.

Studies in this chapter have recently been published in (Sarell, et al. 2009).
3.1. Introduction

3.1.1. Why Determine the Affinity?
Determining the affinity of the Aβ-Cu$^{2+}$ complex is important to give physiological relevance to the hypothesis that Cu$^{2+}$ has a role in AD. A low binding affinity would suggest that the Aβ-Cu$^{2+}$ complex in vivo is unlikely, given the presence of other physiological ligands with a higher affinity and the total levels of extracellular Cu$^{2+}$ ions. Furthermore, understanding affinities can give a greater understanding of how Cu$^{2+}$ might interact with Aβ. For example a recent paper determined the apparent Cu$^{+}$ binding affinities for intracellular copper proteins (such as the cytoplasmic copper chaperones CCS) and showed that affinity gradients are key to driving copper to its destination within the cell (Banci, et al. 2010).

Additionally chelation therapy is used to treat some metal-related illnesses such as β-thalassemia sufferers, where iron chelation drugs are administered. Likewise excess copper in Wilson’s disease can be removed by Triethylenetetramine (TETA) or penicillamine. However selectivity is extremely important, if the chelator’s affinity is too weak it will be inefficient, too high and it might withdraw metal ions from essential metalloproteins, leading to metal deficiency and a range of severe consequences. Thus the chelation therapy ligand is chosen on the basis of the affinity of the complexes formed, and if metal chelators are to be used in AD (see Chapter 7) a reliable affinity of Cu$^{2+}$ for Aβ must be obtained.

Currently there are large discrepancies between the affinities reported for monomeric Aβ, and no reports of Cu$^{2+}$ affinities for fibrillar Aβ. Dissociation constants for monomeric Aβ range from μM to nM (Garzon-Rodriguez, et al. 1999, Hatcher, et al. 2008, Syme, et al. 2004, Tougu, et al. 2008), and in one report up to atto-molar K$_d$ ~ $10^{-18}$ M (Atwood, et al. 2000). In Section 3.5.3 some explanations are offered for these large discrepancies.

3.1.2. Background Theory to Affinity and Dissociation Constants
Any reaction or binding event can be thought of as an equilibrium between the reactants and products. In the case of a metal (M) binding to a ligand (L) to form a metal-ligand complex (ML) then:
Chapter 3. Fibrillar and Monomeric Aβ have a Picomolar Affinity for Cu\(^{2+}\)

\[ M + L \rightleftharpoons ML \]  
(eq 1)

The affinity (\(K_a\)) for this binding event is given by the ratio of the products formed relative to the reactants remaining:

\[
K_a = \frac{[\text{Product}]}{[\text{Reactants}]} = \frac{[ML]}{[M][L]} \]  
(eq 2)

In this case the units of affinity (\(K_a\)) are Molar \(^{-1}\). The reverse reaction, where the metal dissociates from the ligand, is given by:

\[
K_d = \frac{[M][L]}{[ML]} \]  
(eq 3)

Here the dissociation constant (\(K_d\)) is given in Molar concentrations. \(K_a\) and \(K_d\) are related by:

\[
K_a = \frac{1}{K_d} \]  
(eq 4)

The smaller the dissociation constant, the higher the affinity between the two components of the complex. The dissociation constant \(K_d\) is useful as it indicates at what concentration of metal half of the ligand’s (e.g. protein’s) binding site will be occupied by the ion. For example a \(K_d\) of 1 nM for a complex means that the metal concentration must be equal to 1 nM for half the protein binding sites to be filled. If the metal is at a pM concentration the protein binding site will not be significantly occupied, even if the protein is added in excess. Alternatively at metal concentrations greater than nM, additions of the protein will result in the metal occupying the protein binding sites until the binding sites are saturated.

Thus in the case of a single Cu\(^{2+}\) binding site in Aβ:

\[
K_d = \frac{[Cu^{2+}][A\beta]}{[Cu^{2+}A\beta]} \]  
(eq 5)
Chapter 3. Fibrillar and Monomeric Aβ have a Picomolar Affinity for Cu$^{2+}$

3.1.3. Absolute and Apparent Stability Constants

pH independent stability constants

Stability constants can be pH-independent (the *absolute affinity*). For example, in one commonly used reference book the stability constants are expressed as absolute affinities. They are quoted at 25-30 °C at ionic strengths between 0.01 to 0.5 (Dawson 1986), and they are independent of pH. The absolute affinities of glycine, histidine and nitrilotriacetic acid (NTA) are shown in the first column of Table 3.1.

pH dependent stability constants

The pH-dependent stability constant is called the *apparent dissociation constant* or the *apparent affinity*, and in this chapter it is the experimentally measured affinity. It is calculated from the absolute affinity, see experimental. It is more useful to know the affinity at a particular pH, e.g. at physiological pH 7.4, as the affinity of a metal for a protein can be strongly influenced by pH. At lower pH values H$^+$ ions compete with the metal ion for binding sites. Thus the affinity of the metal for a protein can be many orders of magnitude higher at pH 8, for example, compared to pH 6. The apparent affinity and apparent dissociation constants at pH 7.4 are shown in columns 3 and 4 of Table 3.1 respectively. Buffers can also compete for metal ions and although their affinity is usually quite low, they are often present at relatively high concentrations thus they must be accounted for when calculating a meaningful affinity.

3.1.4. Influences on Affinity

The affinity of an ion such as Cu$^{2+}$ for a ligand is strongly influenced by its surroundings. Molecules in solution interact, either to attract or to repel each other. It is generally agreed that this interaction can be ignored if the stability constants are determined in the presence of a background electrolyte at high ionic strength. However, because of this, stability constants will vary depending on the specific electrolyte used, and the concentration of the electrolyte.

The $K_a$ is also temperature dependent, for exothermic reactions $K_a$ will decrease with increasing temperature, but for endothermic reactions, $K_a$ increases with increasing temperature. Finally the *in vivo* environment is more crowded than the dilute conditions that most *in vitro* experiments are performed in. Total
macromolecule concentrations in the cell are as high as 400 mg/mL (Ellis 2001), and although the interactions between Aβ and Cu²⁺ occur extracellularly, which is less crowded than intracellular conditions, this is still a factor that should be considered when assessing the probability that the complex will form.

### 3.1.5. Affinity (K) Vs Reaction Rates (kf/kr)

An interesting side-point is that although affinity is crucial in determining if two species will form a complex, it does not necessarily reflect how fast the reaction will occur or how permanent the complex will be. For example metallothionein has a high affinity for zinc ions (0.1 picomolar), yet will exchange zinc readily with proteins with weaker affinities (Jacob, et al. 1998, Maret, et al. 1999). On the other hand, carbonic anhydrase has a dissociation constant of 4 pM but exchanges zinc extremely slowly, with a half-life of 1 year (Lindskog, et al. 1962).

Reaction rates can be calculated as follows:

The rate of the forward reaction is given by \( v_f \) and the rate of the reverse reaction is given by \( v_r \).

\[
v_f = k_f [M][L] \quad \text{(eq 6)}
\]

Where \( k_f \) is the proportionality constant in the forward direction. Likewise, the rate in the reverse reaction is:

\[
v_r = k_r [M][L] \quad \text{(eq 7)}
\]

Where \( k_r \) is the rate constant for the reverse reaction. At equilibrium \( v_f = v_r \) and:

\[
\frac{k_f}{k_r} = \frac{[ML]}{[M][L]} = K_a
\]

Thus this equation shows that the affinity, \( K_a \) is equal to the ratio of the forward and reverse reaction.
3.1.6. Methods of Calculating Affinity

The methods and equations used to calculate the affinity of Aβ for Cu\(^{2+}\) in this chapter are described in the experimental section. However there are multiple different ways the affinity of a complex can be determined, with any technique that can quantitatively measure the levels of products or reactants present potentially able to measure the affinity. A few common techniques are listed below:

- Equilibrium dialysis
- Isothermal titration calorimetry
- Surface plasmon resonance
- Spectroscopy
- Gel filtration chromatography
- Membrane filtration

**Binding Curves**

A common method of presenting affinity data is as a binding curve. Binding curves are graphs of ligand concentration on the x axis against the saturation of the binding site (\(\theta\)) on the y axis (see Figure 3.1a). The dissociation constant \(K_d\) can be calculated when \(\theta = 0.5\), i.e. half the ligand is bound and half is not. Before nonlinear regression programs were widely available it was necessary to transform the curves into a linear form and then analyse the data by linear regression. The most popular method to do this was to create a Scatchard plot. This is done plotting \(\theta/[L]\) on the y axis and \(\theta\) on the x axis, where [L] is the concentration of free ligand. This gives a straight line slope of \(-1/K_d\) (see Figure 3.1b). A non-linear regression line indicates that there is cooperativity occurring in the binding.

3.1.7. Competitive Metal Capture

To calculate an affinity an equilibrium needs to be achieved where half the Aβ molecules have Cu\(^{2+}\) bound. The equipment used in this thesis to measure affinity requires micromolar concentrations of Aβ and Cu\(^{2+}\). At these concentrations the binding curves show that tight binding is occurring. Examples of weak and strong binding curves are shown in Figure 3.1c.
Chapter 3. Fibrillar and Monomeric Aβ have a Picomolar Affinity for Cu$^{2+}$

The problem with tight binding is that all of the added Cu$^{2+}$ is bound to Aβ as it is titrated in, and no detectable free Cu$^{2+}$ remains in solution, i.e. there is no free Cu$^{2+}$ in equilibrium with bound Cu$^{2+}$, thus the protein binding sites are filled until saturated. This is illustrated in Figure 3.1d, where at a protein concentration of 50 μM, at one equivalent Cu$^{2+}$, the binding site is full, thus no equilibrium is observed and the titration curve can only show that the affinity is higher than 50 nM, but it would be identical if the protein had a pM or fM dissociation constant.

Figure 3.1. Binding curves. (a) shows a typical binding curve with saturation of the binding site $\theta$ on the y axis and ligand concentration ([L]) on the x axis. The $K_d$ is calculated at 50 % binding site saturation. (b) shows a Scatchard plot of the data. Strong and weak binding curves are shown in (c). (d) shows tight binding and illustrates that given the same metal and ligand concentrations the titration curve for a binding equilibrium with even tighter binding, e.g. a $K_d$ of 0.5 pM or 0.05 pM would be identical to this graph (when $K_d = 50$ nM).
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If the spectrometers used in this thesis were more sensitive, very low concentrations of Aβ and Cu$^{2+}$ could be used to observe the affinity, however micromolar concentrations are necessary. Thus to generate an observed equilibrium between free (or chelator bound) Cu$^{2+}$ and Cu$^{2+}$ bound to Aβ, competing chelators with known copper affinities were used.

3.2. Aims

- Compare the affinity of Cu$^{2+}$ for both monomeric and fibrillar Aβ(1-42).
- Compare affinities obtained with three different chelators and from circular dichroism and fluorescence spectroscopy.
- Determine if the affinity of Cu$^{2+}$ for Aβ(1-42) is physiologically relevant.

3.3. Experimental

For peptide synthesis, purification and solubilisation, please refer to Chapter 2.

3.3.1. Peptide and Buffers in both Circular Dichroism and Fluorescence Affinity Experiments

Two different spectroscopic methods – fluorescence quenching and circular dichroism, and three different competitive Cu$^{2+}$ chelators were used, glycine, histidine and NTA, to assess the accuracy and precision of the experimentally determined $K_d$.

50 μM Aβ(1-42) was added to water (ultra high quality, >18 Ω. cm resistivity) with 160 mM NaCl, pH 7.4. For experiments with fibrillar Aβ, the fibril formation method is described in Chapter 2. The pH was monitored after every addition of Cu$^{2+}$ or competing chelator, before and after each spectrum was recorded. The pH was adjusted using small volumes (typically < 10 μL) of 10-100 mM NaOH or HCl. The addition of metal ions or competing ligands to the Aβ peptides was performed using small aliquots from stock aqueous solutions (10 – 50 mM) of CuCl$_2$, glycine, L-histidine or nitrilotriacetic acid (NTA), all obtained from Sigma Aldrich, analar grade or better.
3.3.2. Circular Dichroism Measurements in the near-UV and Visible Region

Circular dichroism (CD) bands associated with Cu\(^{2+}\) bound to A\(\beta\) are used to quantitatively measure the amount of Cu\(^{2+}\) bound to A\(\beta\), the theory of this method is discussed in Chapter 2 Section 2.4.5.

CD spectra were recorded on a Chirascan (Applied Photophysics) CD spectrometer at 25 °C. A cell with a 1 cm path length was used for spectra recorded between 280 and 800 nm with sampling points every 2 nm and 2 seconds recording time per point. A minimum of 3 scans were recorded for all spectra including baseline spectra which were subtracted from each spectra. Applied Photophysics Chirascan Viewer was used to apply minimal smoothing to the data. Direct CD measurements (θ, in millidegrees) were converted to molar ellipticity, Δ\(\varepsilon\) (M\(^{-1}\) cm\(^{-1}\)), using the relationship:

\[
\Delta \varepsilon = \frac{\theta}{(33000 * c * l)}
\]

where \(c\) is the concentration and \(l\) the path length (Greenfield, et al. 1969).

Sequential additions of 0.2 mol eq Cu\(^{2+}\) were added up to a final concentration of 1 mol eq of Cu\(^{2+}\) to 50 \(\mu\)M A\(\beta\)(1-42) (the stoichiometry of Cu\(^{2+}\):A\(\beta\) for full-length A\(\beta\) is 1:1, see Chapter 4 for supporting experimental results). Three competing ligands glycine, histidine and nitrilotriacetic acid (NTA) were titrated in from stocks of between 10 – 50 mM. Glycine and NTA are CD silent and thus as the Cu\(^{2+}\) is removed from A\(\beta\) by these chelators the CD band at 320 nm is correspondingly reduced.

3.3.3. Fluorescence Experimental Specifics

For fluorescence quenching experiments a single cell, Hitachi F-2500 fluorescence spectrophotometer was used with an excitation wavelength of 280 nm, and emission wavelengths starting at 290 nm and finishing at 400 nm. Both the excitation and emission slitwidths were 5 nm. 1 cm pathlength quartz cuvettes were used with no agitation. 4 traces were taken at each reading and readings were taken at room temperature.
Sequential additions of Cu$^{2+}$ were added up to 1 mole equivalent to 50 μM Aβ in the conditions outlined above in Section 3.3.1. This caused a linear reduction of the Aβ tyrosine fluorescence signal. A non-fluorescent competing Cu$^{2+}$ chelator (glycine, histidine or NTA) was then titrated in until the maximal tyrosine fluorescence signal had returned. The affinity can thus be calculated from the tyrosine fluorescence at half the maximal fluorescence value.

**Affinity Calculations**

The fraction of Cu$^{2+}$ bound to Aβ (θ) was calculated assuming this relates directly to either the circular dichroism signal at 320 nm or the fluorescence signal at 307 nm. The affinity can thus be calculated from binding curves of circular dichroism/fluorescence signal intensity at half the maximal value. Under these conditions (θ = 0.5) the dissociation constant ($K_d$) is equal to the concentration of Cu$^{2+}$ free. Free Cu$^{2+}$ refers here to Cu$^{2+}$ not bound to Aβ.

### 3.3.4. Competitive Metal Capture Analysis

The $K_d$ at pH 7.4 of Aβ for Cu$^{2+}$ can be calculated from the observed $K_d$ in the presence of a competing ligand, this is called competitive metal capture analysis (Atwood, et al. 1998). This method uses Cu$^{2+}$ chelators to compete with Aβ to form Cu$^{2+}$ complexes. This method is required because, as mentioned previously, for an affinity to be measured there must be a measurable equilibrium between the free and bound states. Binding curves were constructed with concentration of added ligand on the x axis and the CD or fluorescence signal on the y axis. The binding curves were normalised and at 50 % maximal signal the concentration of ligand added was determined from the binding curves.

### 3.3.5. Calculating the Apparent Affinity of the Competitive Ligands

The absolute (Abs $K_a$) and apparent (App $K_a$ and App $K_d$) stability constants for Cu$^{2+}$(Gly)$_2$, Cu$^{2+}$(His)$_2$ and Cu$^{2+}$(NTA) are shown in Table 3.1.
### Table 3.1. Affinities and Dissociation constants for competing chelators.

Log Absolute affinity (Log Abs $K_a$) is pH-independent and obtained from Dawson 1986. Apparent (App) $K_a$ and $K_d$ values calculated as shown in equations 9 and 10 of text.

Due to differences in experimental details, $Cu^{2+}$(Gly) stability constants are calculated at pH 7.4, pH 7.6 and pH 7.8. Likewise $Cu^{2+}$(His) stability constants are calculated at pH 7.4 and pH 7.8.

To calculate the pH dependent apparent affinity (App $K_a$) of $Cu^{2+}$ for the competing ligands the following calculation is used:

$$\log \alpha = pK_a - pH$$  \hspace{1cm} (eq 9)

And then:

$$\log App\ K_a = \log Abs\ K_a - \log \alpha$$  \hspace{1cm} (eq 10)

E.g. The $pK_a$ of NTA is 9.8 and the absolute affinity of NTA for $Cu^{2+}$ is 13.1 (Dawson 1986) so using the above equations 9 and 10 the apparent affinity (pH dependent) at pH 7.4 can be calculated.

$$\log \alpha = 9.8 - 7.4 = 2.4$$
log \( \text{App } K_a \) at pH 7.4 = 13.1 – 2.4 = 10.7

This gives an apparent affinity at pH 7.4 of 5.0 \( \times 10^{10} \text{ M}^{-1} \) or a dissociation constant of 19.9 picomolar.

### 3.3.6. Calculating the Affinity of the Aβ-Cu\(^{2+}\) Complex

At equilibrium, where 0.5 equivalents of Cu\(^{2+}\) are bound to Aβ and 0.5 equivalents are bound to the competing chelator, it can be assumed that the competing ligand-Cu\(^{2+}\) complexes exist predominantly as Cu\(^{2+}\)(Gly)\(_2\), Cu\(^{2+}\)(His)\(_2\), Cu\(^{2+}\)(NTA).

Equation 11 below was used to calculate the \( K_d \) of the Aβ-Cu complex (the value of \([\text{Cu}^{2+}\text{free}]\)), when NTA is the competing chelator. NTA only forms a 1:1 complex with Cu\(^{2+}\). Alternatively equation 12 was used when glycine or histidine were the competing chelators (Hatcher, et al. 2008).

\[
[\text{Cu}^{2+}\text{free}] = \frac{([\text{Cu}^{2+}\text{total}]-[\text{Cu}^{2+}\text{bound to Aβ}])}{1+(K_{a1}[\text{NTA}_{\text{free}}])} \quad (\text{eq 11})
\]

\[
[\text{Cu}^{2+}\text{free}] = \frac{[\text{Cu}^{2+}\text{total}]-[\text{Cu}^{2+}\text{bound to Aβ}]}{1+(K_{a1}[\text{Gly}_{\text{free}}])+(K_{a1}K_{a2}(\text{Gly}_{\text{free}})^2))} \quad (\text{eq 12})
\]

Additionally the \( K_d \) at pH 7.4 of Aβ for Cu\(^{2+}\) can also be calculated from the observed \( K_d \) in the presence of the competing ligand glycine by using equation 13 (Wells, et al. 2006).

\[
K_d = K_d (\text{observed}) \times \frac{(\text{App} K_{d1})}{[\text{Gly}_{\text{free}}]} \times \frac{(\text{App} K_{d2})}{[\text{Gly}_{\text{free}}]} \quad (\text{eq 13})
\]

**Worked Example for Calculating Aβ Affinity Using Glycine as a Competing Chelator.**

The binding curve in Figure 3.2b shows that at \( \theta = 0.5 \), 55 \( \times \) 50 \( \mu \text{M} \) total glycine has been added. Because:

\[
[\text{Gly}_{\text{free}}] = [\text{Gly}_{\text{total}}]-[\text{Gly bound to Aβ}]
\]
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\[ \text{[Gly}_{\text{free}}] = [55 \times 50 \mu M] - [50 \mu M] \]
\[ \text{[Gly}_{\text{free}}] = 2700 \mu M \text{ is the free glycine present.} \]

Histidine and glycine have two Cu^{2+} binding sites and generally form a Cu(His)\textsubscript{2} and Cu(Gly)\textsubscript{2} complex, thus their affinity is given by \( K_{a1} \) and \( K_{a2} \) (Dawson 1986), see Table 3.1. It is assumed that all Cu\textsuperscript{2+} not bound to A\textbeta forms a Cu(Gly)\textsubscript{2} or Cu(His)\textsubscript{2} complex with the excess chelator present.

Using the above equation (12) and the experimental specifics of one of the fluorescence experiments:

\[ \text{[A\textbeta total]} = 50 \mu M (5 \times 10^{-5} \text{M}) \]
\[ \text{[Cu^{2+} total]} = 50 \mu M (5 \times 10^{-5} \text{M}) \]
\[ \text{[Cu^{2+} boundtoA\textbeta]} = 25 \mu M (2.5 \times 10^{-5} \text{M}) \]

Apparent \( K_{a1} \) at pH 7.4 for glycine = 7.4 \times 10^5 \text{M}^{-1}

Apparent \( K_{a2} \) at pH 7.4 for glycine = 7.4 \times 10^4 \text{M}^{-1}

\[ \text{[Gly}_{\text{free}}] = 2.70 \times 10^{-3} \text{M} \]

\[ \text{[Cu^{2+} free]} = \frac{[\text{Cu^{2+}}_{\text{total}}] - [\text{Cu^{2+} boundtoA\textbeta}]}{1 + (K_{a1} \text{[Gly}_{\text{free}}]) + (K_{a1} K_{a2} ([\text{Gly}_{\text{free}}]^2))} \]

\[ \text{[Cu^{2+} free]} = \frac{5 \times 10^{-5} - 2.5 \times 10^{-5}}{1 + (7.4 \times 10^5)(2.7 \times 10^{-3}) + (7.4 \times 10^5)(7.4 \times 10^4)((2.7 \times 10^{-5})^2))} \]

\[ \text{[Cu^{2+} free]} = \frac{2.5 \times 10^{-5}}{1 + ((1.998 \times 10^3 + 5.476 \times 10^6) \times 7.29 \times 10^6)} \]

\[ \text{[Cu^{2+} free]} = \frac{2.5 \times 10^{-5}}{1 + (2.035 \times 10^3 + 3.992 \times 10^5)} \]

\[ \text{[Cu^{2+} free]} = \frac{0.000025}{401236.4} \]

\[ \text{[Cu free]} = 6.2 \times 10^{-11} = K_d = 62 \text{ pM} \]
Similarly using equation 13:

\[ K_d = K_d(\text{observed}) \times \frac{(\text{AppK}_{d1})}{[\text{Gly}_{\text{free}}]} \times \frac{(\text{AppK}_{d2})}{[\text{Gly}_{\text{free}}]} \]

\[ K_d = 25 \mu M \times \frac{(1.35 \mu M)}{[2700 \mu M]} \times \frac{(13.5 \mu M)}{[2700 \mu M]} \]

\[ K_d = 25 \mu M \times (5 \times 10^{-4} \mu M) \times (5 \times 10^{-3} \mu M) \]

\[ K_d = 6.2 \times 10^{-5} \mu M = 62 \text{ pM} \]

In the original paper published (Sarell, et al. 2009), the concentration of the total ligand added at \( \theta = 0.5 \) was used rather than the concentration of the free ligand in calculating the affinities. This had a subtle effect on the \( K_d \) calculated, for example in the case illustrated above, 60 pM rather than 62 pM was obtained, see (Xiao, et al. 2010).

3.4. Results

3.4.1. Calculating the Affinity from Cu\(^{2+}\) Fluorescence Quenching

The affinity of Cu\(^{2+}\) for A\(\beta\) is vital in understanding the physiological significance of Cu\(^{2+}\) in Alzheimer’s disease. There is currently conflicting experimental data for the affinity of Cu\(^{2+}\) binding to monomeric A\(\beta\) and no data on the Cu\(^{2+}\) affinity of fibrillar A\(\beta\). To determine if the large (eleven orders of magnitude) differences in Cu\(^{2+}\) binding to A\(\beta\) reported (Atwood, et al. 2000, Syme, et al. 2004) might reflect the difference in Cu\(^{2+}\) affinity for different oligomeric forms of A\(\beta\), two independent
methods were used to measure Cu$^{2+}$ affinity to both monomeric and fibrillar Aβ(1-42).

The competitive effects of glycine, L-histidine and NTA were used to measure the Cu$^{2+}$ affinity for both monomeric and fibrillar Aβ(1-42) by fluorescence spectroscopy. Figure 3.2a shows that the addition of increasing amounts of Cu$^{2+}$ to Aβ(1-42) causes marked quenching of the single tyrosine (Tyr10) fluorescence signal at 307 nm. A plot of fluorescence versus mole equivalents of Cu$^{2+}$ (Figure 3.2a insert) indicates a linear reduction in fluorescence up to 1 mole equivalent Cu$^{2+}$ addition. By 1 mole equivalent Cu$^{2+}$ the fluorescence intensity has reduced by ~75%. A breakpoint at 1 mole equivalent indicates 1:1 stoichiometry, as continued addition of Cu$^{2+}$ causes only a slight further reduction in fluorescence attributed to non-specific interaction of Cu$^{2+}$ with the tyrosine. The 1:1 stoichiometry is confirmed by EPR studies (Chapter 4).

Figure 3.2b shows the effect of glycine addition on a 1:1 Cu-Aβ(1-42) complex. As glycine is added, it competes with Aβ(1-42) for the Cu$^{2+}$ ions and the tyrosine fluorescence signal at 307 nm reappears. Maximum tyrosine fluorescence signal, for both soluble and fibrillar Aβ(1-42), only returns after more than 200 mole equivalents of glycine are added. Half the maximal quenching is achieved at 55 equivalents of glycine for soluble Aβ(1-42), and similarly 56 mole equivalents for fibrillar Aβ(1-42). Free glycine will bind to Cu$^{2+}$ ions in a Cu(Gly)$_2$ complex, coordinating via the amino and carboxylate groups. The step-wise apparent affinity for glycine is known to be $K_{a1} = 7.4 \times 10^5$ M$^{-1}$ and $K_{a2} = 7.4 \times 10^4$ M$^{-1}$ at pH 7.4 (Dawson 1986). It is sometimes not appreciated that the affinity of glycine for Cu$^{2+}$ is the product of the affinities of the $K_{1}K_{2}$ and related to [Gly]$^2$. The apparent dissociation constant for the Cu-Aβ(1-42) complex at pH 7.4 may be calculated using equation 12. Using this method a $K_d$ at pH 7.4 of 62 pM and 60 pM for both monomeric and fibrillar Aβ(1-42) respectively was calculated. Almost identical values are calculated using the alternative approach equation 13.

The experiment was then repeated using either the competing ligands L-histidine or NTA, which have a higher affinity for Cu$^{2+}$ than glycine (Figure 3.2c and d). NTA has an apparent dissociation constant for Cu$^{2+}$ at pH 7.4 of 19.9 pM (Dawson 1986).
and binds Cu$^{2+}$ to form a 1:1 complex, making determination of the $K_d$ more straightforward.

It is clear from Figure 3.2d that addition of 1 mole equivalent of NTA removes just over half the Cu$^{2+}$ from Aβ(1-42), indicating that Cu$^{2+}$ has a very similar affinity for
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Aβ(1-42) as for NTA. Using equation 11 and the concentration of NTA added at half the maximal fluorescence (0.70 mole equivalents), the dissociation constant at pH 7.4 for monomeric Aβ(1-42) can be calculated. An apparent Kₐ of 50 pM for both monomeric and fibrillar Aβ(1-42) is determined, which is similar to that determined using glycine as the competitive ligand.

L-Histidine is known to bind Cu²⁺ with step-wise apparent Kₐ’s at pH 7.4 of Kₐ₁ = 3.7 nM and Kₐ₂ = 0.47 μM. Two molecules of histidine will bind a single Cu²⁺ ion using the amino and imidazole nitrogens as ligands. The tyrosine fluorescence signal at 307 nm returns to its maximum value for both soluble and fibrillar Aβ after only 5 mole equivalents of L-histidine are added, as shown in Figure 3.2c. Half-maximal quenching is achieved at close to 1.7 mole equivalents for soluble Aβ(1-42) and 1.5 mole equivalents for fibrillar Aβ. This is below the 2 mole equivalents of histidine required to bind 1 mole equivalent of Cu²⁺ and indicates that Cu²⁺ will bind to Aβ(1-42) with a comparable affinity to that of L-histidine. Again the affinity can be calculated using equation 12, this time using the Kₐ₁ and Kₐ₂ of histidine at pH 7.4 and the concentration of histidine at half maximal quenching. The histidine competition experiment indicates an apparent dissociation constant of 36 pM for monomeric and 69 pM for fibrillar Aβ at pH 7.4. Again these values are similar to those calculated using NTA as the competing ligand. Although there is some variation, the Kₐ values are within a single order of magnitude; between 36-69 pM. All the values discussed here are summarised in Table 3.2a

3.4.2. Calculating the Affinity from CD Measurements

Importantly, similar Cu²⁺ affinities for Aβ were obtained with a second independent method using near-UV-CD spectroscopy. These near-UV experiments have the advantage in that they measure a CD signal directly associated with the Cu-Aβ complex. Figure 3.3 shows the CD spectrum of Aβ(1-28) loaded with 1 mole equivalent Cu²⁺ ions at pH 7.4. It was previously shown that although Cu-Aβ does not give rise to CD bands for d-d electronic transitions, there is a positive CD band at 320 nm assigned to Aβ Cu-imidazole charge-transfer transitions (Syme, et al. 2004). This CD band increases in intensity with Cu²⁺ addition. Figure 3.3 shows that as the competing ligand NTA is added the intensity of the band decreases. A plot of
ellipticity at 320 nm, shown as an insert, indicates the band is reduced to half its maximal intensity only after 0.75 mole equivalents of NTA are added. The binding curve using NTA as a competitor bears a striking resemblance to the NTA competition studies using fluorescence quenching, shown in Figure 3.2d. Like the fluorescence data, the CD data indicates Cu$^{2+}$ has a similar affinity for Aβ as that of NTA, with a $K_d$ of 40 pM.

![Figure 3.3. CD spectra of Aβ(1-28) with Cu$^{2+}$; NTA competition. Increasing mole equivalence of NTA added to 50 µM Aβ(1-28) and 50 µM Cu$^{2+}$ causes a decrease in the intensity of the CD signal at 320 nm. Insert shows the normalized CD signal at 320 nm of Cu$^{2+}$- Aβ(1-28) with increasing mole equivalent additions of NTA.](image)

A similar experiment was performed using glycine. Here the intensity of the Cu-Aβ CD bands at 320 nm were reduced to half their maximal intensity at ca. 35 mole equivalents of glycine. Using equation 12 or 13 the apparent Cu$^{2+}$ dissociation constant for Aβ(1-28) at pH 7.6 was calculated to be 60 pM. This value is comparable to a $K_d$ of 96 pM determined using glycine competition and fluorescence quenching of Aβ(1-28) at pH 7.8 previously reported (Syme, et al. 2004) (Table 3.2b). It was not possible to repeat this experiment for Aβ(1-42) as the CD band at 320 nm was not of sufficient intensity.
3.4.3. Summary of Results

In summary, using both direct measurements of Cu$^{2+}$ from CD bands of the Cu-Aβ complex and fluorescence quenching methods, it is shown that one mole equivalent of Cu$^{2+}$ ions bind to Aβ(1–42) with a dissociation constant similar to NTA. An apparent K\textsubscript{d} at pH 7.4 of between 36-69 pM for Aβ(1-42) is calculated. Significantly, comparisons of Aβ(1-40) and Aβ(1-42) in the monomeric and fibrillar form shows almost identical affinity for a single Cu$^{2+}$ ion at pH 7.4. The apparent K\textsubscript{d}s calculated using the different competitive ligands are given in Table 3.2.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Technique</th>
<th>Chelator</th>
<th>pH</th>
<th>K\textsubscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ(1-28) WT</td>
<td>Fluorescence Quenching</td>
<td>Glycine</td>
<td>pH 7.4</td>
<td>96 pM *</td>
</tr>
<tr>
<td></td>
<td>Fluorescence Quenching</td>
<td>His</td>
<td>pH 7.8</td>
<td>44 pM *</td>
</tr>
<tr>
<td></td>
<td>near-UV CD</td>
<td>NTA</td>
<td>pH 7.4</td>
<td>60 pM</td>
</tr>
<tr>
<td></td>
<td>near-UV CD</td>
<td>Glycine</td>
<td>pH 7.6</td>
<td>60 pM</td>
</tr>
</tbody>
</table>

Table 3.2. Dissociation constants for Aβ. (a) Apparent (conditional) dissociation constants taken from fluorescence quenching experiments using three different chelators: NTA, glycine and histidine. [L\textsubscript{tot}] is the total concentration of ligand at θ=0.5 and [L\textsubscript{free}] is the concentration of ligand not bound to Cu$^{2+}$. All Aβ(1-42) experiments were conducted at pH 7.4. (b) Dissociation constants for Aβ(1-28). Dissociation constants taken from fluorescence quenching and near-UV CD. * denotes a K\textsubscript{d} at pH 7.8, raw data previously reported (Syme et al. 2004).

3.5. Discussion

3.5.1. Affinity of Cu$^{2+}$ for Monomeric and Fibrillar Aβ

Despite the range of studies that link Cu$^{2+}$ with Aβ neurotoxicity, a role for Cu$^{2+}$ ions in the etiology of Alzheimer’s Disease has often been met with some scepticism. A common objection has been the relatively low affinity of Cu$^{2+}$ for Aβ. Typically
micromolar, or nanomolar, \(K_d\)'s are reported (Garzon-Rodriguez, et al. 1999, Guilloreau, et al. 2006, Hatcher, et al. 2008, Tougu, et al. 2008). The re-evaluation of \(Cu^{2+}\) affinity for \(A\beta\) described here places the affinity several orders of magnitude higher than previously believed. A picomolar affinity for \(Cu^{2+}\) now allows \(A\beta\) to compete for \(Cu^{2+}\) ions with other extracellular \(Cu^{2+}\) chelators, such as human serum albumin (1.0 pM \(K_d\)) (Masuoka, et al. 1993), particularly during neuronal depolarisation. The biological significance of a picomolar affinity is discussed later.

Two independent methods of determining the affinity of \(Cu^{2+}\) for monomeric and fibrillar \(A\beta(1-42)\) were used. The fluorescence quenching experiments show that both soluble and fibrillar \(A\beta(1-42)\) have an identical affinity, and are comparable to that of the \(Cu^{2+}\) chelator NTA (20 pM) for \(Cu^{2+}\) ions. There were some small differences in the affinities calculated depending on the competing ligand used (NTA, histidine or glycine) but all three competitors indicated an affinity of \(Cu-A\beta\) to within less than an order of magnitude, placing the apparent \(K_d\) between 36 pM - 69 pM at pH 7.4. Near UV-CD was also used to directly monitor the location of \(Cu^{2+}\) ions competing between NTA or glycine and \(A\beta(1-28)\). Again this indicated an affinity of \(Cu^{2+}\) for \(A\beta(1-28)\) comparable to that of NTA at pH 7.4. Glycine competition detected by fluorescence or near UV-CD revealed very similar affinities validating the fluorescence quenching method.

### 3.5.2. A Ternary Complex is Unlikely

The possibility that a ternary complex is formed between \(Cu^{2+}\), \(A\beta\) and the competing ligands; NTA, L-histidine or glycine was considered, see for example (Rozga, et al. 2009) and (Burns, et al. 2001). However a number of pieces of evidence suggest this is unlikely. In particular, competition for \(Cu^{2+}\) ions between \(A\beta\) and L-histidine detected by Vis-CD indicates that when sufficient (~ 4 mole equiv) L-histidine is added to the \(Cu^{2+}-A\beta\) complex a Vis-CD spectra identical to \(Cu-(His)_{2}\) alone is observed (Figure 3.4.)

The visible CD spectra are very sensitive to \(Cu^{2+}\) coordination geometry and the free \(Cu-(His)_{2}\) complex produces a characteristic visible CD spectra with a positive CD band at 700 nm irrespective of the presence of \(A\beta\), suggesting a ternary complex
does not form. Furthermore the Cu$^{2+}$ induced quenching of the Tyr10 of Aβ almost completely returns upon the addition of NTA (or other competing ligands). If Cu$^{2+}$ remained bound to Aβ forming a ternary complex, one might expect some if not all of the Cu$^{2+}$ induced quenching to persist. In addition, the CD band at 320 nm attributed to the Cu$^{2+}$-Aβ imidazoles is completely lost with the addition of a competing ligand. If a ternary complex remained, one might expect the Cu$^{2+}$ imidazole band from Cu$^{2+}$-Aβ to also be retained.

![Figure 3.4. Vis-CD spectrum of Cu(His)$_2$. Cu(His)$_2$ complex (black) compared to Cu$^{2+}$ with Aβ(1-40) and L-His (grey). 50 µM Cu$^{2+}$ and 50 µM Aβ(1-40) at pH 7.4 in 20 mM ethylmorpholine buffer and 160 mM NaCl.](image)

3.5.3. Accounting for the Discrepancies in the Reported Affinities

Previously reported affinities of Cu$^{2+}$ for Aβ are only for the monomeric form. There are no Cu$^{2+}$ affinity studies for Aβ fibrils. Garzon – Rodriguez et al. (1999) found that Cu$^{2+}$ had an affinity for monomeric Aβ(1-42) of ~2 µM (Garzon-Rodriguez, et al. 1999). However the Garzon-Rodriguez study was performed in Tris-buffer, which has a weak, but appreciable affinity for Cu$^{2+}$ ions (see Table 2.1 in Chapter 2). This would lower the observed affinity. Two recent studies have shown that apparent K$_d$’s reported in the literature of Cu-Aβ, once corrected for coordination by Tris-buffer, produce K$_d$’s of 35 nM or 625 pM depending on the approach (Hatcher, et al. 2008, Tougu, et al. 2008). Hatcher et al. suggest that as many as four step-wise
Cu$^{2+}$ dissociation constants must be considered for Tris-buffer. Hatcher et al used iso-thermal calorimetry (ITC) and determined an apparent dissociation constant of 0.4 nM for the Cu-Aβ complex at pH 7.4. This affinity is ~ 10 times weaker than that determined here. The discrepancy might be explained by the use of high levels of Tris-buffer as a competitor which might exaggerate systematic errors in the measurements. Phosphate buffer is also occasionally used in the literature (Danielsson, et al. 2007, Hou, et al. 2006), however there is the risk of the insoluble Cu$_3$(PO$_4$)$_2$ species forming, especially at high pH. This species is likely to precipitate out, thus reducing the concentration of available Cu$^{2+}$ and invalidating the results. Because of the complications arising from the competing effects of buffers, the experiments in this chapter (and others conducted by various research groups) were conducted in the absence of a buffer (Curtain, et al. 2001, Guilloreau, et al. 2006, Kowalik-Jankowska, et al. 2003, Ma, et al. 2006, Syme, et al. 2004).

An additional complication with these types of experiments, highlighted by Hatcher et al is a failure to account for both of glycine’s affinities (Hatcher, et al. 2008). When the step-wise affinity, $K_{a1} K_{a2}$ for the Cu(Gly)$_2$ is used rather than simply $K_{a1}$, comparable affinities as to those reported here may be calculated.

The in-register stacking of successive monomeric units within fibres results in histidines at positions 13 and 14 aligning adjacent to each other along the length of the fibres, (Tycko 2004, Whittemore, et al. 2005) thus groups of four histidine residues would be in close proximity. A particularly intriguing possibility was that the very different affinities reported for Cu-Aβ within the literature ($K_a$’s between $10^{18}$ and $10^6$) (Atwood, et al. 2000, Garzon-Rodriguez, et al. 1999, Tougu, et al. 2008) might reflect differences in the Aβ oligomeric form studied. Importantly the studies described in this chapter find no difference in affinity between monomeric and fibrillar Aβ. It now seems clear that the atto-molar dissociation constant proposed by Atwood et al. (Atwood, et al. 2000) is out of line with other measurements and can not be explained by the oligomeric state of Aβ.

3.5.4. Biological Significance

The dissociation constant reported here for Aβ(1-42)-Cu$^{2+}$ at pH 7.4 of ~50 pM, is a number of order of magnitudes lower than recent reports. Simplistically, this
dissociation constant suggests that for the Aβ-Cu$^{2+}$ complex to form, only picomolar concentrations of Cu$^{2+}$ are needed. This is considerably lower than the extracellular Cu$^{2+}$ concentration within cerebrospinal fluid (CSF) and extra-cellular brain interstitial fluid, which is typically 250 nM (Geigy 1984, Joergstuerenburg, et al. 1999). Furthermore levels of Cu$^{2+}$ are found to be as much as 1000 times higher at the synapse (Lovell, et al. 1998) and within plaques (Kardos, et al. 1989, Lovell, et al. 1998).

However the dissociation constants of other copper chelators must be considered, and although serum albumin is at relatively low levels in the CSF compared to its concentration in blood plasma, it has a $K_d$ of 1.0 pM for Cu$^{2+}$ ions at pH 7.4 (Masuoka, et al. 1993, Rozga, et al. 2007). Thus much of the Cu$^{2+}$ is still likely to be bound to serum albumin. However it is notable that the difference in affinity between albumin and Aβ is only a single order of magnitude. Additionally at the synaptic cleft fluxes of Cu$^{2+}$ ions may be as high as 250 µM during neuronal depolarisation (Kardos, et al. 1989) which is two orders of magnitude higher than the concentration of albumin. In these instances albumin, and other copper binding proteins such as metallothionein-3, may be saturated with Cu$^{2+}$ ions. Aβ is cleaved from APP at the synapse (Lacor, et al. 2004) and would be able to bind fluxes of Cu$^{2+}$ released during cellular depolarisation.

The strong affinity of Aβ for Cu$^{2+}$ also has implications for the current interest in Cu$^{2+}$ chelators, which have shown some efficacy as a treatment for AD (Bush 2002, Cherny, et al. 2001, Ritchie, et al. 2003). Potential chelators should have an affinity for Cu$^{2+}$ an order of magnitude higher than Aβ, placing an ideal affinity at $10^{12}$ M$^{-1}$, comparable to human serum albumin.

These studies have made a significant contribution to understanding the Cu$^{2+}$ affinity of Aβ in both the monomeric and fibrillar form. The re-evaluation of the dissociation constant of Cu$^{2+}$ for Aβ to pM levels adds weight to Cu$^{2+}$ playing a central role in the formation of fibres, as well as the neurotoxic affects of redox active Cu$^{2+}$ ions bound to diffusible Aβ oligomers.
4. The Structure and Stoichiometry of the Cu\(^{2+}\)-Aβ Complex for Monomeric and Fibrillar Aβ
Abstract

Cu$^{2+}$ ions are found concentrated within senile plaques of Alzheimer’s disease patients directly bound to amyloid-β peptide (Aβ), and are linked to the neurotoxicity and self-association of Aβ. However the nature of the Aβ-Cu$^{2+}$ complex for both monomeric and fibrillar Aβ fibres is not fully resolved. Continuous wave-electron paramagnetic resonance (cw-EPR) spectroscopy is used in this chapter to show that Aβ fibres are able to bind a full stoichiometric complement of Cu$^{2+}$ ions. This suggests that Cu$^{2+}$ ions are able to diffuse into all parts of the fibre to give precisely a 1:1 stoichiometry and with little change in fibre structure. EPR spectra are typical for a type II axial spectra indicating tetragonal geometry. Additionally the $A_{II}$ and $g_{II}$ values from the EPR spectra show that fibrillar Aβ has identical coordination geometry to monomeric Aβ. The pH dependence of EPR spectra indicates two tetragonal complexes are present at pH 7.4 containing N and O ligands.

EPR of Aβ analogs, where histidine residues are substituted for alanines, were also studied using EPR. The spectra suggest a dynamic model of the tetragonal Cu$^{2+}$ complex, with axial as well as equatorial coordination of imidazole nitrogens, creating an ensemble of coordination geometries in exchange between each other. Furthermore the N-terminal amino group is essential for the formation of the high pH complex. Finally this study examines why the Aβ(1-28) fragment binds an additional Cu$^{2+}$ ion compared to full length Aβ. This second binding site is revealed in Aβ(1-42) upon addition of methanol, indicating hydrophobic interactions from the long hydrophobic tail of full length Aβ blocks the formation of this second, weaker carboxylate-rich binding site.

The data presented in this chapter was recently published in (Sarell, et al. 2009).
4.1. Introduction

There has been extensive examination of the coordination geometry and stoichiometry of the Cu$^{2+}$ binding site of Aβ, especially in truncated peptides such as Aβ(1-16) and Aβ(1-28). There has been a lack of consensus however on the nature of both the binding ligands and the number of binding sites, especially in full length Aβ. This is likely to be due to a variety of reasons, including aggregation of the full length peptide, the buffer used, solvents present and pH effects.

4.1.1. Reasons for Resolving the Cu$^{2+}$-Aβ Complex

A clear understanding of the coordination geometry of the Aβ-Cu$^{2+}$ complex includes defining the nature of the binding ligands, stoichiometry, and the pH-dependency of the complex.

The coordination geometry will influence the affinity of a complex, by determining the ligands involved, the geometry in which they form bonds with the ion and also how tightly the ion is bound. Additionally coordination of copper may involve the backbone of Aβ, which will influence secondary structure and possibly nucleus formation, fibril elongation or fibril stability. Moreover, knowing which ligands are involved in the complex can aid in understanding how the overall charge of the protein will change when Aβ binds Cu$^{2+}$.

Finally the coordination geometry can also affect the redox properties of the protein by determining whether the copper is free to cycle or not. For example, plastocyanin is a copper binding protein with distorted trigonal pyramidal coordination geometry (Gewirth, et al. 1988). Two nitrogen atoms from two separate histidines and a sulfur from a cysteine (S$_1$) compose the trigonal plane of the pyramidal base. Sulfur from a methionine (S$_2$) forms the apex of the complex. The Cu-S$_1$ bond is shorter (207 picometres) than Cu-S$_2$ (282 picometres). The elongated Cu-S$_2$ bonding increases the redox potential of the protein by destabilising the Cu$^{2+}$ form. The redox potential is highly important as it affects the ability of the copper to undergo redox cycling, and thus influences free radical production. For example metallothionein will undergo a metal swap with Aβ, but in contrast to Aβ, metallothionein binds copper in a redox inactive form and thus has been suggested to be neuroprotective (Meloni, et al. 2008).
4.1.2. Previous Research on the Monomeric Aβ-Cu$^{2+}$ Complex

The Aβ-Cu$^{2+}$ complex has been studied in some detail. It is generally agreed that the Cu$^{2+}$ binding ligands of Aβ all reside in the first 16 residues of the peptide (Karr, et al. 2005, Kowalik-Jankowska, et al. 2003, Minicozzi, et al. 2008, Syme, et al. 2004), thus many of the studies have been done on either Aβ(1-16) or Aβ(1-28), which are more soluble than the longer Aβ(1-40) or Aβ(1-42) peptides.


Previous examination of the coordination complex of monomeric Aβ suggest that the peptide can bind two mole equivalents of Cu$^{2+}$ sequentially, with the first site having a much stronger affinity than the second (Atwood, et al. 2000, Karr, et al. 2008, Syme, et al. 2004). A dimeric complex, composed of two metal ions bridged by an imidazole (Cu$_2$(Aβ)$_2$) has been suggested (Curtain, et al. 2001), but this is disputed by other group’s data, including the work in this chapter. Further details on the nature of the Cu-Aβ complex and how the work described in this chapter relates to other studies are discussed in Section 4.6.

4.1.3. Previous Research on the Fibrillar Aβ-Cu$^{2+}$ Complex

Potentially the Cu$^{2+}$ complex formed by Aβ in a fibrillar state may be very different from that formed by monomeric Aβ in solution. Current models of Aβ fibrils indicate in-register stacking of Aβ monomers (Petkova, et al. 2002). This causes histidine side chains (which have a high affinity for Cu$^{2+}$ ions) at positions 13 and 14 to stack close together in successive peptides creating a potential Cu$^{2+}$ coordination site containing four histidine residues. Thus potentially, fibrils may be created or be stabilised by inter-residue histidines linking together via Cu$^{2+}$ ions. Data on the coordination of Cu$^{2+}$ binding to Aβ(1-42) in its fibrillar form has been obtained.
Chapter 4. Structure and Stoichiometry of the Cu$^{2+}$-Aβ Complex

Previously by EPR (Antzutkin 2004, Karr, et al. 2005, Karr and Szalai 2008), solid state NMR (Antzutkin 2004) and Raman spectroscopy (Miura, et al. 2000) (performed on aggregates rather than fibrils). However the pH dependence of Cu$^{2+}$ coordination to Aβ fibrils has not been reported, and it is still unclear whether one or two Cu$^{2+}$ ions bind to full-length Aβ, and whether this differs depending on the oligomeric state of Aβ. Thus many questions regarding the structure and stoichiometry of the Aβ-Cu$^{2+}$ complex remain unanswered, and this chapter attempts to shed some light on this area.

4.2. Aims

• Determine the pH dependence of Cu$^{2+}$ binding in full length Aβ(1-42) and Aβ(1-28)

• Investigate if the oligomeric state of Aβ(1-42) affects the coordination geometry of the Aβ(1-42)-Cu$^{2+}$ complex

• Determine the coordination ligands of the Aβ(1-28)-Cu$^{2+}$ complex

• Compare the stoichiometry of fibrillar Aβ, monomeric Aβ and Aβ(1-28)

4.3. Experimental

Peptide preparations, fibril formation and the theory of electron paramagnetic resonance (EPR), and transmission electron microscopy (TEM) and circular dichroism (CD) experimental details are discussed in Chapter 2.

In these studies 50 μM Aβ(1-42) was used, in both monomeric and fibrillar forms. A range of buffers was used; phosphate buffer, ethylmorpholine buffer, and HEPES, all at 10 mM with 160 mM NaCl. The structures of the buffers and further details are described in Chapter 2. Aβ(1-42) samples were not incubated with Cu$^{2+}$ during fibril growth, thus for studies with monomeric Aβ(1-42) Cu$^{2+}$ was added to the peptide then the sample was frozen immediately. For studies with fibrillar Aβ(1-42) Cu$^{2+}$ was added after fibril formation had occurred. Likewise in studies with Aβ(1-28) Cu$^{2+}$ was added to the freshly solubilised peptide then the sample was frozen immediately.
4.3.1. Electron Paramagnetic Resonance

Continuous wave X-band EPR data was recorded using a Bruker ELEXSYS 500 spectrometer, operating at a microwave frequency of ~9.3 GHz. Spectra were recorded using a microwave power of 4.012 mW across a sweep width of 2000 G (centred at 3200 G) with a modulation amplitude of 7 G. Samples were frozen in quartz tubes and experiments conducted at typically 20 K using a liquid helium cryostat. A minimum of four scans were recorded per spectrum. All EPR spectra shown have been background subtracted from a buffer blank with subsequent baseline correction in the EPR software package using third or fourth-order polynomial splines.

To analyze EPR data using the method described by Peisach and Blumberg (Peisach, et al. 1974), it is necessary to convert values from gauss (G) to millikaysers (mK) by using the formula $A(mK) = 0.046686g\Delta H$, where $g = 2.0023$ and $\Delta H$ is the $A_{II}$ splitting measured in gauss. Double integration was carried out on the signal between 3200 and 3400 G to determine signal intensity.

4.4. Results

4.4.1. pH Dependence of Cu$^{2+}$ Binding

The Cu$^{2+}$-Aβ(1-42) coordination geometry may be affected by changes in pH. However preliminary studies suggested that the choice of buffer affects the pH dependence behaviour of the complex. There are a variety of buffers commonly used when studying the coordination chemistry of Cu-Aβ (Karr and Szalai 2008, Streltsov, et al. 2008, Syme, et al. 2004). EPR spectra of monomeric Aβ(1-42) loaded with one mole equivalent of Cu$^{2+}$ ions were therefore obtained over a range of pH values under four different sample conditions: 10 mM HEPES, 10 mM ethylmorpholine (EM), 10 mM phosphate buffer or just water as shown in Figure 4.1 and Figure 4.2. It is notable that repeat EPR (or CD) scans recorded over time showed no change in the appearance of the spectra indicating Cu$^{2+}$ coordination to Aβ(1-42) reaches a thermodynamic equilibrium rapidly, within minutes.

The EPR spectrum at pH 5 for Cu-Aβ(1-42) under all four buffer conditions gives a single set of signals typical of type II Cu$^{2+}$, square-planar or square pyramidal coordination geometry, as shown in Figure 4.1a. Regardless of the buffer, the $A_{II}$, $g_{II}$
and $g_\perp$ values are 171 gauss (16.0 mK), 2.27 and 2.02 respectively (designated complex I). At pH 7.4 a new set of hyperfine peaks can be observed at a higher field with $A_{II}$, $g_{II}$ and $g_\perp$ values of 154 gauss (14.4 mK), 2.23 and 2.01 respectively (designated complex II), as shown in Figure 4.1b.

At pH 7.4 complex I remains the more intense of the two under all four conditions and notably in water, phosphate or HEPES buffer ~ 80 % of the Cu\textsuperscript{2+} forms complex
I. At pH 9 (Figure 4.1c) the low and high-field sets of signals of complex I and II are of comparable intensity in water, phosphate and HEPES buffers. However for ethylmorpholine buffer (EM) the low-field species observed exclusively at pH 5 is considerably less intense than the high-field species. The relative intensities of the two complexes is shown in Figure 4.1 d, e and f. The pH dependence of the formation of complex I and II is very similar for phosphate, water and HEPES (Figure 4.1d and e) with a midpoint of pH ~9, however the midpoint in EM buffer (Figure 4.1f) is lower at pH 8.0.

This behaviour is also observed for Aβ(1-28) (Figure 4.2). Comparison of EPR spectra for Aβ(1-28) and Aβ(1-42) show a close correspondence at all pH values. The midpoint of the transition for Aβ(1-28) was ~ pH 9, similar to Aβ(1-42), and again in EM buffer this was reduced to pH 8.0.

The effect of EM buffer on the mid-point of the transition between complex I and II was unexpected as EM has a very low affinity for Cu$^{2+}$ ions. Buffers can have a temperature dependence to their pH. A recent study has shown that some buffers pH can vary by as much as one pH unit between room temperature and 10 K (liquid helium temperatures) (Sieracki, et al. 2008, Williams-Smith, et al. 1977). This may well account for the discrepancies between EM buffer and the other buffers tested.

Peisach and Blumberg have shown that a combination of $A_{||}$ and $g_{||}$ values can indicate the ligand type that coordinates the Cu$^{2+}$ in tetragonal/ square-planar complexes (Peisach and Blumberg 1974), see Chapter 2 for more details. The $A_{||}$ and $g_{||}$ values for complex I and II are shown in Figure 4.2d. Complex I is most typical of two nitrogen and two oxygen ligands (2N2O) although 3N with 1O coordination cannot be ruled out. The $A_{||}$ and $g_{||}$ values of the high-field species observable at pH 7.4 and pH 9 (complex II) do not fall into the standard Peisach and Blumberg plot areas suggesting some distortion in the axial plane (perhaps suggesting a more square pyramidal character). The $A_{||}$ and $g_{||}$ values are shifted away from the oxygen boundaries suggesting a probable 3N1O or 4N coordination. Details of proposed coordinating ligands are described in Section 4.5.2.
Figure 4.2. pH dependence of A\(\beta\)(1-28)-Cu binding. One mole equivalent Cu\(^{2+}\), 50 \(\mu\)M A\(\beta\)(1-28) at (a) pH 5 (b) pH 7.4 and (c) at pH 9 measured by EPR. Spectra were recorded in three different buffering conditions at each pH: i) water ii) 10 mM Ethylmorpholine and iii) 10 mM phosphate. Spectra were recorded at 20 K. The low pH complex is highlighted in purple, the high pH complex in green. (d) Peisach-Blumberg plot for the \(A_{II}\) and \(g_{II}\) values of A\(\beta\)(1-28), Complex I (purple) and Complex II (green), see Table 4.1 for exact values.

4.4.2. Comparison of A\(\beta\) Monomeric and A\(\beta\) Fibrillar
The in-register stacking of A\(\beta\) monomers in amyloids has raised the possibility that the Cu\(^{2+}\) coordination formed with amyloid fibres could be very different from the

![Figure 4.2](image-url)
coordination with Aβ monomers. Figure 4.3 compares the EPR spectra of both monomeric and fibrillar Aβ loaded with 1 mole equivalent of Cu\(^{2+}\) ions.

![EPR spectra comparison](image)

**Figure 4.3. Cu\(^{2+}\) and different oligomeric species of Aβ(1-42).** (a) EPR spectra of monomeric (dashed line) and fibrillar (solid line) Aβ(1-42) 50 μM with 50 μM Cu\(^{2+}\) in 10 mM phosphate buffer with 160 mM NaCl at pH 7.4, 20 K. Insert (b) shows additions of Cu\(^{2+}\) to 50 μM Aβ(1-42) at 0, 3 and 8 days incubation. The Cu\(^{2+}\) coordination for monomeric and fibrillar Aβ is strikingly similar. Figure (c) shows a UV-CD spectra of the predominantly β-sheet Aβ(1-40) fibrils with the addition of up to 15 mole equivalents (750 μM) Cu\(^{2+}\). Aβ(1-40) fibrils without (d) and with (e) Cu\(^{2+}\) present. Cu\(^{2+}\) is added in a 1:1 stoichiometry once fibrils are formed. TEM Images achieved with assistance of Louise Serpell and Kyle Morris, University of Sussex.
It is clear almost identical EPR line shapes are obtained for the fibrillar and monomeric forms of Aβ(1-42), as shown in Figure 4.3a. Of particular note is that there is no detectable line broadening of the fibrillar form compared to monomeric Aβ. EPR spectra reveal a type II axial coordination with an A\(\text{II}\) of 16.0 mK (171 gauss) and a g\(\text{II}\) of 2.27 which are typical for a 3N1O or 2N2O complex at pH 7.4. There are no significant differences in the hyperfine splitting between monomeric and fibrillar Aβ(1-42) and the A\(\text{II}\) and g\(\text{II}\) are the same for both forms of Aβ(1-42).

It is notable that Cu\(^{2+}\) addition has little detectable affect on the secondary structure of fibres once formed, as determined by UV-C. The CD spectra of fibres are dominated by a strong negative band at 217 nm, indicative of β-sheet. The appearance of the spectra is unaffected by the addition of up to 15 mole equivalents of Cu\(^{2+}\) ions as shown in Figure 4.3c.

Figure 4.3b shows the effect of different oligomeric states of Aβ(1-42) on the Cu\(^{2+}\) coordination environment. At different times during Aβ(1-42) fibril growth a sample of the peptide preparation was removed and Cu\(^{2+}\) added, then the samples frozen. By monitoring growth using ThT fluorescence an estimate of the degree of oligomerisation could be obtained at different time points (Naiki, et al. 1989). At zero days, fresh Aβ(1-42) represents the largely monomeric form of Aβ(1-42), with very low ThT fluorescence signal. The Aβ(1-42) species at 3 days represents early fibres, while 8 days represents more mature fibres. Again the spectra are almost identical for all three forms of Aβ. A TEM image of Aβ(1-40) fibres is shown in Figure 4.3d. The fibres were grown without Cu\(^{2+}\) present, then one mole equivalent of Cu\(^{2+}\) added to the Aβ(1-40) sample shown in Figure 4.3e. Fibrils with and without Cu\(^{2+}\) present show a periodic twist, characteristic of amyloid fibres.

4.4.3. pH Dependence and Coordination Geometry of Aβ(1-28) and Analogs

To identify the residues directly involved in the coordination of Cu\(^{2+}\), five peptide analogs have been synthesised. In three analogs of Aβ(1-28) one of the three histidines have been replaced with an alanine residue, Aβ(H6A), Aβ(H13A) and Aβ(H14A). An additional peptide has also been synthesised in which the N-terminus of Aβ(1-28) is blocked by acetylation. Under physiological conditions it is believed
that nitrogens from these four loci (the three imidazole rings and the N-terminal amino group) are the most likely candidates for Cu²⁺ coordination. Comparisons of EPR spectra shown in this chapter, and previously by Karr et al, confirms that Aβ(1-28) contains all the ligands involved in coordinating the first mole equivalent of Cu²⁺ in Aβ(1-42). Finally a shorter fragment, Aβ(Ac10-16), was also studied in which His6 and the N-terminal amino group are absent. The sequence of all five analogs is shown in Chapter 2.

The EPR spectra of all five analogs with one mole equivalent of Cu²⁺ added were obtained over a range of pH values to probe the nature of the coordinating ligands and are shown in Figure 4.4. The Cu²⁺ complex for each analog was studied at pH 5, 6, 7, 8, 9 and 10. All spectra indicate axial Cu²⁺ coordination containing N and O ligands. All but two of the analogs produced two sets of axial peaks.

At pH 5 only one set of A_H and g_II values is present in all five peptide analogs. All have a similar appearance to the low-pH species described for Aβ(1-28), Aβ(1-40) and Aβ(1-42). There are however subtle variations in the appearance of the spectra. The A_H and g_II values for the five peptides and wild-type Aβ(1-28) are given in Table 4.1. All fall within a small range of A_H values between 170 to 164 gauss for complex I (predominant at pH 5 and pH 7.4) except for Aβ(Ac10-16) which has a larger A_H of 178 gauss. The g_II of complex I ranges from 2.26 for Aβ(Ac10-16) to 2.29 for N-terminally acetylated Aβ(Ac1-28).

As the pH is raised to pH 7.4 the appearance of the EPR spectra differs between the analogs. A second, higher field species is obtained for Aβ(1-28) wild-type sequence and all the His substituted analogs, with an A_H and g_II of ~ 165 gauss (15.4 mK) and 2.22, but significantly it is not apparent in the N-terminally acetylated analog Aβ(Ac1-28) or Aβ(Ac10-16). Furthermore the relative intensities of the two species is different between the analogs. The pH dependencies for complex I and II are plotted as relative intensity versus pH for the five analogs and compared to the wild type Aβ(1-28), as shown in Figure 4.4d and e.
At pH 9 the high-field species (complex II) are of similar or greater intensity than the low-field species for wild-type Aβ(1-28), H6A, and H13A. But for H14A ~ 60 % remains as complex I and there is still no observable signal for the higher pH complex II spectra in the N-terminally acetylated analogs.

Figure 4.4. Cu²⁺-pH dependence of Aβ(1-28) analogues. Cu-EPR spectra of Aβ(ac10-16) (purple), Aβ(ac1-28) (orange), Aβ(H14A) (red), Aβ(H13A) (blue), Aβ(H6A) (black), and Aβ(1-28) wild type (WT) (green) at 50 μM with one mole equivalent of Cu²⁺ at (a) pH 5, (b) pH 7.4 and (c) pH 9. Figures (d) and (e) show the relative percentage intensities of complex I (d) and complex 2 (e) of the six peptides over a wider range of pH values. It is clear the N-terminally acetylated analogs will not form the high pH, complex II.

In Table 4.1 the $A_{II}$ and $g_{II}$ values for the five analogs and wild-type Aβ(1-28) are given and also the affinities of Cu²⁺ for the Aβ analogs studied, obtained from fluorescence quenching experiments in Chapter 3 and also from raw data previously reported (Syme, et al. 2004). The dissociation constants were calculated from the glycine concentration at half-maximal quenching at pH 7.8 using equation 13 in Chapter 3. It is notable that all analogs have reduced affinity for Cu²⁺ ions,
indicating that all four nitrogen ligands identified have a role in stabilising the Cu$^{2+}$-Aβ complex. The N-terminal blocked and His6Ala analogs show the most marked reduction in affinity, while the H13A and H14A analogs show less pronounced change in affinity for Cu$^{2+}$ ions relative to the wild-type Aβ(1-28).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Complex I (pH 7.4)</th>
<th>Complex II (pH 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A$_H$</td>
<td>g$_H$</td>
</tr>
<tr>
<td>Aβ(1-42) mono</td>
<td>16.0 mK (171 G)</td>
<td>2.27</td>
</tr>
<tr>
<td>Aβ(1-42) fibrillar</td>
<td>16.0 mK (171 G)</td>
<td>2.27</td>
</tr>
<tr>
<td>Aβ(1-28) WT</td>
<td>15.9 mK (170 G)</td>
<td>2.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ(1-28) NB</td>
<td>15.7 mK (168 G)</td>
<td>2.29</td>
</tr>
<tr>
<td>Aβ(1-28) H6A</td>
<td>15.7 mK (168 G)</td>
<td>2.28</td>
</tr>
<tr>
<td>Aβ(1-28) H13A</td>
<td>15.3 mK (164 G)</td>
<td>2.28</td>
</tr>
<tr>
<td>Aβ(1-28) H14A</td>
<td>15.5 mK (166 G)</td>
<td>2.28</td>
</tr>
<tr>
<td>Aβ(10-16)</td>
<td>16.7 mK (178 G)</td>
<td>2.26</td>
</tr>
<tr>
<td>Aβ(1-11)</td>
<td>13.6 mK (146 G)</td>
<td>2.30</td>
</tr>
<tr>
<td>Aβ(1-28) 2nd mole eq</td>
<td>16.4 mK (175 G)</td>
<td>2.28</td>
</tr>
</tbody>
</table>

Table 4.1. A$_H$ and g$_H$ values and dissociation constants for Aβ fragments and analogs. Complex I pH 5 - 8, complex II > pH 9. Apparent (conditional) dissociation constants taken from Gly competition fluorescence quenching data at pH 7.4.

* denotes a Kd at pH 7.8, raw data previously reported (Syme et al 2004). Values shown were calculated using equation 13 in Chapter 3.

♦ denotes where the Kd at pH 7.6 has been calculated, using glycine competition and near-UV – CD.

In summary all the peptide analogs have slightly different A$_H$ and g$_H$ values, and perturbed pH dependence compared to wild type Aβ(1-28). In addition all analogs have reduced affinity for Cu$^{2+}$ ions. Most striking is the N-terminally blocked analogs, Aβ(Ac1-28) and Aβ(Ac10-16), both of which will not form complex II even at pH 9.

4.4.4. Stoichiometry of Cu$^{2+}$ Binding to Monomeric and Fibrillar Aβ(1-40) and Aβ(1-42).

The process of fibril formation could lead to an intermolecular Cu$^{2+}$ complex, for example fibrillar Aβ might potentially form a Cu(Aβ)$_2$ 1:2 complex. To identify any
differences in Cu$^{2+}$ binding stoichiometry for monomeric and fibrillar forms of Aβ(1-42) and Aβ(1-40), EPR spectra were obtained with increasing mole equivalents of Cu$^{2+}$ at pH 7.4, shown in Figure 4.5 and 4.6.

**Figure 4.5. Cu$^{2+}$ titration of monomeric Aβ(1-40) and Aβ(1-42).** (a) EPR spectra recorded with 0.4, 0.6, 0.8, 1.0 and 2.0 mole equivalents of Cu$^{2+}$ and 50 µM Aβ(1-42) in 10 mM phosphate buffer at pH 7.4, 20 K. Double integration of the EPR spectra of monomeric Aβ(1-40) (circles) and Aβ(1-42) (squares) with increasing mole equivalents of Cu$^{2+}$ is shown in panel (b). Best fit straight lines are fitted through 0-1 mole equivalents Cu$^{2+}$ and 1-4 mole equivalents Cu$^{2+}$ data points. The EPR signal intensity is ($I/I_{max}$). Both Aβ(1-40) and Aβ(1-42) monomeric indicate that saturation of Aβ occurs at 1 mole equivalent Cu$^{2+}$.

Increasing amounts of Cu$^{2+}$ added to monomeric Aβ(1-40) and Aβ(1-42), (Figure 4.5), gave identical line shapes up to one mole equivalent, with a commensurate increase in intensity indicating a single binding mode. Addition of a further mole equivalent of Cu$^{2+}$ (and up to ten mole equivalents) results in no further increase in the intensity of the EPR spectra for either Aβ(1-40) or Aβ(1-42) in its monomeric form. This was a surprise as previous studies within our lab on Aβ(1-28) reported a 2:1 stoichiometry; 2 Cu$^{2+}$ ions binding a single Aβ(1-28) molecule (Syme, et al. 2004). Spin integration of the EPR spectra has been plotted versus Cu$^{2+}$ addition as
shown in Figure 4.5b. Both Aβ(1-40) and Aβ(1-42) show a linear increase in intensity up to one mole equivalent of Cu$^{2+}$ ions followed by saturation of the signal. The clear break point at one mole equivalent Cu$^{2+}$ indicates that at 50 µM Aβ(1-40) and Aβ(1-42) Cu$^{2+}$ binds tightly with a 1:1 stoichiometry. It should be noted that free Cu$^{2+}$ ions in water at neutral pH give a largely EPR silent signal (Aronoff-Spencer, et al. 2000). This observation was confirmed by obtaining EPR spectra of 50 µM CuCl$_2$ in EM, PB, HEPES and water buffer at pH 7.4 which showed that Cu$^{2+}$ EPR signals are drastically attenuated relative to Cu$^{2+}$ bound to Aβ(1-40) and Aβ(1-42).

Next, the stoichiometry of Cu$^{2+}$ binding to the fibrillar form of Aβ(1-40) and Aβ(1-42) was investigated, as shown in Figure 4.6. Cu$^{2+}$ binding curves (Figure 4.6c-d) for fibrillar Aβ(1-40) and Aβ(1-42) also show a maximal Cu$^{2+}$ EPR signal at one mole equivalent. After one mole equivalent of Cu$^{2+}$, both Aβ(1-40) and Aβ(1-42) in their fibrillar form became saturated with Cu$^{2+}$ at physiological pH. Because of the necessity of the long incubation of Aβ(1-40) and Aβ(1-42) at 37 °C, 0.05 % (v/v) sodium azide was added to some samples, this had no effect on stoichiometry or the g$_{II}$ value, although the A$_{II}$ was reduced by approximately 2 gauss. Aβ(1-42) shows a slight reduction in signal with further Cu$^{2+}$ addition (above 1 mole equivalent) due to some precipitation (~ 20 %) of the less soluble Aβ(1-42) prior to freezing. As with monomeric Aβ, the EPR data indicates fibrillar Aβ(1-40) and Aβ(1-42) also bind a single Cu$^{2+}$ ion. This is in contrast to the shorter soluble Cu$^{2+}$ binding fragment Aβ(1-28). This was suspected to be due to the hydrophobic tail of Aβ(1-42) blocking the second Cu$^{2+}$ binding site. Thus to investigate this, methanol, an intramolecular hydrogen bond promoter was added.
Figure 4.6. Cu\(^{2+}\) titration of fibrillar A\(\beta\)(1-40) and A\(\beta\)(1-42). (a) Increasing mole equivalents of Cu\(^{2+}\) with 50 \(\mu\)M A\(\beta\)(1-42) in phosphate buffer solution up to two mole equivalents at pH 7.4, 20 K. (b) hyperfine splitting showing the A\(n\) of fibrillar A\(\beta\)(1-42). Panel (c) shows the double integration of the EPR spectra of fibrillar A\(\beta\)(1-40) with sodium azide (open circles) and fibrillar A\(\beta\)(1-40) with no sodium azide (squares). Panel (d) shows the double integration of fibrillar A\(\beta\)(1-42) with no sodium azide (black circles) plotted versus mole equivalent of Cu\(^{2+}\) ions. All indicate that saturation occurs at 1 mole equivalent of Cu\(^{2+}\).

4.4.5. Differences in Stoichiometry Between Full Length A\(\beta\)(1-42) and Fragments

The addition of 20 \% (v/v) methanol has a profound influence on the stoichiometry of full-length A\(\beta\)(1-42). It is clear from the Cu\(^{2+}\) binding curve in Figure 4.7a that A\(\beta\)(1-42) has a 2:1 stoichiometry in the presence of methanol rather than the 1:1 stoichiometry observed in 100 \% water.
Figure 4.7. Effect of methanol on Aβ(1-42) stoichiometry. (a) A double integration of Aβ(1-42) in 100 % water (squares) and water/methanol mixture (80/20 % v/v) (circles). (b) Double integration of Aβ(1-28) with (circles) and without (squares) 20 % methanol. Cu²⁺-Aβ(1-42) changes from 1:1 to 2:1 stoichiometry in the presence of methanol. Cu²⁺-Aβ(1-28) retains 2:1 stoichiometry irrespective of the presence of methanol. The concentration of Aβ(1-42) and Aβ(1-28) was 50 µM.

As a direct comparison Aβ(1-28) was also studied in 20 % (v/v) methanol (Figure 4.7b) but this had no effect on the binding stoichiometry, and Aβ(1-28) still bound two Cu²⁺ ions both with or without 20 % v/v methanol present. The EPR spectra for Cu²⁺-Aβ(1-28) in methanol/water are identical to Cu²⁺-Aβ(1-28) in water alone.

The effect of methanol on the secondary structure of monomeric Aβ(1-42) was further studied by UV-CD. Measurements indicated no significant change in secondary structure from the peptide’s random coil conformation with addition of 20 % methanol (Figure 4.8a). Affinity of the complex in 20 % methanol was also measured using fluorescence with L-His as a competing ligand (Figure 4.8b). The half-maximal quenching occurs at close to 3 (~3.25) mole equivalents of L-His for soluble Aβ(1-42) in 20 % methanol, which is comparable to soluble Aβ(1-42) without methanol (1.7 mol equivalents).
Figure 4.8. Aβ(1-42) with 20 % (v/v) methanol. (a) UV-CD spectra of the secondary structure of Aβ(1-42) with 20 % (v/v) methanol (pink) or with no methanol (black). 50µM Aβ(1-42) in 10 mM phosphate buffer at pH 7.4. The spectra indicate monomeric Aβ(1-42) remains predominantly random coil even after the addition of 20 % (v/v) methanol. (b) Fluorescence spectra of Cu²⁺-monomeric Aβ(1-42) in 20 % methanol L-histidine competition. The tyrosine fluorescence signal at 307 nm returns with additions of L-histidine. Only half of the maximal fluorescence intensity has returned after 2 mole equivalents of histidine is added.

4.4.6. Cu²⁺ binding of the Second Mole Equivalent to Aβ(1-28)

The nature of the binding site for the second mole equivalent of copper bound to Aβ(1-28) also warranted further investigation. Figure 4.9 shows EPR spectra of one and two mole equivalents of Cu²⁺ ions bound to Aβ(1-28), at pH 7.4. As Cu²⁺ was titrated in beyond one equivalent a new set of signals appear to low field, while signals for the first equivalent of Cu²⁺ remain unaffected. A difference spectra is shown of Cu₂Aβ(1-28) minus Cu₁Aβ(1-28), emphasising the appearance of the new set of signals at 2 mole equivalents of Cu²⁺. The copper type-II spectrum gives $A_{II}$ and $g_{II}$ values of 16.36 mK (175 gauss) and 2.28 respectively. These values are more typical of an oxygen rich complex such as 1N3O. An interesting possibility is that this second, weaker binding site originates from ligands found in the first 11 residues of Aβ. Thus for comparison an EPR spectra of Aβ(1-11) with 1 mole equivalent of Cu²⁺ added is also shown in Figure 4.9.
Figure 4.9. Comparison of the second Cu$^{2+}$ binding site with Aβ(1-11). Aβ(1-28) with 0.5, 1.0, 1.5 and 2.0 mole equivalents Cu$^{2+}$ present. The difference spectra is the two mole equivalent spectra minus the 1 mole equivalent spectra. The difference spectra highlights the 3O1N nature of the second binding site which is very different from Aβ(1-11). See Table 4.1 for $A_H$ and $g_H$ values.

It is clear that the weaker second binding site does not, in fact, mimic binding to the Aβ(1-11) fragment, which has $A_H$ and $g_H$ values of 144 gauss (13.6 mK) and 2.30 respectively, typical of 2N2O. Indeed the weaker second binding site does not resemble EPR spectra of any of the analogs studied (Table 4.1).

4.4.7. Temperature Dependence of Cu$^{2+}$-Aβ(1-28) EPR; A Bridging Cu-His-Cu Dimer is Unlikely

It has previously been suggested that there is histidine bridging between two Cu$^{2+}$ ions in the Aβ complex (Curtain, et al. 2001, Smith, et al. 2006). A $g$ value of $\sim 4$ in the EPR spectra of Aβ-Cu$^{2+}$ in addition to a broad $g \sim 2$ line was observed. Such EPR spectra are diagnostic for the existence of binuclear Cu$^{2+}$ centers where the Cu$^{2+}$ ions are coupled by dipolar interactions, and the authors suggest the atoms are 6.2 Å apart. This study was repeated using the same conditions used by Smith et al (Smith, et al. 2006) but no line broadening in the spectra was observed, shown in Figure 4.10. EPR spectra in which electron coupling is taking place are very temperature dependent and will be effectively decoupled at lower temperatures (20
K) (Valentine, et al. 1979). The Aβ(1-28)-Cu²⁺ complex was observed at a range of temperatures and no resonances at $g \sim 4$ were observed. Without such an observation the possibility of a bridge complex must be questioned.

![Figure 4.10. Temperature dependence of Aβ(1-28). 100 µM Aβ(1-28) and 0.6 mole equivalents Cu²⁺ (Cu²⁺ stock used was 1 part Cu²⁺ to 6 parts glycine) in phosphate buffered saline (PBS) to mimic the parameters of (Smith et al 2006). Spectra at 20 K and 40 K were taken at 2 mW microwave power. All subsequent spectra were at 4 mW microwave power.](image)

### 4.5. Discussion

#### 4.5.1. Cu²⁺ Binding to Fibrils

Fibrils, once formed, retain their structure with Cu²⁺ additions, as judged by UV-CD and unperturbed ThT binding, and also TEM (Karr and Szalai 2008). In agreement with previous studies (Karr and Szalai 2008) the EPR spectra shown here suggest that the fibrillar Cu²⁺ complex forms the same coordination geometry and ligands as observed with monomeric Aβ. This is strongly supported by the observation that Cu²⁺ affinity for the two Aβ forms is almost identical (discussed in Chapter 3). Interestingly, Aβ fibres are able to accommodate a full stoichiometric complement of Cu²⁺ ions, suggesting Cu²⁺ ions are able to diffuse into all parts of the fibre giving precisely a 1:1 stoichiometry. The nature of the ligand's coordination geometry and
pH dependence of the Aβ-Cu\(^{2+}\) complex is discussed later but is shown in Figure 4.11. The data shown in this chapter does not suggest an inter-molecular Cu-Aβ complex for the fibrillar form of Aβ.

Karr et al. have reported Cu\(^{2+}\)–EPR spectra of fibrils for the low pH species similar to those observed here (Karr, et al. 2005, Karr and Szalai 2008). However, they also argue, based on the solid state NMR models of Aβ fibres, that both His13 and His14 cannot bind a single Cu\(^{2+}\) ion in Aβ(1-40) because the β-sheet structure would be disrupted by the simultaneous coordination of two adjacent residues (Karr, et al. 2005). However the data in this chapter shows that the Cu\(^{2+}\) affinity is the same in the fibres as in the monomers, which would be unlikely if either His13 or His14 were not part of the complex. Indeed, deuterium exchange experiments indicate that stable hydrogen bonds are not present between residues 1-14, suggesting a more flexible picture of Aβ fibres up to and including residue His14 (Olofsson, et al. 2007, Whittemore, et al. 2005). This explains why the Cu\(^{2+}\) coordination geometry is unaffected by the formation of fibrils and can contain both His13 and His14.

The β-strands are 4.7 Å apart in the fibril β-sheets (Serpell 2000). This, on average, would place adjacent His residues 4.7 Å apart. The EPR studies shown here demonstrate that Aβ(1-40) and Aβ(1-42) can bind up to 1 equivalent of Cu\(^{2+}\) ions. Dipolar through-space interactions of Cu\(^{2+}\) ions are typically felt between Cu\(^{2+}\) ions that are 7 Å or less apart and usually result in broadened spectra. It is therefore perhaps a surprise that some broadening of the EPR spectra is not observed, although it is certainly possible that the broadening is too small to be detected.
Figure 4.11. Proposed model of high affinity Cu$^{2+}$ binding site to Aβ(1-42) in both fibrillar and monomeric forms. Complex I and II are tetragonal and complex I dominates below pH 9. Complex I is square planar while complex II may be more square pyramidal. Coordinating ligands include all three histidine imidazole nitrogens but with two histidine ligands in the equatorial plane and a third as a weaker axial ligand. In addition, the N-terminal amino group coordinates at pH 7.4 as well as pH 9, but as an axial ligand at pH 7.4. The oxygen ligands are likely to arise from the carboxylate side-chains of Asp1 and Asp7. At room temperature the Cu$^{2+}$ complex may be quite dynamic exchanging between ligands in the equatorial and axial plane. Without crystallographic data this proposed coordination geometry is tentative.

4.5.2. Coordination Geometry

The EPR studies suggest two pH dependent species are formed with square-planar or tetragonal geometry. Based on $g_{II}$ and hyperfine splitting values complex I (at low pH) is composed of 2N2O or 3N1O ligands while complex II (at high pH) gives EPR spectra more typical of a complex with 3N1O or 4N ligands. A range of Aβ analogs have been used to further probe the nature of the coordination geometry. It is commonly believed that all three histidines within Aβ form equatorial imidazole nitrogen ligands with the Cu$^{2+}$ ion (Shin and Saxena 2008). It is therefore surprising
that upon replacement of His6, His13, or His14 with alanine there is not more of a significant effect on the appearance of the EPR spectra, as alternative imidazole nitrogens are not available to compensate for the alanine substitution. In contrast, removal of His6, His13, His14 or N-terminal acetylation, all reduce affinity for Cu$^{2+}$ (Table 4.1) and in addition cause perturbed Cu$^{2+}$ loaded UV-CD spectra compared to the Cu$^{2+}$-wild type spectra (Syme, et al. 2004). It is notable that the N-terminus has the most reduced affinity for Cu$^{2+}$ followed by the H6A analog, while the affinity reduction observed for H13A or H14A is much less marked. An explanation for these seemingly conflicting observations is that the EPR spectra attributed to complex I is actually the result of a superposition of a mixture of species containing only two histidine ligands in the equatorial plane with a third histidine in the axial position. Removal of one of the histidine residues, or the N-terminal amino coordination, from the equatorial plane is replaced by the histidine that had originally occupied an axial position. At room temperature it might be more appropriate to think of the Cu$^{2+}$ complex as quite dynamic with two histidines in the equatorial plane and a third histidine axially coordinating, exchanging between each other, as shown in Figure 4.11. Without crystallographic data the proposed coordination geometry is only a tentative model of the Cu-Aβ complex. In support of the model, the EPR spectrum of Aβ(Ac10-16), which only has two potential nitrogen ligands in the equatorial plane has a similar appearance to complex I.

The presence of axial ligands contributing to the tetragonal Cu$^{2+}$ complex has largely been ignored in the literature, as they have little influence on EPR spectra. However axial ligands will stabilise the Cu$^{2+}$ complex and affect main-chain conformation and biophysical properties. A study using extended X-ray absorption fine structure spectroscopy (EXAFS) found a distorted six-coordinated (3N3O) geometry around copper in the Aβ-Cu$^{2+}$ complex, including three histidines, glutamic, or/and aspartic acid, and axial water. Thus supporting a potentially important role for axial ligands in the complex (Streltsov, et al. 2008).

A recent study using cw-EPR and $^{15}$N labelled His analogs together with HYSCORE-EPR presents some similarities with the model in Figure 4.11. The low pH complex observed contained a mixture of species with only two of the three imidazoles coordinating in the equatorial plane, and His6 was described as the
anchor in their complex (Drew, et al. 2009). Interestingly the results in this chapter show that the H6A analog had a more significant reduction in affinity for Cu$^{2+}$ ions than H13A or H14A (Table 4.1), which supports this assertion. Other conclusions drawn by Drew et al for the equatorial ligands appear not to be compatible with this data, in particular, the lack of the inclusion of the N-terminal amino group in the high pH complex (Drew, et al. 2009, Drew, et al. 2009). The evidence in this chapter strongly supports the role of the N-terminus in complex II, as EPR spectra of two peptides which are N-terminal acetylated show that they will not form the high pH complex. This strongly indicates that complex II involves coordination from the N-terminal amino group presumably within the equatorial binding plane, as shown in Figure 4.11.

The pK$_a$ of N-terminal amino groups are typically 8.0. The pH at which the N-terminal amino group deprotonates may match the formation of complex II (dominant at pH 9), particularly if the pK$_a$ for the N-terminus of Aβ is a little higher than is normally observed. A study by Karr and Szalai (Karr, et al. 2007) found that when Asp1 was replaced with Asn it favoured the formation of complex II. This might be explained by the stabilizing effect of the negative carboxylate of Asp1, which will raise the pK$_a$ of the N-terminal amino group closer to pH 9 compared to an Asn side chain at the same position. It seems probable that the mutation of Asp to Asn promotes the change of the N-terminal amino coordination from an axial position to the equatorial plane, thus supporting the model of coordinating ligands shown in Figure 4.11.

Previous studies using visible-CD at physiological pH and below suggest main chain coordination is not taking part in the complex (Syme, et al. 2004). The CD spectrum at neutral pH shows a lack of d-d transition CD bands ~ 600 nm, suggesting minimal vicinal effects, which occur when the asymmetric α-carbon is held in a chelate ring between two chelating donor atoms (e.g. adjacent main-chain amides) (discussed in Chapter 2). This is indicative that main-chain amide coordination is not taking part in the complex at physiological pH and below, although it is possible it might take part at higher pHs.
There is much debate in the literature as to the source of the oxygen ligand in the equatorial plane, water is a possibility but Karr and Szalai found that substituting $H_2^{17}O$ for $H_2^{16}O$ did not affect hyperfine splitting in their spectra suggesting that water is not an equatorial ligand to Cu$^{2+}$ in $\alpha$B(1-40). The Tyr10 hydroxyl group has previously been suggested (Miura, et al. 2000) as a ligand but has since been shown to be unlikely by $^1H$ NMR (Syme, et al. 2004) which shows no significant broadening of the tyrosine ring protons. This is also supported by the lack of Cu$^{2+}$ charge-transfer bands associated with Cu-Tyr (Guilloreau, et al. 2006, Kowalik-Jankowska, et al. 2003). Carbonyl main chain Cu$^{2+}$ coordination is also a possibility, however coordination of oxygen from one of the four carboxyl side-chains (Asp1, Glu3, Asp7 and Glu11) in the first sixteen residues is most probable. Karr et al. showed that the EPR spectra for D1N and E3Q analogs left complex 1 unchanged (Karr and Szalai 2007), although removal of Asp1 might simply result in it being replaced by an alternative carboxylate ligand which would give a very similar EPR spectrum. Asp1 and Asp7 (adjacent to His6) are favoured as potential oxygen ligands for complex I, as shown in Figure 4.11. This is because the H6A analog is more destabilised than H13A or H14A (see Figure 4.4 for pH dependence and Table 4.1 for affinities), and coordination of the carboxyl and N-terminal amino group of Asp1 will form a stable six-membered chelate ring with the Cu$^{2+}$ ion. A very recent study using $^{13}C$ labelled Asp1 and HYSCORE EPR suggested Asp1 carboxylate coordination in the low pH complex (Drew, et al. 2009).

Ab-initio molecular modelling of the His13-His14 Cu$^{2+}$ binding site suggests a trans arrangement of the two imidazole rings with a main-chain carbonyl of His13 coordinating between the two His residues (Raffa, et al. 2005, Rauk 2008). However this type of complex is likely to generate appreciable d-d transition signals which are not observed by CD (Syme, et al. 2004). In contrast a crystal structure of a model compound containing a di-peptide analog of two adjacent histidine residues has been reported (Hori 1979). In this compound, Cu$^{2+}$ chelates to the eN of both imidazole rings making a cis-arrangement rather than trans coordination (90° rather than 180°) to the Cu$^{2+}$ ion in a square-planar arrangement. Sterically, this type of ligand arrangement could facilitate the coordination of the N-terminus and His6 without any obvious restrictions, but would stop main-chain coordination to Cu$^{2+}$, as implied
by the lack of visible-CD signal from d-d transitions lending support to the proposed model shown in Figure 4.11.

4.5.3. Stoichiometry and the Second Binding Site
It is well established that two Cu$^{2+}$ ions bind to fragments Aβ(1-16) and Aβ(1-28) (Curtain, et al. 2001, Syme, et al. 2004). It was therefore surprising that the longer full length Aβ only binds one mole equivalent of Cu$^{2+}$ ions with appreciable affinity, with a clear break point and saturation of the signal at one mole equivalent (at 50 µM peptide concentration). The difference between Aβ(1-28) and Aβ(1-42) is the 14 additional residues in the hydrophobic tail of Aβ(1-42); however the potential two Cu$^{2+}$ binding sites are in the first 16 residues. A possible explanation for the decreased binding stoichiometry of Aβ(1-42) compared to Aβ(1-28) may be hindrance to the second Cu$^{2+}$ binding site by the hydrophobic tail in Aβ(1-42). To test this hypothesis the hydrophobic interactions were disrupted. Alcohols, such as methanol, are thought to disrupt tertiary interactions and allow local interactions (hydrogen bonding) to dominate within the polypeptide chain (Kony, et al. 2007). After adding 20 % (v/v) methanol, Aβ(1-42) bound a second Cu$^{2+}$ ion as seen for the shorter Aβ(1-28) peptide.

In agreement with this study (in the absence of methanol) others have indicated a 1:1 stoichiometry for Aβ(1-40) (Garzon-Rodriguez, et al. 1999, Tougu, et al. 2008). In contrast a recent study reported that Aβ(1-40) can bind two Cu$^{2+}$ ions (Atwood, et al. 2000). However for this study a cryoprotectant (50 % v/v glycerol) was used. This alcohol (a triol), like methanol is known to reduce hydrophobic interaction and promote intra-molecular hydrogen bonding (Jones, et al. 1996). Thus the glycerol has the same affect shown here for 20 % (v/v) methanol and explains the discrepancy in the stoichiometries reported.

The nature of the second Cu$^{2+}$ binding site is not well understood. It is clear that it is contained within the first 16 amino acids (Syme, et al. 2004). EPR suggests that this binding site is also tetragonal or square-planar with the A_II and g_II shifted to values more typical for oxygen than nitrogen ligands, perhaps 3O1N. It is possible that the first equivalent of Cu$^{2+}$ binds to all 3 histidine residues, although one His is probably an axial ligand, it is notable that as the second mole equivalent of Cu$^{2+}$ is added the
binding mode of the first equivalent does not appear to be perturbed. It is therefore likely that the second mode of binding is rich in carboxylate ligands (D1, E3, D7 and E11) and perhaps a single nitrogen ligand; the N-terminus or His13/14. Support for this comes from comparison of the EPR spectra of Aβ(1-11), which contains two potential nitrogen ligands. This fragment reveals a very different spectrum from that of the second mole equivalent spectra of Aβ(1-28). Thus this suggests a complex containing the N-terminus and His6 can be ruled out for the second weak-binding site on Aβ. The affinity of the second mole equivalent has been determined to be $10^{-5}$ M (Guilloreau, et al. 2006).

4.5.4. Biological Significance

It seems clear that Aβ fibres once formed are able to accommodate the coordination of Cu$^{2+}$ ions. Indeed Cu$^{2+}$ ions are found concentrated within amyloid plaques bound directly to Aβ (Dong, et al. 2003, Miller, et al. 2006). The affinity, coordination geometry, and stoichiometry are unaffected by the form of Aβ; monomeric or fibrillar. In particular Aβ fibrils are able to accommodate a full stoichiometric complement of Cu$^{2+}$ ions, and the β-sheet secondary structure is unperturbed.

The studies described in this chapter have made a significant contribution to understanding Cu$^{2+}$ coordination in both monomeric and fibrillar form. It is known that Cu$^{2+}$ homeostasis within the brain is impaired in AD patients (Bush 2003, Cuajungco, et al. 1997). It is possible that increasing Cu$^{2+}$ levels may promote Aβ oligomerisation and Cu$^{2+}$ loaded fibres may be more resistant to clearance, thus explaining the elevated level of Cu$^{2+}$ ions in amyloid plaques (Lovell, et al. 1998, Miller, et al. 2006).

The data in this chapter suggests Cu$^{2+}$ ions are not involved in Aβ cross-linking within fibres. Cu$^{2+}$ is certainly able to bind Aβ fibres once formed, less well understood is the potential effect Cu$^{2+}$ has on inducing fibre formation. It is well established that in vitro Cu$^{2+}$ significantly reduces the solubility of Aβ (Atwood, et al. 1998, Bush, et al. 1994, Huang, et al. 2004, Sengupta, et al. 2003), but only induces the formation of amorphous aggregates rather than amyloids (Raman, et al.
2005, Yoshiike, et al. 2001). In the next chapter the effect of Cu$^{2+}$ ions on the rate of formation of fibres is investigated.
5. \( \text{Cu}^{2+} \) ions Accelerate Fibril Growth
Chapter 5. Cu$^{2+}$ ions Accelerate Fibril Growth

Abstract

The role of Cu$^{2+}$ ions in AD is often disputed, as it has previously been shown in vitro that Cu$^{2+}$ ions inhibit fibre formation and only result in non-toxic amorphous aggregates of A$\beta$. In contrast, in this chapter it is shown that the presence of stoichiometric and sub-stoichiometric levels of Cu$^{2+}$ ions profoundly increases the rate of production of amyloid fibres, doubling the rate of nucleation and elongation of amyloid formation. The stoichiometry of Cu$^{2+}$ ions is shown to be crucial, up to and including 1 mole equivalent accelerates fibril formation. However at Cu$^{2+}$ concentrations higher than 1 mole equivalent amorphous aggregation is favoured and fibril formation does not occur.

An explanation for the effect of Cu$^{2+}$ ions on fibril growth is developed through pH studies. Fibril growth with or without Cu$^{2+}$ is highly pH-dependent, strongly influencing the likelihood of A$\beta$ self-association. Additionally Cu$^{2+}$ coordinates to A$\beta$ through A$\beta$’s histidines, and it is shown that the rate of fibril formation is closely related to the protonation state of histidine residues within A$\beta$. Thus it is found that the binding of Cu$^{2+}$ ions at a physiological pH causes A$\beta$ to approach its isoelectric point, inducing self-association and fibre formation. Finally it is found that as the pH drops closer to the pl of A$\beta$, or alternatively as the stoichiometry of Cu$^{2+}$ binding increases, the rate of fibril formation increases until it is over-taken by amorphous aggregation. Thus a delicate balance between fibril formation and amorphous aggregation is found.
5.1. Introduction

The factors affecting the rate of fibril growth are described in this introduction. Fibril formation, including detection methods, oligomeric species and monomeric and fibrillar structures are discussed in Chapter 1.

5.1.1. Influences on Fibril Formation

Determining the mechanisms of fibril formation is crucial in understanding and perhaps controlling AD onset and progression. Formation of fibrils requires a considerable amount of organisation of Aβ monomers, and unfavourable forces that would destabilise Aβ fibrils must be offset by forces that stabilise Aβ fibrils. There are a range of factors that can influence whether or not a protein will form fibrils, and how rapidly it will do so. Some of these factors have been optimised in the fibril growth studies described in this chapter, and so are discussed here in more detail.

The propensity of fibril formation to occur is based on a range of intrinsic and extrinsic factors. Intrinsic factors refer to properties of the peptide or protein itself which favour fibril formation, whereas extrinsic factors refer to external conditions such as pH, ionic strength, and protein concentration. These factors have been studied in some depth by various groups, and equations have been developed which can predict the aggregation rate of a protein.

DuBay et al included both intrinsic and extrinsic factors in their equation and found an impressive correlation coefficient of 0.92 of the predicted aggregation rate and the actual experimental aggregation rate. (DuBay, et al. 2004). A more recent algorithm is AGGRESCAN, which predicts aggregation-prone segments of protein sequences (Conchillo-Sole, et al. 2007). This method identifies areas of the polypeptide which have been identified experimentally as ‘hot spots’. An impressively large list of 24 different disease-linked polypeptides were studied and compared to existing experimental data. The authors conclude that blocking the hot spots of aggregation may be a strategy for designing therapeutics. A second method for predicting aggregation is the Zyggregator method, which uses three physico-chemical properties, hydrophobicity, charge and the propensity to form α-helical or β-sheet structure in an equation (Tartaglia, et al. 2008). The Zyggregator algorithm
determines the effect of mutations in the polypeptide sequence upon protein aggregation rates.

\[
\log(k/k') = \alpha_{\text{hydr}} \Delta I^{\text{hydr}} + \alpha_{\text{ss}} \Delta I^{\text{ss}} + \alpha_{\text{ch}} \Delta I^{\text{ch}}
\]

Where the parameters \( \alpha \) were obtained through fitting the above equation to a database of mutational variants with known aggregation rates from \textit{in vitro} experiments. \( \log(k/k') \) is the logarithm in base 10 of the ratio of \( k \), the aggregation rate of the wild type and \( k' \), the aggregation rate of the mutant, and the change in hydrophobicity (\( \Delta I^{\text{hydr}} \)), secondary structure propensity (\( \Delta I^{\text{ss}} \)) and charge (\( \Delta I^{\text{ch}} \)). Experimental changes in aggregation rates correlated well (\( R = 0.85 \)) with predictions produced through the Zyggregator method. Both AGGRESCAN and the Zyggregator method assume that the sequence of a protein is wholly responsible in determining the likelihood of aggregation, in contrast to DuBay et al.’s equation which includes external factors such as ionic strength and pH.

### 5.1.2. Intrinsic Factors

Hydrophobic interactions play an important role in folding and misfolding. Protein folding is thermodynamically driven to result in the most stable structure, and subsequently hydrophobic residues are clustered and buried, rather than on the protein surface. Replacement of nonpolar residues with polar residues results in inhibition of \( \text{A}\beta(1-42) \) fibril formation and an increase in the peptide’s solubility (Wurth, et al. 2002). Additionally Wurth et al.’s study showed that replacing nonpolar residues with other nonpolar residues had little or no effect, suggesting the hydrophobicity of residues is the most important factor in promoting fibril formation rather than specific amino acid interactions.

NH and CO groups in the peptide backbone are thermodynamically more favourable in water compared to a nonpolar environment, which causes conflict when they are next to hydrophobic side chains. To solve this thermodynamic mismatch, NH and CO groups form hydrogen bonds. Hydrogen bonds play a crucial part in influencing the propensity of proteins with \( \beta \)-sheet secondary structure to form fibrils. Rational design of peptides shows that \( \beta \)-sheet proteins favour intermolecular aggregation over intramolecular folding (Wang, et al. 2002). This is due to the difference in
hydrogen bonding between the two different secondary structures. Whereas
backbone hydrogen bonding in an α-helix can be mainly “satisfied” without needing
to oligomerise, in β-strands the C=O and N-H groups form hydrogen bonds to N-H
and C=O bonds on neighbouring strands.

Hydrophobic patterns are also important in predicting aggregation rates. DuBay et al
found that patterns of five consecutive hydrophobic and hydrophilic alternating
residues correlated significantly with the experimental values of aggregation rates,
i.e. the more patterns of this type the faster the aggregation rate. Based on this, it is
unsurprising that β-strands which avoid hydrophobic patterns are much more
common in nature than those which contain them (DuBay, et al. 2004).

Charge is also highly important in affecting the propensity for fibril formation. Two
reasons why this could be are:

(1) Specific charge-charge interactions between imidizolium groups on histidine
residues and the carboxylate groups on aspartate and glutamate could
stabilise oligomers.

(2) Electrostatic repulsion: when the protein has a net positive or negative charge
it is less likely to dimerise.

Experimental evidence suggests that electrostatic repulsion is more influential than
specific charge-charge interactions. Fraser et al showed that substituting amino acids
with opposite charges, e.g. histidine to aspartate, did not prevent fibril formation
(Fraser, et al. 1994), suggesting a loss of specificity will not prevent fibril formation.
Likewise four of the mutations that cause familial Alzheimer’s disease: Iowa Asp23
→ Asn, Arctic Glu22 → Gly, Dutch Glu22 → Gln and Italian Glu22 → Lys result in
a decrease in the net charge of Aβ at physiological pH. DuBay et al also found that
fibril formation rates were inversely proportional to the absolute value of the net
charge, i.e. the lower the net charge (the closer to 0) the faster the aggregation rates
(DuBay, et al. 2004). Thus electrostatic repulsion must be overcome for fibril
formation to occur.
5.1.3. Extrinsic Factors

The influence of pH on fibril formation is closely linked to the importance of charge, as the external pH approaches a protein’s pI, i.e. a net charge of 0, electrostatic repulsion will decrease and aggregation will increase. Salt can also effect fibril formation: as salt concentration increases, protein solubility decreases (salting out). This is due to increasing additions of salt reducing the number of water molecules available to the hydrophilic residues on the surface of the protein, causing protein-protein interactions to become stronger than solvent-protein interactions. Additionally salt ions can shield the ionic charges of the protein, reducing electrostatic repulsion. These two effects will increase protein self-association and the likelihood of fibril formation or protein aggregation. This is supported by fibril growth studies where monomer oligomerisation was found to be highly dependent on ionic strength (Johansson, et al. 2006) and protofibril association required salt to minimise electrostatic repulsion between the two axial sides of the fibrils and allow lateral association to take place (Lin, et al. 2008, Nichols, et al. 2002). DuBay et al found that the accuracy of the fibril growth rate predictions from their equation improved when ionic strength was included (DuBay, et al. 2004).

In addition to controlling the salt concentration, temperature and agitation are often manipulated in fibril growth experiments. Specific stages of Aβ fibril formation are highly temperature dependent. Raised temperatures shorten the lag phase and accelerate nucleation, but do not affect the elongation stage of fibril formation (Lin, et al. 2008). Agitation is performed widely in the literature to aid formation of fibrils. Lee et al found an increase in fibril formation in the presence of agitation by a factor of 2-4 (Lee, et al. 2007). Additionally agitation can affect fibril structure (Petkova, et al. 2005), possibly because agitation accelerates nucleation, preventing intramolecular structural rearrangement and instead favouring rearrangement on an intermolecular scale. In contrast quiescent conditions allow longer time for intramolecular rearrangements, resulting in increased stability and more energetically favourable structures to form (Lee, et al. 2007).

DuBay et al found that the rate of fibril formation increased with peptide concentration (DuBay, et al. 2004). A higher protein concentration increases the likelihood of protein association. This could effect nucleus formation, if the nucleus
The concentration dependent nature of fibril formation can be likened to the crystallization of proteins, in which overly precipitative conditions for self-association will cause amorphous aggregates rather than ordered crystals to form. This fine line between fibril formation and aggregation may explain why some studies found no effect on fibril formation with increased protein concentration (Johansson, et al. 2006).

Disagreements over which factors are more important in aiding or hindering fibril formation are common; this is especially true for metal ions. Studies showed more than a decade ago that Zn$^{2+}$ and Cu$^{2+}$ ions cause marked aggregation of Aβ (Atwood, et al. 1998, Bush, et al. 1994). However, further investigations suggested that Zn$^{2+}$ and Cu$^{2+}$ promote amorphous aggregation of Aβ and actually inhibit fibre formation and cell toxicity (Raman, et al. 2005, Yoshiike, et al. 2001). In this study, temperature, agitation, salt concentration, pH, metal and peptide concentration are all optimised and manipulated to promote fibril formation and minimise aggregation.

5.2. Aims

- Optimise the fibril growth assay to promote fibril formation and minimise amorphous aggregation.
- Determine if there is a role for Cu$^{2+}$ ions in promoting fibre formation.
- Extract rate constants from fibril growth curves to allow comparison between lag times and elongation rate.
- Ascertain any differences between Aβ(1-40) and Aβ(1-42) fibril formation.
- Determine the effect of peptide and metal concentrations and pH on fibril formation rates.

5.3. Experimental

For peptide solubilisation, the theoretical background of the use of Thioflavin T, and the specifics of transmission electron microscopy experiments please refer to Chapter 2.
5.3.1. Fibril Growth

Fibre growth kinetics are very sensitive to a number of factors that must be carefully controlled. They include the pH, concentration, agitation, temperature and ionic strength. Solubilisation of Aβ into a seed-free form is also important; the method for peptide solubilisation, and evidence to suggest it is seed-free, is discussed in Chapter 2. Fibre growth experiments were typically with 5 µM Aβ(1-40) or 3 µM Aβ(1-42) and the peptide was incubated at 30 °C in 160 mM NaCl, 50 mM HEPES buffer. This temperature and salt concentration allowed reproducible fibril formation growth curves, with a lag phase and a high concentration of fibrils at the end of incubation (as determined by ThT fluorescence). Additionally 160 mM NaCl is physiologically relevant (Lazarus-Barlow 1896). HEPES was used for its low affinity for Cu$^{2+}$ and Zn$^{2+}$ ions (see Table 2.1 in Chapter 2). Agitation was optimised to 30 seconds orbital shaking every 30 minutes.

Fibril formation was monitored by a BMG Fluostar Galaxy fluorometer. This fluorometer allowed 96 well plates to be used and temperature and agitation to be controlled. An excitation filter wavelength of 440 nm and an emission filter wavelength of 490 nm were used. 50-100 flashes (readings) were taken, and shaking was orbital and over a shaking width of 3 mm. 96 well plates used were sterile, flat bottomed and from Becton Dickinson. To minimise evaporation in the experiments the plates were sealed with Starseal polyolefin film sealing tape (Starlabs) and the wells were filled to 300 µL to reduce the volume of air in the plate.

Small adjustments were made with 10 mM NaOH or HCl to the stock Aβ solutions. The pH, a critical parameter in fibre growth rates, was measured before and after each fibre growth experiment; variations were ±0.05 pH units or less over the course of the experiment. Metal stock solutions were CuCl$_2$ 25 mM and ZnCl$_2$ 20 mM or as a Cu(Gly)$_2$ chelate. The Thioflavin T (ThT) stock was dissolved in water to 2 mM, and kept foil wrapped and at 5 °C, then a small volume added to the experimental sample to give a final concentration of 10 µM. UHQ water ($10^{-18}$ Ω$^{-1}$cm$^{-1}$ resistivity) was used at all times.
5.3.2. Growth Curve Analysis

Conversion of essentially monomeric Aβ to fibrillar Aβ follows a characteristic sigmoidal growth curve, Figure 5.1a. This is characterised by an initial lag phase followed by a rapid growth phase in which ThT fluorescence markedly increases, and a final equilibrium phase where ThT fluorescence reaches a plateau.

A number of empirical parameters can be obtained from a fibre growth curve. To aid comparison of fibril growth rates under different conditions Origin 7 was used to fit the experimental data to two different equations. These equations are widely used in the literature and were found to give the best fit to the experimental data. Figure 5.1b and 5.1c show the parameters used in both equations.

Equation 1 includes the time needed to reach half maximal ThT intensity ($t_{50}$) and the lag-time. The $t_{50}$ is influenced by both the nucleation and elongation phase. These values can readily be extracted from the data by fitting the growth curve to the following equation (Uversky, et al. 2001).

\[
Y = (y_i + m_i x) + \frac{\nu_f + m_f x}{1 + e^{-(x-xo/\tau)}} \quad \text{(eq 1)}
\]

Where $Y$ is the fluorescence intensity, $x$ is the time, $X_o$ is the time at half height of fluorescence ($t_{50}$). The apparent fibre growth rate is $k_{app} = 1/\tau$ and the lag time ($t_{lag}$) is $X_o - 2\tau$. Equation 1 allows for a slope in the initial and final parts of the growth curve, ($y_i + m_i x$), ($\nu_f + m_f x$), rather than forcing these to be horizontal. Table 5.1 shows the $t_{50}$, $X_o$, $t_{lag}$, ($X_o - 2dx$) and $k_{app}(1/dx)$ derived from three data sets.
Chapter 5. Cu$^{2+}$ ions Accelerate Fibril Growth

Figure 5.1. Fibril growth curves. (a) A typical fibril growth curve, monitored by ThT fluorescence, with a lag phase and elongation phase. (b) Key parameters used in equation 1 (Uversky et al. 2001), $Y$ is the fluorescence intensity, $x$ is the time, $X_o$ is the time at half height of fluorescence ($t_{50}$). (c) shows parameters from equation 2 (Morris et al. 2008), where $[B]_t$ is the fibrillar concentration as measured by the ThT signal and $k_1$ (units of hrs$^{-1}$) is proportional to the lag time and $k_2$ is proportional to the slope during elongation.

Equation 2 describes two rate constants for the initial nucleation and elongation process (Morris, et al. 2008). Firstly $k_1$ describes the monomer converting into a nucleating species. The second rate constant ($k_2$) describes monomer addition on to the nucleating species (elongation), see Table 5.2. It should be noted that $k_2$ is simply an average rate of multiple additions and assumes that the rate constant is independent of fibre size.
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$$\frac{[B]_t}{[A]_0} = \frac{k_1/k_2+[A]_0}{1+(k_1/k_2[A]_0)e^{(k_1+k_2[A]_0)t}} \quad (eq \ 2)$$

Where $[B]_t$ is the fibrillar concentration as measured by the ThT signal and $[A]_0$ is the total concentration of Aβ in monomeric and fibrillar form: $[A]_0 = [B]_t + [A]_t$ (where $[A]_t$ is the amount of monomer at time $t$). $k_1$ (units of hrs$^{-1}$) is proportional to 1/lag time and $k_2[A]_0$ is proportional to the slope during elongation. These parameters are shown in Figure 5.1c. Thus $k_1$ describes the lag-phase (nucleation) and $k_2$ describes the growth phase (elongation).

Kinetic parameters have been extracted from between 6 and 9 raw traces. Mean values with 1 standard error (SE) are given in Tables 5.1 and 5.2. A paired t-test was used to confirm the significance of the difference between the kinetics with and without Cu$^{2+}$ ions.

5.3.3. $^1$H NMR

The pH dependent protonation state of the three histidine side-chains of Aβ were determined from $^1$H NMR chemical shifts of the εH and δH protons, recorded at 0.5 pH unit intervals between pH 5 and 9. The singlet chemical-shift of the εC and δC protons were readily identified from the 1D $^1$H NMR spectra, His εC and δC assignments were confirmed using 2D $^1$H Total Correlation Spectroscopy (TOCSY) spectra, using standard acquisition parameters and a spin-lock of 60 ms. Assignment of the His6, His13 and His14 residues was based on previous $^1$H NMR studies with H6A H13A and H14A analogs (Syme, et al. 2006). The Aβ(1-28) fragment was used rather than full-length Aβ(1-40) to improve solubility. 10 µM DSS (sodium 2,2 dimethyl-2-silapentane -5-sulphonate) was used as a reference. 0.1 mM Aβ(1-28) in 50 mM phosphate buffer, in 90 % H$_2$O, 10 % D$_2$O, at 25 °C. The pH dependent shifts for ε and δ protons were fitted to a modified Hill equation to determine pK$_a$ for His6, His13 and His14.
5.4. Results and Discussion

5.4.1. Copper Accelerates Fibril Growth Rates

Using the well-established amyloid binding Thioflavin T (ThT) fluorescence assay, fibre formation was investigated over a range of Aβ(1-40) concentrations, with and without the presence of Cu\(^{2+}\) ions. Aβ(1-40) concentrations of greater than 10 µM, pH 7.4, showed no detectable amyloid fibrils in the presence of 1 mole equivalent Cu\(^{2+}\) ions, as previously reported (Raman, et al. 2005, Yoshiike, et al. 2001). However, when more dilute conditions of Aβ(1-40) were investigated, of between 5 and 2 µM, rapid fibre formation was detected in the presence of Cu\(^{2+}\) ions. Figure 5.2 shows that Cu\(^{2+}\) ions significantly increase the rate of Aβ(1-40) fibre formation at pH 7.4. In Figure 5.2a multiple ThT fluorescence traces are shown, with and without Cu\(^{2+}\) ions present, the ThT readings are absolute (not normalised). Figure 5.2b shows normalised data from the mean of nine measurements repeated on two separate occasions (individual fluorescence traces are shown in Figure 5.3b and c). Metal-free Aβ(1-40) preparations typically take more than 70 +/- 2 hours to reach half maximal fluorescence (t\(_{50}\)), while the same Aβ(1-40) preparations with 0.5 or 1 mole equivalent of Cu\(^{2+}\) ions cause fibres to form in nearly half the time; 38 +/- 2 hours, (Figure 5.2c). A paired t-test confirms that Cu\(^{2+}\) ions significantly increase fibre growth rates with 99.9 % confidence. Kinetic parameters taken from the fibre growth curves are given in Tables 5.1 and 5.2.
Figure 5.2. Cu$^{2+}$ accelerates fibre growth. (a) Fibre growth curves with 0.5 mole equivalent Cu$^{2+}$ (blue traces) and without Cu$^{2+}$ (red traces) at pH 7.4. Six traces are shown for each condition and the ThT fluorescence values are absolute. Aβ(1-40) 5 µM, HEPES buffer 50 mM, 160 mM NaCl at 30 ºC. (b) Average of 9 growth curves recorded on two separate occasions, apo in red, 1 mole equivalent Cu$^{2+}$ in blue. The fluorescence signal is normalised at maximal intensity. (c) Time to reach half-maximal fluorescence ($t_{50}$) in two experiments with 0 (red), 1 (mid-blue) or 0.5 (dark blue) mole equivalents of Cu$^{2+}$ ions. The presence of Cu$^{2+}$ typically halves the time taken to form fibres. Error bars are for standard error from nine traces.

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Figure 5.3. Cu$^{2+}$ accelerates Aβ fibril growth. Comparison of Cu$^{2+}$ loaded (blue traces) and Cu$^{2+}$-free (red traces). Aβ(1-40) at 5 µM, pH 7.4 in the presence of (a) 2.5 µM Cu$^{2+}$, or 5 µM Cu$^{2+}$ in (b) and (c). Figures (b) and (c) are from two separate experiments. All carried out in 50 mM HEPES, 160mM NaCl, 30º C, with agitation for 30 seconds every 30 minutes. The ThT fluorescence intensities have been normalized. Averages of the nine traces are shown in Fig. 4.2(b).
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### Table 5.1. Kinetic Data from fibril growth curves using equation 1.

Data obtained from fitting equation 1 to the graphs shown in Figure 5.2 and 5.3, with the standard error shown in parentheses. (a) is taken from the data shown in Figure 5.2a, (b) is taken from the data shown in Figure 5.3a and b and (c) obtained from fitting to Figure 5.3c. Equation 1 determines the kinetics parameters: $t_{50}$ ($X_o$), $t_{lag}$ ($X_o - 2dx$) and $k_{app}$ (1/dx) with the number of traces shown in n.

<table>
<thead>
<tr>
<th></th>
<th>Apo</th>
<th>$t_{50}$ (hrs)</th>
<th>$t_{lag}$ (hrs)</th>
<th>$k_{app}$ (hrs$^{-1}$)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>Apo</td>
<td>72 (2)</td>
<td>55 (3)</td>
<td>0.120 (0.007)</td>
<td>6</td>
</tr>
<tr>
<td>0.5 Cu$^{2+}$</td>
<td>42.6 (0.6)</td>
<td>32.2 (0.8)</td>
<td>0.20 (0.01)</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td>Apo</td>
<td>68 (2)</td>
<td>53 (8)</td>
<td>0.09 (0.03)</td>
<td>9</td>
</tr>
<tr>
<td>0.5 Cu$^{2+}$</td>
<td>20 (3)</td>
<td>14 (3)</td>
<td>0.20 (0.06)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>1.0 Cu$^{2+}$</td>
<td>41 (3)</td>
<td>28 (3)</td>
<td>0.17 (0.03)</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>(c)</td>
<td>Apo</td>
<td>72 (1)</td>
<td>49 (2)</td>
<td>0.091 (0.006)</td>
<td>9</td>
</tr>
<tr>
<td>1.0 Cu$^{2+}$</td>
<td>36 (2)</td>
<td>16 (4)</td>
<td>0.12 (0.02)</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

Inspection of the growth curves indicates that both the nucleation and elongation rate are accelerated by Cu$^{2+}$ ions for Aβ(1-40). The average $t_{50}$ time, based on the data sets shown in Figure 5.2 and 5.3, is 71 hours for apo-Aβ, 31 hours for Aβ-0.5 mol eq Cu$^{2+}$, and 39 hours for Aβ-1 mol eq Cu$^{2+}$. The average $t_{lag}$ time is 52 hours for apo-Aβ, and 23 hours for Aβ-0.5 eq Cu$^{2+}$ and 22 hours for Aβ- 1 eq Cu$^{2+}$ (Table 5.1). The lag-time is particularly reduced by Cu$^{2+}$ ions.

Using the second equation, where the nucleating rates and elongation rates are obtained. The average apo nucleating rate is $0.21 \times 10^{-3}$ h$^{-1}$, whereas in the presence of 1 mol eq Cu$^{2+}$ it is three times faster, at $0.63 \times 10^{-3}$ h$^{-1}$. The elongation rate is twice as fast in the presence of 1 mol equivalent Cu$^{2+}$. The results of these equations
suggest that the effect of Cu\(^{2+}\) ion on A\(\beta\)(1-40) fibril formation is to accelerate both the nucleation and elongation stages of fibril growth.

<table>
<thead>
<tr>
<th></th>
<th>Nucleating Rate (x 10(^{-3}))</th>
<th>Elongation Rate (x10(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Apo</td>
<td>0.037 (0.017)</td>
<td>0.117 (0.007)</td>
</tr>
<tr>
<td>50% Cu(^{2+})</td>
<td>0.16 (0.03)</td>
<td>0.147 (0.004)</td>
</tr>
<tr>
<td>(b) Apo</td>
<td>0.19 (0.07)</td>
<td>0.098 (0.008)</td>
</tr>
<tr>
<td>100% Cu(^{2+})</td>
<td>0.15 (0.06)</td>
<td>0.19 (0.00)</td>
</tr>
<tr>
<td>(c) Apo</td>
<td>0.41 (0.08)</td>
<td>0.073 (0.005)</td>
</tr>
<tr>
<td>100% Cu(^{2+})</td>
<td>1.1 (0.4)</td>
<td>0.14 (0.02)</td>
</tr>
</tbody>
</table>

**Table 5.2. Nucleating and elongation rate obtained from equation 2.** Data obtained from fitting to the data shown in (a) Figure 5.2a, (b) Figure 5.3b and (c) Figure 5.3c. The standard error is shown in parentheses.

Interestingly, in studies with A\(\beta\)(1-42), at 1, 3 and 5 \(\mu\)M, the growth curves in Figure 5.4 show that the lag phase is of a similar length both in the presence and absence of Cu\(^{2+}\), however the elongation rate is faster when Cu\(^{2+}\) is present. A possible reason for the nucleation rate of A\(\beta\)(1-42) being unaffected by the presence of Cu\(^{2+}\), whereas in A\(\beta\)(1-40) it is notably reduced, is the formation of a different nucleating species. Different fibril forming nuclei for A\(\beta\)(1-40) and A\(\beta\)(1-42) have been suggested (Bitan, et al. 2003) and are discussed further in Chapter 1 Section 1.6.3.

The second interesting aspect of Cu\(^{2+}\) induced fibril formation of A\(\beta\)(1-42) is that total A\(\beta\) fibre content is significantly enhanced by the presence of Cu\(^{2+}\) ions at 3 and 5 \(\mu\)M in contrast to A\(\beta\)(1-40), where, as seen in Figure 5.2a, fibrils formed faster but the end ThT signal was lower in the presence of Cu\(^{2+}\). It is possible that this is a result of Cu\(^{2+}\) not accelerating the nucleating stage of A\(\beta\)(1-42) fibril growth. This allows more time for intramolecular organisation of the fibril and subsequently more stable fibre formation, whereas in A\(\beta\)(1-40) Cu\(^{2+}\) accelerates nucleation to the point where there is some aggregate formation, even at low Cu\(^{2+}\) concentrations.
Figure 5.4. Cu^{2+} accelerates Aβ(1-42) fibril growth. Aβ(1-42) at (a) 1 µM, (b) 3 µM and (c) 5 µM with and without 0.5 mole equivalents Cu^{2+} ions. Growth curves are a sum of 6 traces. Absolute ThT fluorescence intensity is shown. All carried out in 50 mM HEPES, 160 mM NaCl, 30°C, with agitation for 30 seconds every 30 minutes.

Copper was also added bound to a chelate to mimic in vivo conditions where there is very little free Cu^{2+}. In the experiments shown in Figure 5.5 Cu^{2+} was added as a Cu(Glycine)_2 chelate. However this produced identical results to adding Cu^{2+} alone.
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5.4.2. Morphology of the Cu$^{2+}$ Induced Fibrils

The nature of the Cu$^{2+}$ promoted amyloids was then characterized. Transmission electron microscopy (TEM) images indicate the presence of fibres (Figure 5.6). Based on TEM images, under these conditions, the morphology of the fibres generated appears quite similar for fibres formed with and without the presence of Cu$^{2+}$ ions. It is noted that previous studies by other groups using TEM did not reveal fibres in the presence of Cu$^{2+}$ because the high Aβ and Cu$^{2+}$ concentrations used caused amorphous precipitation (Raman, et al. 2005, Yoshiike, et al. 2001).
Figure 5.6. TEM images of Aβ(1-42) fibrils formed in the absence (a) and presence (b) of Cu²⁺ ions. The morphology of fibres formed with and without Cu²⁺ ions present are similar, typically long, straight, unbranched fibres are observed often stacked together. The scale bar is 50 nm. The negatively stained fibres are generated from 3 μM Aβ(1-42) with and without 0.5 mole equivalent Cu²⁺ ions, over 200 hours with agitation every 30 minutes, at 30 °C in 50 mM HEPES, 160 mM NaCl. The fibrils were then freeze-dried to approximately 0.5 mg/mL.

A further test to ensure that fibrils had been made in the presence of Cu²⁺ was to see if the Cu²⁺ generated fibres were capable of seeding fibre formation of fresh, metal-free Aβ(1-40). Figure 5.7 shows that seeding did occur, indicated by a reduction in the lag-time.

5.4.3. pI and Fibril Growth
How does the presence of Cu²⁺ ions accelerate the rate of fibre formation? At μM concentrations of Aβ Cu²⁺ does not form crossed-linked species (Drew, et al. 2009, Syme, et al. 2004) and the Cu²⁺ coordination geometry is identical in the monomer and fibre (Karr, et al. 2008, Sarell, et al. 2009). This rules out copper bridging to form cross-linked Aβ as a possible mechanism of accelerated fibre formation. It is also possible that Cu²⁺ coordination may trigger the Aβ misfolding that nucleates fibre assembly, however the conformational changes in Aβ upon Cu²⁺ binding are small and outside of the fibre core (Sarell, et al. 2009, Syme, et al. 2004). Instead, the pH dependency of fibril formation, and Cu²⁺’s effect on the net charge of the protein was investigated.
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Figure 5.7. Cu$^{2+}$ induced fibrils will seed fibril formation. Comparison of 5.5 µM apo Aβ(1-40) (red traces) and 5 µM apo-Aβ with 10 % preformed (Cu$^{2+}$ promoted) Aβ-fibrils (green traces). All carried out in 50 mM HEPES, 160mM NaCl, pH 7.4, 30º C, with agitation for 30 seconds every 30 minutes. The fluorescence intensities are absolute values.

Fibre growth experiments were performed at a number of pH values (8.0, 8.5, and 9.0) to see if Cu$^{2+}$ enhanced fibril growth at higher pHs. In each case Cu$^{2+}$ increases the rate of fibre formation compared to fibril growth of apo-Aβ (Figure 5.8). Overall fibril growth rates become slower as the pH was increased away from the pI of Aβ. At pHs below 7.4, amorphous aggregation was favoured over fibril formation and subsequently in the presence of Cu$^{2+}$ no fibril growth was observed.

The effect of the net charge of Aβ(1-40) on the rate of fibre formation was investigated further and in more detail by monitoring fibre growth of apo-Aβ over a wider range of pHs (Figure 5.9). It is clear that as the pH drops from 8.3 to 5.9 the rate at which fibres form significantly increases. Figure 5.9b is a plot of lag-times versus pH, lag times ($t_{lag}$) reduce from 170 (+/- 8) hours at pH 8.3 to 23 (+/- 4) hours at pH 5.9.
Figure 5.8. Cu$^{2+}$ accelerates Aβ fibril growth over a range of pH’s. (a) pH 8 (b) pH 8.5 and (c) pH 9. Blue traces are for Aβ(1-40) with 1 mole equivalent Cu$^{2+}$, red traces are for apo Aβ(1-40). (a) and (c) are means of the fluorescence from six growth curves. Aβ(1-40) at 5 µM in 50 mM HEPES, 160 mM NaCl.

The reason for the pH-mediated increase in fibril growth shown in Figure 5.9 is well documented. Intermolecular self-association of peptides and proteins is strongly influenced by the net charge of the protein. As Aβ approaches its isoelectric point, a pI of 5.3, and an overall neutral charge, its solubility decreases (Hortschansky, et al. 2005). Histidine is the only amino acid with an ionisable side-chain in the physiological pH range, and is also involved in the Cu$^{2+}$ binding complex. To determine if the pKa of histidine residues were related to fibre growth, the protonation states of histidines within Aβ were investigated and compared to fibril formation rates.
Figure 5.9. pH dependence of Aβ fibre formation. (a) Fibre growth curves (average from 6 traces) between pH 5.5 and 8.7, for Aβ(1-40) at 5 μM with 160 mM NaCl. The maximal ThT fluorescence signal has been normalized. (b) The lag-times for fibril growth ($t_{lag}$) versus pH. The insert indicates the protonation state of the three histidine residues of Aβ versus pH, data is based on $^1$H NMR chemical shift. The rate fibres form increases as Aβ approaches its isoelectric point and this is dependent on histidine protonation. Error bars are standard error based on 6 traces.
The protonation state of the three His residues within Aβ(1-40) (His6, His13 and His14) was determined over a range of pH values using $^1$H NMR chemical shift measurements (Figure 5.9b insert and Figure 5.10).

**Figure 5.10.** pK$_a$ measurements of Aβ histidine residues. (a) A selection of 1D $^1$H NMR spectra from a pH titration, 100 µM Aβ(1-28) in 20 mM phosphate buffer at 25 ºC. CδH and CεH singlets from H6 (blue), H13 (red) and H14 (purple) are highlighted. (b) 2D TOCSY spectrum of histidine region of Aβ(1-28), pH 7, 60 ms spin-lock mixing time. (c) pH dependence of CδH and CεH $^1$H chemical shift. pKa are: 6.67, 6.67 and 6.64 at 25 ºC in 90% H$_2$O 10% D$_2$O.
The $pK_a$ of the His residues at 25°C are 6.7. A 2D $^1$H TOCSY was also performed (Figure 5.10b) to confirm the His $\text{C}^\varepsilon\text{H}$ and $\text{C}^\delta\text{H}$ assignments. It is noted that the pH dependence of the fibre growth rates bears a strong resemblance to the protonation state of the histidine residues within Aβ. From this data it appears that the protonation state of the three imidazole rings and consequently the net charge of Aβ is crucial to its amyloidogenicity.

In addition to pH, the binding of metal ions will also perturb the net charge of Aβ. It is known that Cu$^{2+}$ (and Zn$^{2+}$) ions bind to the three histidine residues within Aβ (Dong, et al. 2003, Drew, et al. 2009, Faller, et al. 2008, Faller, et al. 2009, Karr and Szalai 2008, Sarell, et al. 2009, Syme, et al. 2004, Syme and Viles 2006). At pH 7.4 Aβ’s histidine residues are predominately (80 %) deprotonated and neutrally charged, thus coordination of a divalent Cu$^{2+}$ (or Zn$^{2+}$) ion to Aβ’s histidines adds two positive charges. Adding two positive charges to Aβ at pH 7.4 makes the Aβ peptide complex more neutral in overall net charge. These results suggest that the coordination of Cu$^{2+}$ to the histidine residues of Aβ results in faster fibril formation.

5.4.4. The Balancing Act of Fibril Formation: Effect of Cu$^{2+}$ Stoichiometry on Fibril Growth

Having established a mechanism for Cu$^{2+}$ accelerated fibril formation, the stoichiometric effect of Cu$^{2+}$ on fibre growth was investigated in more detail. All concentrations of Cu$^{2+}$ up to 1 mole equivalent caused the rate of fibrillisation to increase. Interestingly, sub-stoichiometric amounts of Cu$^{2+}$ between 0.2-0.4 mole equivalents, display the greatest increase in fibre growth rates (Figure 5.11).

This supports the observation that Cu$^{2+}$ accelerates nucleation (as well as elongation) as this suggests sub-stoichiometric amounts of Cu$^{2+}$ can “seed” fibre formation. Further addition of Cu$^{2+}$ beyond one mole equivalent caused precipitation of Aβ(1-40) and markedly reduces the amount of fibres generated, as previously noted (Jun, et al. 2009). The total amount of fibres generated in the presence (or absence) of Cu$^{2+}$ ions can be quite variable, presumably reflecting competition between fibril formation and amorphous aggregation.
Figure 5.11. Effect of Cu^{2+} concentration on fibril growth rates. Normalised fibril growth curves with different mole equivalents of Cu^{2+} ions. The insert shows that fibre growth times (t_{50}) all decrease in the presence of Cu^{2+} ions, from 150 hours for the apo (red) to 50 hours with 0.4 mole equivalents of Cu^{2+} ions (cyan). The maximal effect is between 0.2 and 0.4 mole equivalent Cu^{2+}. With excess, 2 and 4 mole equivalents, of Cu^{2+} ions the fibril growth is completely inhibited. Growth curves represent the mean of 6 traces, standard error is shown. Aβ(1-40) at 5 µM in 50 mM HEPES, 160 mM NaCl, at pH 7.4, 30 °C, with intermittent agitation.

Figure 5.12a illustrates that the effect of Cu^{2+} ions on Aβ(1-40) does not only accelerate fibril formation, but as Cu^{2+} concentration increases, the balance between fibril formation and amorphous aggregation increasingly favours the latter. Likewise in Figure 5.12b, as the pH increases from pH 8.3 to tal fibre formation decreases despite the increase in fibril formation rate. Presumably as the rate of fibril formation increases there is less time available for stable contacts to form between sidechains and the structure of the fibrils is less stable, resulting in increased amorphous aggregation. This effect is reduced with dilution but 2-5 µM levels of Aβ(1-40) are required for reliable timely detection. A limitation in previous experiments that showed only amorphous aggregates was the high concentration of Aβ(1-40) and Cu^{2+} ions used (Raman, et al. 2005, Yoshiike, et al. 2001) (typically 50 µM, 100 µM respectively), much higher than that found in vivo. As can be seen from these experiments, the effect of Cu^{2+} on Aβ fibril formation favours rapid fibril formation, and if conditions such as pH or concentration are also too “favourable,”
fibril formation becomes too fast to result in stable fibrils and this may explain why the predominant view in the literature is that Cu\(^{2+}\) ions only result in amorphous aggregates.

**Figure 5.12. Effect of Cu\(^{2+}\) concentration and pH on total fibril levels.** (a) Effect of Cu\(^{2+}\) on the maximal ThT fluorescence signal of Aβ fibres. At 2 and 4 mole equivalent Cu\(^{2+}\) the fibril growth is inhibited. b) The effect of pH on the maximum fluorescence. 5 µM Aβ(1-40) in 50 mM HEPES, 160 mM NaCl, at pH 7.4, 30 °C, with intermittent agitation.

**5.4.5. Effect of Zn\(^{2+}\) on Fibril Growth**

Interestingly accelerated fibril formation appears to be quite specific for Cu\(^{2+}\) ions, Zn\(^{2+}\) ions completely inhibit fibre formation even at 3 µM of Aβ(1-40) (Figure 5.13). This may be due to the very different coordination geometry (at µM concentration) between the two metal ions. Cu\(^{2+}\) ions form an intra-molecular complex with Aβ, (Drew, et al. 2009, Karr and Szalai 2008, Sarell, et al. 2009, Syme, et al. 2004) while at µM levels current data suggests zinc ions will form an inter-molecular complex, cross-linking between histidine residues on multiple Aβ molecules (Faller and Hureau 2008, Syme and Viles 2006). These cross-linked Zn\(^{2+}\)-
Aβ species will inhibit amyloids forming by interfering with the regular cross-beta assembly.

**Figure 5.13. Zn$^{2+}$ inhibits fibril growth.** Fibril growth curves with 1 mole equivalent of Zn$^{2+}$ ions (green traces) and metal free (red traces) (a) 3 µM Aβ(1-40), pH 7.4 (b) 4 µM Aβ(1-40), pH 7.4 (c) 5 µM Aβ(1-40), pH 7.4 (d) 5 µM Aβ(1-40), pH 8.0. All carried out in 50 mM HEPES, 160mM NaCl, 30º C, with agitation, 3-6 traces for each experiment are shown.

5.4.6. Conclusions

This chapter suggests that Cu$^{2+}$ ions increase the rate of fibre formation, at pH 7.4, by causing Aβ to approach its isoelectric point. To put these observations in context, the increase in fibre growth rates measured here due to Cu$^{2+}$ ions are comparable to those observed for (metal free) Aβ(1-40) mutants associated with familial early onset AD (E22K/G/Q), where a halving of the growth times ($t_{50}$) of fibre formation is also reported (Murakami, et al. 2003).
Currently the vast majority of studies examining the effect of \( \text{Cu}^{2+} \) on A\( \beta \) suggest \( \text{Cu}^{2+} \) either inhibits fibril formation or causes amorphous aggregates to form (Chauhan, et al. 1997, Ha, et al. 2007, Yoshiike, et al. 2001). One of the only studies to date that supports the findings in this chapter is Huang et al, who studied the effects of \( \text{Cu}^{2+}, \text{Zn}^{2+} \) and \( \text{Fe}^{3+} \) on seeding experiments of A\( \beta \)(1-40) with A\( \beta \)(1-42) and found that trace levels of zinc, copper, and iron initiated A\( \beta \) oligomerisation (Huang, et al. 2004). However, in their experiments with ThT they do not show data for A\( \beta \)(1-40), only a seeded mixture of A\( \beta \)(1-40)/A\( \beta \)(1-42), which makes comparisons with the results in this chapter difficult. Additionally with their ThT data they show only one time point, at 10 days, which suggests highest fluorescence for A\( \beta \)(1-40)/A\( \beta \)(1-42) in the presence of \( \text{Cu}^{2+} \) and surprisingly \( \text{Zn}^{2+} \), in contrast with the results in Figure 5.13. The data shows total fibres generated rather than the kinetics of fibre formation. The effect of metal ions on A\( \beta \)(1-40) alone was studied using turbidometric analysis only, which would also be affected by amorphous aggregation and thus is not reflective of only fibril formation. Additionally they suggest trace metals accelerate fibril formation through acting as a seed. The findings in this chapter partly support this hypothesis, as sub-stoichiometric \( \text{Cu}^{2+} \) levels were most effective at accelerating fibril formation, but the additional effect of metal ions on the pI of A\( \beta \) is not investigated by Huang et al.

Metals have also been proposed as triggers for other misfolding and assembly diseases such as dialysis-related amyloidosis (Calabrese, et al. 2008), Parkinson’s disease (Uversky, et al. 2001) and prion diseases, although it remains to be established if the mechanisms by which metals induce fibrillisation are shared. The observations in this chapter provide a rationale for the in vivo findings in Drosophila and mammals which link the AD phenotype with impaired \( \text{Cu}^{2+} \) homeostasis (Sanokawa-Akakura, et al., Sparks, et al. 2003). It is known that \( \text{Cu}^{2+} \) levels in the brain increase with age (Barnham, et al. 2008), thus the observations in this chapter should refocus attention on loss of \( \text{Cu}^{2+} \) homeostasis as a possible risk factor in AD. \( \text{Cu}^{2+} \) chelators are now being investigated in clinical trials as a potential therapy for Alzheimer’s disease (Barnham and Bush 2008, Cherny, et al. 2001).
In the next chapter, the toxicity of Aβ fibres, with and without Cu\(^{2+}\), is investigated, to determine if the concentration-dependence of Cu\(^{2+}\) accelerated fibril formation shown in this chapter, is mirrored in the toxic effects exerted by Aβ fibrils.
Chapter 6. Toxicity of Cu-A\(^{2+}\) to PC12 cells

6. Toxicity of Cu\(^{2+}\) -A\(\beta\) to PC12 Cells
Abstract

Recent *in vivo* studies using animal models of Alzheimer’s disease (AD) have shown that impaired copper homeostasis enhances the toxic effects of amyloid beta-peptide (Aβ). In the experiments described here, a clonal neuronal cell line was used as a model to assess Aβ toxicity. Fibrillar and monomeric Aβ were added to the cell culture and cell viability monitored with a fluorescent dye (alaran blue). The results show that Cu²⁺ ions bound to Aβ are consistently more toxic to neuronal cells than Aβ in the absence of Cu²⁺ ions, while the same levels of Cu²⁺ in the absence of Aβ are not toxic to the cells. Additionally Cu²⁺ binding to the non-amyloidogenic Aβ(1-16) fragment is not toxic.

The degree of Cu-Aβ cyto-toxicity correlates with the levels of Cu²⁺ ions that accelerate fibre formation as described in Chapter 5, with the greatest toxicity observed at sub-stoichiometric levels of Cu²⁺. Additionally monomeric Aβ requires longer incubation with the cells to exert toxicity than fibrillar Aβ, suggesting self-association may be necessary for Aβ toxicity. An active role for Cu²⁺ ions in promoting cell death suggests impaired copper homeostasis may be a risk factor for Alzheimer’s disease.
6.1. Introduction

*In vitro* and *in vivo* studies performed since the early 1990s have supported the hypothesis that Aβ has a direct role in the neurodegeneration that occurs in AD. However a clear idea of the molecular events and mechanisms for Aβ toxicity has yet to be established. In this introduction some of the different models that can be used to study toxicity and different measures of toxicity are discussed.

6.1.1. *In Vitro* Toxicity Models


Cell lines will differ in their sensitivity to Aβ. Aβ(25-35) was over 200 fold more toxic to PC12 cells than Neuro-2A cells (Calderon, et al. 1999). PC12 cells have 4-5 fold lower concentrations of glutathione than Neuro-2A cells, which makes PC12 cells more sensitive to Aβ-induced oxidative stress. Additionally Aβ showed a higher propensity to bind to PC12 cell membranes than a range of other models, including a neuronal cell line, cortical primary neurons and hippocampal primary neurons (Simakova, et al. 2007). Thus the PC12 cell line was chosen to be the model for Aβ toxicity in this thesis.

6.1.2. Toxicity Assays *In Vitro*

There are numerous methods to evaluate the toxicity of a compound to cells. Although none of these assays can reliably predict toxicity *in vivo* they are useful tools to compare different treatments and are widely used to indicate toxicity at a basic level. An overview is given below:

**Cell Counting**

The number of surviving cells are quantitated as a percentage of the number of live cells counted after plating (Whitson, et al. 1989). Counting cells stained with trypan
blue can also be used as a measure of cell viability (Pike, et al. 1993). Here, the exclusion of trypan blue (trypan negative) indicates cells with an intact cell membrane.

**Lactate Dehydrogenase Release Assay**

The lactate dehydrogenase (LDH) assay measures the number of viable cells, either by measuring the number of cells via total cytoplasmic LDH or membrane integrity as a function of the concentration of cytoplasmic LDH that leaks into the extracellular medium (Koh, et al. 1987). LDH in the extracellular medium will reduce nicotinamide adenine dinucleotide (NAD) to NADH. NADH then reacts stoichiometrically with a yellow tetrazolium salt to produce a red, formazan-class dye which is measured by absorbance at 492 nm. The amount of formazan is directly proportional to the amount of LDH in the culture, which is in turn directly proportional to the number of dead or damaged cells. Formazan dyes are commonly used in cell viability assays as they are formed by the reduction of tetrazolium salts by dehydrogenases and reductases, and thus are a good indicator of cell viability. They vary in colour depending on the original tetrazolium salt.

**The 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is traditionally the most widely used measure of cell viability since it was developed by Mosmann in 1983 (Mosmann 1983). MTT is a tetrazolium salt that produces a yellow solution when dissolved in water. Active mitochondrial dehydrogenases in metabolically active cells will convert MTT to insoluble purple formazan crystals. This change will not occur in dead cells. The insoluble formazan is solubilised by the addition of cell lysis buffer, and the absorbance at 570 nm measured.

**Alamar Blue**

Alamar blue is used to measure cell viability in this chapter, so it will be discussed in more detail. The alamar blue assay uses the reactions of the electron transport chain as a measure of cell proliferation and cyto-toxicity. Alamar blue is an oxidation-reduction indicator. The active ingredient of alamar blue (resazurin) is a blue, nontoxic, cell permeable compound that is nonfluorescent. Upon entering cells, resazurin is reduced by mitochondrial enzymes to resorufin, which produces very
bright red fluorescence (Figure 6.1). Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of cell viability. Excitation of the dye occurs at ~560 nm with the subsequent emission at ~590 nm.

![Figure 6.1. Structure and metabolism of the cell viability dye alamar blue (Invitrogen). Resazurin is reduced by mitochondrial enzymes to the strongly fluorescent resorufin.](image)

The redox potential of alamar blue illustrates why it is a good indicator for detecting oxidation of the electron transport chain (Table 6.1). The redox potential is a measure of the tendency of a chemical species to acquire electrons and be reduced. Each species has its own intrinsic reduction potential; the more positive the potential, the greater the species' affinity for electrons and tendency to be reduced.

Table 6.1 shows that alamar blue has a midpoint potential greater than the midpoint potential of the cytochromes. This allows alamar blue to detect oxidation by all the components of the electron transport chain. MTT, also shown in Table 6.1, has a lower midpoint potential than alamar blue. MTT’s midpoint potential is higher than the electron donors FMNH₂, FADH₂, NADH and NADPH, but lower than the cytochromes. Because of this MTT will interrupt the respiratory chain, whereas alamar blue does not interfere with any of the redox reactions and thus allows the cells to remain functional and healthy.

Alamar blue also performs with equal or better sensitivity than MTT. Human hepatoma cells (HepG2) were exposed to 117 compounds and the effects measured with MTT and alamar blue (Hamid, et al. 2004). The majority of the compounds performed consistently in both assays, regardless of the underlying mechanism of the compounds toxicity. However for a minority of compounds alamar blue outperformed MTT in sensitivity.
Chapter 6. Toxicity of Cu-Aβ to PC12 cells

\[ \text{Half-reaction} \quad \text{Eo'} (\text{mV}) \quad \text{pH} \quad 7.0 \quad 25 \degree \text{C} \]

<table>
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<tr>
<th>\text{Half-reaction}</th>
<th>\text{Eo'} (\text{mV})</th>
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<th>7.0</th>
<th>25 \degree \text{C}</th>
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<tr>
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<td>-320</td>
<td></td>
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<tr>
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<tr>
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<td>-210</td>
<td></td>
<td></td>
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<tr>
<td>MTT_{ox}^+ 2H^++2e^- ↔ MTT_{RED}</td>
<td>-110</td>
<td></td>
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<tr>
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<td>+80 to +290</td>
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<tr>
<td>Alamar blue_{ox}+2H+2e^- ↔ Alamar blue_{RED}</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>O_2+4H^++4e^- ↔ 2H_2O</td>
<td>820</td>
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**Table 6.1. Redox potentials of components of the electron transport system and alamar blue and MTT.** The midpoint redox potentials (Eo’) values were determined at pH 7.0 at 25 \degree \text{C}. The midpoint potential (Eo’) for any half reaction is defined as the voltage at which equilibrium is achieved between the concentration of oxidized and reduced products (AbD Serotec 2010).

Alamar blue is frequently used in cell toxicity assays of PC12 cells, (Blum, et al. 2000, Magliaro, et al. 2009, Seyfried, et al. 2007) with no confounding effects reported. Although MTT is commonly used to observe the effects of Aβ on cells in the AD field, the difficulties in solubilising the formazan which forms during the assay has resulted in other tests becoming more popular, and alamar blue has been used recently to study Aβ antimicrobial activity in Alzheimer’s disease (Soscia, et al. 2010) and Aβ toxicity to neuronal cultures (Chung, et al. 2010). Further details on the specific use of alamar blue are given in the experimental section of this chapter.
6.2. Aims

- Develop a suitable system for monitoring Aβ(1-40) and Aβ(1-42) and Cu²⁺ toxicity in PC12 cells.
- Determine what concentrations of Cu²⁺ alone are toxic.
- Determine how Cu²⁺ influences the toxicity of both monomeric and fibrillar Aβ(1-40) and Aβ(1-42).
- Ascertain if there is a relationship between fibril growth and toxicity.

6.3. Experimental

6.3.1. Cell Culture Protocol

PC12 cells were obtained from the Health Protection Agency (HPA) Culture Collections, lot number 08F011. The predominant culture medium used was as recommended by the HPA: RPMI 1640 + 2 mM Glutamine + 10 % Horse Serum (HS) + 5 % Fetal Bovine Serum (FBS). Cultures were maintained at approximately 6 x 10⁶ cells/mL and incubated at 5 % CO₂, 37 °C in 25 cm² cell culture flasks (Iwaki). Cells were loosely adherent and gentle pipetting was sufficient to dislodge them. Cells were centrifuged at 1000 RPM (95 x g) for 5 minutes and resuspended in fresh medium every 3-4 days to an approximate cell concentration of 2 x 10⁵ cells/mL.

To determine the stock cell concentration, a 10 µL aliquot was added to 10 µL of 3 mg/mL trypan blue. 10 µL from this solution was added to a haemocytometer and the cells counted. The cells were diluted in Opti-MEM and added to the wells of a 96 well plate, see individual experiments in the results section for exact concentrations. The cells were typically incubated with the test samples for 24 hours, prior to alamar blue addition.

20 µL alamar blue (Invitrogen) was added to each 200 µL well volume. The wells were incubated at 37 °C for 4-6 hours to allow colour development. The fluorescence was measured in a Molecular Devices SpectraMax Gemini XPS spectrofluorometer, excitation 530 nm, emission 585 nm with a cut off at 550 nm.
6.4. Results

Optimisation of Cell Toxicity Assay

6.4.1. Morphology and Behaviour of PC12 Cells

The PC12 cell line was established from rat adrenal pheochromocytoma cells (Greene, et al. 1976). In the growth medium used in this thesis the PC12 cells have a round shape and tend to grow in small clumps. The doubling time of the cells is 92 hours. The cells synthesise and store sizeable quantities of the neurotransmitters dopamine and norepinephrine. Consequently PC12 cells resemble chromaffin cells and sympathetic neurons making them a useful model for studying the toxic effect of Aβ preparations in vitro.

6.4.2. Choice of Cell Media

The media used for normal cell growth (RPMI 1640 + 2 mM Glutamine + 10 % HS + 5 % FBS) contains high levels of competing copper ligands, such as serum albumin. The levels of albumin in blood plasma, e.g. in FBS and HS, is much higher than found in the CSF and brain interstitium, where Aβ-Cu²⁺ interactions are thought to take place. For example in humans albumin is present at 600 μM in blood plasma, but only at ~3 μM in the CSF (Carter, et al. 1994, Licastro 1993). Consequently, Opti-MEM media (Invitrogen) with no added serum was used as an alternative.

Opti-MEM is a modification of Eagle’s minimal essential medium, buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine (purine derivative), sodium pyruvate (source of energy) and L-glutamine (amino acid). The protein level of Opti-MEM is 15 μg/mL, with insulin and transferrin as the only protein supplements. More information on the exact protein and trace metal concentrations was requested from Invitrogen but was refused due to the confidential nature of this information. In most of the experiments described in this thesis, 50 μL of Opti-MEM was used in each 200 μL well, thus the protein concentration contributed from the Opti-MEM was 0.75 μg/200 μL compared to a typical concentration of 8.66 μg/200 μl of Aβ. Thus competing ligands for Cu²⁺ outside the cell are minimised.
6.4.3. Alamar Blue Experimental Specifics and Definitions

Alamar blue is a quantitative dye, the greater the fluorescence, the greater the number of viable cells. In the majority of experiments in this chapter, the effect of treatments on the cells is described by percentage cell growth, i.e. the ability of the cells to metabolise alamar blue. Alamar blue was added at different time points and the cell growth, or lack of it determined. Cell viability is used to describe the effect of treatments when a single alamar blue addition was carried out. The effect of treatments on PC12 cells are described as “toxic” occasionally in this chapter, but this refers to the effect of the treatments in impairing cell viability and cell growth, rather than necessarily resulting in cell death. Alamar blue only acts as a measure of cell metabolism, and thus is not a direct measure of cell toxicity.

It is also important to note that alamar blue metabolism is unidirectional, i.e. once it is metabolised by living cells, it can not then indicate if the cells begin to die. Thus when the alamar blue readings plateau, it may be that the cells are dying. Because of this the data shown in this chapter is the first point where the alamar blue fluorescence began to plateau. Where the data is presented as percentage cell viability (or percentage cell growth), the values were calculated using:

\[
\text{Percentage cell viability} = \left( \frac{F - F_{\text{buff}}}{F_{\text{cells}} - F_{\text{buff}}} \right) \times 100
\]

Where \( F \) is the alamar blue fluorescence, \( F_{\text{buff}} \) is the buffer fluorescence only and \( F_{\text{cells}} \) is the fluorescence of cells with no treatments.

6.4.4. Using Opti-MEM Media

Opti-MEM was used as an alternative media to RPMI + HS + FBS, RPMI alone was tried but cell viability was poor. Firstly the optimum cell concentration for Aβ toxicity experiments was investigated. PC12 cells were serially diluted in Opti-MEM, and alamar blue added at either day 0 or day 4. The bargraph in Figure 6.2a shows the alamar blue fluorescence of cells seeded at \( 3 \times 10^6 \) cells/mL after 4 hours incubation with alamar blue. These wells have the highest alamar blue fluorescence compared to starting cell concentrations of (b) \( 3 \times 10^5 \), (c) \( 3 \times 10^4 \) and (d) \( 3 \times 10^3 \) cells/mL. After 4 days incubation, Figure 6.2a shows alamar blue fluorescence at this seeding concentration has reduced to ~ 50 % of the cell growth at 0 days.
In contrast, cells seeded at $3 \times 10^5$ cells/mL in (b) and $3 \times 10^4$ cells/mL in (c) show an increase in alamar blue fluorescence over the same period. The most dilute starting concentration of $3 \times 10^3$ cells/mL (Figure 6.2d) shows only a small increase in cell growth, suggesting this concentration of PC12 cells is too dilute.

**Figure 6.2. Effect of cell concentration on cell growth.** Alamar blue was added at 0 days or 4 days to PC12 cells in Opti-MEM cell medium, pH 7.4. PC12 cells starting concentrations were (a) $3 \times 10^6$ cells/mL (b) $3 \times 10^5$ cells/mL (c) $3 \times 10^4$ cells/mL and (d) $3 \times 10^3$ cells/mL. Average data shown calculated from three wells for day 0 and two wells for day 4. Error bars are standard error.
From this data it was found that cells at concentrations of $10^5$ and $10^4$ grew well in Opti-MEM media. Additionally cells seeded at $10^4$ cells/mL were monitored for longer and it was found that up to 7 days incubation in Opti-MEM media did not shown a decrease in cell viability. Thus further experiments were performed using starting cell concentrations of $10^5$ cells/mL to allow maximum growth over the time course of the experiment.

6.4.5. Effect of Diluting Opti-MEM with HEPES/NaCl

The degree of dilution of Opti-MEM by the addition of Aβ preparations was investigated next. Ideally, minimal dilution of the peptide preparations would be necessary. However Aβ fibres are grown in 50 mM HEPES + 160 mM NaCl (see Chapter 5 Section 5.3.1) and it was not known what effect dilution of Opti-MEM with this buffer would have on PC12 survival. Thus PC12 cells were grown for 4 days in different ratios of the two media (Opti-MEM and HEPES/NaCl) and their viability examined (Figure 6.3).

![Figure 6.3. Cell viability at 4 days in Opti-MEM dilutions. 2.5 x 10^4 cells/mL PC12 cells were incubated in different concentrations of Opti-MEM diluted with 50 mM HEPES + 160 mM NaCl, both at pH 7.4. Metabolism of alamar blue by PC12 cells is greatest in the presence of 50 % or above Opti-MEM. 0 % and 10 % Opti-MEM show low cell viability. Averages of 3 wells and error bars are standard error.](image-url)
Figure 6.3 shows that alamar blue fluorescence was highest in 100 % and 75 % Opti-MEM. Cell viability was greatly reduced when the media contained more HEPES than Opti-MEM. This decrease was even more pronounced at 10 % and 0 % Opti-MEM. From this experiment it was concluded that the cells would optimally be grown in 100 %, 75 % or 50 % Opti-MEM. However to maintain an A\(\beta\) concentration that was toxic, a media combination of 25 % Opti-MEM, 75 % HEPES/NaCl was typically used. Control cells not incubated with A\(\beta\) had the media diluted to the same extent as A\(\beta\)-treated cells.

6.4.6. Free Cu\(^{2+}\) and A\(\beta\)(1-16) bound Cu\(^{2+}\) Toxicity

To determine whether Cu\(^{2+}\) alone is toxic, PC12 cells were incubated with a range of Cu\(^{2+}\) concentrations.

![Graph showing cell growth with different concentrations of Cu\(^{2+}\) and A\(\beta\)](image)

**Figure 6.4. Free Cu\(^{2+}\) ions are not cyto-toxic.** Cu\(^{2+}\) ions were added as CuCl\(_2\) and incubated with 5.9 x 10\(^4\) cells/mL in Opti-MEM cell medium, pH 7.4. (a) Alamar blue was added after 1 day incubation and cell viability monitored. Data is shown after 70 hours total incubation. (b) At 6 days alamar blue was added again and additional readings taken. Cell viability in the presence of Cu\(^{2+}\) was comparable to the viability of cells without Cu\(^{2+}\) ions in (a) and Cu\(^{2+}\) increased cell growth in (b). Averages calculated from 3 wells. Error bars are standard error.
Figure 6.4 shows that all Cu²⁺ concentrations up to 2.5 μM Cu²⁺ had no detectable effect on cell growth. Surprisingly the presence of additional Cu²⁺ appears to enhance cell viability.

The effect of Cu²⁺ bound to the non-amyloidogenic Cu²⁺ binding fragment, Aβ(1-16), was also studied (Figure 6.5).

Figure 6.5. Cu²⁺ bound to Aβ(1-16) is not toxic. Cu²⁺-Aβ(1-16) was added to 5.9 x 10⁴ cells/mL immediately (t0) or incubated for 200 hours (t200) under fibril forming conditions before addition to PC12 cells. Alamar blue was added after 1 day Aβ incubation with the cells. Data is shown after 3 days total incubation, averages calculated from 3 wells, error bars are standard error.

The use of Aβ(1-16) is a particularly good control as Cu²⁺ binds to this shorter fragment of Aβ with the same affinity and coordination geometry (Drew, et al. 2009,
Chapter 6. Toxicity of Cu-Aβ to PC12 cells

Karr, et al. 2008, Sarell, et al. 2009, Syme, et al. 2004) as full-length Aβ, but Aβ(1-16) lacks the amyloidogenic region and does not form fibrils. Figure 6.5 shows that Cu$^{2+}$ ions when bound to Aβ(1-16) do not affect cell viability. The interaction of Aβ(1-16) with and without Cu$^{2+}$ was studied for both freshly solubilised Aβ(1-16) (t0 in Figure 6.5) and also Aβ(1-16) which had undergone the standard fibril growth procedure (t200 in Figure 6.5). This data shows that addition of Cu$^{2+}$ bound protein is not generically toxic to cells, and that any detrimental effects of Cu$^{2+}$ are specific to their interaction with Aβ(1-42) and Aβ(1-40).

**Cu$^{2+}$ and Aβ(1-40) and (1-42) Toxicity**

**6.4.7. Cu$^{2+}$ and Aβ(1-42) Fibre Toxicity**

The effect of Aβ(1-42) fibrils formed in the presence or absence of Cu$^{2+}$ on the cell viability of PC12 cells was investigated. Figure 6.6 shows PC12 cell viability after 3 days incubation with Aβ(1-42) fibrils. Two findings are evident from this data. Firstly, it can clearly be seen that cells incubated with Aβ(1-42) fibres, in both the presence or absence of Cu$^{2+}$, show a decrease in cell viability. This is especially evident at lower concentrations, i.e. 0.9 μM Aβ(1-42) decreases cell viability more than 4.5 μM. This may be because at low concentrations of Aβ(1-42), fibres formed may be smaller than fibres formed at higher Aβ(1-42) concentrations. The smaller fibres could diffuse to the PC12 cell membrane more easily and rapidly than the larger fibres, resulting in greater cell toxicity.

The second finding from Figure 6.6 is that the presence of Cu$^{2+}$ consistently results in a larger decrease in cell viability at all concentrations than apo-Aβ(1-42). For example, 2.7 μM apo-Aβ(1-42) fibres decreased cell viability to 40 % viability. However the same concentration of Aβ(1-42) fibres in the presence of half a mole equivalent Cu$^{2+}$ ions results in only 4 % viability. This experiment was repeated on a number of occasions and each time the presence of Cu$^{2+}$ ions consistently enhanced Aβ(1-42)’s negative effect on PC12 cell viability. This suggests that in PC12 cells, Cu$^{2+}$ ions bound to Aβ(1-42) impair cell viability more than Aβ(1-42) in the absence of Cu$^{2+}$ ions, while the same levels of Cu$^{2+}$ in the absence of Aβ(1-42) are not toxic to the cells.
Chapter 6. Toxicity of Cu-Aβ to PC12 cells

Figure 6.6. Cu$^{2+}$-Aβ(1-42) fibrils are more cyto-toxic than Aβ(1-42) fibrils. 1, 3 and 5 μM Aβ(1-42) fibrils were formed with and without 0.5 mol eq Cu$^{2+}$ ions. After fibril formation (monitored by ThT) the fibrils were added to 10% (v/v) PC12 cells. Cell concentration was 5.9 x 10^4 cells/mL. 2.5 μM Cu$^{2+}$ was added to the cells to check for free Cu$^{2+}$ toxicity. All preparations were incubated with the cells for 24 hrs then alamar blue added. The data shown here is after a total incubation time of 3 days. Error bars are standard error.

6.4.8. Effect of Cu$^{2+}$:Aβ Ratio on Cell Toxicity

Next the ratios of Cu$^{2+}$ to Aβ(1-40) that cause toxicity were investigated. This was examined in light of the results from the fibril formation studies described in Chapter 5, which showed that fibril formation was accelerated by the presence of Cu$^{2+}$ ions, but only at stoichiometric and substoichiometric Cu$^{2+}$ concentrations. Thus cell assays were carried out to see if the same concentration-dependence of Cu$^{2+}$-Aβ(1-40) affected PC12 cell viability.

Figure 6.7 shows that 10 μM Aβ(1-40) bound to as little as 0.01 mole equivalent Cu$^{2+}$ (100 nM) is significantly more effective at impairing cell growth than the same concentration of Aβ(1-40) in the absence of Cu$^{2+}$ ions (Figure 6.7). 0.1 mole equivalent Cu$^{2+}$ bound Aβ(1-40) had an even more pronounced affect on cell viability, and 0.5 mole equivalents of Cu$^{2+}$ ions were also more toxic than Aβ(1-40).
in the absence of Cu\(^{2+}\) ions. However at the Cu\(^{2+}\) ratio of 1:1 mole equivalents (10 \(\mu\)M Cu\(^{2+}\)), cell growth is no longer impaired. Indeed at 5 mole equivalents of Cu\(^{2+}\), relative to \(\alpha\)β(1-40), Cu\(^{2+}\) is actually protective.

These observations complement the optimum ratio of Cu\(^{2+}\)-\(\alpha\)β(1-40) that generates amyloid fibres, as shown in Chapter 5. Sub-stoichiometric amounts of Cu\(^{2+}\) (0.2-0.4 mole equivalents) were shown in the previous chapter to be more effective at generating amyloid fibres, while Cu\(^{2+}\) levels above 1 mole equivalent will actually inhibit fibre formations. These results show that the influence of Cu-\(\alpha\)β(1-40) ratios on toxicity in PC12 cells must relate closely to the ability of Cu\(^{2+}\) to accelerate (or inhibit) fibre formation, this is discussed further in the discussion section of this chapter.

![Figure 6.7](image)

**Figure 6.7.** Cu\(^{2+}\)-\(\alpha\)β and \(\alpha\)β added as monomers inhibit growth after 6 days incubation. 10 \(\mu\)M monomeric \(\alpha\)β(1-40) with a range of Cu\(^{2+}\) concentrations were incubated with 5.4 x 10^4 cells/mL in Opti-MEM cell medium, pH 7.4. Alamar blue was added after 6 days incubation. Averages calculated from 3 wells and error bars are standard error.
6.4.9. Monomer vs. Fibrillar Toxicity

The results described in Figure 6.7 show Aβ(1-40) added as a monomer to PC12 cells and alamar blue added after 6 days incubation. To observe if Aβ(1-40) also affected cell viability after a shorter incubation, in the same experiment alamar blue was added after only 2 days incubation and cell viability monitored for a further 2 days (Figure 6.8). However addition of monomeric Aβ(1-40) shows no impairment of cell viability within 4 days of addition (Figure 6.8).

Figure 6.8. Cu\(^{2+}\)-Aβ and Aβ added as monomers do not inhibit growth after 4 days incubation. Monomeric Apo and Cu- Aβ(1-40) were incubated with 5.4 x 10^4 cells/mL PC12 cells in Opti-MEM at pH 7.4. Alamar Blue added after 48 hr Aβ incubation with cells, data shown is after 4 days total incubation. Error bars are standard error based on 3 wells.

This suggests that it takes longer than 4 days incubation for monomeric Aβ(1-40) to cause toxicity to PC12 cells. This is in contrast with the toxicity seen with Aβ(1-42) fibrils in Figure 6.6 where toxicity was observed within 3 days. When Aβ(1-42) is added to PC12 cells as a monomer it may remain monomeric during incubation, and in this form take longer to have a negative effect on cell growth than when applied as fibrils. Alternatively the longer incubation time before toxicity is observed may
be due to the time taken for Aβ to form a toxic species. This is supported by fibre growth trials in the cell media using a ThT assay (Figure 6.9) which shows that both apo-Aβ(1-42) and Cu²⁺-Aβ(1-42) will form fibrils in Opti-MEM. Although the experimental conditions are slightly different to those in the cell viability assays, the fibre formation curves suggest that the time taken to form fibrils in Opti-MEM may be an influencing factor on Aβ toxicity.

![Figure 6.9. Aβ and Cu²⁺-Aβ added as monomers to Opti-MEM form fibrils. 4 µM apo-Aβ(1-42) (red trace) and 0.5 mol eq Cu²⁺ (blue trace) in 25 % (v/v) Opti-MEM, 75 % (v/v) 50 mM HEPES/160 mM NaCl in standard fibril growth conditions (see Chapter 5 Section 5.3.1). Fibril growth is monitored by ThT and shows that both Apo and Cu-Aβ(1-42) form fibrils under these conditions. Average of 6 traces shown and the data is zeroed.]

6.5. Discussion

The results in this chapter show that both pre-formed fibrils and monomeric Aβ(1-40) or Aβ(1-42) have detrimental effects on PC12 cells. There are a few studies already published investigating the relationship between Cu²⁺ and Aβ cell-toxicity, but these studies appear to show conflicting results. Opazo et al agreed with the studies in this chapter and found that copper promoted toxicity however the toxicity experiments were carried out with excess EDTA present (Opazo, et al. 2002). This metal chelator has a 10¹⁸ affinity for Cu²⁺ ions compared to the 10¹⁰ affinity of
Aβ(1-42) for Cu²⁺ (Sarell, et al. 2009), thus it seems unlikely the Cu²⁺ ions were bound to Aβ. Yoshiike et al found that Cu²⁺ appeared to protect against toxicity (Yoshiike, et al. 2001). But in both Yoshiike and Opazo’s studies the nature of the Aβ preparation was not well defined (i.e. amorphous aggregate, monomer or fibre) and this may be the source of the discrepancies. In contrast the nature of the Aβ preparation in this chapter was known, as evident by the toxicity of fibrillar full length Aβ(1-40) or Aβ(1-42) with Cu²⁺ bound compared to the lack of toxicity observed with non-fibrillar Aβ(1-16). Additionally monomeric Aβ-Cu²⁺ was only toxic after 6 days incubation, suggesting that some degree of oligomerisation may be necessary for toxicity. A third study by Meloni et al did not show conclusively whether the observed cell toxicity of Aβ-Cu²⁺ was simply due to the presence of Cu²⁺ ions or specifically Cu²⁺ bound to Aβ in a oligomeric/fibrillar form (Meloni, et al. 2008). The results in this chapter suggest that at the experimental Cu²⁺ concentrations used free-Cu²⁺ has no toxic effects on the PC12 cells.

The toxic effect of Cu²⁺-Aβ warrants further discussion. Cu²⁺-Aβ detrimentally affects cell viability more than apo-Aβ. There are potentially two reasons for the enhanced toxicity of Aβ in the presence of Cu²⁺ ions:

**Hypothesis 1: Increased oxidative stress at the membrane.** Aβ has been shown to bind to PC12 cell membranes with high affinity (Simakova and Arispe 2007). If Aβ is also bound to Cu²⁺ this would result in an accumulation of Cu²⁺ ions at the neuronal cell surface, where Cu²⁺ could generate toxic hydrogen peroxide and hydroxyl radicals. Lipid peroxidation of the membrane will compromise membrane integrity and result in cell death.

**Hypothesis 2 Formation of toxic species.** The results in this chapter and those in Chapter 5 suggest that Cu²⁺’s role in promoting not only fibril formation, but also smaller species that form along the fibril formation pathway could promote the formation of Aβ species that are toxic to cells. The rate, quantity or morphology of the Cu²⁺ promoted fibres or prefibrillar oligomers may cause the heightened cytotoxicity.
If it was simply a matter of reactive oxygen species (ROS) generation by Cu$^{2+}$ ions bound to Aβ (hypothesis 1) then one might expect the more Cu$^{2+}$ bound to Aβ, the greater the toxicity due to increased levels of ROS from cycling of the Cu$^{2+}$. However, in Figure 6.7 small concentrations of Cu$^{2+}$ ions (0.1 μM) are more toxic than much higher concentrations (5 μM) of Cu$^{2+}$ ions. In light of this, the ability of Cu$^{2+}$ to promote fibres (described in Chapter 5), appears to be the significant factor in Cu$^{2+}$ promoted Aβ cell toxicity, supporting hypothesis 2. This is supported by a study with human islet amyloid polypeptide (IAPP), the fibril forming protein in type-2 diabetes mellitus, which showed that the kinetic profile of IAPP-induced membrane damage is characterized by a lag phase and a sigmoidal transition, which matches the kinetic profile of IAPP fibril growth. Thus the process of fibril formation on the cell membrane was directly responsible for damage to the membrane (Engel, et al. 2008). It is possible that a similar mechanism occurs with Aβ fibrils. However hypothesis 1 and 2 could both be valid, as it is possible that the fibrillar species formed with Cu$^{2+}$ present, not only form faster, but also result in Aβ species with a different underlying structural form to species formed by apo-Aβ. The alternative structure of Aβ species could bind Cu$^{2+}$ in a redox active form, thus facilitating Cu$^{2+}$ redox cycling.

To investigate these hypotheses further tests could be performed. To see if more lipid peroxidation occurs in PC12 cells incubated with Cu$^{2+}$ bound fibrils (hypothesis 1) there is a large range of tests that could be conducted (Halliwell 2007). The thiobarbituric acid (TBA) assay is one of the oldest and most frequently used methods for measuring peroxidation. It measures the concentration of malondialdehyde (MDA). MDA arises from peroxidation of polyunsaturated fatty acids in membrane lipids. The TBA in the assay reacts with any MDA present to form a red complex with an absorbance maximum at 532 nm, and thus allows comparisons of the degree of lipid peroxidation under different conditions. However the TBA assay has a number of flaws associated with it and is vulnerable to misinterpretation, so it is instead now recommended that MDA concentrations are measured directly by HPLC or GC-MS (Halliwell 2007).

To study the Cu$^{2+}$ induced fibrils more closely (hypothesis 2) a variety of different structural techniques could be used. Morphology of the fibers could be examined by
transmission electron microscopy more closely and in further detail than has been performed in this thesis, atomic force microscopy could also be used for this purpose. Additionally antibodies could be used to identify if there were any specific morphological differences, such as Kayed et al’s antibody which differentiates between fibrils and oligomers (Kayed, et al. 2003). Alternatively a more detailed atomic level model could be developed from solid state NMR.

In conclusion, the results presented here show Cu$^{2+}$ decreases cell viability in PC12 cells through the affect of Cu$^{2+}$ on Aβ fibril formation. It will be of interest to see if the precise mechanism is identified, and if there are differences between Cu$^{2+}$ loaded fibrils and apo-fibrils. The results described here are used to help build a hypothesis for Aβ-Cu$^{2+}$ toxicity in AD in the next, and final, chapter of this thesis.
7. Conclusion
In this chapter three models of potential modes of Aβ-Cu$^{2+}$ toxicity are outlined. These models are based on changes in copper homeostasis both within the cell and extracellularly. The three main sources of metal ions in the brain are 1) a vesicular pool located in synaptic vesicles of nerve terminals, 2) an ionic pool of free or loosely bound ions and 3) a protein-metal complex pool (Frederickson 1989). The three models focus predominantly on the first two sources of copper. Disruption to protein-metal complexes will not be discussed in detail as a potential mechanism for AD onset, as this pool of protein-metal complexes has extremely high affinities, for example superoxide dismutase has a dissociation constant of 6 fM (Rae, et al. 1999) and it is thus unlikely that Aβ would reach levels high enough within the cell to compete with this pool of copper. However protein-metal complexes will be touched upon in all three hypotheses.

**Model 1. The Synaptic Hypothesis.**

Model one examines the vesicular pool of copper at the synapse, and a possible mode of interaction with Aβ in the synaptic cleft due to Cu$^{2+}$ dyshomeostasis. This model is based on the work of Schlief et al, who found a role for copper in preventing NMDA receptor-mediated neuronal toxicity (Schlief, et al. 2006) and Snyder et al who found that NMDA receptor trafficking is regulated by Aβ (Snyder, et al. 2005). These two findings provide evidence for an interaction between Aβ and Cu$^{2+}$ at the synapse.

The synapse is a good place to hypothesise a model for the trigger for AD for several reasons: Aβ fibrils first appear at the synapse, and synaptic damage and loss is a good correlation to the severity of AD symptoms (Terry, et al. 1991). This model suggests a cascade of events arising from intracellular Cu$^{2+}$ deficiency at the synapse which results in AD onset and progression.

Copper is essential for nervous system development and continuing function, with a range of roles including acting as an enzyme cofactor, peptide amidation and neurotransmitter synthesis (Culotta 2001). Nowhere is this more evident than in the fatal neurodegenerative Menkes disease, which occurs due to a loss-of-function mutation in the gene encoding a copper-transporting ATPase, Atp7a. (Vulpe, et al. 1993).

Strikingly, recent research has shown that Atp7a has a critical role in the release of a pool of copper in neurons (Schlief, et al. 2006). Synaptic NMDAR activation results in
the rapid trafficking of Atp7a to the somato-dendritic and axonal compartments of neurons followed by rapid release of a vesicular pool of copper from the neuronal membrane. Once released, endogenous copper catalyses the S-nitrosylation of NMDA receptors, resulting in a down-regulation of their activity (Schlief, et al. 2006). Thus copper acts as a neuroprotectant, preventing NMDA-mediated excitotoxicity (Schlief, et al. 2006). Excitotoxicity can occur if NMDAR and other receptors are over-stimulated in response to neurotransmitter (glutamate) release. Over-activation of NMDAR results in high levels of calcium entering the cell which go on to activate a range of enzymes that damage the cell, resulting in cell death (Choi 1992).

Strikingly another important neuroprotectant that prevents NMDAR over-stimulation is Aβ. Aβ promotes down-regulation of NMDA receptors through endocytosis of the receptors (Snyder, et al. 2005). This is further supported by a study showing that activation of NMDA receptors results in increased levels of Aβ, through a favouring of the amyloidogenic pathway over the non-amyloidogenic pathway (Lesne, et al. 2005), thus suggesting a feedback mechanism between NMDA stimulation, Aβ production and NMDA down-regulation. Thus balanced Aβ and copper homeostasis are both essential in preventing excitotoxicity at the synapse. A possible model for Aβ and Cu$^{2+}$ at the synapse is shown in Figure 7.1.

So what might be the trigger for the formation of Aβ-Cu$^{2+}$ complexes at the synapse and the development of AD pathology? This model hypothesises that large changes in Cu$^{2+}$ homeostasis in AD are responsible. In AD Cu$^{2+}$ is present in extracellular plaques and in the serum in AD at high concentrations (Lovell, et al. 1998, Squitti, et al. 2002), conversely intracellular copper levels decrease (Deibel, et al. 1996). The effects that copper dyshomeostasis at the synapse could have in regards to promoting Aβ-Cu$^{2+}$ complex formation are discussed below:

1. Intracellular copper deficiency has been shown to alter the structure of lipid rafts (Hung, et al. 2009) which may favour γ-secretase cleavage at the Aβ(1-42) cleavage site instead of the Aβ(1-40) site.

2. Intracellular copper deficiency would lead to Cu$^+$-free APP molecules, which may have a different structure to the Cu$^+$-loaded APP that is present in non-AD
brains. This change in APP structure could also lead to an increased propensity for γ-secretase cleavage at the Aβ(1-42) cleavage site.

3. In addition to increasing the ratio of Aβ(1-42) to Aβ(1-40) through effects 1 and 2, a decrease in intracellular Cu\(^{2+}\) may result in a small decrease in Cu\(^{2+}\) release at the synapse. This would result in a slight loss of Cu\(^{2+}\)-mediated downregulation of NMDA receptors. The subsequent overstimulation of the NMDAR would result in sustained Aβ release and thus an increase in Aβ concentrations at the synapse. Regulatory mechanisms for degradation of Aβ would become overwhelmed, especially as neprilysin levels decrease as we age (Iwata, et al. 2005) and Aβ clearance has been shown to be impaired in AD (Mawuenyega, et al. 2010). This would result in Aβ accumulation at the synapse and an increased propensity for copper binding.

4. Once Aβ concentrations reached a critical level at the synapse, Aβ could begin to outcompete other copper binding proteins to bind copper at the synapse, especially as a major one, metallothionein, is down regulated in AD (Meloni, et al. 2008). Copper-seeded fibril formation could then occur, which would have two toxic effects. One is the physical damage caused by fibrils at the synapse. The fibrils could pierce the neuronal membrane (Engel, et al. 2008), or allow the cycling of copper resulting in production of free radicals (Butterfield, et al. 2001). The second is that newly released Aβ and copper would be rapidly recruited into fibrils at the synapse, and thus they would no longer function in their neuroprotectant roles against NMDAR over-activation. NMDAR activation would not be prevented and thus the signal for the neuroprotectants copper and Aβ to be released would continue. A positive feedback loop would be initiated with more and more copper and Aβ being released to try and prevent NMDAR excitotoxicity resulting in further fibril formation. These two effects of physical damage by fibrils and excitotoxicity due to the loss of function of copper and Aβ as neuroprotectants, would produce the synaptic damage and loss that is characteristic of early signs of AD.

Thus in conclusion, the disrupted copper homeostasis that occurs in the AD brain is hypothesised to effect copper’s role as an important neuroprotectant at the glutamatergic synapse. Downstream effects of copper dyshomeostasis include an
increase in Aβ(1-42) levels, an increase in Aβ concentrations in the synaptic cleft, and an increase in the likelihood of an Aβ:Cu$^{2+}$ complex formation, resulting in an AD pathology.

**Figure 7.1. Model of hypothetical interaction of Aβ and copper at the glutamergic synapse.** Aβ is secreted into the synaptic cleft from APP and acts to down regulate NMDA receptors. Additionally NMDA receptor activation by glutamate results in calcium release into the cell, which results in the release of copper which blocks NMDA receptors from glutamate limiting further calcium entry into the cell, preventing excitotoxicity. Neprilysin normally degrades Aβ, and metallothionein normally binds copper. But both are downgraded in AD. Adapted from Tanzi 2005 and Schlief 2006 and Bush 2008.

**Model 2. The Ligand Hypothesis**

Model two focuses on the ionic/loosely-bound pool of copper, and emphasises the importance of non-protein Cu$^{2+}$ ligands, specifically carnosine in the olfactory bulb. A
decrease in the carnosine concentration in the brain is hypothesised here to result in an increase in free Cu\(^{2+}\) in the olfactory bulb and result in amyloid formation.

Carnosine (β-alanyl-histidine) is one of a group of ω-aminoacylamino acids and is highly expressed in the olfactory bulb at 2-5 mM concentrations (Ferriero, et al. 1975).

![Figure 7.2. Structure of Carnosine (Baran 2000)](image)

Carnosine is of interest in relation to this thesis for three major reasons:
Firstly, carnosine will chelate copper and the copper-carnosine complex has been studied in some detail. There are three groups within carnosine that undergo acid base reactions between pH 1 and 10, the carboxylic acid group, an ammonium group, and the protonated N\(^3\) imidazole group (Figure 7.2). Interestingly EPR data shows that there is a concentration dependence to the stoichiometry of the Cu\(^{2+}\):carnosine complex (Brown, et al. 1979). When carnosine is present in excess a monomeric complex forms. However as the carnosine concentration reduces the monomeric complex begins to be in equilibrium with a dimeric complex, until at equimolar concentrations formation of a dimeric complex is favored (Brown and Antholine 1979). Another group found that at equimolar concentrations of copper and carnosine the dimeric complex (Cu\(_2\)L\(_2\)H\(_2\))\(^0\) is the only species (Sovago, et al. 1982).

The dissociation constant of the carnosine-copper complex has not been definitively characterised, one suggestion is that the binding constant is surprisingly low, at only 1.1 M\(^{-1}\) for a 1:1 complex (determined at pH 7.8) (Velez, et al. 2008). However it is possible that complexes formed at different stoichiometries of carnosine and copper.
may have very different stability constants as shown in Table 7.1 (Baran 2000). Importantly the very high concentrations of carnosine in the olfactory bulb (2-5 mM) in non-AD cases (Ferriero and Marogolis 1975) means carnosine may have an important role in forming copper-carnosine complexes in the olfactory bulb and skeletal muscle.

The predominant role suggested for carnosine is as a biologically significant antioxidant due to the imidazole ring- carnosine was shown to prevent the oxidative damage of deoxyguanosine induced by copper ions (Kohen, et al. 1988) and the copper-carnosine complex has been shown to possess SOD-like activity (Kohen, et al. 1991). Carnosine can scavenge peroxyl radicals and singlet oxygen and significantly protects olfactory bulb neurons from copper-mediated toxicity, although only at relatively high levels of carnosine (1 mM or above) (Horning, et al. 2000). This suggests that it is no coincidence that carnosine is at its highest levels in two of the tissues with the highest active oxidative metabolism – the brain and skeletal muscle (Boldyrev 2000).

<table>
<thead>
<tr>
<th>Equilibrium</th>
<th>Log $k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Cu}^{2+} + \text{L}^- + \text{H}^+ = [\text{CuLH}]^{2+}$</td>
<td>$13.30 \pm 0.03$</td>
</tr>
<tr>
<td>$\text{Cu}^{2+} + \text{L}^- = [\text{CuL}]^+$</td>
<td>$8.47 \pm 0.01$</td>
</tr>
<tr>
<td>$\text{Cu}^{2+} + \text{L}^- = [\text{CuLH}]_1^{4+} + \text{H}^+$</td>
<td>$2.44 \pm 0.07$</td>
</tr>
<tr>
<td>$2\text{Cu}^{2+} + 2\text{L}^- = [\text{Cu}_2\text{L}_2\text{H}_2]^{4+} + 2\text{H}^+$</td>
<td>$8.35 \pm 0.02$</td>
</tr>
<tr>
<td>$2\text{Cu}^{2+} + \text{L}^- = [\text{Cu}_2\text{LH}_1]\text{H}^{2+} + \text{H}^+$</td>
<td>$5.37 \pm 0.02$</td>
</tr>
</tbody>
</table>

Table 7.1 Stability constants in the Cu(II)/Carnosine system (at 25 ºC) (Baran 2000)

Secondly, olfactory dysfunction is a significant aspect of AD pathology. Loss of smell is one of the first symptoms of AD, and plaques and neurofibrillary tangles occur in the olfactory bulb (Murphy 1999). Immunostaining of Aβ in transgenic rats shows amyloid plaques form early on in the olfactory bulb, Figure 7.3 (Badea, et al. 2010). In fact, a potential method for early diagnosis of AD is the use of olfactory sensory dysfunction experiments with studies using a transgenic AD mouse model showed that progressive olfactory impairment corresponded with an increasing Aβ burden in the olfactory region (Wesson, et al. 2010).
Figure 7.3. Immunostaining for Aβ reveals progressive accumulation of amyloid plaques (brown) throughout the forebrain of transgenic APP mice. Initial amyloid deposits can be clearly seen in the cortex, hippocampus, and olfactory bulb by 4 months in this highly overexpressing transgenic model; pathology worsens significantly with time Badea et al 2009.

Carnosine may have an important role in copper homeostasis in the brain: neurological damage observed in Wilson’s disease, which is a disease involving incorrect copper transport, is highly similar neurological injury produced by improper metabolism of carnosine (Bessman, et al. 1962). This suggests that carnosine has an important role in preventing copper-related toxicity, via being a major chelator of “free” copper within the olfactory bulb and skeletal muscle. Interestingly carnosine is currently being studied as a potential therapeutic for pathologies related to increased oxidative stress and metal dyshomeostasis (Lanza, et al. 2011).

Significantly in respect to model one, carnosine has also been shown to act antagonistically towards copper’s effects on NMDA receptors – voltage clamp experiments showed that whereas copper blocked most of the current evoked by NMDA, carnosine was found to completely prevent this effect (Trombley, et al. 2000). Thus there appears to be a role for carnosine as a neuromodulator of copper at the synaptic cleft.
Another reason for including carnosine in a model for AD onset is the effect of carnosine on Aβ fibrillisation and toxicity. Carnosine has been shown to inhibit advanced glycation end-product (AGE) formation. AGEs significantly accelerate fibril formation through cross linking Aβ. Inhibitors of AGE have been shown to result in a marked improvement in cognition and memory in AD patients after treatment (Munch, et al. 1997), thus suggesting that carnosine has an effect on inhibiting Aβ fibril formation. Additionally 5 µM Aβ(1-42) addition to PC12 cells caused a concentration-dependent reduction of viability. However pre-treatment with 5 mM carnosine ameliorated this impairment, suggesting that as well as inhibiting fibril formation, carnosine also protects against Aβ toxicity (Fu, et al. 2008).

So how might carnosine have a role in AD? Interestingly in a study of changes in amino acid and dipeptide levels in AD, carnosine was highlighted as undergoing one of the biggest decreases in plasma levels, when the plasma of patients with probable AD was compared to that in control subjects there was a decrease in carnosine levels by ~ 50 % (Fonteh, et al. 2007). Thus this model suggests that decreases in carnosine levels in AD result in a decreased ability for carnosine to form a complex with extracellular copper. This would have two detrimental effects: 1) In the absence of being bound to carnosine, Cu²⁺ would be free to undergo redox cycling and produce ROS, resulting in membrane damage. 2) Low carnosine levels would result in an increased propensity for Aβ to compete with carnosine for this loosely bound pool of copper, increasing the likelihood for Aβ-Cu²⁺ formation. Figure 7.3 shows that once amyloid forms in the olfactory bulb, it rapidly spreads throughout the brain, suggesting that once the critical stage of amyloid formation in the olfactory bulb has occurred, AD pathology throughout the brain occurs.

**Model 3. Hypothesis based on the data in this thesis**

In this final model, the main findings outlined in Chapters 3, 4, 5 and 6 are briefly summarised and then a model with these findings and parts of model one and two is suggested.

In this thesis the interaction between Aβ and Cu²⁺ was studied in detail. In Chapter 3 it was found that Aβ has an affinity for Cu²⁺ of approximately 50 pM. When this is compared to other copper binding proteins in the brain, such as human serum albumin (HSA), which has a high affinity for Cu²⁺ (a K_d of 1 pM) (Masuoka, et al. 1993) it can
be clearly seen that the affinity of HSA is higher than the affinity of Aβ. Additionally, concentration is important: although Aβ may have a higher affinity for Cu²⁺ than carnosine, the level of carnosine is ~ 1000 000 (Ferriero and Marogolis 1975) higher than Aβ (Vigo-Pelfrey, et al. 1993) under non-disease conditions. Thus Cu²⁺ in the synaptic cleft is unlikely to ever be “free” but bound to either proteins with a high affinity for Cu²⁺ such as metallothionein, or to ligands present at a high concentration, such as carnosine.

Chapter 5 showed rapid Aβ fibrillisation in the presence of Cu²⁺, however as stated above under non disease conditions as Aβ-Cu²⁺ complex is unlikely, and Aβ alone is inhibited from forming fibrils under normal disease conditions by carnosine inhibiting AGE formation (Munch, et al. 1997) and by the low Aβ concentrations (Vigo-Pelfrey, et al. 1993) which are controlled by effective Aβ clearance from the synaptic cleft by neprilysin (Iwata, et al. 2001).

So what could happen under AD conditions? This model is set in the olfactory bulb glutamatergic synapse:

1. Copper dyshomeostasis begins, with a decrease in the intracellular Cu⁺ pool (Deibel, et al. 1996)
2. Decreased release of Cu⁺ into the synaptic cleft results in decreased inhibition of NMDAR (Schlief, et al. 2005)
3. Decreased inhibition of NMDAR by Cu²⁺ results in increased Aβ release as compensation to avoid excitotoxicity (Snyder, et al. 2005)
4. Decreased intracellular Cu²⁺ results in a favouring of amyloidogenic processing of Aβ, with an increase in the Aβ(1-42):Aβ(1-40) ratio
5. Aβ clearance is impaired (Mawuenyega, et al. 2010), due to a decrease in neprilysin, resulting in a further increase in Aβ concentration in the synaptic cleft
6. Carnosine levels decrease (Fonteh, et al. 2007), increasing the potential for Aβ:Cu²⁺ complex formation. The carnosine decrease also increases the likelihood of AGE formation and an increase in oxidative stress at the synapse.
7. Aβ reaches a critical concentration at the synapse where it can begin to compete with remaining carnosine ligands for Cu²⁺, due to Aβ’s high affinity for Cu²⁺, as shown in Chapter 3.
Chapter 7. Conclusions

8. Sub-stoichiometric Cu$^{2+}$ binding to Aβ results in rapid Cu$^{2+}$-induced Aβ fibril formation, as shown in Chapter 5, where Cu$^{2+}$-bound Aβ formed fibrils twice as fast as apo-Aβ.

9. Once formed, these fibrils seed monomeric Aβ to result in further fibril formation. Additionally, the results in Chapter 4 show that once formed, fibrils can continue to bind Cu$^{2+}$, as Cu$^{2+}$ can diffuse through the fibril structure to form a 1:1 stoichiometry with the fibres. Importantly, once Cu$^{2+}$-bound Aβ aggregates are formed, metallothionein is unable to aid deaggregation of, or extract copper from, the aggregates (Chung, et al. 2010).

10. Newly released Cu$^+$ or Aβ in response to NMDAR activation is ineffective as instead of binding to the NMDAR, they are sequestered into the fibrils, resulting in prolonged NMDAR activation, and further release of Cu$^+$ and Aβ in response.

11. In Chapter 6 it was shown that Aβ-Cu$^{2+}$ fibres were significantly more toxic to PC12 cells than apo-Aβ fibres or Cu$^{2+}$ bound to the soluble fragment Aβ(1-16). These fibres result in physical damage to the neuronal cell membrane.

12. Redox cycling of Cu$^{2+}$ bound to Aβ and the low levels of carnosine result in increased oxidative stress at the synapse.

13. Inefficient inhibition of NMDAR by Cu$^+$ and Aβ results in excitotoxicity.

14. Neuronal cell death and synapse loss begin to occur, resulting in olfactory dysfunction. Amyloid plaques spread from the olfactory bulb through the brain, eventually resulting in widespread memory loss and loss of brain function and severe AD pathology.

This hypothesis aims to combine the results found in this thesis and current literature into a hypothetical model that may explain parts of the bigger picture of AD. If Cu$^{2+}$ dyshomeostasis is responsible for the onset of AD, it will be interesting to see the results of the phase III clinical trial of the metal chelator PTB2 (Lannfelt, et al. 2008). This chelator is derived from 8-hydroxyquinoline, which will bind Cu$^{2+}$ at pH 7.4 with stepwise apparent $K_d$'s of $K_{d1} = 0.2$ nM and $K_{d2} = 2$ nM, giving an overall $K_d$ of $4 \times 10^{-19}$ M (Dawson 1986). This is a much higher affinity than the $5 \times 10^{-11}$ M dissociation constant determined for Aβ in Chapter 3, and suggests that this compound would remove Cu$^{2+}$ from Aβ, thus suggesting it could be an effective therapeutic.
In summary, the data in this thesis suggests that Aβ has the potential to bind Cu\(^{2+}\) with a high affinity, accelerate fibril formation, continue to bind Cu\(^{2+}\) once in the fibrillar form, and cause toxicity \textit{in vitro}. The literature also seems to support a role for Cu\(^{2+}\) in AD pathology. However a great deal of scepticism remains in the AD field as to whether there is a role for metal ions in AD, thus whether the results of the trial will be compelling enough to change the current “Amyloid cascade hypothesis” to the “Amyloid and metal cascade hypothesis” remains to be seen.


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