Fluorescence Studies of the Interactions of Amyloid-β with Metal Ions and Lipids

A thesis submitted for the degree of

Doctor of Philosophy

By

Thomas Branch

Department of Chemistry,
Imperial College London

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Declaration

I declare that this thesis, submitted to fulfill the requirements for the degree of Doctor of Philosophy at Imperial College London, titled “Fluorescence Studies of the Interactions of Amyloid-β with Metal Ions and Lipids” represents my own work, unless otherwise stated.

Thomas Branch
May 2015
Abstract

Amyloid-β (Aβ) metal interactions promote aggregation and produce reactive oxygen species, hallmarks of Alzheimer’s disease (AD). Oligomerisation of Aβ has been proposed to be modulated by copper and zinc ions. In environments of fast molecular turnover, such as the synaptic cleft, the kinetics of molecular interactions are important. To assess the roles of Cu$^{2+}$ and Zn$^{2+}$ in the early molecular events of AD, the kinetics of Aβ binding to both ions were determined. Metal binding was monitored using the quenching of a fluorescent dye covalently linked to Aβ by Cu$^{2+}$, enabling measurements to be performed at physiologically relevant Aβ concentrations.

The binding of monomeric Aβ to Cu$^{2+}$ was nearly diffusion limited with a lifetime of a few seconds. Two forms of Aβ·Cu were found that interconverted, and at least two further Cu$^{2+}$ ions could bind. The protonated form of Aβ·Cu was capable of dimerisation. Cu$^{2+}$ assisted dimerisation is two orders of magnitude faster than without Cu$^{2+}$. A metric was devised to measure the effectiveness of the removal of Cu$^{2+}$ from Aβ·Cu by a ligand. The kinetics of Cu$^{2+}$ binding to Aβ pre-bound to GM1 micelles were similar to unbound Aβ, however GM1 micelles protected the Aβ complex from Cu$^{2+}$ binding ligands. Zn$^{2+}$ binding to Aβ·Cu, was a further three orders of magnitude slower.

A reaction-diffusion simulation of the repeated release of neurometals into a synaptic cleft suggested that Aβ·Cu is two orders of magnitude more likely to form than Aβ·Zn under physiological conditions. This suggests that Cu$^{2+}$ rather than Zn$^{2+}$ is responsible for the dimerisation of Aβ in the synaptic cleft.

The methodology applied here is applicable to determine the Cu$^{2+}$ binding kinetics of other peptides or proteins.
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Chapter 1

Introduction

Dementia is a decline in cognitive ability such that it interferes with daily life and a growing global problem. The number of people worldwide estimated to be living with dementia is 45 million people, and it is expected to increase to over 130 million by 2050\(^1\). In 2010 the cost of care was estimated to be USD 604 billion\(^2\).

Of the dementias, Alzheimer’s disease is the most common form, making up 60% to 80% of cases\(^3\) and is associated with being of older age\(^4\). In older adults, dementia is the second most prevalent disease in contributing significantly to death (13.6%)\(^5\). Alzheimer’s disease begins 10 to 15 years before the symptoms become apparent, and is characterised by abnormal levels of senile plaques and neurofibrillary tangles. Although there are drugs available that reduce the apparent effects of Alzheimer’s disease, none exist that combat the cause, neither preventing nor slowing continued progression of Alzheimer’s disease. The difficulty in developing effective therapeutics comes in part from a lack of understanding of the disease mechanism at a molecular scale and thus finding targets with which drugs can interfere.

Currently the main hypothesis for the cause of Alzheimer’s disease is the amyloid cascade hypothesis\(^6,7\) (see Fig. 1.1). Central to the cascade is the thought that the peptide known as Amyloid-\(\beta\) (A\(\beta\)), which is the main component in senile plaques, is thought to be the main culprit in Alzheimer’s disease. However, monomeric A\(\beta\) and A\(\beta\) in fibrils are not thought to be the cause of the neurotoxicity. Studies suggest
that it is instead the small Aβ oligomers that are toxic\cite{8}. Isolating the mechanism of oligomer formation and the structure of toxic aggregates would provide targets for drug development.

One explanation for the cause of the amyloid cascade hypothesis is the amyloid-metal hypothesis. Metal ions are found at high concentrations in senile plaques, so it is thought that metal ions bind to Aβ promoting oligomerisation and aggregation. The binding of metal ions to Aβ may also produce reactive oxygen species, damaging Aβ, proteins or lipids nearby.

This thesis investigates the amyloid-metal hypothesis, that neurometal ions mediate the aggregation and toxicity in Alzheimer’s disease\cite{9}, by elucidating the kinetic mechanism of the binding of metals to Aβ. Understanding the kinetics of Aβ-metal interactions allows for the lifetime and formation of complexes to be predicted in vivo. This can then be used to determine the probability of oligomers forming and gives a basis system for their study.

### 1.1 Alzheimer’s disease

Alzheimer’s disease was first reported by Alois Alzheimer in 1907, when a patient showed the characteristic symptoms of memory loss, neurofibrillary tangles (formed from hyperphosphorylated tau protein) and miliary foci (senile plaques formed from Aβ)\cite{10;11}, shown in figure 1.2.
The criteria for the clinical diagnosis of Alzheimer’s disease were established in 1984\cite{13} and have since been updated in 2011\cite{14}. The main issue in the diagnosis of Alzheimer’s disease is differentiating it from other forms of dementia, which have similar symptoms, but a different underlying biological cause.

The progression of Alzheimer’s disease is separated into three phases: preclinical, mild cognitive impairment (MCI), and full Alzheimer’s disease. However, these are part of a continuum and not clear cut.

The preclinical stage (before apparent symptoms) is thought to begin one to two decades before the diagnosis of Alzheimer’s disease\cite{15,16}. This is because the characteristic features of Alzheimer’s disease, such as memory loss, only occur once sufficient neuronal matter is destroyed by the disease. This complicates drug trials for a cure for Alzheimer’s disease, as once it is diagnosed it may already be too late to reverse the changes, i.e. the irreversibly damaged neurons. It is therefore important to understand the initial stages of Alzheimer’s disease, so that effective therapeutics can be developed.
1.1.1 Diagnosis and Symptoms

For a diagnosis of Alzheimer’s disease\textsuperscript{[14]} the clinical criteria of ‘all-cause dementia’ must be met, with a set of further criteria for the three classifications of dementia caused by Alzheimer’s disease. They are (1) Probable Alzheimer’s disease dementia, (2) Possible Alzheimer’s disease dementia, and (3) Probable or possible Alzheimer’s disease dementia with evidence of the Alzheimer’s disease pathophysiological process\textsuperscript{*}.

All-cause dementia

A summary of the criteria for diagnosis are:

1. Interference with function at work or usual activities.
2. A decline from previous performance levels and function.
3. When not explained by delirium or major psychiatric disorder.
4. A detectable and diagnosed cognitive impairment.
5. A cognitive or behavioral impairment of at least two of:
   \begin{enumerate}
   \item Acquiring and remembering new information.
   \item Reasoning and handling of complex tasks.
   \item Visuospatial abilities.
   \item Language functions.
   \item Changes in personality or behavior.
   \end{enumerate}

For the exact criteria, see reference\textsuperscript{[14]}.

Probable Alzheimer’s disease

Here all the ‘all-cause dementia’ criteria are met with, and the following criteria (summary):

1. Gradual onset over months to years.
2. Clear cut history of worsening cognition.
3. The first and most prominent deficits are of either category:
   \begin{enumerate}
   \item Amnestic presentation: Impairment in learning and recall of recently learned information. With further evidence of cognitive dysfunction in at least one other cognitive domain.
   \end{enumerate}

\textsuperscript{*}The third classification is only for research purposes, not clinical diagnosis.
(b) Nonamnestic presentations: Deficits in one of the following, with further deficits in other cognitive domains:

- Language
- Visuospatial
- Executive

4. Diagnosis should not be applied when there is evidence of:

(a) Cognitive impairment from a cerebrovascular disease, such as a stroke.
(b) Features of Lewy body dementia (excluding dementia itself).
(c) Features of frontotemporal dementia.
(d) Prominent features of semantic or nonfluent/agrammatic variants of primary progressive aphasia.
(e) Evidence of another disease or medication that could substantially effect cognition.

The diagnosis can be made with increased certainty when there is evidence of a causative genetic mutation (i.e. in amyloid precursor protein, presenilin-1, or presenilin-2).

Carrying the $\epsilon 4$ allele of the apolipoprotein E gene, was explicitly excluded from this list as it was not sufficiently specific[17]. For the exact criteria, see reference[14].

**Possible Alzheimer’s disease**  The diagnosis is made if the disease follows an atypical course, or there is mixed aetiology.

For a definitive diagnosis of Alzheimer’s disease, a histopathological examination is required[18].

### 1.1.2 Treatments for the Symptoms of Alzheimer’s Disease

There are currently four* drugs approved in the US to temporarily slow the worsening of the symptoms of Alzheimer’s disease thus improving the quality of life. There are three cholinesterase inhibitors (donepezil, galantamine and rivastigmine), and

---

*The drug tacrine, a cholinesterase inhibitor, has been discontinued.
one NMDA (N-methyl-D-aspartate) receptor antagonist (memantine)\(^{[19]}\). Both these categories affect the communication between neurons via synaptic proteins.

**Cholinesterase inhibitors** Acetylcholine (ACh) is a neurotransmitter, released into the synaptic cleft during neurotransmission. In Alzheimer’s disease its receptors (alpha-4 beta-2 nicotinic receptors), which has been implicated in learning\(^{[20]}\), are impaired\(^{[21]}\). The cholinesterase enzymes in the synapse break down the ACh. The method of action of the drug is to inhibit the enzymes that break down ACh, increasing the activity of the ACh receptors.

**NMDA receptor antagonists** NMDA receptors are activated by the neurotransmitter glutamate. However, in Alzheimer’s disease abnormally prolonged release of glutamate causes excitotoxicity, resulting in the death of neurons\(^{[22]}\). Unlike ACh, there are no enzymes in the synapse to degrade glutamate\(^{[23]}\). Thus the mechanism of action of NMDA receptor antagonists is thought to be to protect neurons from damage, by inhibiting the response of NMDA receptors.

### 1.1.3 Progression of Alzheimer’s Disease Pathology

Alzheimer’s disease is thought to begin one to two decades before cognitive impairment shows\(^{[15]}\). There are five well established biomarkers for Alzheimer’s disease:

1. Decrease in CSF (cerebrospinal fluid) A\(_{\beta 42}\) concentration.
2. Increase in PET (positron emission tomography) imaging of amyloid.
3. Increased CSF concentrations of tau and phosphorylated tau.
4. Hypometabolism on fluorodeoxyglucose (a fluorescent glucose analog).
5. Atrophy on structural MRI, showing large scale neuronal loss.

This list also corresponds to the hypothesised temporal ordering of biomarkers in patients\(^{[24]}\) (see Fig. 1.3). This suggests that in order to understand the cause of Alzheimer’s disease, the cause of the decrease in A\(_{\beta 42}\) and increase in larger amyloid structures should be investigated from their formation as small oligomers.

The second category of biomarker change is the increase in tau proteins. The A\(_{\beta}\) and tau proteins are the basis for the two main hypotheses for Alzheimer’s disease.
1.1.4 Alzheimer’s Disease Hypotheses

Alzheimer’s disease is caused by the atrophy of neuronal matter. Once sufficient matter is destroyed, then the clinical symptoms occur. However, the underlying disease mechanism of the atrophy is unknown. The hypotheses fall broadly into two main categories, the amyloid hypothesis (related to the aggregation of Aβ) and the tau hypothesis (related to the aggregation of tau proteins). Better understanding of the cause of aggregation may allow the underlying cause of Alzheimer’s disease to be determined and for the development of therapeutics to combat it.\cite{25}

**Tau Hypothesis**

The purpose of tau proteins in healthy cells are to stabilise microtubules which form the cell’s cytoskeleton. The binding of tau to microtubules is controlled in the cell by their phosphorilation state. Phosphorilation of tau causes decreased association with the microtubules. There is redundancy in this stabilisation mechanism, in that two other proteins are known to also stabilise microtubules (MAP1 & MAP2). In Alzheimer’s disease, the tau proteins become hyperphosphorylated, becoming less soluble and aggregate forming neurofibrillary tangles within cells.\cite{26} It is thought that tau oligomers are more toxic than the tau in filaments. Filaments themselves

---

[Figure 1.3: Model of the dynamics of biomarkers in the Alzheimer’s disease pathological cascade. Reprinted from The Lancet, 12, Clifford R Jack, et al., Tracking pathophysiological processes in Alzheimer’s disease: an updated hypothetical model of dynamic biomarkers, 207-216, 2013, with permission from Elsevier.\cite{24}]
may be considered protective, as they sequester tau oligomers. However, the loss in microtubules independently burdens the cell.\textsuperscript{[26,27]} It has been proposed that the tau mediates A\textsubscript{\(\beta\)} toxicity\textsuperscript{[28]} and vice versa\textsuperscript{[29]}.

**Amyloid Hypothesis**

The amyloid cascade hypothesis\textsuperscript{[6;30–32]} is a sequence of events in the development of Alzheimer’s disease which are:

1. Increase in levels of A\(\beta\), possibly through over production, reduced clearance, or increased aggregation propensity of A\(\beta\) (mechanism unknown).
2. Oligomerisation, aggregation and deposition of A\(\beta\) (mechanism unknown).
3. Aggregate stress (mechanism unknown).
4. Formation of neurofibrillary tangles (mechanism unknown).
5. Neuronal dysfunction and death (mechanism unknown).

There are multiple hypotheses for all the ‘unknown mechanisms’ of the amyloid hypothesis. More recently there has been a consensus towards the toxicity being in some way related to A\(\beta\) oligomers. The A\(\beta\) related hypotheses seem to suggest that it is not A\(\beta\) itself that is neurotoxic. Therefore, this thesis works towards the end goal to gain an understanding of how the initial oligomerisation process occurs, starting with how dimerisation can occur. The hypothesis of aggregation investigated is the metal-amyloid hypothesis, which suggests that it is metal cations that are responsible for the dimerisation and aggregation of A\(\beta\).

### 1.2 Amyloid-\(\beta\)

A\(\beta\) mainly exists in two major forms A\(\beta\)\textsubscript{40} & A\(\beta\)\textsubscript{42}, having 40 and 42 amino acid residues, respectively. The production of A\(\beta\) occurs widely in the body by most cell types\textsuperscript{[33]}.

In neurons A\(\beta\) is released into the extracellular space at a rate of 2 to 4 A\(\beta\) molecules per neuron per second\textsuperscript{[34]}.

In Alzheimer’s disease patients, the concentrations of A\(\beta\) have been found to be in the low nanomolar regime, in the cerebrospinal fluid (CSF) and tens of picomolar in the blood plasma (see table 1.1).
Aβ$_{42}$ is thought to be more related to neurotoxicity than Aβ$_{40}$.

<table>
<thead>
<tr>
<th>Sample size</th>
<th>Control</th>
<th>MCI</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF Aβ$_{40}$/nM</td>
<td>1.4(5)</td>
<td>1.6(7)</td>
<td>1.4(5)</td>
</tr>
<tr>
<td>CSF Aβ$_{42}$/nM</td>
<td>0.20(8)</td>
<td>0.2(1)</td>
<td>0.12(6)</td>
</tr>
<tr>
<td>Plasma Aβ$_{40}$/pm</td>
<td>60(20)</td>
<td>60(20)</td>
<td>60(10)</td>
</tr>
<tr>
<td>Plasma Aβ$_{42}$/pm</td>
<td>30(30)</td>
<td>20(10)</td>
<td>20(20)</td>
</tr>
</tbody>
</table>

Table 1.1: Levels of Aβ$_{40}$ & Aβ$_{42}$ in the cerebrospinal fluid (CSF) and blood plasma for cognitively healthy patients (Control), mild cognitive impairment (MCI), and Alzheimer’s disease (AD) patients. [35]

Other than in Alzheimer’s disease, Aβ has been seen to accumulate in inclusion-body myositis, an inflammatory muscle disease leading to muscular degeneration [36;37].

1.2.1 Production of Amyloid-β

Aβ is produced by the cleavage of the amyloid precursor protein (APP) by β-secretase (BACE-1), γ-secretase and ε-secretase producing varying lengths of Aβ’s [38;39]. Alternatively, APP can be cleaved by α-secretase to produce p3 (Aβ$_{17-40}$/42). The cleavage occurs within the Aβ region of APP which prevents Aβ from being produced [40;41]. Figure 1.4 shows an overview of the process.

APP is a transmembrane protein of mass 110 kDa to 135 kDa. The variations in mass arise from alternative splicing and post-translational modifications. Alternative splicing produces mainly three different APP’s (695, 751 & 770 amino acids) [42].

The first cleavage is by β-secretase between Met$_{596}$ and Asp$_{597}$ [43]. The cleavage is external to the cell’s plasma membrane to which β-secretase is bound [44]. It is this cleavage from which Aβ gets its name.

The second cleavage is by ε-secretase which produces two lengths of Aβ, 48 & 49 residues in length [39;45].
Finally, $\text{\(A\beta_{48/49}\)}$ is cleaved successively by $\gamma$-secretase, releasing tripeptides, i.e.

$$
\text{\(A\beta_{49}\)} \xrightarrow{\text{-ITL}} \text{\(A\beta_{46}\)} \xrightarrow{\text{-IVV}} \text{\(A\beta_{43}\)} \xrightarrow{\text{-IAT}} \text{\(A\beta_{40}\)} (1.1)
$$

to produce $\text{\(A\beta_{40}\)}$, and

$$
\text{\(A\beta_{48}\)} \xrightarrow{\text{-VIT}} \text{\(A\beta_{45}\)} \xrightarrow{\text{-TVI}} \text{\(A\beta_{42}\)} (1.2)
$$

to produce $\text{\(A\beta_{42}\)}$\textsuperscript{[39]}. $\gamma$-secretase is membrane bound and the cleavage occurs within the membrane\textsuperscript{[46]}. This suggests that longer variants of $\text{\(A\beta\)}$ may exist on the membrane, and only when sufficiently short can they detach into the CSF (Cerebrospinal fluid). It is possible that these are involved in the initial aggregation.

Alternatively, $\alpha$-secretase (ADAM10) can cut the APP within the $\text{\(A\beta\)}$ region, producing $\text{\(p3\)}$\textsuperscript{[41]}. The cleavage occurs between LYS\textsubscript{16} and LEU\textsubscript{17} in the $\text{\(A\beta\)}$ region\textsuperscript{[47]}.

### 1.2.2 Transport of Amyloid-$\beta$

$\text{\(A\beta\)}$ may be moved across the blood brain barrier by $\text{\(A\beta\)}$-pumps, such as ABCB1 (ATP (Adenosine triphosphate binding) binding cassette (ABC), subfamily B, number 1)\textsuperscript{[48]}. Whether toxic oligomers form in the brain or elsewhere, such as the muscles...
(as in sporadic inclusion-body myositis) and then act as a template for their further production in the brain is an open question.

Studies have shown that neurons may internalise Aβ using various lipid raft associated receptors[49].

1.2.3 Structure of Amyloid-β

Aβ is peptide, typically of 40 or 42 amino acids in length and is thought to be the culprit in Alzheimer’s disease. The primary structure of Aβ is shown in table 1.2. The amino acids are joined by peptide bonds forming the backbone, with the sidechains determining the amino acid’s properties. Aβ_{40} has a mass of 4330.9 Da, and Aβ_{42} of 4515.1 Da. In solution, Aβ has a hydrodynamic radius of 0.9(1) nm[50].

In aqueous solution (pH 7), the majority of the Aβ is found to have a median net electrostatic charge of −3 e, however there is further evidence for a −4 e charged state and a very small population of Aβ in a −2 e charged state[51].

Aβ has three histidines (his_{6}, his_{13} & his_{14}) which are good ligands for binding Cu^{2+}. The acid dissociation constant (pK_a) of histidine is approximately 6. When Cu^{2+} binds to amino acids, it has the effect of lowering the pK_a of a nearby amide group into physiological range[52].

Aβ has one tyrosine (tyr) with an absorbance of 1280 cm^{-1} M^{-1} at 280 nm. The tyrosine absorbance may be used to quantify the amount of the peptide for experiments.

<table>
<thead>
<tr>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP⁻</td>
<td>ALA</td>
<td>GLU⁻</td>
<td>PHE</td>
</tr>
<tr>
<td>16</td>
<td>20</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>LYS⁺</td>
<td>LEU</td>
<td>VAL</td>
<td>PHE</td>
</tr>
<tr>
<td>31</td>
<td>35</td>
<td>40</td>
<td>(41 42)</td>
</tr>
</tbody>
</table>

Table 1.2: The primary structure of Aβ_{40} & Aβ_{42}. Colour scheme: AGILPV - Amino acids with non-polar sidechains, FYW - Aromatic amino acids, CM - Sulfur containing amino acids, DENQRHSTK - Amino acids with polar sidechains (the charged residues are denoted with + or −, respectively.)
Of the two most common forms of Aβ, Aβ_{42} has been found to be more neurotoxic and aggregate more rapidly\cite{53}. Aβ_{42} contains two extra C-terminal amino acids isoleucine (ILE) and alanine (ALA) which make the peptide more hydrophobic\cite{8}. Aβ_{43} has a further polar C-terminal threonine (THR) and has been found to have a even higher propensity to aggregate and to be more neurotoxic than Aβ_{42}\cite{54}.

Aβ_{40} has been shown to precipitate in vitro, for concentrations greater than 14 µM\cite{55}, therefore the concentration for oligomerisation is less than this value.

**Secondary & Tertiary Structure**

In aqueous solution Aβ is thought to be mostly unstructured, in a flexible collapsed coil\cite{50}.

A structure has also been found by NMR (nuclear magnetic resonance) when in an aqueous solution of fluorinated alcohols\cite{56}. Figure 1.5 shows there are two α-helical regions, linked by a more flexible region between them (approximately residues 27 to 29). This causes the side chains to point away from the core of the helix. The main differences between the NMR structures found were small fluctuations in the angle between the helical sections and the positions of the ends of the peptide chain. They were both quite varied suggesting that they are unaffected by structural interactions. The aqueous fluorinated alcohol solution was chosen such that Aβ would adopt the helical structure and so may not reflect in vivo conditions\cite{56}. Aβ

![Figure 1.5: Conformation of Aβ_{42} in an aqueous solution of fluorinated alcohols from the Protein Data Bank (PDB). The thicker red part shows the α-helix structure. The N-terminus is on the right of the diagram. (PDB ID: 1IYT)\[56\]](image)
in membranes is also thought to be in an $\alpha$-helical conformation$^{[57]}$. In amyloid plaques it has a $\beta$-sheet conformation. Within a membrane, conformational change may be difficult as the hydrogen bonds that hold the core $\alpha$-helix shape would need to be broken in the apolar environment of the membrane.

**Quaternary Structure**

**Fibrils**  NMR has been used to find the to structure of A$\beta$ fibrils (see Fig. 1.6, in vitro). A$\beta$ is layered in a $\beta$-sheet formation. There are two $\beta$-sheet regions, residues 18-26 and 31-42. The N-terminus (residues 1-16) could not be located using NMR which suggests that they do not hold a relatively fixed position in space. The stagger between the layers of the two $\beta$-sheets may vary.

In fibres, these $\beta$-sheet structures pack together as shown in figure 1.7. Longer fibrils have been observed using electron cryomicrography (see Fig. 1.8) by embedding the fibrils in ice$^{[61]}$. The fibrils were shown to be helical in shape$^{[61]}$.

Alternative conformations have also been shown. In Alzheimer’s disease patients, fibrils consist of mostly A$\beta_{40}$, such as in figure 1.9. However, the number of clinical...
(a) Two-fold symmetry (2LMO)  
(b) Three-fold symmetry (2LMP)

Figure 1.7: Cross-section of Aβ_{40} in fibrils determined via NMR and electron microscopy. (PDB ID: 2LMO & 2LMP)

Figure 1.8: Electron micrograph of an Aβ_{40} fibril embedded in ice (top). Projection of the 3D reconstructed fibril (middle). Side-view surface rendering of the reconstructed fibril (bottom). Copyright 2015 National Academy of Sciences, USA.

(a) Coloured by β-sheet or loop  
(b) Coloured by Aβ monomer

Figure 1.9: Aβ_{40} fibril from Alzheimer’s disease brain, all residues located. (PDB ID: 2M4J)
studies of fibrils from patients is too low to determine whether the fibril properties are fundamentally different from that in vitro. Understanding the role of fibrils, their formation and evolution is further complicated by the purity and composition of the fibrils. There may be modifications to peptides in vivo that alter the way in which fibres are formed, such as oxidative damage, truncation \[^{[63]}\] or binding to metals.

The dissociation constant of A\(\beta\) from A\(\beta\)\(_{40}\) fibrils was measured to be 0.6 \(s^{-1}\) and from A\(\beta\)\(_{42}\) fibrils to be \(10^{-2} \, s^{-1}\) \[^{[64]}\]. The rate of disaggregation into soluble species was measured by a further group to be 9(3) \(s^{-1}\) \[^{[65]}\].

Super resolution images of fibres have been observed on HeLa cells showing different morphologies \[^{[66]}\].

**Oligomers**

Oligomers are thought to be the cause of neurotoxicity in Alzheimer’s disease and have been shown to affect long term potentiation in the hippocampus \[^{[67]}\]. Though their mechanism of action is unclear with multiple hypotheses proposed \[^{[68]}\], such as interactions with receptors, channel formation, and oxidative stress. It is possible to detect femtomolar concentrations of A\(\beta\) oligomers in the CSF of patients \[^{[69]}\].

Molecular dynamics simulations suggest that the structure of the oligomers is quite polymorphic \[^{[70]}\]. It has been found that A\(\beta\)\(_{40}\) (10 \(\mu\)m) rapidly forms spherical oligomers which then slowly convert into fibrils \[^{[71]}\].

Identifying the structure and formation of toxic oligomers is fundamental to understanding the molecular mechanisms behind Alzheimer’s disease. However, just being able to reliably produce toxic species of oligomer would allow for drug discovery and optimisation.

### 1.2.4 The Evolution of Amyloid-\(\beta\) and Murine Amyloid-\(\beta\)

The A\(\beta\) sequence in the APP arose approximately \(5 \times 10^8\) years ago in the common ancestor of jawed vertebrates, and the \(\beta\)-cut site approximately \(4 \times 10^8\) years ago in the common ancestor of tetrapods and lobe-finned fish \[^{[72]}\]. A\(\beta\) is well conserved across a variety of species (see Fig. 1.10).
Interestingly in non-human primates, the APP sequence is homologous to that in humans with developing Aβ plaques. However, they do not appear to have any neurodegeneration related to Alzheimer’s disease\textsuperscript{[73]}. It has been suggested that this is due to structural variation in multimeric Aβ\textsuperscript{[74]}.

Of further interest is the murine (mouse/rat) sequence of Aβ. The sequence has only three mutations, all in the N-terminal region: arg\textsubscript{5} → gly, tyr\textsubscript{10} → phe, and his\textsubscript{13} → arg. However, murines do not show the Aβ deposits\textsuperscript{[75,76]}. The positively charged arginine is moved from residue 5 to 13, removing a histidine. The loss of a histidine is thought to change the binding of Aβ to metal cations, such as zinc and copper\textsuperscript{[76]}. Therefore, identifying differences between human and murine Aβ, may give insight into the mechanism of Aβ aggregation.

There are a number of familial mutations in and around the Aβ sequence in the human genome that further increase the probability of getting Alzheimer’s disease\textsuperscript{[77–79]}.
1.3 Toxicity and Aggregation

The mechanism of the toxicity of oligomers in Alzheimer’s disease is currently unknown. However, there are many proposed mechanisms\cite{80}, these include interacting with receptors on and in the cell causing them to malfunction, leading to cell death\cite{80,81}. Alternatively, Aβ oligomers may form pores in the membrane of the cells, upsetting ion homeostasis leading to cell death\cite{82,83}.

1.3.1 Interactions with Receptors

Extracellular Aβ oligomers are thought to bind to receptors on the cell surface interfering with their downstream pathways, see figure 1.11\cite{80}. However, there have not been any receptors identified that are highly specific to Aβ oligomers, nor have the signalling mechanisms been fully elucidated for those that have been found to interact\cite{80}.

Figure 1.11: Putative Aβ oligomer binding receptors and potential signalling pathways thought to be involved in the synaptotoxicity of Aβ oligomers. In this model there are two general categories of signalling pathways: those that signal via tyrosine kinase (tyr k, fyn, and c-Abl), and those that hyperactivate CDK5/glycogen synthase kinase (GSK3β).\cite{81} Copyright 2015 National Academy of Sciences, USA.
1.3.2 Channel Formation in Cell Membranes

It has been proposed that Aβ oligomers form pores in the cell membrane\cite{84,85}. These pores may then upset the membrane’s permeability and the cell’s ion homeostasis. It has been shown that Aβ may form Ca^{2+} pores\cite{86,87}. These Ca^{2+} channels have been shown to be blocked by Zn^{2+}\cite{88}. Ca^{2+} is important in neurotransmission as an influx of Ca^{2+} causes vesicles to release neurotransmitters into the synaptic cleft. In physiology, Ca^{2+} flows into the cell via voltage-gated Ca^{2+} channels, which activate when an action potential reaches them.

1.3.3 Oxidative Stress

One of the characteristics of brains in Alzheimer’s disease patients is oxidative stress\cite{89}. This is proposed to be caused by redox chemistry of metal ions with Aβ\cite{90,91}. Aβ has been shown to catalyse the reduction of Cu^{2+} & Fe^{3+} to Cu^{+} & Fe^{2+}, producing hydrogen peroxide, which may then lead to the Fenton reaction

\begin{align*}
\text{Me}^{n+} + \text{H}_2\text{O}_2 & \longrightarrow \text{Me}^{(n+1)^+} + \text{HO}^- + \text{OH}^- \quad (1.3) \\
\text{Me}^{(n+1)^+} + \text{H}_2\text{O}_2 & \longrightarrow \text{Me}^{n+} + \text{HOO}^- + \text{H}^+ \quad (1.4)
\end{align*}

producing oxygen-radical species\cite{92,93}. This redox cycling, which produces ROS (reactive oxygen species) may then lead to oxidative damage of surrounding material\cite{94}. The damage to Aβ itself, has been used to determine the coordination modes to the redox active Aβ·Cu coordinations\cite{95}. It has been proposed that the purpose of Aβ may be as part of a copper regulatory system that reduces oxidative stress (see Fig. 1.12)\cite{96}.

Also, it has been proposed that this redox chemistry may turn Tyr_{10} into a tyrosyl radical allowing it to cross-link with a tyrosine on another Aβ, forming a covalently linked dimer\cite{97}.
Figure 1.12: Proposed mechanisms for the effects of metal ions in the brain and their effects on Aβ production and aggregation. SOD (superoxide dismutase) is an anti-oxidant which reduces oxidative stress (ox-stress). This is thought to be down-regulated as intracellular (IC) levels of copper are decreased. Copper is exported from cells when APP is expressed, increasing the levels in the CSF (cerebrospinal fluid) forming a negative feedback loop. In Alzheimer’s disease the feedback loop is insufficient to prevent APP expression and thus Aβ production & accumulation. Therefore it is thought that an intracellular copper deficiency may cause the over-production of Aβ and then Alzheimer’s disease. Copyright (c) 2005 John Wiley & Sons. Metals and Amyloid-β in Alzheimer’s disease, Christa J. Maynard, et al., International Journal of Experimental Pathology, 86:3. [96]

1.4 Therapeutic Strategies Against Alzheimer’s Disease

There are currently 4 drugs that help with the symptoms of Alzheimer’s disease (see Section 1.1.2). However, there are no drugs that prevent or cure Alzheimer’s disease. In the US, there are approximately 1500 completed and ongoing clinical trials of which 64 are completed with results from phase 3 or 4[98].

In clinical trials based on the Aβ cascade hypothesis, there are three main approaches: preventing the production of Aβ, inhibiting the formation of oligomers, and promoting the clearance or degradation of Aβ. However, there have not been any successful phase 3 trials. Furthermore, some trials have been terminated as the effects of the drugs were worse than the placebo. This may be because there is a lack of understanding of the molecular mechanisms behind Alzheimer’s disease.
1.4.1 Inhibiting Aβ Production

The therapeutic strategies applied to inhibiting the production of Aβ are to prevent the cleavage of Aβ from being completed by preventing cleavage by β-secretase or γ-secretase, or alternatively to promote the cleavage by α-secretase to produce p3 instead.

α-Secretase Modulators These increase the cleavage by α-secretase, cutting APP in the Aβ region to form the shorter peptide p3.

**Acitretin** (Soriatane, Neotigason, RO 101670. Phase 2, Completed) Increases expression of ADAM10 (α-secretase). In the trial, α-secretase-derived APP was increased by 25%, but β-secretase-derived APP was unchanged. It was expected to decrease as the alternative cleavage pathway was promoted. Aβ42 levels were unchanged, though Aβ40 levels were not measured. The trial was completed in 2011.[99]

β-Secretase Inhibitors The development of β-secretase inhibitors has been difficult due to problems with brain access, cell penetration, and oral bioavailability[100]. Furthermore, neuregulin-1 is also a substrate of β-secretase. Neuregulin-1 is essential for the normal development of the nervous system and the heart, also effecting the myelination of neurons[101;102].

**LY2886721** (Phase 2, Failed) Terminated before completion due to abnormal liver biochemical tests[103]. The study was terminated in mid 2013 however the results are as of yet still unpublished. LY2886721 was a derivative of LY2811376, the first β-secretase inhibitor.

**MK-8931** (Phase 2/3, Ongoing) The phase I trial showed a dose dependent decrease of Aβ in the CSF by up to 92% and was completed in Jun 2012, but the results are still unpublished[104].

γ-Secretase Inhibitors Development of γ-secretase inhibitors has been most problematic as inhibiting γ-secretase also interferes with Notch signalling. Notch has many physiological functions in regulating cell growth and catabolising
membrane protein fragments. This in turn causes serious side effects such as an increased risk of skin cancers and infections.\cite{105}

**Semagacestat** (LY450139 Dihydrate, hydroxylvaleryl monobenzocapro-lactam. Phase 3, Terminated) Terminated before completion due to increased weight loss, increase in skin cancers and infections. Patients in the semagacestat groups showed greater worsening of dementia than the placebo group.\cite{106;107}

**Avagacestat** (BMS-708163. Phase 2, Failed) Terminated before completion as the required efficacy was not observed\cite{108}. Avagacestat was designed to selectively avoid Notch interference\cite{109} though others disagree\cite{110}. The study was terminated in Nov 2012 however the results are still unpublished.

**Tarenflurbil** (Flurizan, R-flurbiprofen, MPC-7869, (R)-2-(3-Fluoro-4-phenyl-phenyl) propanoic acid. Phase 3, Failed) No improvement in cognitive ability or ability to carry out daily activities\cite{111}. Thought to lower Aβ\textsubscript{42} and act as an anti-inflammatory through allosteric modulation of γ-secretase\cite{112;113}.

**NIC5-15** (Pinitol, D-Pinitol. Phase 2, Ongoing) Thought to modulate γ-secretase to reduce Aβ production whilst sparing the cleavage of Notch.

### 1.4.2 Inhibiting Oligomerisation

The development of inhibitors of Aβ oligomerisation has been challenging due to the unknown structure of Aβ oligomers and lack of a formation mechanism.

**PBT2** (Phase 2, Failed) A derivative of clioquinol (CQ), and is thought to reduce Aβ aggregation by interfering with its interactions with copper and zinc. There was no evidence for the reduction of amyloid plaques or relative improvement in cognition and function. However, there was a trend showing hippocampal brain volume was preserved. This aspect of the study is still ongoing.\cite{114} CQ is used in the studies in section 3.6.
ELND005 (AZD-103, Scylloinositol, cyclohexane-1,2,3,4,5,6-hexol. Phase 2, Completed) Thought to neutralise small toxic oligomers and prevent them aggregating\(^{[115;116]}\). Aβ levels in a CSF were reduced in the mild Alzheimer’s disease subgroup. ELND005 had serious adverse affects.\(^{[117]}\) The study is being continued for the mild Alzheimer’s disease subgroup.

1.4.3 Promoting Clearance or Degradation

This is the largest category of drugs, attempting to intervene in the amyloid hypothesis, typically applying either a passive immunotherapy (giving the patient antibodies\(^*\)) or an active immunotherapy (giving the patient a vaccine).

**Solanezumab** (LY2062430. Phase 3, Failed) No significant improvement in worsening of dementia, compared to the placebo group\(^{[118]}\). There was decreased unbound A\(\beta\)\(_{40}\) in the CSF, but increased levels of A\(\beta\)\(_{42}\)\(^{[119]}\). Trials are still ongoing testing its effect on familial mutations and mild Alzheimer’s disease patients.

**Gantenerumab** (RO4909832, RG1450. Phase 3, Ongoing) Human IgG1 (Immunoglobulin G subclass 1) antibody with specific binding to A\(\beta\) in plaques.\(^{[120]}\)

**Simvastatin** (Phase 4, Complete) Has been shown to lower levels of A\(\beta\) in the brain and CSF in animals models\(^{[121]}\). Simvastatin improved some measures of cognitive function, but did not decrease CSF levels of A\(\beta\)\(_{42}\)\(^{[122]}\).

**Bapineuzumab** (Phase 3, Failed) IgG1 antibody that binds soluble and fibrillar A\(\beta\). Six phase 3 trials, four of which were terminated before completion. The four later trials were terminated after there was no improvement seen in the initial two trials. No treatment differences in CSF levels of A\(\beta\).

**CAD-106** (Phase 2, Complete) A\(\beta\) vaccine recognising A\(\beta\)\(_{3–6}\), binding to monomers and oligomers\(^{[123]}\). Results not yet published.

**AN1792** (AIP 001. Phase 2, Failed) Vaccine recognising full length A\(\beta\). The follow up to the trial showed less decline in cognitive measures, but also decrease in

\(*\)Drugs ending in -mab are monoclonal antibodies (mAb).
brain volume. Dosing was halted in the trial after some patients developed brain inflammation.[124]

**Affitope AD02** (Phase 2, Failed) Aβ vaccine recognising Aβ6. In this trial their ‘placebo’ (AD04) showed more stabilisation than AD02.[125]

**BAN2401** (Phase 2, Ongoing) Humanized IgG1 monoclonal antibodies that binds selectively to large, soluble Aβ.

**Crenezumab** (MABT5102A, RG7412. Phase 2, Ongoing) Monoclonal antibody against Aβ.

**Octagam** (Intravenous Immunoglobulin, NewGam. Phase 2, Failed) Uses naturally occurring polyclonal autoantibodies against Aβ. The CSF levels of Aβ were unchanged with no cognitive or functional benefit shown.[126]

**Ponezumab** (PF-04360365. Phase 2, Failed) Passive immunotherapy treatment against Aβ40. No change in brain or CSF Aβ burden.[127]

**Vanutide Cridificar** (ACC-001, PF-05236806. Phase 2, Failed) Vaccine against Aβ42. Designed to avoid the safety concerns of AN1792. No apparent clinical benefit seen.[128]

1.5 The Role of the Membrane

The role of the membrane is implicit in the formation of Aβ from APP given that Aβ is part of the transmembrane region of APP, and that the secretases that cleave it are also membrane bound[129]. The N-terminus of Aβ consists of predominantly hydrophobic residues which have been shown to interact with and disrupt membranes[130]. However, it is oligomers that are thought to be neurotoxic rather than monomers or fibrils.

Aβ oligomers have been shown to cause cognitive loss in rats[131]. Oligomers have been shown to affect synaptic plasticity, whereas undissolved fibrils and monomers do not[132]. These oligomers increase the conductance of the membrane, possibly by forming Ca2+-permeable channels[133]. This increase in conductance is
also seen for other amyloid forming proteins\textsuperscript{[134]}. Even picomolar concentrations of A\textsubscript{\textbeta}40 or A\textsubscript{\textbeta}42 oligomers increase intracellular Ca\textsuperscript{2+} in astrocytes. However, this was not observed in neurons\textsuperscript{[135]}. This may be due to differences in the membrane composition, such as the decreased level of cholesterol in neurons as compared to astrocytes\textsuperscript{[136]}. Cholesterol levels in neurons have been previously linked to Alzheimer’s disease\textsuperscript{[137]}. The early stages of Alzheimer’s disease have been thought to be linked with synaptic loss rather than neuronal death\textsuperscript{[138]}. With regards to metals, Cu\textsuperscript{2+} was found to destabilise lipid bilayers, but the membrane could be stabilised by the addition of A\textsubscript{\textbeta}42\textsuperscript{[139]}.

On the cell membrane there are regions known as lipid rafts, which are microdomains containing sphingomyelin and cholesterol, and are enriched with gangliosides (sialic acid-containing glycosphingolipids)\textsuperscript{[140–142]}. The ganglioside GM1 has been shown to bind to A\textbeta in vivo\textsuperscript{[143]} and to catalyse A\textsubscript{\textbeta}40 aggregation\textsuperscript{[144]}, possibly with the A\textbeta·GM1 complex acting as a seed\textsuperscript{[145]}. It has been proposed that GM1 sequesters A\textbeta after its cleavage\textsuperscript{[146]}. The A\textbeta fibrils formed in the presence of GM1 were of a different morphology to those without GM1\textsuperscript{[144]}.

GM1, when in aqueous solutions of 0.8 \textmu M to 156 \textmu M, spontaneously forms micelles of 168(4) GM1 units\textsuperscript{[147]}. The \textit{K}_d (equilibrium dissociation constant) of A\textsubscript{\textbeta}40 (with a \textit{tyr}\textsubscript{10} \textit{→} \textit{trp} mutation, to increase intrinsic fluorescence) was found to be 1.4 \textmu M\textsuperscript{[148]}. Using an N-terminal dye label, the \textit{K}_d was measured to be 0.11(2) \textmu M\textsuperscript{[149]}. Using NMR the \textit{K}_d was measured to be approximately 5 \textmu M\textsuperscript{[150]}. This suggests that the \textit{K}_d of A\textbeta to GM1 micelles is in the hundreds of nanomolar to low micromolar regime. For monomeric GM1 the \textit{K}_d is approximately 1 mM\textsuperscript{[150]}.

On micelles and membrane-mimics, A\textbeta has been shown to adopt an \alpha-helical conformation, however the regions that form \alpha-helices are strongly dependent on experimental conditions\textsuperscript{[151]}. NMR has been used to determine the relative hydrophobicity of the environment of the residues of A\textbeta to determine their position relative to the micellar-water interface\textsuperscript{[152]}. Residues 1–11 are in solution, 12–25 are in the membrane, 26–30 are in solution, 31–36 are in the membrane, 37–38 are in solution, and 39–40 are in the membrane\textsuperscript{[152]}. On GM1 micelles, A\textbeta forms two \alpha-helical segments from his$_{14}$-val$_{24}$ and ile$_{31}$-val$_{36}$\textsuperscript{[152;153]}. The positions of
the residues in a GM1 micelle have been determined by applying FRET (Förster resonance energy transfer) techniques, to determine the distance of the mutated residues from the micellar centre (see Fig. 1.13). Furthermore, it has been shown

Figure 1.13: Illustration of the position of Aβ in GM1 micelles, by using FRET to measure the distance of different amino acids (C12, C26) from the micellar centre\textsuperscript{[154]}. Reprinted from Biophysical Journal, 99:5, I. Mikhalyov, et al., Designed Fluorescent Probes Reveal Interactions between Amyloid-β(1–40) Peptides and GM1 Gangliosides in Micelles and Lipid Vesicles, 1510–1519, Copyright 2010, with permission from Elsevier.

that once Aβ is bound, it was able to undergo conformational change from α-helix to β-sheet with cholesterol\textsuperscript{[149,155]}. These may act a seed for aggregation and suggests that cholesterol is a risk factor.

Sodium dodecyl sulfate (SDS) micelles have also been used as a membrane mimic, although its composition is of less physiological relevance\textsuperscript{[156,157]}.

The kinetics of binding of Aβ\textsubscript{40} to a supported lipid bilayer of the phosphatidylcholine phospholipid POPC and the phosphatidylglycerol phospholipid POPG was determined,

\[
{\text{Membrane binding site}} + \text{Aβ}_{40} \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} \{\text{Membrane binding site}\} \cdot \text{Aβ}_{40},
\]

(1.5)

where \(k_{\text{on}}\) was \(4.3(3) \times 10^3\text{M}^{-1}\text{s}^{-1}\), and \(k_{\text{off}}\) was \(<2 \times 10^9\text{s}^{-1}\)[158].

\textsuperscript{*}Note the units of \(k_{\text{on}}\) given are incorrect as it is reaction between components on a surface and in solution, whereas the units are for both components being in solution.
1.6 The Role of Metal Ions

Transition metals, in particular Zn$^{2+}$, Cu$^{2+}$ & Fe$^{2+}$, have been shown to be connected to multiple neurodegenerative diseases, including Alzheimer’s disease$^{[159]}$. Aβ is an intrinsically disordered peptide fluctuating amongst different transient structures$^{[160]}$. Upon metal binding, the structure of Aβ is thought to become more defined. However, unlike typical metalloproteins, Aβ has several binding modes to the metal ions and a much lower binding affinity.

The equilibrium dissociation constant ($K_d$) of Aβ to Zn$^{2+}$ has been measured to be in the range of 1 µm to 100 µm, whereas values the $K_d$ for Cu$^{2+}$ range from 10 pm to 100 nm$^{[161;162]}$. For comparison, the $K_d$ of HSA (human serum albumin), a common protein in the CSF to Zn$^{2+}$ is approximately 30 nm and for Cu$^{2+}$ is approximately 1 pm$^{[163]}$.

HSA is the most common protein in blood plasma with many known roles$^{[164]}$. In the CSF, HSA is at concentrations of approximately 3 µm$^{[165;166]}$, significantly higher than the concentrations of Aβ. This, combined with its stronger $K_d$’s for Zn$^{2+}$ (~30 nm) & Cu$^{2+}$ (~7 pm)$^{[167]}$, suggest that for these ranges of the metal ion-Aβ $K_d$, monomeric Aβ cannot compete with HSA for binding to these metal cations, unless HSA is already saturated with metal ions. The concentrations of Zn$^{2+}$ in the CSF is 160(40) nm ($n = 52$)$^{[168]}$, whereas Cu$^{2+}$ is 220(90) nm ($n = 113$)$^{[169]}$, well below the concentration of HSA. Therefore, if, as in the Aβ metal hypothesis that Aβ oligomerisation is caused by metals, where and when can this happen?

During the process of synaptic transmission Zn$^{2+}$ and Cu$^{2+}$ are transiently released into the synaptic cleft (see Fig. 1.14)$^{[170]}$. Zn$^{2+}$ concentrations may reach 200 µm to 300 µm in the synaptic cleft$^{[172]}$ when it is released from synaptic vesicles into the cleft$^{[173]}$, whereas Cu$^{2+}$ may reach concentrations of approximately 15 µm$^{[174;175]}$. These values are above the range of the $K_d$’s determined. Is it here where the oligomers first form resulting in Alzheimer’s disease, after binding to metal ions? The $K_d$ suggests they could bind to metals at these equilibrium concentrations. However, due to the transient nature of synaptic release only the kinetics can determine whether they do bind.
It has also been shown that increased synaptic transmission activity correlates to an increase in release of Aβ via endocytosis-associated mechanisms.\textsuperscript{176}

### 1.6.1 Metal Ions and Alzheimer's Disease

Studies of Aβ plaques have shown elevated levels of Zn\(^{2+}\), Cu\(^{2+}\) and Fe\(^{3+}\), with dry weight concentrations of approximately 70 µM, 340 µM and 350 µM, respectively, in healthy brains.\textsuperscript{177} Whereas in brains with Alzheimer’s disease the concentrations were elevated to approximately 400 µM, 1 mM and 1 mM for Zn\(^{2+}\), Cu\(^{2+}\) and Fe\(^{3+}\), respectively.\textsuperscript{177} In the CSF, the concentrations of zinc and copper are nanomolar (see table 1.3), but are elevated in Alzheimer’s disease patients. It has been hypothesised that Alzheimer’s disease is caused by the dishomeostasis of Zn\(^{2+}\) in the brain.\textsuperscript{179}

There has been shown to be an overlap between the areas of the brain rich in glutamatergic receptors, free vesicular zinc and Aβ plaques, in certain APP transgenic mice.\textsuperscript{180}
Table 1.3: Concentration of metal ions in the CSF in control patients ($n = 15$) and patients with Alzheimer’s disease ($n = 21$).

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Control</th>
<th>Alzheimer’s disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc / $\mu$M</td>
<td>$0.08(5)$</td>
<td>$0.1(1)$</td>
</tr>
<tr>
<td>Copper / $\mu$M</td>
<td>$0.16(3)$</td>
<td>$0.3(2)$</td>
</tr>
<tr>
<td>Iron / $\mu$M</td>
<td>$4(1)$</td>
<td>$4.3(7)$</td>
</tr>
</tbody>
</table>

Metal ions have been shown to enhance the fibrillisation rate of A$\beta$, and metal chelators can reverse the effect\cite{76,181}. However, metal-induced aggregates are mainly non-fibrillar\cite{182,183}, but small metal-induced aggregates may act as seeds for the A$\beta$ fibrillisation process\cite{184,185}. It has also been proposed that the different coordinations of A$\beta$ with Cu$^{2+}$, or Zn$^{2+}$ are related to the different morphologies and toxicities of aggregates\cite{186}.

It was also shown that Cu$^{2+}$ or Zn$^{2+}$ binding to fibrils does not induce structural changes in the $\beta$-sheet structure of the hydrophobic core residues\cite{187,188}. However, for Zn$^{2+}$ there was some evidence of breaking the salt bridge between ASP$_{23}$ and LYS$_{28}$.

Coordination of Cu$^{2+}$ to fibrils is similar to that of monomeric A$\beta$, with a $K_d$ of approximately $10\text{ pM}$\cite{189}.

1.6.2 Zinc

Zn$^{2+}$ coordination typically involves four or six ligands. It is believed that all three histidines and glutamate$_{11}$ are involved in the coordination of Zn$^{2+}$ to A$\beta$\cite{190,195}. It has been shown that A$\beta$ may coordinate with up to three Zn$^{2+}$’s\cite{186}. It has been found possible for intermolecular His-Zn$^{2+}$-His bridges to be formed between two A$\beta$’s\cite{196}. This allows for Zn$^{2+}$ assisted dimers to form, but also for the transfer of Zn$^{2+}$ (see Fig. 1.15)\cite{197}. Zn$^{2+}$ has been shown to stabilise oligomers on short timescales\cite{198}.

The $K_d$ of Zn$^{2+}$ binding to A$\beta$ has been measured by many groups (see table 1.4), with values ranging from 1 $\mu$M to 300 $\mu$M. The methods used are described below:

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Figure 1.15: Schematic of the exchange of Zn$^{2+}$ between two Aβ’s, and transient dimer formation. Copyright (c) 2009. Copper and Zinc Binding to Amyloid-β: Coordination, Dynamics, Aggregation, Reactivity and Metal-Ion Transfer, P. Faller, ChemBioChem.

$^{65}$Zn$^{2+}$ displacement. Aβ is immobilised onto a surface and immersed in a solution of Zn$^{2+}$ and $^{65}$Zn$^{2+}$. The solution is then washed off and the radioactivity of the bound Aβ-$^{65}$Zn$^{2+}$ is measured, so that the proportion of Aβ bound to Zn$^{2+}$ can be calculated$^{[199;200]}$. This method will underestimate the $K_d$ if the dissociation rate of Aβ-$^{65}$Zn$^{2+}$ is close to or faster than the washing time.

Tyr fluorescence. Tyr$_{10}$ has an intrinsic fluorescence which is enhanced when Aβ binds to Zn$^{2+}$$^{[201]}$. Zn$^{2+}$ is then titrated into the solution of Aβ and the increase in fluorescence is fitted to a binding curve, obtaining the $K_d$. However, the intrinsic fluorescence of tyr is very weak with an absorbance $\varepsilon$ of 1280 m$^{-1}$ cm$^{-1}$ and quantum yield $\phi$ of 0.14$^{[202]}$. Thus, in order to obtain sufficient signal to noise for the measurement, micromolar concentrations of Aβ are required, possibly leading to aggregation.

ITC. Isothermal titration calorimetry is performed by measuring the power required to heat a reaction when the solution is kept at a constant temperature. The heat loss of the solution (known) and the energy given off by the reaction (unknown) is balanced against the input power (known). For a known heat loss, this allows for the determination of the Gibbs energy ($\Delta G$) of the reaction and thus the $K_d$ via

$$\Delta G = K_B T \ln(K_d)$$  \hspace{1cm} (1.6)
where $T$ is the temperature and $K_B$ is Boltzmann’s constant. Due to the small energy change of the reaction per molecule, high numbers of reactions and thus concentrations are required. The high concentrations (>10 µm) of Aβ & Zn$^{2+}$ are likely to induce rapid aggregation.

**Zincon competition**  Zincon binding to Zn$^{2+}$ ($K_d = 12.6$ µm) introduces a distinct absorption band at 620 nm ($\varepsilon_{\text{max}} = 23 200$ M$^{-1}$ cm$^{-1}$) which can be used to determine the concentration of Zincon·Zn$^{2+}$ by absorbance. Then by titration of Zincon into a solution of Aβ·Zn$^{2+}$, the $K_d$ of Aβ with Zn$^{2+}$ can be found relative to that of Zincon with Zn$^{2+}$ by the equilibrium of

\[
\text{Zincon·Zn}^{2+} + \text{Aβ} \rightleftharpoons \text{Zincon + Aβ·Zn}^{2+} \tag{1.7}
\]

reached. This requires the $K_d$ of Zincon·Zn$^{2+}$ to be known accurately, be similar to the $K_d$ of Aβ·Zn$^{2+}$ and for negligible free Zn$^{2+}$, i.e. the concentration of Aβ must be much greater than the $K_d$ of Aβ·Zn$^{2+}$ and the total concentration of Zn$^{2+}$. $10$ µm $\gg$ (7 µm to 14 µm). [203–205]

**Tyr fluorescence by Cu$^{2+}$ Competition**  Tyr$_{10}$ is quenched by paramagnetic Cu$^{2+}$. Therefore, by fluorescence of tyr$_{10}$ the concentration of Aβ·Cu can determined. Given that the binding sites are the same[206], the $K_d$ of Aβ·Zn relative to that of Aβ·Cu can be determined from the competition

\[
\text{Aβ·Cu + Zn}^{2+} \rightleftharpoons \text{Aβ + Cu}^{2+} + \text{Zn}^{2+} \rightleftharpoons \text{Cu}^{2+} + \text{Aβ·Zn} \tag{1.8}
\]

This requires an accurate determination of the $K_d$ of Aβ·Cu which varies in the literature from 10 pM to 100 nM (see §1.6.3).

**NMR**  In nuclear magnetic resonance, molecules are placed in a magnetic field, so that the magnetic quantum ($m_l$) energy levels of nuclei with an intrinsic magnetic moment (non-zero spin) are split, and they then try to align to the magnetic field. The unpaired nucleons are excited with a 90° radio-frequency pulse at the resonant frequency of the difference in the energy levels caused by the magnetic field. This
causes the magnetic moment of the spins to become perpendicular to the applied magnetic field and align the phases of the moments as they precess around the applied magnetic field vector. The signal obtained by NMR is from the spins realigning to the applied magnetic field emitting photons. However, the signal is reduced by the spins dephasing. The environment around the spins, i.e. caused by the surrounding electrons and other atoms in the molecule, perturbs the local magnetic field, and thus the frequency of photon given off and dephasing. These variations in the signal allow probing of molecular structure. Changes in the signal can be correlated to Zn$^{2+}$ binding to Aβ. However, to obtain sufficient signal for NMR high concentrations are required.
<table>
<thead>
<tr>
<th>$K_d/\mu m$</th>
<th>Aβ</th>
<th>[Aβ]/\mu m</th>
<th>Conditions</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2</td>
<td>Aβ40</td>
<td>20 mM Tris, pH 7.4</td>
<td>$^{65}$Zn$^{2+}$ displacement</td>
<td>[199]</td>
</tr>
<tr>
<td>3.2</td>
<td>Aβ40</td>
<td>~ 0.5</td>
<td>10 mM Tris, pH 7.4</td>
<td>$^{65}$Zn$^{2+}$ displacement</td>
</tr>
<tr>
<td>3.2</td>
<td>Aβ40</td>
<td>~ 0.5</td>
<td>10 mM Hepes, pH 7.4</td>
<td>$^{65}$Zn$^{2+}$ displacement</td>
</tr>
<tr>
<td>300</td>
<td>Aβ40</td>
<td>3</td>
<td>10 mM Tris, 100 mM NaCl, pH 7.4</td>
<td>Tyr fluorescence</td>
</tr>
<tr>
<td>57</td>
<td>Aβ42</td>
<td>3</td>
<td>10 mM Tris, 100 mM NaCl, pH 7.4</td>
<td>Tyr fluorescence</td>
</tr>
<tr>
<td>22(15)</td>
<td>Aβ16</td>
<td>~15</td>
<td>20 mM Tris, 100 mM NaCl, pH 7.4</td>
<td>ITC</td>
</tr>
<tr>
<td>71(5)</td>
<td>Aβ16</td>
<td>140</td>
<td>20 mM Tris, 100 mM NaCl, pH 7.4</td>
<td>ITC</td>
</tr>
<tr>
<td>10(8)</td>
<td>Aβ28</td>
<td>~15</td>
<td>20 mM Tris, 100 mM NaCl, pH 7.4</td>
<td>ITC</td>
</tr>
<tr>
<td>30(4)</td>
<td>Aβ28</td>
<td>140</td>
<td>20 mM Tris, 100 mM NaCl, pH 7.4</td>
<td>ITC</td>
</tr>
<tr>
<td>7(3)</td>
<td>Aβ40</td>
<td>10</td>
<td>20 mM Tris, 100 mM NaCl, pH 7.4</td>
<td>ITC</td>
</tr>
<tr>
<td>3(2)</td>
<td>Aβ40</td>
<td>70</td>
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<td>ITC</td>
</tr>
<tr>
<td>14(5)</td>
<td>Aβ16</td>
<td>10</td>
<td>20 mM HEPES, 100 mM NaCl, pH 7.4</td>
<td>Zincon competition</td>
</tr>
<tr>
<td>12(5)</td>
<td>Aβ28</td>
<td>10</td>
<td>20 mM HEPES, 100 mM NaCl, pH 7.4</td>
<td>Zincon competition</td>
</tr>
<tr>
<td>7(3)</td>
<td>Aβ40</td>
<td>10</td>
<td>20 mM HEPES, 100 mM NaCl, pH 7.4</td>
<td>Zincon competition</td>
</tr>
<tr>
<td>7(3)</td>
<td>Aβ42</td>
<td>10</td>
<td>20 mM HEPES, 100 mM NaCl, pH 7.4</td>
<td>Zincon competition</td>
</tr>
<tr>
<td>6.6(2)</td>
<td>Aβ28</td>
<td>10</td>
<td>10 mM HEPES, pH 7.2</td>
<td>Tyr fluo, Cu$^{2+}$ comp</td>
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<td>1.10(8)</td>
<td>Aβ28</td>
<td>10</td>
<td>10 mM Na Phosphate, pH 7.2</td>
<td>Tyr fluo, Cu$^{2+}$ comp</td>
</tr>
<tr>
<td>1.20(3)</td>
<td>Aβ40</td>
<td>50</td>
<td>10 mM Na Phosphate, pH 7.2</td>
<td>NMR</td>
</tr>
<tr>
<td>60(14)</td>
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<td>10 mM Tris, 100 mM NaCl, pH 7.4</td>
<td>Tyr fluorescence</td>
</tr>
<tr>
<td>184(30)</td>
<td>Aβ40</td>
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<td>100 mM Tris, 100 mM NaCl, pH 7.4</td>
<td>Tyr fluorescence</td>
</tr>
<tr>
<td>65(3)</td>
<td>Aβ40</td>
<td>4</td>
<td>20 mM HEPES, 100 mM NaCl, pH 7.4</td>
<td>Tyr fluorescence</td>
</tr>
<tr>
<td>91(16)</td>
<td>Aβ42</td>
<td>4</td>
<td>20 mM HEPES, 100 mM NaCl, pH 7.4</td>
<td>Tyr fluorescence</td>
</tr>
<tr>
<td>11</td>
<td>Aβ16</td>
<td>&gt;50</td>
<td>50 mM HEPES, pH 7.1</td>
<td>Competition</td>
</tr>
<tr>
<td>210(20)</td>
<td>Aβ40</td>
<td>75</td>
<td>10 mM HEPES, pH 7.4, 281 K</td>
<td>NMR</td>
</tr>
</tbody>
</table>

Table 1.4: Measurements of the $K_d$ of Aβ with Zn$^{2+}$, at near physiological pH. Sorted by publication date. Abbreviations: fluo - fluorescence, comp - competition. [161;206]
1.6.3 Copper

The coordination of Cu$^{2+}$ to A$\beta$ is dependent on the pH, which varies the level of protonation of A$\beta$ (see Fig. 1.16). This is also true for murine A$\beta$·Cu, with mutations causing different protonation states to form (see Fig. 1.16). At physiological pH, there are two dominant forms of A$\beta$·Cu for human A$\beta$, termed ‘Component I’ ([A$\beta$·Cu]$_i$) and ‘Component II’ ([A$\beta$·Cu]$_{ii}$)$^{[210–212]}$, with a $pK_a$ of pH 7.7$^{[213]}$, caused by the deprotonation of the asp$_1$–ala$_2$ peptide bond$^{[214]}$. For murine A$\beta$, the $pK_a$ is pH 6.2 for the two components, but their coordination spheres are different$^{[213]}$.

Coordination spheres have been proposed (see Fig. 1.17)$^{[159;215–217]}$. For both human and murine A$\beta$, the binding of Cu$^{2+}$ is to the N-terminal region. The coordination of Cu$^{2+}$ to A$\beta_{16}$ has been shown to be the same as to full length A$\beta$$^{[218–220]}$. Thus shorter lengths of A$\beta$ are an acceptable model for the interactions of A$\beta$ with Cu$^{2+}$.

Cu$^+$ has been shown to bind to A$\beta$ (see Fig. 1.17) with a $K_d$ of approximately 50 nM$^{[221]}$. Copper whilst bound to A$\beta$ is capable of undergoing redox chemistry and the intermediate redox active states have been determined$^{[95]}$. 

(a) Human A$\beta_{16}$$^{[209]}$  
(b) Murine A$\beta_{16}$$^{[209]}$ 

Figure 1.16: Relative concentration of A$\beta_{16}$·Cu complexes at different pH’s, where L is A$\beta$. Reprinted from Coordination Chemistry Reviews, 256:19-20, T. Kowalik-Jankowska, et al., Coordination abilities of the 1–16 and 1–28 fragments of $\beta$-amyloid peptide towards copper(II) ions: a combined potentiometric and spectroscopic study, 270–282, Copyright 2003, with permission from Elsevier.$^{[209]}$
Aβ has been shown to bind multiple Cu$^{2+}$ with to up to four Cu$^{2+}$’s bound\cite{222,223}. Furthermore it was shown that oligomers of Aβ$\text{16} \cdot$Cu up to a 17-mer may be formed\cite{222}. However, the measurements used 0.2 mM Aβ$\text{16}$ and 0 mM to 4 mM Cu(Gly)$_2$, at pH 6.5 in NEM buffer.

For the measurement of the $K_d$ of Aβ with a single Cu$^{2+}$ ion there have been a large number of inconsistent measurements spread over approximately four orders of magnitude. Three methods have been applied to determining the $K_d$: Quenching of intrinsic tyr$_{10}$ fluorescence, ITC, and potentiometry. These methods are reasonably (approximately one order of magnitude) consistent with themselves, but differ between them (see Fig. 1.18). One possible reason for the discrepancy between measurement methods is due to their sensitivity and thus the different concentrations required for a measurement. At higher concentrations Aβ will aggregate and it is not clear from the measurements if the binding affinity measured is purely that of monomeric Aβ, but also that of oligomers, which likely have a lower $K_d$.

The $K_d$ of Cu$^{2+}$ binding to Aβ has been measured by many groups (see tables 1.5 & 1.6) with values ranging from approximately 10 pM to 100 nM. The measurements are further complicated by the weak binding of Cu$^{2+}$ to components in the buffer, reducing the apparent concentration of Cu$^{2+}$ and increasing the apparent $K_d$. Phosphate buffers such as PBS (phosphate buffered saline) should not be used at all as the phosphates in the buffer form insoluble Cu$_3$(PO$_4$)$_2$ with Cu$^{2+}$, which precipitates.
and may act as seeds for aggregation.

Figure 1.18: Relationship between Aβ peptide concentrations and reported $\kappa_{\text{ML}}$ ($\kappa_{\text{ML}} = \frac{[\text{M}\cdot\text{L}]}{[\text{M}][\text{L}]}$, conditional binding constant for a metal-ligand (M·L) interaction) values for Cu$^{2+}$ binding, according to various experimental methodologies. Reprinted from Coordination Chemistry Reviews, 256:19-20, I. Zawisza, et al., Affinity of copper and zinc ions to proteins and peptides related to neurodegenerative conditions (Aβ, APP, α-synuclein, PrP), 2297–2307, Copyright 2012, with permission from Elsevier. [161]
<table>
<thead>
<tr>
<th>$K_d$/нм</th>
<th>Aβ</th>
<th>$[A\beta]/\mu$m</th>
<th>Conditions</th>
<th>C</th>
<th>$K_d^{\text{App}}/\mu$m</th>
</tr>
</thead>
<tbody>
<tr>
<td>73</td>
<td>Aβ40</td>
<td>3</td>
<td>10 мм Tris, 100 мм NaCl, pH 7.4</td>
<td>1.34</td>
<td>1.6(9) [201]</td>
</tr>
<tr>
<td>91</td>
<td>Aβ42</td>
<td>3</td>
<td>10 мм Tris, 100 мм NaCl, pH 7.4</td>
<td>1.34</td>
<td>2.0(8) [201]</td>
</tr>
<tr>
<td>53</td>
<td>Aβ16</td>
<td>10</td>
<td>100 мм Tris, 150 мм NaCl, pH 7.4</td>
<td>2.94</td>
<td>47(5) [219]</td>
</tr>
<tr>
<td>32</td>
<td>Aβ28</td>
<td>10</td>
<td>100 мм Tris, 150 мм NaCl, pH 7.4</td>
<td>2.94</td>
<td>28(5) [219]</td>
</tr>
<tr>
<td>21</td>
<td>Aβ40</td>
<td>10</td>
<td>100 мм Tris, 150 мм NaCl, pH 7.4</td>
<td>2.94</td>
<td>11(1) [219]</td>
</tr>
<tr>
<td>120</td>
<td>Aβ40</td>
<td>10</td>
<td>50 мм PBS, 100 мм NaCl, pH 7.4</td>
<td>1.82</td>
<td>8 [224]</td>
</tr>
<tr>
<td>370</td>
<td>Aβ28</td>
<td>10</td>
<td>50 мм HEPES, pH 7.2</td>
<td>0.87</td>
<td>2.5(2) [192]</td>
</tr>
<tr>
<td>77</td>
<td>Aβ28</td>
<td>10</td>
<td>10 мм HEPES, pH 7.2</td>
<td>1.51</td>
<td>2.5(2) [192]</td>
</tr>
<tr>
<td>370</td>
<td>Aβ40</td>
<td>4</td>
<td>20 мм Tris, 100 мм NaCl, pH 7.3</td>
<td>1.10</td>
<td>0.047(23) [206]</td>
</tr>
<tr>
<td>35</td>
<td>Aβ40</td>
<td>4</td>
<td>20 мм Tris, 100 мм NaCl, pH 7.3</td>
<td>1.53</td>
<td>1.21(41) [206]</td>
</tr>
<tr>
<td>24</td>
<td>Aβ40</td>
<td>4</td>
<td>50 мм Tris, 100 мм NaCl, pH 7.3</td>
<td>2.20</td>
<td>3.82(89) [206]</td>
</tr>
<tr>
<td>54</td>
<td>Aβ40</td>
<td>4</td>
<td>100 мм Tris, 100 мм NaCl, pH 7.3</td>
<td>2.74</td>
<td>30.1(57) [206]</td>
</tr>
<tr>
<td>36</td>
<td>Aβ40</td>
<td>4</td>
<td>20 мм HEPES, 100 мм NaCl, pH 7.4</td>
<td>1.19</td>
<td>0.57(23) [206]</td>
</tr>
<tr>
<td>24</td>
<td>Aβ40</td>
<td>4</td>
<td>50 мм HEPES, 100 мм NaCl, pH 7.4</td>
<td>1.57</td>
<td>0.90(21) [206]</td>
</tr>
<tr>
<td>34</td>
<td>Aβ40</td>
<td>4</td>
<td>100 мм HEPES, 100 мм NaCl, pH 7.4</td>
<td>1.86</td>
<td>2.5(6) [206]</td>
</tr>
<tr>
<td>48</td>
<td>Aβ42</td>
<td>4</td>
<td>20 мм HEPES, 100 мм NaCl, pH 7.4</td>
<td>1.19</td>
<td>0.76(100) [206]</td>
</tr>
<tr>
<td>57</td>
<td>Aβ40</td>
<td>8.2</td>
<td>20 мм to 100 мм HEPES, pH 7.4</td>
<td>0.19</td>
<td>0.6(32) [225]</td>
</tr>
<tr>
<td>0.09</td>
<td>Aβ16</td>
<td>10</td>
<td>100 мм HEPES, pH 7.4</td>
<td>3.5</td>
<td>[226]</td>
</tr>
<tr>
<td>0.04</td>
<td>Aβ40</td>
<td>10</td>
<td>100 мм HEPES, pH 7.4</td>
<td>3.5</td>
<td>[226]</td>
</tr>
<tr>
<td>0.1</td>
<td>Aβ16</td>
<td>0.2</td>
<td>10 мм MOPS, pH 7.4</td>
<td>0</td>
<td>0.1 [227]</td>
</tr>
</tbody>
</table>

Table 1.5: Measurements of the $K_d$ of Aβ with Cu$^{2+}$ from using the intrinsic fluorescence of tyr10. $K_d = K_d^{\text{App}}/10^C$ [228]. Sorted by publication date. [161,206]
<table>
<thead>
<tr>
<th>$K_d$/нм</th>
<th>Aβ</th>
<th>[Aβ]/μм</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Isothermal Titration Calorimetry:</td>
</tr>
<tr>
<td>91</td>
<td>Aβ₁₆</td>
<td>70</td>
<td>50 мм HEPES, pH 7.4 [229]</td>
</tr>
<tr>
<td>67</td>
<td>Aβ₂₈</td>
<td>70</td>
<td>50 мм HEPES, pH 7.4 [229]</td>
</tr>
<tr>
<td>0.67</td>
<td>Aβ₁₆</td>
<td>20</td>
<td>50 мм HEPES, pH 7.2 [230]</td>
</tr>
<tr>
<td>0.34</td>
<td>Aβ₁₆</td>
<td>20</td>
<td>50 мм HEPES, pH 7.4 [230]</td>
</tr>
<tr>
<td>0.33</td>
<td>Aβ₁₆</td>
<td>20</td>
<td>50 мм PIPES, pH 7.2 [230]</td>
</tr>
<tr>
<td>0.91</td>
<td>Aβ₄₀</td>
<td>20</td>
<td>50 мм HEPES, pH 7.2 [230]</td>
</tr>
<tr>
<td>0.42</td>
<td>Aβ₄₀</td>
<td>20</td>
<td>50 мм HEPES, pH 7.4 [230]</td>
</tr>
<tr>
<td>1.1</td>
<td>Aβ₄₀</td>
<td>20</td>
<td>50 мм PIPES, pH 7.2 [230]</td>
</tr>
<tr>
<td>0.67</td>
<td>Aβ₄₀</td>
<td>20</td>
<td>50 мм HEPES, pH 7.2 [231]</td>
</tr>
<tr>
<td>0.95(10)</td>
<td>Aβ₁₆</td>
<td>150</td>
<td>20 мм ACES, 100 мм NaCl, pH 7.4 [232]</td>
</tr>
<tr>
<td>0.96(10)</td>
<td>Aβ₁₆</td>
<td>150</td>
<td>20 мм ACES, 100 мм NaCl, pH 7.4 [232]</td>
</tr>
<tr>
<td>0.95(10)</td>
<td>Aβ₁₆</td>
<td>150</td>
<td>100 мм ACES, 100 мм NaCl, pH 7.4 [232]</td>
</tr>
<tr>
<td>1.7(3)</td>
<td>Aβ₂₈</td>
<td>150</td>
<td>20 мм ACES, 100 мм NaCl, pH 7.4 [232]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Potentiometry:</td>
</tr>
<tr>
<td>0.21</td>
<td>Aβ₁₆</td>
<td>1100</td>
<td>100 мм KNO₃ [209]</td>
</tr>
<tr>
<td>0.024</td>
<td>Aβ₂₈</td>
<td>1100</td>
<td>100 мм KNO₃ [209]</td>
</tr>
<tr>
<td>0.12</td>
<td>Aβ₁₆PEG</td>
<td>2000</td>
<td>200 мм KCl [223]</td>
</tr>
</tbody>
</table>

Table 1.6: Measurements of the $K_d$ of Aβ with Cu$^{2+}$ by ITC and potentiometry. Sorted by method, then publication date.[161;206]
1.6.4 Mixed Zn\(^{2+}\) & Cu\(^{2+}\) Complexes with A\(\beta\)

Zn\(^{2+}\) has been shown to influence the sub-conformations of (A\(\beta\)·Cu)\(_i\), but not that of (A\(\beta\)·Cu)\(_{ii}\)\[^{194}\]. However, this does not affect reactive oxygen species production\[^{233}\].

Mixed Zn\(^{2+}\) & Cu\(^{2+}\) complexes have been shown to form on A\(\beta\)\(_{16}\)-PEG which are stable at physiological pH\[^{234}\]. At high concentrations of Zn\(^{2+}\), the Zn\(^{2+}\) binding pushes the Cu\(^{2+}\) to the N-terminus freeing up his\(_{13}\) and his\(_{14}\) to bind to the excess Zn\(^{2+}\).

1.6.5 Metal Binding Kinetics

Very little is known about the kinetics of A\(\beta\) with Zn\(^{2+}\) or Cu\(^{2+}\). It has been proposed that their association is likely to be diffusion limited\[^{198;235}\].

It has been suggested that the movement of the metal ion between different binding sites is on the time scales of milliseconds or faster, as it is faster than the time resolution of NMR.\[^{159;197}\] This has been suggested to still be as fast as when A\(\beta\) is bound in fibrils\[^{187}\].

1.7 Summary

A\(\beta\) is a small peptide of 40/42 residues, that is thought to be involved in Alzheimer’s disease and is found aggregated in fibrils and plaques. However, it is thought that small oligomers of A\(\beta\) are toxic, rather than in monomeric or fibrillar form. The mechanism of the toxicity and the mechanism of oligomer formation is unclear.

One hypothesis is that the oligomerisation is caused by A\(\beta\) binding to metal ions including Zn\(^{2+}\) and Cu\(^{2+}\). The equilibrium dissociation constants (\(K_d\)) for A\(\beta\) with a Zn\(^{2+}\) & Cu\(^{2+}\) are across many orders of magnitude, 1 \(\mu\)M to 100 \(\mu\)M for A\(\beta\)·Zn and 10 \(p\)M to 100 \(n\)M Cu\(^{2+}\). However, the binding of A\(\beta\) to Zn\(^{2+}\) or Cu\(^{2+}\) is too weak to compete against common metal binding proteins, such as HSA, in the cerebrospinal fluid. This suggests that A\(\beta\)·Zn and A\(\beta\)·Cu do not form in equilibrium in the CSF.

Zn\(^{2+}\) and Cu\(^{2+}\) are released during neurotransmission, reaching concentrations of 300 \(\mu\)M and 15 \(\mu\)M respectively. The concentrations are transiently above the \(K_d\) of A\(\beta\) with Zn\(^{2+}\) or Cu\(^{2+}\) suggesting that complexes may be able to form. However,
the kinetic parameters required to estimate the magnitude of complex formation are unknown.

**Amyloid-β, Metal Ions, Membranes, and this Thesis**

The aim of this work is to investigate the amyloid-metal hypothesis, with Zn\(^{2+}\) or Cu\(^{2+}\) as the initial steps in the formation of Aβ dimers. This thesis concentrates on the determination of the kinetics of Aβ binding to Cu\(^{2+}\) and Zn\(^{2+}\) in vitro. As the kinetics are fundamental biophysical properties their values measured in vitro, should be equally applicable in vivo.

The principles and methods applied, lists of the materials used in this thesis are described in chapter 2. In particular, stopped flow is the main technique used to determine kinetics. Stopped flow is used to measure the change in fluorescence of a reaction as it progresses in time.

In chapter 3, the kinetics of Aβ binding to a single Cu\(^{2+}\) are determined. The removal of Cu\(^{2+}\) from Aβ·Cu by various ligands is determined, which may provide a methodology for drug optimisation. The kinetics of Aβ binding to more than one Cu\(^{2+}\) is also investigated.

In chapter 4, initially the binding of Aβ to HSA and the aggregation of Aβ with Zn\(^{2+}\) in thermodynamic equilibrium is investigated. The kinetics of Aβ binding to Zn\(^{2+}\) and the rates of formation of mixed metal Aβ·Cu·Zn complexes is determined.

In chapter 5, Aβ is shown to bind to various model membranes and an order of magnitude estimate of the number of Aβ on synaptic membranes is made. The kinetics of Aβ bound to a GM1 micelle with to a single Cu\(^{2+}\) are determined.

In chapter 6, a toy model of a synapse is developed to gain some perspective on the parameters obtained in the previous chapters, by making a rough estimate of the binding of Aβ to metal ions under approximate synaptic conditions.

Chapter 7 outlines some of the implications of Aβ and metals from a kinetics point of view in physiology and the pathology of Alzheimer’s disease.
Chapter 2

Methods and Materials

2.1 Fluorescence

Fluorescence occurs when an electron in a molecule absorbs a photon, causing it to move into an excited state, and then later emits some of the energy as a lower energy (longer wavelength) photon (see Fig. 2.1). This difference in excitation and emission photon energy allows filters to be used to separate the emitted light from the excitation source. This phenomena is the basis on which the interactions of Amyloid-β are probed in this thesis, by labelling one of the components of the system with a small molecule fluorescent dye. Other types of fluorophore are available, such as quantum dots and fluorescent proteins. Although their fluorescent properties may be better, they are both considerably larger than Aβ, and may have a larger adverse influence on the system.

The electrons of a molecule are primarily separated into different energy levels by the apparent electrostatic field from the nuclei. These electronic energy levels are sub-divided by the vibration and rotation modes accessible to the molecule. In fluorescence, an electron in its ground state can absorb a photon exciting it into a higher energy state, when the energy of the photon matches the difference in energy between the two states. The absorbance spectrum of a molecule shows the relative ease at which different photon energies (wavelengths) can be absorbed. Once the electron is in an excited state, there are multiple pathways by which it can return
Figure 2.1: A Jablonski diagram illustrating some of the possible transitions for a fluorophore. The solid lines are photon absorption or emission processes, the wavy lines are internal processes in the fluorophore, where ‘IC’ is internal conversion, and ‘ISC’ is intersystem crossing. $S_n$ are the singlet states the $n^{th}$ energy level, and $T_n$ are the triplet states of the $n^{th}$ energy level. The vertical axis represents the energy. Adapted from [236].

To the ground state. Typically, for fluorescence, the electron undergoes vibrational relaxation (in approximately a picosecond) into the lowest energy excited state of the vibrational band. It then emits a photon (after approximately a nanosecond), to move into one of the vibrational modes of the ground state, before undergoing vibrational relaxation again into the ground state. The variation in available modes gives the characteristic emission spectrum for fluorescent molecules.

One alternative to the molecule undergoing fluorescence is internal conversion. Here the electron moves directly into one of the vibrational modes of the ground state, however this is relatively rare. The internal conversion between excited states (in approximately a picosecond) is much more probable, and faster than fluorescent emission, so typically the electron relaxes into the lowest energy excited state before emitting.

Another significant relaxation pathway is intersystem crossing. It is caused by the electron undergoing a ‘forbidden transition’ from a singlet state into a triplet state. Forbidden, as it requires the spin state of the electron to change.
In the triplet state, the electron may emit a photon to return to the ground state (phosphorescence, at least microseconds), as in fluorescence. Alternatively it may again undergo intersystem crossing into one of the vibrational modes of the ground state. The long lived nature of this state gives rise to blinking, as the fluorophore is dark whilst in this state. Whilst in this state the fluorophore is ‘deactivated’, and the molecule is dark. This phenomena is used for some super-resolution imaging techniques, to aid with the localisation of molecules.

One major problem for fluorescence studies is photobleaching, in which the fluorophore is permanently damaged preventing further fluorescence. This is usually caused when the fluorophore is in the triplet state. This state can then interact with the triplet ground state of molecular oxygen \( \text{O}_2 \), which results in the oxidation of the fluorophore. This process limits the useful lifetime of the fluorophore, in particular with higher excitation intensities, such as when imaging.

2.1.1 Förster Resonance Energy Transfer

Förster resonance energy transfer or fluorescence resonance energy transfer (FRET) is a distance dependent non radiative energy transfer between two different fluorophores\(^{[237]}\). It is used to show the co-localisation of the two fluophores (~10 nm) and allows for the distance between the two fluorophores to be determined.

FRET can occur between two fluorophores when the emission spectrum of one (donor fluorophore) overlaps with the absorption of the other (acceptor fluorophore). The donor absorbs a photon, and the energy is non-radiatively transfered to the acceptor, by a dipole-dipole interaction between the transition dipoles. The acceptor can then emit the photon by fluorescence. The energy transfer efficiency \( E \)

\[
E = \frac{1}{1 + \left( \frac{r}{R_0} \right)^6}
\]

is very dependent on the distance between the fluorophores \( r \). This allows FRET to be used as a ‘molecular ruler’, for distances of a few nanometers. The Förster radius
(\(R_0\)) is given by

\[
R_0 = \sqrt{\frac{9000Q_0 \ln(10)\kappa^2 J}{128\pi^3 n^4 N_a}},
\]

where \(Q_0\) is the quantum efficiency of the donor, \(\kappa^2\) is the dipole orientation factor (typically \(2/3\) for freely rotating dyes), \(n\) is the refractive index of the medium, \(N_a\) is Avogadro’s number, and \(J\) is the spectral overlap integral

\[
J = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda,
\]

where \(F_D\) is the normalised donor emission spectrum, and \(\varepsilon_A\) is the acceptor molar extinction coefficient. For the FRET pair BODIPY-FL\(^{[238]}\) (donor) and Hilyte Texas red (acceptor) used in these studies, \(R_0 \approx 5.6\) nm in water (\(n = 1.33\)), see figure 2.2.

\(\text{(a) Normalised donor emission spectrum and acceptor molar extinction coefficient} \quad \text{(b) Spectral overlap}\)

Figure 2.2: Normalised donor emission spectrum, acceptor molar extinction coefficient and spectral overlap for BODIPY-FL\(^{[238]}\) (donor) and Hilyte Texas red (acceptor).

2.2 Experimental Methods

2.2.1 Cleaning and Decontamination

In order for experimental data to be reproducible it is vital that equipment is thoroughly cleaned, such that the experiments are not affected by the history of the equipment. To clean the equipment, initially it is rinsed with MQ. Then left in 1 mM EDTA solution for a few minutes. The latter removes metal ions adsorbed to
surfaces. After that, it is left in 1 v/v% decon 90 overnight. This removes surface adsorbed proteins and peptides. Finally, it is flushed with copious amounts of MQ. This method of cleaning is applied to both quartz cuvettes and the stopped flow machine.

2.2.2 Stopped Flow

A stopped flow machine (KinetAsyst SF-61DX2, Hi-Tech Scientific) is used to measure the progress of chemical reactions in time. It is able to measure fluorescence, FRET, adsorbance and anisotropy. There are two modes available, ‘single mixing’ mode, in which there is one mixing step of two solutions immediately before measuring, and ‘double mixing’ mode. In double mixing mode, two samples are mixed into an ‘age loop’, and left to react for a set amount of time. This solution is then mixed with a third solution and the fluorescence is measured. The concentration is halved in each mixing step. A diagram of the flow circuit is shown in figure 2.3.

Samples are were excited at 488 nm by a xenon lamp via a monochromator or a fiber coupled laser diode (MCLS1-473-20, Thorlabs) at 473 nm. The advantage of the laser is that higher intensities are available. However, due to the laser power’s digital feedback system it is unstable at >100 ms timescales, as it causes steps in the intensity. The light sources are fiber coupled to the optical cell. For the detectors, photon multiplier tubes are used. For fluorescence measurements the emission is filtered with a 510 nm long pass filter (510ALP, Omega Filters), to remove the excitation light. Alternatively a 515 nm long pass filter is used (515 GY, Comar). For anisotropy measurements, in addition to the long pass filter, there are two polarising filters (half-wave plate). The data-points were taken using the log time-scale scheme. This gives batches of linearly spaced points, logarithmically distributed. For analysis, the data points are weighted by the difference in time between them, as this is likely to be proportional to the error. This is used as the PMT (photon multiplier tube) does not give the number of photons, nor does the stopped flow machine give the integration time used. The dead time of the system is ~1 ms, so only the data points after 2 ms are used.
Figure 2.3: Schematic of the stopped flow machine.

**Single Mixing**  Samples are placed into syringes C & D, from the sample reservoir. The valves for A & B are left open, disconnecting the syringes from the flow circuit. The syringes are driven by pneumatic piston. This pushes the solutions through the flow circuit, mixing just before the optical cell, filling the stop syringe. The solution in the optical cell, while flowing, has only had about 1 ms since mixing to reach the optical cell. Once the stop syringe is filled with $\sim 90 \mu l$, it hits the stop stop, ceasing the flow, and the data recording starts. This allows the progress of the reaction to be followed in time, from freshly mixed solution.

**Double Mixing**  Samples for the first mixing are placed in syringes A & B. Buffer is placed into syringe D, and the sample for the second mixing is placed into syringe C. When double mixing, all the valves are closed, to connect all four sample syringes to the flow circuit. Firstly, the front stop is moved in front of drive 1, such that $\sim 150 \mu l$
of solution can be pushed through the circuit. The back stop is placed behind drive 2, to prevent any solution being pushed into C & D. When the stops are in place, drive 1 is powered, mixing solutions A & B together, to fill and flush the age loop. Once it hits the front stop, a timer is started, allowing the sample to age. Once the set time has elapsed, drive two is powered. This pushes sample C into the second mixer, and uses the buffer in D to push the aged solution through into the second mixer. Once the stop syringe is filled with ~240 µl, it hits the stop stop, and the data recording starts with the actual age time calculated.

2.2.3 Fluorometry

A spectrofluorometer (Fluoromax-3, Horiba or Fluoromax-4, Horiba) is used to measure the output fluorescence of samples, with different incident wavelengths.

The light source used in the fluorometer is a xenon arc lamp to produce visible and UV light by bremsstrahlung. A diffraction grating separates the light into different wavelengths, and the chosen excitation wavelength is centred on a slit. The slit width selects the bandwidth of wavelengths to excite the sample. The windows of the cuvette which holds the sample, require at least 110 µl. The cuvette has a maximum capacity of ~2 ml. The fluorescence is collected perpendicular to the excitation beam. This is then diffracted off another grating, and put through the emission slit to select the emission bandwidth of the light of interest. A PMT (photon multiplier tube) is used to count the number of photons over a time interval. The PMT is less sensitive at longer wavelengths, so does not give an absolute count of photons, but this can be corrected for.

2.2.4 Ultraviolet-Visible Spectrophotometry

UV/Vis Spectroscopy (ultraviolet-visible spectrophotometry) is used to measure the absorption spectrum of a sample. This can then be used to determine the concentration of substances that absorb light.

The UV/Vis spectrometer (Lambda 25, PerkinElmer) uses two lamps to produce the different range of wavelengths needed. Deuterium for UV, and tungsten mainly for visible light. UV/Vis spectrometers are similar in operation to fluorometers.
using gratings to separate out the spectrum. The quartz cuvette holds approximately 600 µl of sample, with a path length of 10 mm. The absorbance \( A \) is calculated from the amount of light that is transmitted through the sample, by

\[
A = -\log(T) ,
\]

where \( T \) is the fraction of transmitted light. As the sample may fluoresce, the beam wavelength is selected to measure the transmitted proportion of light. The bandwidth of the system is fixed at 1 nm.[239]

As transmittance is measured, it is important to ‘zero’ the spectrometer with respect to the buffer solution. This removes background absorbance from the cuvette and components of the buffer. ‘Scatterers’ in solution may also appear to absorb light. One source of ‘scatterers’ are bubbles in the cuvette. These may be from when solution is initially put into the cuvette, or when a cold solution increases in temperature it becomes less soluble to gases forming bubbles. Another source is the aggregation of proteins. Changes of concentrations of proteins in the buffer solution, or proteins adsorbed to the surface of the cuvette from a previous measurement will also affect the absorbance spectrum.

**Determining Sample Concentration**

The concentration of a sample \( C \) can be determined from the absorbance \( A \) and known extinction coefficient \( \varepsilon \) at a specific wavelength, using Beer’s Law

\[
C = \frac{A}{\varepsilon L} ,
\]

where \( L \) is the path length of the light through the cuvette. For dye labelled samples, the peak absorbance \( A_{\text{peak}} \) of the dye is used to determine the concentration of the dye. For unlabelled proteins, the concentration can be determined if it contains UV absorbing amino acids, such as tyrosine or tryptophan, typically measured at 280 nm. Human Aβ contains one tyrosine at position 10. When a peptide is dye labelled, the label usually contributes some absorbance at 280 nm, by a factor \( \varphi \) proportional to \( A_{\text{peak}} \). The concentration of the peptide can thus be determined.
from
\[ C = \frac{A_{280nm} - \varphi A_{peak}}{\varepsilon_{280nm}L} \] . \hspace{1cm} (2.6)

However, this does not give the proportion of labelled peptide. Here it will be assumed that the concentration of the dye is the concentration of the dye labelled peptide.

### 2.2.5 Microscopy Methods

#### Confocal Microscopy

Confocal microscopes are used to observe femtolitre volumes, and unlike conventional microscopes, use an aperture to block some of the out of focus light not from the focal plane (see Fig. 2.4)\superscript{[240]}. The aperture reduces the apparent emission volume in the direction of the beam. This allows for higher spatial frequencies to be observed with less background, out of focus light. In this thesis, confocal microscopy is used for fluorescence correlation spectroscopy (FCS) and measuring FRET.

![Figure 2.4: Illustration of how the detector aperture blocks out of focus light from out of focus planes in a confocal microscope. Adapted from\superscript{[240]}.](image-url)
Microscopy Setup  The custom-built microscope setup used is shown in figure 2.5, and was built by Dr. Liming Ying. The argon ion laser (35LAP321-230, Melles Griot, USA) was used to excite fluorophores in the confocal volume. The laser is tunable to different wavelengths. 488 nm and 514 nm light is used to excite the Hilyte 488 and Hilyte 555 dyes, respectively. In the figure 2.5, the first mirror actually is a pair of mirrors, to redirect and lift the beam to the height of the microscope. The neutral density filter reduces the laser intensity by a factor of $10^{-D}$, where $D$ is the optical density. The Dove prism totally internally reflects the beam, and is used to remove the laser’s plasma lines. The beam expander focuses the beam to a point and the collimating lense recollimates the beam after it has expanded. The spatial filter, an aperture at the focus, ‘cleans up’ the beam by removing aberrations, i.e. the ring-shaped pattern around the main beam in the Fourier plane (one focal length from the lense). In the next pair of mirrors, the first changes the position that the beam will hit the second mirror, changing the entry position into the inverted microscope. The second mirror corrects the entry
angle. The aperture on entry to the microscope reduces the size of the beam such that it under fills the back-aperture of the objective. This elongates and enlarges the confocal volume, and reduces diffraction fringes, to produce a nearly Gaussian illumination profile[^244]. Under filling the back aperture of the objective has been shown experimentally by Hess et al. to improve FCS measurements[^244]. The laser power is measured after the aperture and before entry into the microscope. The inverted microscope (Nikon Eclipse TE2000-U) uses a high numerical aperture oil-immersion objective (Apo TIRF 60×, NA 1.49, Nikon, UK). This produces less reflected light than a water-immersion objective due to the similarities in the refractive indexes of the oil and glass[^245]. Oil immersion objectives are also better at light collection[^246]. Type FF immersion oil (Cargille, USA) is used, as it has virtually no fluorescence. The microscope is used to focus the beam 6 µm into the solution from the surface of the slide for the experiments. 6 µm is used so that the confocal intensity profile is consistent across measurements. The excitation filter is a bandpass filter to let through the beam. The dichroic separates the excitation and emission beam, reflecting the excitation beam towards the objective, and allowing the emission fluorescence to pass through. The lense within the microscope focuses the light to a point where the confocal pinhole is placed. The dichroic is used in FRET experiments, to separate the donor and acceptor fluorescence signal for detection. In FCS experiments, the beam splitter is used to increase temporal resolution. The emission filters reduce misdirected photons, and photons that are not in the fluorophore’s main emission range. The final lenses focus the emission to an approximately 80 µm spot on the APD (avalanche photodiode) (single photon counting module spcm-aqr-14). The sensor is approximately 180 µm across, however the edges are less sensitive[^247]. They have a dead time of 50 ns between detecting pulses[^247]. The APD’s are connected to a digital hardware correlator (flex02-01d/c) with a sample rate of 640 MHz[^248]. They are also connected to a multichannel scaler (MCS-pci) in a computer, which records counts, with rates of up to 150 MHz[^249].
**TIRF / Wide Field Microscopy**

TIRF (Total Internal Reflection Fluorescence) and wide field microscopes are similar in setup to confocal microscopes, but do not have the pinhole to block out of focus light.

In wide field microscopy the beam goes through the centre of the objective exciting the fluorophores directly (see Fig. 2.6a). This illuminates a large amount of the solution, increasing the background noise due to out of focus fluorophores.

In TIRF microscopy an evanescent wave is produced, from total internal reflection (TIR) of the beam in the objective. This is used to excite the fluorophores. The intensity of the evanescent wave exponentially decreases into the sample, typically propagating 100 nm into the sample from the glass slide/sample interface\textsuperscript{251}. The intensity \( I \) is given by

\[
I(z) = I(0)e^{-\frac{z}{d}}, \quad \text{where} \quad d = \frac{\lambda}{4\pi} \left( n_1^2 \sin^2(\theta) - n_2^2 \right)^{-\frac{1}{2}},
\]

\( z \) is the distance from the slide, \( \lambda \) is the wavelength, \( \theta \) is the angle of incidence and \( n_1 \) & \( n_2 \) are the refractive indices of the slide & sample, respectively\textsuperscript{252}. The propagation distance can be decreased by increasing the incidence angle\textsuperscript{250}. 

---

Figure 2.6: The difference in beam configuration for wide field and TIRF microscopy. Adapted from\textsuperscript{250}. 

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\textsuperscript{250} Adapted from.  
\textsuperscript{251} Typically propagating 100 nm into the sample from the glass slide/sample interface.  
\textsuperscript{252} The propagation distance can be decreased by increasing the incidence angle.
The lateral resolution of confocal, TIRF and wide field is diffraction limited to approximately $\frac{\lambda}{2n}$\[^{253}\]. TIRF has the highest axial resolution ($\sim$100 nm), then confocal ($\sim$1 μm), and wide field with the least\[^{254}\].

**Microscopy Setup** The custom-built microscope setup used is shown in figure 2.7\[^{241}\], built by Dr. Liming Ying. The equipment and setup is similar to the confocal setup in figure 2.5, so only the differences will be described. The shutter is used to control the blocking of the beam, and is unblocked just after a series of images starts to be taken. This reduces the amount of photobleaching that occurs before acquisition. To switch between TIRF and wide field mode there are a pair of flip mirrors, which are raised or lowered. These are also used during alignment, allowing the TIRF and wide field to show the same lateral position on the slide. Type DF oil (Cargille, USA) is used for the objective. It has slightly more fluorescence than FF oil, but has a higher viscosity which more closely matches the refractive index of cover slips. The EMCCD Camera (CoolView EM 1000, Photonic Science) records the image.

Figure 2.7: TIRF/Wide field microscopy setup.
2.3 Analytical Methods

2.3.1 Fluorescence Correlation Spectroscopy

The confocal microscope setup is used to monitor the intensity fluctuations as fluorophores diffuse across the confocal volume. In fluorescence correlation spectroscopy (FCS), the signal from the APD is auto-correlated, which looks for repeating patterns in the signal. FCS can give the average diffusion time for a fluorophore to cross the confocal volume and the average concentration of molecules in the confocal volume.

The normalised autocorrelation \( G(\tau) \) function correlates the time series with itself, shifted by time \( \tau \):

\[
G(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t)^2 \rangle} - 1,
\]

where \( I(t) \) is the photon count at time \( t \)\(^{[255]} \), and

\[
\langle f(t) \rangle = \frac{1}{T} \int_0^T f(t) \, dt,
\]

where \( T \) is the measurement time. This produces traces such as in figure 2.8. The different time scales in an FCS curve correspond to different types of behaviour.

![Figure 2.8](image.png)

Figure 2.8: The different timescales over which the FCS curve is affected by different processes.\(^{[256]} \)
in the system. The system described in section 2.2.5 is only capable of detecting the longer scale behaviour due to the dead time of the detectors. The diffusion component (\(>10^{-2}\) ms) can be measured with one APD, and the photophysical behaviour of the fluorophores (\(10^{-4}\) ms to \(10^{-2}\) ms), such as triplet state blinking, with two APD’s and a beam splitter. Under this setup the signals are pseudo-autocorrelated (cross-correlation with signals that are similar). If two APD’s are not used then the autocorrelation curve rises very steeply at the ‘triplet’ timescales, as the APD’s dead time correlates. Using two APD’s reduces the effective dead time of the detector.

When comparing the signal with itself at long time scales, the signal from different molecules diffusing through the confocal volume may or may not ‘line up’ as diffusion is a random process. The rise in correlation when moving to shorter time scales is due to signal from the same molecule starting to align. The length of time spent in the confocal volume varies for different molecules as they random walk through the focus. Therefore initially (from decreasing \(\tau\)) the signal from the molecules that spent longest in the focus overlap, increasing \(G(\tau)\), then the ones which spent less and less time, until \(\tau\) is much shorter than the diffusion time, then there is effectively no difference in \(G(\tau)\). The position of the \(\xi\)-shape depends on the diffusion time of the molecules. The slower the molecules, the longer the diffusion time, and the further the curve is shifted to longer \(\tau\). The amplitude of the \(\xi\)-shape is inversely proportional to the concentration of fluorescent particles.

Autocorrelation is related to, and can be calculated by way of a Fourier transform, as
\[
G(\tau) = I(\tau) \star I(\tau) = F^{-1}[F(I(\tau))^2].
\]
It therefore suffers from some of the same problems as Fourier transforms, in particular time information about when something happened is lost.

The diffusion part of FCS curves can be fitted with
\[
G_{2D}(\tau) = \frac{\gamma}{N \left(1 + \frac{\tau}{\tau_D}\right)} + C,
\]
where \(\gamma\) is the shape factor, \(N\) is the average number of molecules in the focus and \(\tau_D\) is the average time for a molecule to traverse the radial dimension of the
volume\textsuperscript{[255]}. This assumes that the diffusion is two dimensional. When using the form for a three dimensional confocal volume, one of the parameters cannot be fitted. This is due to the length of the confocal volume (approximately 1 \( \mu \)m), over which there is little signal fluctuation for movement in the direction of the beam axis. \( G(0) = \gamma/N \) is the amplitude of the correlation function at \( \tau = 0 \textsuperscript{[255]} \). This can be used to estimate the concentration (\( C \))

\[
C = \frac{N}{N_A \times V} \approx \frac{1}{G(0) \times N_A \times V}
\]

(2.11)

where \( N_A \) is Avogadro’s number and \( V \) is the focal volume (approximately 1 fl). The diffusion time

\[
\tau_D = \frac{\omega_0^2}{4D}, \text{ where } \omega_0 = \frac{\text{FWHM}}{\sqrt{2\ln(2)}}
\]

(2.12)

\( D \) is the diffusion coefficient, \( \omega_0 \) is the beam waist and FWHM is the full width half maximum (the diameter of the confocal volume at half of the maximal intensity)\textsuperscript{[255]}.

Multiple component curves, due to multiple fluorescent species with different \( \tau_D \)’s (\( \tau_1 \) & \( \tau_2 \)), can be fitted with

\[
G(\tau) = \frac{G_1(0)}{1 + \frac{\tau}{\tau_1}} + \frac{G_2(0)}{1 + \frac{\tau}{\tau_2}} + C
\]

(2.13)

where

\[
F_1 = \frac{G_1(0)}{G_1(0) + G_2(0)} \quad \text{and} \quad F_2 = \frac{G_2(0)}{G_1(0) + G_2(0)}
\]

(2.14)

are the relative population fractions of type 1 and type 2, respectively. This can then be used to calculate the mean diffusion time

\[
\tau_D = F_1 \tau_1 + F_2 \tau_2
\]

(2.15)

for both species diffusing through the confocal volume.

One problem with FCS is its inability to separate components with similar diffusion times. It has been shown for a two component system that the \( \tau_D \)’s need to differ by a factor of 1.6 to distinguish the two components\textsuperscript{[257]}.
When there is triplet state behaviour, $G(\tau)$ can be modified to

$$G(\tau) = \left( \frac{G_1(0)}{1 + \frac{\tau}{\tau_1}} + \frac{G_2(0)}{1 + \frac{\tau}{\tau_2}} \right) \left( 1 - F + Fe^{-\frac{\tau}{t_m}} \right) + C,$$  \hspace{1cm} (2.16)

where $F$ is the fraction of molecules in the dark state and $t_m$ is mean triplet state lifetime\textsuperscript{1256}. Triplet state behaviour in typically in the $10^{-4}$ ms to $10^{-2}$ ms region, and is dependent on the excitation intensity. This is because increasing the intensity increases the cycling rate of the fluorophore, increasing the probability that the transition is into the dark triplet state, making it more prominent in the FCS curve. If the behaviour is not intensity dependant it may be due to other processes, such as the movement of the fluorophore if it causes it to be quenched by the molecule it is attached to. Increasing the excitation intensity can also photobleach the fluorophores more quickly, decreasing their apparent diffusion time.

2.3.2 Binding Reactions and Equilibria

The law of mass action can be used to predict the progress and behaviour of a chemical reaction in time. The law of mass action states that the rate of production of products is proportional to the product of the concentrations of the constituents, and vice versa for the reverse reaction. That is

$$A + B \xrightleftharpoons{k_{\text{on}}}{k_{\text{off}}} C$$ \hspace{1cm} (2.17)

gives us

$$\frac{d[C]}{dt} = k_{\text{on}}[A][B] - k_{\text{off}}[C], \quad \text{and} \quad \frac{d[A]}{dt} = \frac{d[B]}{dt} = k_{\text{off}}[C] - k_{\text{on}}[A][B],$$  \hspace{1cm} (2.18)

where $t$ is time, and $[x]$ denotes the concentration of $x$. When reactions are performed the total amounts of $A$ and $B$ are known in all its forms. Conservation of matter can then be used to give

$$[A]_T = [A] + [C], \quad \text{and} \quad [B]_T = [B] + [C],$$  \hspace{1cm} (2.19)
where \([x]_T\) is the total concentration of \(x\). These equations can be used to determine the reaction’s transient and steady state behaviour.

The reaction will eventually reach steady state, that is

\[
\frac{d[A]}{dt} = \frac{d[B]}{dt} = \frac{d[C]}{dt} = 0 ,
\]

as \(t \to \infty\). Therefore the equilibrium dissociation constant \(K_d\)

\[
K_d = \frac{k_{\text{off}}}{k_{\text{on}}} = \frac{[A][B]}{[C]}
\]

can be determined from the \(k_{\text{on}}\) and \(k_{\text{off}}\). The thermodynamic parameter \(K_d\) can thus be used to determine the concentration of reactants and products in equilibrium. In general, equilibrium constants are denoted with uppercase letters, whereas rates are denoted with lowercase letters.

To determine the transient state behaviour of the reaction, the differential equations are solved. The experiments in this thesis are performed under pseudo first order conditions, that is when one of the reactants is in greater excess, i.e. \([A] \ll [B]\). Here we will assume that to be true when less than 5% of one of the reactants is consumed, i.e. \(20 \times [A] < [B]\). This produces simpler analytical solutions, but is also beneficial under practical conditions.

In the above model, the pseudo-first order condition reduces the equations to

\[
\frac{d[A]}{dt} = k_{\text{off}}[C] - k_{\text{on}}[A][B] , \quad \frac{d[C]}{dt} = k_{\text{on}}[A][B] - k_{\text{off}}[C],
\]

\[
[A]_T = [A] + [C] , \quad \frac{d[B]}{dt} = 0 .
\]

To which the solutions are

\[
[A] = \frac{[A]_T k_{\text{off}}}{k_{\text{off}} + k_{\text{on}}[B]} + \left( [A]_{t=0} - \frac{[A]_T k_{\text{off}}}{k_{\text{off}} + k_{\text{on}}[B]} \right) e^{-\left(k_{\text{off}} + k_{\text{on}}[B]\right)t},
\]

\[
[C] = \frac{[A]_T [B] k_{\text{on}}}{k_{\text{off}} + k_{\text{on}}[B]} + \left( [A]_{t=0} - \frac{[A]_T k_{\text{off}}}{k_{\text{off}} + k_{\text{on}}[B]} \right) e^{-\left(k_{\text{off}} + k_{\text{on}}[B]\right)t},
\]

which are exponentials with linear dependence on \([B]\) for the rates.

For comparison without the pseudo-first order condition the differential equa-
tion are non-linear, with the solutions

\[ [A] = \frac{-k_{\text{off}} - k_{\text{on}} c_1 + \alpha \tan \left( \frac{1}{2} \alpha (c_3 - t) \right)}{2k_{\text{on}}} \], \quad (2.26)

\[ [B] = \frac{-k_{\text{off}} + k_{\text{on}} c_1 + \alpha \tan \left( \frac{1}{2} \alpha (c_3 - t) \right)}{2k_{\text{on}}} \], \quad (2.27)

\[ [C] = \frac{k_{\text{off}} k_{\text{on}} (c_1 + 2c_2) - \alpha \tan \left( \frac{1}{2} \alpha (c_3 - t) \right)}{2k_{\text{on}}} \], \quad (2.28)

where

\[ \alpha = \sqrt{-(k_{\text{off}} + k_{\text{on}} c_1)^2 - 4k_{\text{off}} k_{\text{on}} c_2} \ ], \quad (2.29)\]

and \( c_1, c_2 \) & \( c_3 \) are the constants of integration. However, when determining the constants of integration using the initial conditions, the equations are transcendental making solutions difficult to find algebraically.

**The Hill Equation**

The Hill equation is a phenomenological equation commonly used to describe cooperative binding of a ligand \( L \) to a receptor. At equilibrium it gives the ratio of ligand bound receptors to total receptors (\( \theta \))

\[ \theta = \frac{1}{1 + K_A/[L]^\beta} = \frac{1}{1 + (K_A/[L])^\beta} \], \quad (2.30)\]

where \( K_A \) is the microscopic dissociation constant and \( \beta \) is the Hill coefficient. If \( \beta > 1 \), then there is positive cooperativity, that is when one ligand has bound, it increases affinity for the next ligand to bind to it. If \( \beta = 1 \) then there is no effect on the next ligand binding, and if \( \beta < 1 \) then it is harder for the next ligand to bind.

This model assumes that \([L] \) is the free ligand concentration, which is true when the concentration of ligand is much greater than the concentration of receptors. It can be derived from the model

\[ R + L \overset{K_d}{\underset{K_1}{\rightleftharpoons}} R \cdot L \] \quad (2.31)
when $\beta = 1$, for a receptor $R$ and ligand $L$. The ratio of bound receptors is

$$\theta = \frac{[R\cdot L]}{[R] + [R\cdot L]} = 1 - \frac{[R]}{[R]_T}, \quad (2.32)$$

where $[R]_T$ is the total concentration of receptor. Given the assumption that the ligand concentration is constant then

$$K_d = \frac{[R][L]}{[R\cdot L]} = \frac{[R][L]}{[R]_T - [R]} \implies \frac{[R]}{[R]_T} = \frac{K_d}{[L] + K_d}, \quad (2.33)$$

and thus

$$\theta = 1 - \frac{K_d}{[L] + K_d} = \frac{[L]}{[L] + K_d} = \frac{1}{1 + K_d/[L]} . \quad (2.34)$$

If the receptor is fluorescent and quenches when a ligand binds, then the fluorescence ($F$) is

$$F = F_b + \lambda(1 - \theta)[R]_T, \quad (2.35)$$

where $F_b$ is the background fluorescence, $\lambda$ is a constant proportional to the amount of fluorescence per receptor, and $[R]_T$ is the total concentration of the receptor. Then the maximal fluorescence ($F_{\text{max}}$), when no ligand is bound ($\theta \to 0$) is

$$F_{\text{max}} = \lim_{\theta \to 0} (F_b + \lambda(1 - \theta)[R]_T) = F_b + \lambda[R]_T \implies \lambda[R]_T = F_{\text{max}} - F_b . \quad (2.36)$$

Therefore the fluorescence traces may be fitted with the form

$$F = F_b + \frac{F_b - F_{\text{max}}}{1 + ([L]/K_d)^{\beta}} . \quad (2.37)$$

This function is sigmoidal, with amplitude $F_b - F_{\text{max}}$ and offset $F_b$.

**1 : 1 Stoichiometry Binding Model**

1 : 1 stoichiometry binding is

$$A + C \xrightleftharpoons{K_d} A\cdot C , \quad (2.38)$$
for reactants A & C and product A·C, with an associated $K_d$. When the total concentrations of A is approximately that of C, the Hill equation cannot be applied. However, there is an analytical solution to the model.

The model gives the equations

$$K_d = \frac{[A][C]}{[A\cdot C]} \quad \text{and} \quad [A]_T = [A] + [A\cdot C]$$

$$[C]_T = [A] + [A\cdot C]$$

(2.39)

which may be solved to give

$$[A] = \frac{[A]_T - [C]_T - K_d \pm \sqrt{(K_d + [C]_T - [A]_T)^2 + 4K_d[A]_T}}{2}.$$  

(2.40)

Taking the limit $[C]_T \to \infty$, shows that the $+$ form is the relevant form.

If unbound A is fluorescent, the total fluorescence ($F$) is

$$F = F_b + \lambda[A], \quad \text{and} \quad \lambda = \frac{F_{\text{max}} - F_b}{[A]_T},$$

(2.41)

where $F_b$ is the background fluorescence, $\lambda$ is a constant proportional to the amount of fluorescence per free A, and $F_{\text{max}}$ is the maximal fluorescence when no C is bound to A. Therefore the fluorescence as a function of $[A]_T$ is

$$F = F_b + \frac{F_{\text{max}} - F_b}{2[A]_T} \left([A]_T - [C]_T - K_d + \sqrt{(K_d + [C]_T - [A]_T)^2 + 4K_d[A]_T}\right),$$

(2.42)

which may be used to fit fluorescence binding curves.

2.4 Materials and Sample Preparation

2.4.1 Amyloid-β Samples

Aβ stock solutions should be dissolved at high pH ($\sim$ pH 9), to prevent aggregation, aliquoted, and stored at $-20^\circ$C. All Aβ samples were purchased from AnaSpec, via Cambridge Bioscience. They have a purity of $\geq 95\%$, obtained from the peak area by HPLC (high-performance liquid chromatography), except when custom synthesised.
Unlabelled Amyloid-β’s:

$\text{A}_\beta_{16}$ Beta-Amyloid (1–16), Human  
Sequence: DAEFRHDSGYEVHHQK

$\text{A}_\beta_{40}$ Beta-Amyloid (1–40), Human  
Sequence: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGVVV

$\text{A}_\beta_{42}$ Beta-Amyloid (1–42), Human  
Sequence: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGVVVIA

C-Terminally or Mid-Labelled Amyloid-β’s:

$\text{A}_\beta_{16}\text{LysHL488}$ Beta-Amyloid (1–15)-Lys16(HiLyte Fluor 488), Human  
Sequence: DAEFRHDSGYEVHHQ-K(HiLyte Fluor 488)

Rat$\text{A}_\beta_{16}\text{LysHL488}$ CBDM-5/Beta-Amyloid (1–15)-Lys16(HiLyte Fluor 488). This peptide was custom synthesised and has a purity of > 90%, peak area by HPLC.  
Sequence: DAEFGHDSGFVRH-Q-K(HiLyte Fluor 488)

$\text{A}_\beta_{28}\text{LysHL488}$ Beta-Amyloid (1–28)-Lys(HiLyte Fluor 488), Human  
Sequence: DAEFRHDSGYEVHHQKLVFFAEDVGSN-K(HiLyte Fluor 488)

Aβ$_{40}$Cys$_{20}$HL488 CBDM-6/(HiLyte488)Cys20-BetaAmyloid (1–40). This peptide was custom synthesised and has a purity of > 90%, peak area by HPLC.  
Sequence: DAEFRHDSGYEVHHQKLVVF-C(HiLyte Fluor 488)-AEDVGSNKGAIIGLMVGVVV

N-Terminally Labelled Amyloid-β’s:

$\text{A}_\beta_{16}\text{HL555}$ Beta-Amyloid (1–16), HiLyte Fluor 555-labeled, Human  
Sequence: HiLyte Fluor 555-DAEFRHDSGYEVHHQK

$\text{A}_\beta_{40}\text{HL488}$ Beta-Amyloid (1–40), HiLyte Fluor 488-labeled, Human  
Sequence: HiLyte Fluor 488-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGVVV
**Aβ_{40}CysHL555** [Cys(HiLyte Fluor 555 C2 maleimide)]-Beta-Amyloid (1–40), Human

Sequence: C(HiLyte Fluor 555 C2 maleimide)-DAEFRHDSGYEVHHQKLVFF-AEDVGSNKGAIIGLMVGGVV

**Aβ_{40}HLTR** Beta-Amyloid (1–40), HiLyte Fluor TR-labeled

Sequence: HiLyte Fluor TR-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGA-IIGLMVGGVV

**Aβ_{40}HL647** Beta-Amyloid (1–40), HiLyte Fluor 647-labeled, Human

Sequence: HiLyte Fluor 647-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGA-IIGLMVGGVV

**Aβ_{42}HL488** Beta-Amyloid (1–42), HiLyte Fluor 488-labeled, Human

Sequence: HiLyte Fluor 488-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGA-IIGLMVGGVVIA

### 2.4.2 Serum Albumins

HSA (human serum albumin) is found in the CSF in the range of 1 µm to 6 µm, and is an important carrier protein and metal binder. BSA (bovine serum albumin) is the equivalent in cows. It is also used to block surfaces, such as glass or quartz, to prevent the surface adsorption of other proteins.

**BSA** Albumin from Bovine serum. Purity ≥ 99% for agarose gel electrophoresis. Purchased from Sigma-Aldrich.

**BSA-AL488** Albumin from Bovine Serum (BSA), Alexa Fluor 488 conjugate. The specification for the maximum degree of labelling is 5-8 moles of dye per mole protein. Purchased from Life Technologies.

**HSA** Albumin from human serum Purity ≥ 99% for agarose gel electrophoresis. Purchased from Sigma-Aldrich.
2.4.3 Metal Chlorides

The metal chlorides were dissolved in MQ at 20 m. The exception was NaCl, which was dissolved at 2 m in MQ, as it was used to make buffer solutions. The divalent metal chlorides can cause buffers to precipitate, in particular with PBS, and thus Good’s buffers are recommended. For this reason Hepes was chosen as the buffer for the metal experiments. However, millimolar concentrations of Cu$^{2+}$ and Zn$^{2+}$ still cause precipitation, and should still be stored in MQ.

**NaCl** Sodium chloride. Purity ≥ 99.5%. Purchased from Sigma-Aldrich.

**MgCl$_2$** Magnesium chloride hexahydrate. Purity > 99%. Purchased from Sigma-Aldrich.

**CaCl$_2$** Calcium chloride dihydrate. Purity > 99%. Purchased from Sigma-Aldrich.

**MnCl$_2$** Manganese(II) chloride tetrahydrate. Purity > 99.9%. Purchased from Sigma-Aldrich.

**FeCl$_2$** Iron(II) chloride tetrahydrate. Purity > 99%. Purchased from Sigma-Aldrich.

**CoCl$_2$** Cobalt(II) chloride, anhydrous. Purity 99.7%. Purchased from Alfa Aesar.

**NiCl$_2$** Nickel(II) chloride hexahydrate. Purchased from Sigma-Aldrich.

**CuCl$_2$** Copper(II) chloride dihydrate. Purity > 99%. Purchased from Alfa Aesar.

**ZnCl$_2$** Zinc chloride. Purchased from Fluka Analytical.

**CdCl$_2$** Cadmium chloride. Purity > 99.99%. Purchased from Sigma-Aldrich.

2.4.4 Model Membrane Components

**GM1** Monosialoganglioside $G_{M1}$ from bovine brain. Also known as Ganglioside GM1, and monosialo. Purity ≥ 95%, by TLC (thin layer chromatography). Purchased from Sigma-Aldrich.

**GM1 Bodipy** BODIPY FL C5-Ganglioside $G_{M1}$. Purity ≥ 90%, by HPLC. Purchased from Invitrogen.
Cholesterol  Cholesterol. Also known as 3β-Hydroxy-5-cholestene, and 5-Cholen-3β-ol. Purity ≥ 90%, by gas chromatography. Purchased from Sigma-Aldrich.

Sphingomyelin  Sphingomyelin from bovine brain. Also known as N-Acyl-4-sphingenyl-1-O-phosphorylcholine, and N-Acyl-D-sphingosine-1-phosphocholine. Purity ≥ 97%, by TLC. Purchased from Sigma-Aldrich.

POPC  2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine. Also known as (7R,17Z)-4-Hydroxy-N,N,N-trimethyl-9-oxo-7-[[1-oxohexadecyl]oxy]methyl]-3,5,8-trioxa-4-phosphahexacos-17-en-1-aminium 4-oxide, inner salt, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-Hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine, 1-Hexadecanoyl-2-(cis-9-octadecenoyl)-sn-glycero-3-phosphocholine, 3-sn-Phosphatidylcholine, 2-oleoyl-1-palmitoyl, L-β-Oleoyl-γ-palmitoyl-α-lecithin, PC(16:0/18:1(9Z)), PC(16:0/18:1), PC(16:0/18:1w9), and POPC. Purity ≥ 95%, by gas chromatography, and ≥ 98%, by TLC. Purchased from Sigma-Aldrich.

POPG  2-Oleoyl-1-palmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt. Also known as (9Z)-9-Octadecenoic acid (1R)-1-[[[(2,3-dihydroxypropoxy)... hydroxyphosphinyl]oxy]methyl]-2-[(1-oxohexadecyl)oxy]ethyl ester monosodium salt, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) sodium salt, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol sodium salt, 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1′-rac-glycerol) sodium salt, L-α-Phosphatidyl-DL-glycerol, β-oleoyl-γ-palmitoyl sodium salt, PG(16:0/18:1(9Z)), POPG-Na, and POPG. Purity ≥ 98%, by TLC. Purchased from Sigma-Aldrich.

2.4.5 Buffers and Solvents

Buffers are used to keep the pH of the solutions at a known concentration, as the pH can be affected by reactions, and can effect the protonation states of molecules. The buffers also contain NaCl, to keep the ionic strength at near physiological conditions.
Buffers:

**Hepes**  HEPES. Also Known as 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid. Purchased from Alfa Aesar or Sigma-Aldrich, as buffer solutions at pH 6.5, 7.5 & 8.0.

**Hepes NaCl**  50 mM Hepes with 100 mM NaCl, at pH 7.5 unless otherwise stated.

**Hepes BSA**  Hepes NaCl with 0.3 g l⁻¹ BSA (4.32 μM).

**PBS**  Phosphate buffered Saline. The 10× solution was diluted in MQ to contain 11.9 mM phosphates, 137 mM sodium chloride, and 2.7 mM potassium chloride at pH 7.4(1). Purchased from Fisher Scientific. PBS should not be used with divalent metal cations as it will precipitate.

**PBS BSA**  PBS with 0.3 g l⁻¹ BSA (4.32 μM).

**Tween**  TWEEN 20, for molecular biology. Also known as Polyethylene glycol sorbitan monolaurate, and Polyoxyethylenesorbitan monolaurate. Purchased from Sigma-Aldrich. This was used in some buffers at 0.05% volume.

Solvents:

**MQ**  Purified and deionised water, with a resistivity of 18.2 MΩ cm.

**Methanol**  Methanol. Purity 99.8%, by gas chromatography. Purchased from VWR.

**Chloroform**  Chloroform, anhydrous. Purity > 99%. Purchased from Sigma-Aldrich.

**DMSO**  Dimethyl Sulfoxide. Purity ≥ 99.9%. Purchased from Sigma-Aldrich.

**Ethanol**  Ethanol, 190 proof, for molecular biology. Purchased from Sigma-Aldrich.

**Ammonium Hydroxide**  Ammonium hydroxide. Purchased from Sigma-Aldrich.

2.4.6 Other Small Molecules

**EDTA**  Ethylenediaminetetraacetic acid. Purchased from Fisher Scientific.
Fluozin  FluoZin-1, Tripotassium Salt, cell impermeant. It is based on the N-(2-methoxyphenyl)iminodiacetate chelator. Purity $\geq 90\%$, by HPLC. Purchased from Invitrogen.

Glycine  Glycine. Purity $\geq 99\%$. Purchased from Sigma-Aldrich.

CQ  Clioquinol. Also known as 5-Chloro-7-iodo-8-quinolinol, 5-Chloro-8-hydroxy-7-iodoquinoline, and Iodochlorhydroxyquin. Purity 98.9\%, by HPLC. Purchased from Sigma-Aldrich. CQ was dissolved at 20 mM in DMSO, or 2 mM in Ethanol.

L2-b  L-2b. Also known as N1,N1-dimethyl-(pyridin-2-ylmethyl)benzene-1,4-diamine. Acquired from Prof. Mi Hee Lim, University of Michigan. L2-b solution was prepared by dissolving in methanol, and leaving it to evaporate, then dissolving in Hepes NaCl at 4 mM. It is important that the L2-b is prepared fresh, as otherwise it will oxidise and change colour.
Chapter 3

Interactions of Amyloid-β with Cu^{2+}

Cu^{2+} is thought to be implicated in Alzheimer’s disease as it is found bound to Aβ plaques in vivo, and also found to bind in vitro. However, whether Cu^{2+} binding plays a critical role in the events leading to Alzheimer’s disease is still undetermined. It is thought that Cu^{2+} bound to Aβ may play a role in promoting Aβ oligomerisation and aggregation, or producing reactive oxidative species. Thus studying the binding of Cu^{2+} to Aβ may give insight into physiological events leading to Alzheimer’s disease.

Over the past 20 years the studies of Cu^{2+} binding to Aβ have given an estimate of the equilibrium dissociation constant (K_d) to be between 10 pm to 100 nm\textsuperscript{161}, showing that there are two forms of Aβ bound Cu^{2+}, and binding coordinations have been suggested. This, combined with the concentration of Cu^{2+} along with the known concentrations and K_d’s of competitors in the CSF, eliminates the possibility of monomeric Aβ binding Cu^{2+} in the CSF. This leaves areas with higher concentrations of Cu^{2+} or a lack of competitors. One such example is the synaptic cleft. However, the possibility of binding there, given the transient nature of its Cu^{2+} release and diffusion, cannot be determined from thermodynamics alone. The knowledge of the kinetics of the interactions, from which the thermodynamics may be derived, is required. Further to this, the parameters determining the binding of
multiple Cu$^{2+}$ ions to Aβ, although shown, are yet to be addressed.

Here the quenching of a covalently attached external fluorophore to Aβ by Cu$^{2+}$ is exploited to study the binding and kinetics of Aβ to a single, and then multiple, Cu$^{2+}$ ions. The competitiveness of Aβ compared to HSA or glycine is determined, and the copper assisted dimerisation rate is measured. This is mainly carried out by using stopped-flow methodologies to look at the temporal change in fluorescence as reactions progress.

3.1 Quenching of Labelled Aβ by Cu$^{2+}$

One of the methods used in the literature to measure the $K_d$ of Aβ binding to Cu$^{2+}$ involves measuring quenching of the intrinsic fluorescence of the tyrosine in Aβ, once Cu$^{2+}$ is bound. The main drawback with this methodology is the low sensitivity in measuring the fluorescence. This is due to tyrosine’s small absorbance ($\varepsilon = 1400\text{ cm}^{-1}\text{ m}^{-1}$ at 274 nm), combined with its low quantum efficiency ($\phi = 0.14$). This therefore requires high concentrations of Aβ to obtain sufficient signal, complicating experiments by unquantifiable amounts of oligomerisation and aggregation. Furthermore, this method cannot be applied to murine Aβ, given its lack of an intrinsic tyrosine.

It is known that engineered fluorescent dyes are also quenched by Cu$^{2+}$, due to its paramagnetic* properties. This poses the question as to whether engineered dyes can also be used to investigate Aβ binding to Cu$^{2+}$, given their increased brightness. For comparison, HL488 has $\varepsilon = 68000\text{ cm}^{-1}\text{ m}^{-1}$ (at 503 nm) and $\phi = 0.91$. This is over two orders of magnitude brighter for a similar excitation intensity at its absorption maxima. It is also sufficiently bright to be suitable for single molecule studies.

To determine the quenching selectivity of Cu$^{2+}$ by labelled Aβ, compared to other common divalent cations (M$^{2+}$), the fluorescence of 100 nM of labelled Aβ was measured with ($F$), and without ($F_0$) 100 µM of divalent cations, in Hepes NaCl. 50 s was allowed for the Aβ·M$^{2+}$ reaction to complete. The fluorescence was measured

*Atoms or ions with unpaired electrons in the d-orbital are paramagnetic.
using the fluorometer. The samples were left to equilibrate with the quartz cuvettes for 2 h at 25°C before measurement. The excitation and emission wavelengths for data collection were 480 nm/527 nm, 520 nm/564 nm, and 605 nm/670 nm, for Hilyte 488, Hilyte 555 and Hilyte 647, N-terminally labelled Aβ respectively.

Figure 3.1: Quenching selectivity of 100 nM N-terminally labelled Aβ, by 100 µM of chosen common divalent cations. $F_0$ is the initial fluorescence intensity without the cations, whereas $F$ is the the fluorescence in the presence of the cations. Fe$^{2+}$ was not used as it oxidises very quickly in Hepes NaCl.

The results shown in figure 3.1 show that Cu$^{2+}$ does bind and quench labelled Aβ. Excluding the labelled Aβ$_{42}$, the dye is almost completely quenched to 98.7(5) %, whereas the other metal cations were quenched to 14(8) %. The Cu$^{2+}$ quenches a range of dyes, allowing any of these to be used for Cu$^{2+}$ binding studies. This data also shows that Cu$^{2+}$ could interfere in studies which do not specifically account for this effect, also employing labelled Aβ, and would likely prevent FRET studies. Free Alexa Fluor 488 C5 maleimide (similar to HL488) did not quench with 100 µM Cu$^{2+}$, and free Hilyte Fluor 488 maleimide was not quenched by 10 µM CuCl$_2$. At higher concentrations, other mechanisms of quenching, such as Stern-Volmer quenching$^{[258]}$ may occur.

Aβ$_{42}$ is known to aggregate with Cu$^{2+}$ and Zn$^{2+}$. This suggests that the increased quenching for Zn$^{2+}$, Co$^{2+}$, Ni$^{2+}$ and Cd$^{2+}$ may be due to aggregates bringing the dyes close together, allowing quenching via Homo-FRET. The decreased quenching of Aβ$_{42}$ by Cu$^{2+}$ could be due to larger structures forming in solution, preventing some of the Aβ’s from binding to Cu$^{2+}$.
Apart from Cu\(^{2+}\), the other metal cations also appear to reduce the fluorescence of labelled A\(\beta\)\(_{16/40}\), by about 15\%. As surface adsorption of A\(\beta\) is a problem, the A\(\beta\) samples were left in the cuvettes for 1 h to equilibrate. To test the effect of mixing, the experiment was performed with and without mixing, without adding sample to the cuvette. For A\(\beta\)\(_{40}\)HL647, the intensity was found to drop by approximately 13\%, where as without mixing there was little difference. This suggests that the small decreases in figure 3.1 are an artifact of the method used. For future experiments it suggests that when 100 nm concentrations of A\(\beta\) or less are used, the actual concentration may be lower than expected, and that surface adsorption of A\(\beta\) onto pipette tips may also be a problem.

The quenching of fluorescently labelled A\(\beta\) by Cu\(^{2+}\) is the basis for the rest of the work in this chapter. This phenomenon will be used to investigate the thermodynamics and kinetics of A\(\beta\) binding to Cu\(^{2+}\) ions, and is why Cu\(^{2+}\) is preferentially studied, rather than Zn\(^{2+}\). It has been previously shown that dyes may be quenched by Cu\(^{2+}\)\(^{[259]}\), and other paramagnetic metal ions\(^{[260]}\), however it seems that this phenomenon has not been exploited to understand the binding of metals to proteins and peptides.

### 3.2 Multiple Binding Phases of A\(\beta\) to Cu\(^{2+}\)

To investigate the binding of Cu\(^{2+}\) to labelled A\(\beta\), titrations were performed. This would allow for the equilibrium dissociation constant (\(K_d\)) to be measured. Knowing the \(K_d\) allows the concentration of the A\(\beta\)·Cu complexes to be predicted, given the concentration of A\(\beta\) and Cu\(^{2+}\). The \(K_d\) can then be used to calculate the concentration of A\(\beta\)·Cu formed in equilibrium in the CSF. It also gives the minimal concentration required for transient concentrations of Cu\(^{2+}\) to reach for binding to occur. However, whether binding does or does not occur, would still depend on the kinetics of the reaction.

To determine the quenching profile of labelled A\(\beta\) by Cu\(^{2+}\), CuCl\(_2\) was titrated into 133 nm of A\(\beta_{16}\)LysHL488 in Hepes NaCl, measuring the fluorescence. The A\(\beta\) sample was left to equilibrate in the quartz cuvettes for 2 h at 25 °C, before titration.
The sample was excited at 480 nm, and the emission intensity was integrated across 500 nm to 600 nm.

The results shown in figure 3.2 show that there are three apparent phases of Cu$^{2+}$ binding to Aβ, and that the quenching level is similar to that of the N-terminally labelled Aβ’s with 100 µm Cu$^{2+}$. Fitting the binding curves with Hill functions (see Eqn. 2.30), gives the $K_A$’s and $\alpha$’s shown in table 3.1. The first phase appears to be standard binding, as $\alpha \approx 1$. However, as the $K_d \gg [A\beta]$, approximations of the Hill function are no longer valid, and it overestimates the value of the $K_d$. Refitting with the 1:1 binding equation, a Hill function gives $K_d$’s of 165(9) nm & 9.0(4) µm, with an $\alpha$ for the second phase of 2.0(1). In section 3.5, the $K_d$ of the first copper binding
Table 3.1: Parameters of the multiple Hill fits of the data shown in figure 3.2. For the fitting of (b) & (c), the parameters of the first & second phase were fixed, respectively. The values are not corrected for the Hepes-Cu$^{2+}$ interaction, this underestimates the concentration of Cu$^{2+}$ by a factor of approximately 3 (see §3.3 & §3.5).

<table>
<thead>
<tr>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_A/\text{nm}$</td>
<td>$\alpha$</td>
<td>$K_A/\mu\text{m}$</td>
</tr>
<tr>
<td>10 nM to 200 $\mu\text{m}$</td>
<td>250(20)</td>
<td>1.0(1)</td>
</tr>
<tr>
<td>1 $\mu\text{m}$ to 100 $\mu\text{m}$</td>
<td>9.0(3)</td>
<td>4.3(3)</td>
</tr>
<tr>
<td>10 $\mu\text{m}$ to 100 $\mu\text{m}$</td>
<td>52(1)</td>
<td>5.2(4)</td>
</tr>
</tbody>
</table>

is derived from the kinetics to be approximately 1 nM, over an order of magnitude lower than measured here. This suggests that the measurement here is an artifact, possibly due to surface adsorption of A$\beta$ or Cu$^{2+}$. The later phases are likely more reliable as the concentration of Cu$^{2+}$ is much higher, and is much greater than the concentration of A$\beta$, negating some of the problems of surface adsorption.

The second and third phases have $K_A$’s of approximately 9 $\mu\text{m}$, and 50 $\mu\text{m}$. However, the phases are much steeper than expected for standard stoichiometric binding. In the Hill model, $\alpha > 1$ suggests that cooperative ligand binding may be occurring, but here the number of Cu$^{2+}$ binding at each phase is unknown. Given that Cu$^{2+}$ has a strong positive charge, and that A$\beta$ is a short peptide, it is unlikely that multiple Cu$^{2+}$ ions would bind with a similar $K_d$. An alternative possibility is that the Cu$^{2+}$ is causing the A$\beta$ to oligomerise.

To investigate the possibility of oligomerisation the same titration was performed using the FCS setup. However, 14 nM of labelled A$\beta$ was used, as 133 nM is too concentrated to perform FCS. This allows for the concentration of fluorescent particles to be measured, via $G_0$, and also the mean diffusion time of the complex. If all the A$\beta$’s form dimers, the $G_0$ should double, as it is inversely proportional to the concentration of fluorescent particles.

Figure 3.3 shows that intensity of the FCS experiments still shows two phases, in approximately the same position as the fluorometer measurement, even with the order of magnitude reduction in A$\beta$ concentration. The graph of $G_0$ is flat across the
Figure 3.3: The quenching of 14 nM Aβ16LysHL488 by Cu^{2+}, the fluorescence intensity, \( G_0 \) and mean diffusion time of the complex, measured using FCS. a) The fitted intensity curve is a two Hill function with \( K_A \)'s of 0.5(1) \( \mu \text{m} \) & 14 \( \mu \text{m} \), with \( \alpha \)'s of 5(6) & 2.4(9). The fluorometer fit was scaled to the data range from the FCS measurement, for comparison. b) For \( G_0 \) the fit gives \( K_A = 41(1) \mu \text{m} \) and \( \alpha = 2.5(1) \). c) For the mean diffusion time, the fit gives \( K_A \)'s of 0.14(2) \( \mu \text{m} \) & 5.5(8) \( \mu \text{m} \), with \( \alpha \)'s of 2.1(4) & 1.8(3).

First two phases, showing that the first two phases are not oligomerisation. However, there is drop in the concentration of fluorescent particles in line with the third phase. In section 3.3.1 it is shown that the third phase is also not oligomerisation. Therefore, the third phase is either deposition of Aβ onto the surface, or the labelled Aβ is quenched to a point such that FCS no longer works. The diffusion time also seems to follow a trend towards the labelled molecules getting smaller as the Cu^{2+} concentration increases. Assuming the molecules are spherical, there is approximately a 15\% total decrease in the hydrodynamic radius. Interestingly, the phases for decrease in mean diffusion time are not aligned with the phases for decrease in intensity, but are shifted to lower concentrations. This suggests that
transient binding of Cu$^{2+}$ to $\alpha\beta$ is causing the $\alpha\beta$ to spend more time in a more compact state, even when most of the time is not spent bound to Cu$^{2+}$.

The $K_d$’s measured here show that $\alpha\beta$·Cu complexes cannot form in the steady state conditions of the CSF, due to it being unable to compete with other metal binding proteins, such as HSA, which is more prevalent and has a picomolar $K_d$.

### 3.2.1 Comparison with N-Terminally Labelled $\alpha\beta$

N-terminal modifications on $\alpha\beta$ are known to reduce the binding affinity of Cu$^{2+}$ to $\alpha\beta$[210;261], turning the amine into an amide∗. Here Cu$^{2+}$ was titrated into 100 nM N-terminally labelled $\alpha\beta$ in Hepes NaCl. Again the binding curves were steeper than expected for standard 1 : 1 binding stoichiometry, and thus the Hill equation was used. The results shown in figure 3.4, show that the baseline is not flat, in particular for $\alpha\beta_{40/42}$, whereas $\alpha\beta_{16}$ appears flat. This is likely due to the hydrophobic tail on $\alpha\beta_{40/42}$ increasing surface adsorption. To account for this behaviour an exponential term is put into the Hill Equation ($F([\text{Cu}])$

$$F'([\text{Cu}]) = F_b + (1 + (Ae^{-b[Cu]} - 1)) \times (F([\text{Cu}]) - F_b) , \quad (3.1)$$

where $A$ and $b$ are parameters for the exponential term, and $F_b$ is the baseline of the quenched fluorescence.

The results in figure 3.4 and table 3.2 show that N-terminally labelled $\alpha\beta$ binding to Cu$^{2+}$ is considerably weaker. This suggests that the N-terminal amine is involved in the coordination with Cu$^{2+}$, or that there is some steric hindrance from

<table>
<thead>
<tr>
<th>Solution</th>
<th>$K_d$/$\mu$m</th>
<th>$\alpha$</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha\beta_{16}$HL555</td>
<td>4.3(6)</td>
<td>1.5(1)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>$\alpha\beta_{40}$HL488</td>
<td>3.5(8)</td>
<td>1.3(2)</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>$\alpha\beta_{42}$HL647</td>
<td>5.6(6)</td>
<td>1.5(1)</td>
<td>(n = 7)</td>
</tr>
</tbody>
</table>

Table 3.2: Fitted parameters for the Hill function in figure 3.4.

∗Loss of the lone pair of electrons on the N-terminal Nitrogen.
the attached dye. It also suggests that the hydrophobic C-terminal region does not affect Cu\(^{2+}\) binding, given that the measured \(K_A\)'s are within error of each other.

These results show that N-terminally labelled A\(\beta\) should not be used to investigate unmodified A\(\beta\).

![Figure 3.4: Fluorescence Titration curves for 100 nm labelled A\(\beta\) with Cu\(^{2+}\), with the microscopic dissociation constant (\(K_A\)) closest to the mean of multiple runs. Fitted parameters for the Hill function are shown in table 3.2. The corresponding fluorescence spectra are shown as inset, for selected copper concentrations. They are normalised by \(F \mapsto F/(F_{\max}(1 - b))\)](image)

### 3.3 Association Kinetics of A\(\beta\) Binding to Cu\(^{2+}\)

To determine whether A\(\beta\) is able to bind Cu\(^{2+}\) in the transiently high concentrations of Cu\(^{2+}\) during synaptic transmission, the association rate \(k_{on}\) is required. After release, the ‘free’ Cu\(^{2+}\) concentration is depleted by binding to other Cu\(^{2+}\) binding proteins, and diffusion across and out of the synapse.

To measure the A\(\beta + Cu^{2+} \longrightarrow A\beta \cdot Cu\) association rate, 25 nm of HL488 labelled
human or murine A\beta, was mixed with 500 nM CuCl₂, in Hepes NaCl (except for A\beta₁₆LysHL488, which was performed with 20 nM and 400 nM CuCl₂). The fluorescent traces were fitted with exponentials, and the rate of the largest and fastest was taken to be the apparent association rate (k\textsubscript{on(App)}). This is true when the binding is assumed to be irreversible. If the reaction is reversible, the reaction is

\[
A\beta + Cu^{2+} \xrightarrow{k_{on}} A\beta \cdot Cu .
\]

Under pseudo-first-order conditions, this gives the differential equations

\[
\frac{d[A\beta]}{dt} = -k_{on}[A\beta][Cu^{2+}] + k_{off}[A\beta \cdot Cu] ,
\]

\[
\frac{d[A\beta \cdot Cu]}{dt} = +k_{on}[A\beta][Cu^{2+}] - k_{off}[A\beta \cdot Cu] ,
\]

and

\[
[A\beta]_T = [A\beta] + [A\beta \cdot Cu] .
\]

The solutions are therefore

\[
[A\beta] = [A\beta]_T - [A\beta \cdot Cu] = e^{-k_{off}[Cu^{2+}]t} ,
\]

for a constant c. Thus the irreversibility assumption is valid when

\[
k_{off} \ll k_{on}[Cu^{2+}] .
\]

In section 3.5, k\textsubscript{off} is shown to be approximately 0.5 s\textsuperscript{-1}, and therefore the irreversibility of the reaction is a valid assumption. Due to the weak binding of Hepes to Cu\textsuperscript{2+}, the Hepes concentration was varied in the Hepes NaCl from 10 mM to 100 mM, to determine the effect of Hepes on the binding. The resulting rates of k\textsuperscript{-1\textsubscript{on(App)}} against Hepes concentration were phenomenologically fitted with a zero centered parabola

\[
k_{on(App)}^{-1} = A[Hepes]^2 + C ,
\]

where A describes the shape of the parabola, and C the intercept. C is thus the
The inverse of the Hepes independent association rate constant \( (k_{on}) \).

\[ k_{-1}^{\text{on(App)}} / \text{ms} \]

\[ [\text{Hepes}] / \text{mm} \]

Figure 3.5: Determination of the Hepes independent association rate. 25 nm labelled A\( \beta \) was reacted with 500 nm Cu\(^{2+}\) with various Hepes concentrations, all with 100 mm NaCl. The A\( \beta_{16} \) was 20 nm A\( \beta \) with 400 nm Cu\(^{2+}\). The fitting parameters are summarised in table 3.3.

![Graphs showing association rate constants](image)

(a) A\( \beta_{16} \) LysHL488  
(b) A\( \beta_{28} \) LysHL488  
(c) A\( \beta_{40} \) Cys\( \_20 \) HL488  
(d) Murine A\( \beta_{16} \) LysHL488

Table 3.3: Summary of Hepes independent A\( \beta + \text{Cu}^{2+} \rightarrow \text{A} \cdot \text{Cu} \) association rate constants \( k_{on} = C^{-1} \) and fitting parameters from figure 3.5. \( K_{\text{Hepes}} @50 \text{mm} \) represents the factor of the apparent reduction in Cu\(^{2+}\) concentration at 50 mm Hepes.

<table>
<thead>
<tr>
<th></th>
<th>( A/\text{mm}^{-2} \text{s} )</th>
<th>( k_{on}/10^6 \text{M}^{-1} \text{s}^{-1} )</th>
<th>( K_{\text{Hepes}} @50 \text{mm} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A( \beta_{16} ) LysHL488</td>
<td>3.5(5)</td>
<td>5.1(6)</td>
<td>3.0(6)</td>
</tr>
<tr>
<td>A( \beta_{28} ) LysHL488</td>
<td>6.3(5)</td>
<td>4.9(1)</td>
<td>4.1(7)</td>
</tr>
<tr>
<td>A( \beta_{40} ) Cys( _20 ) HL488</td>
<td>4.1(3)</td>
<td>4.9(5)</td>
<td>3.5(4)</td>
</tr>
<tr>
<td>Murine A( \beta_{16} ) LysHL488</td>
<td>3.4(1)</td>
<td>6.1(3)</td>
<td>3.6(2)</td>
</tr>
</tbody>
</table>

The results in figure 3.5 and table 3.3 show that the mean Hepes independent...
association rate is $5.0(3) \times 10^8 \text{ M}^{-1} \text{s}^{-1}$. This is close to the diffusion limit of approximately $10^9 \text{ M}^{-1} \text{s}^{-1}$ and much faster than initially expected. This may possibly be due to electrostatic interactions between the positively charged Cu$^{2+}$ and the negatively charged Aβ. The near diffusion limited nature suggests that Aβ is able to bind to Cu$^{2+}$, whilst the concentration is dropping due to diffusion. Therefore the limiting factor as to whether Aβ is able to bind in the transient Cu$^{2+}$ concentration, is the depletion by other Cu$^{2+}$ binding molecules, such as HSA or the Cu$^{2+}$ binding amino acids.

3.3.1 The Binding of the Second Cu$^{2+}$ to Aβ

Are the Second and Third Phases Dimerisation or Aggregation?

It was shown in figure 3.3, using FCS, that the second phase was not dimerisation of Aβ, but another copper binding to Aβ. This is assumed to be the binding of a second copper ion. To confirm these results, the concentration of Aβ was varied to look for changes in the rate of fluorescent quenching. If dimerisation is occurring, the rate should be highly dependent on Aβ concentration. This methodology can simultaneously be applied to determining whether the third phase is aggregation.

To investigate the dependence of the second and third phase on Aβ concentration, 25 nM, 50 nM and 100 nM Aβ$_{16}$LysHL488 was mixed with 10 μM CuCl$_2$, in Hepes NaCl.

The results in figure 3.6 show that with 10 μM Cu$^{2+}$, the second and third phases are visible. Normalising out the difference in brightness due to the different concentrations of labelled Aβ, shows that the rates are indistinguishable across a factor of 4 change in the Aβ concentration. Therefore neither the second, nor third phase is dimerisation of the Aβ.

Kinetics of the Second Cu$^{2+}$ Binding to Aβ

To investigate the binding rate of the second Cu$^{2+}$ to Aβ, 50 nM of Aβ$_{16}$LysHL488 was mixed with varying concentrations of CuCl$_2$ from 5 μM to 20 μM, and the apparent association rate $k_{on}^{App}$ was measured by fitting the phase with an exponential.
Equation 3.6 shows, that assuming the same model for binding, the solution is exponential with the rate

\[ k_{\text{App}}^{\text{on}} = k_{\text{off}} + k_{\text{on}}[\text{Cu}^{2+}] \] .

That is ignoring the reaction \( A\beta + \text{Cu}^{2+} \longrightarrow A\beta\cdot\text{Cu} \), as the rate is \( >800\text{s}^{-1} \), and only considering \( A\beta\cdot\text{Cu} + \text{Cu}^{2+} \rightleftharpoons A\beta\cdot\text{Cu}_{2} \). Therefore, when varying the \( \text{Cu}^{2+} \) concentration near the \( K_{d} \),

\[ k_{\text{off}} \approx k_{\text{on}}[\text{Cu}^{2+}] \] ,

and thus plotting \( k_{\text{App}}^{\text{on}} \) against \([\text{Cu}^{2+}]\) should be linear, where the intercept is the dissociation rate constant of \( A\beta\cdot\text{Cu}_{2} \longrightarrow A\beta\cdot\text{Cu} + \text{Cu}^{2+} \) \( (k_{\text{off}}) \), and the gradient is the association rate constant of \( A\beta\cdot\text{Cu} + \text{Cu}^{2+} \longrightarrow A\beta\cdot\text{Cu}_{2} \) \( (k_{\text{on}}) \).

The results in figure 3.7 show that the apparent rate appears to be linear with \( k_{\text{off}} = 7.3(7)\text{s}^{-1} \), and \( k_{\text{on}} = 4.2(6) \times 10^{5}\text{M}^{-1}\text{s}^{-1} \) (the rate is not corrected for the effect of the buffer on apparent \( \text{Cu}^{2+} \) concentration). The second copper binding \( K_{d} \) is therefore \( 17(3)\mu\text{M} \). This is approximately half \( (2\sigma \text{ to } 3\sigma) \) the previous measurement in table 3.1, but confirms that the \( K_{d} \) is in the low 10’s of micromolar regime. However, the association rate is four orders of magnitude below the diffusion limited rate. This suggests that a second or third copper is unlikely to bind in the transient \( \text{Cu}^{2+} \) release of neurotransmission, given the diffusion of copper out of
the synapse.

![Graph](image)

Figure 3.7: The apparent rate of the second phase of 50 nM $A\beta_{16}$LysHL488 with copper. This gives the association rate to be $4.2(6) \times 10^5$ M$^{-1}$ s$^{-1}$ and the dissociation rate to be $7.3(7)$ s$^{-1}$.

### 3.3.2 Activation Energy

To confirm the near diffusion limited rate of the first Cu$^{2+}$ binding to $A\beta$, the activation energy was measured. 100 nM of $A\beta_{16}$LysHL488 or 20 nM $A\beta_{28}$LysHL488 was mixed with 1 µM or 400 nM CuCl$_2$ respectively and the temperature was varied.

![Graphs](image)

Figure 3.8: Arrhenius plots of $A\beta$ binding to Cu$^{2+}$. The rate constant $K_{App}$ is the reaction rate divided by the concentration of Cu$^{2+}$. a) 100 nM $A\beta_{16}$LysHL488 mixed with 1 µM Cu$^{2+}$. This gives an $E_A = 27(2)$ kJ mol$^{-1}$ for the association rate constant. b) 20 nM $A\beta_{28}$LysHL488 mixed with 400 nM Cu$^{2+}$. This gives an $E_A = 32(1)$ kJ mol$^{-1}$ for the association rate constant. The rates are not corrected for the effect of Cu$^{2+}$ binding to Hepes.
The results in figure 3.8 show that the activation energy for Aβ\textsubscript{16} is 27(2) kJ mol\textsuperscript{-1}, and for Aβ\textsubscript{28} is 32(1) kJ mol\textsuperscript{-1}, within 2 σ. Here, the activation energy is the activation energy of Aβ binding to Cu\textsuperscript{2+} as the dissociation rate is negligible. The activation energy of the association is small, given that the contribution from the viscosity of water is approximately 17 kJ mol\textsuperscript{-1}. Correcting for this, gives activation energies of 10(2) kJ mol\textsuperscript{-1} (∼4 k\textsubscript{B} T) & 15(1) kJ mol\textsuperscript{-1} (∼6 k\textsubscript{B} T), for Aβ\textsubscript{16} & Aβ\textsubscript{28}, respectively. These are very low, further suggesting that the binding of Aβ to Cu\textsuperscript{2+} is near diffusion limited.

For comparison, the activation of the second Cu\textsuperscript{2+} binding to Aβ was measured. 200 nM Aβ\textsubscript{16}LysHL488 was mixed with 10 μM CuCl\textsubscript{2}, in Hepes NaCl, and the temperature was varied.

![Figure 3.9: Arrhenius plot of the binding of a second Cu\textsuperscript{2+} to Aβ-Cu. \(k_{\text{App}}\) is the apparent rate of this reaction. 200 nM of Aβ\textsubscript{16}LysHL488 was mixed with 10 μM Cu\textsuperscript{2+}, giving an \(E_A = 43(2)\) kJ mol\textsuperscript{-1}. The rates are not corrected for the binding of Cu\textsuperscript{2+} to Hepes.](image)

The results in figure 3.9, give the activation energy to be 43(2) kJ mol\textsuperscript{-1}. However, as the 10 μM Cu\textsuperscript{2+} used is close to the \(K_d\), the \(k_{\text{App}}\) is made up of a mixture of the dissociation rate, and the apparent association rate. The activation energy is therefore likely to be an overestimate of the activation energy of the second Cu\textsuperscript{2+} binding, as typically intermolecular processes, such as dissociation, are more strongly dependent on temperature.
3.3.3 Association of N-Terminally Labelled Aβ

To determine whether the difference in $K_d$’s for the N-terminally labelled and C-terminally labelled Aβ peptides was due to the association, dissociation or both, the Cu$^{2+}$ binding to Aβ$_{16}$HL488 was measured.

![Graph showing the association rate constant of Aβ$_{16}$HL488 with Cu$^{2+}$ by varying the HEPES concentration in the buffer.](image)

Figure 3.10: Determination of the association rate constant of Aβ$_{16}$HL488 with Cu$^{2+}$ by varying the HEPES concentration in the buffer. 50 nM Aβ$_{40}$HL488 was mixed with 1 µM Cu$^{2+}$ and fitted with an exponential to obtain $k_{on(App)}$. This gives a rate constant $k_{on} = 4.6(2) \times 10^8$ M$^{-1}$ s$^{-1}$.

The results in figure 3.10 show that the association rate constant to be $4.6(2) \times 10^8$ M$^{-1}$ s$^{-1}$, similar to the C-terminally labelled Aβ. The difference in $K_d$’s is therefore due to a difference in dissociation rate constants. This also shows that the N-terminal amine is not involved in the initial binding to Cu$^{2+}$, but is involved in the stability of the Aβ-Cu complex. Using the $K_d$ for the second binding phase from table 3.2, 3.5(8) µM, gives the $k_{off}$ to be 1.4(4) ms$^{-1}$.

These results suggest that N-terminal modifications to Aβ such as acetylated Aβ or pyroglutamate-modified Aβ affect the length of time that Cu$^{2+}$ is bound to Aβ, rather than whether or how fast it binds.

3.4 Competition for Cu$^{2+}$ from HSA and Glycine

Although the binding of Cu$^{2+}$ to Aβ is near diffusion limited, there are other components in the CSF that also bind copper and are found at orders of magnitude higher concentrations than Aβ. Some examples are proteins such as HSA, and amino
acids, such as glycine. The binding of glycine is known to be near diffusion limited for the anionic form\textsuperscript{[262]}.\\

3.4.1 HSA\\

To determine whether A\textsubscript{β} could compete against HSA in binding Cu\textsuperscript{2+}, 50 nM A\textsubscript{β16} was premixed with 5 µM HSA (approximately physiological concentration in the CSF), this solution was then mixed with varying concentrations of Cu\textsuperscript{2+}.\\

![Figure 3.11: Competition of A\textsubscript{β} & HSA for Cu\textsuperscript{2+}. 5 µM HSA was premixed with 50 nM A\textsubscript{β16}LysHL488, and then mixed with various concentrations of Cu\textsuperscript{2+} using stopped flow. a) Normalised raw traces. b) Maximal proportion of A\textsubscript{β} bound to Cu\textsuperscript{2+}, assuming that the fluorescence of A\textsubscript{β}·Cu is 2/3 of free A\textsubscript{β}. 2/3 was chosen as it was similar to the apparent quenching level of the first phase in the fluorimeter titration experiments (Fig. 3.2). Determination of the exact value is not necessary given the qualitative nature of this experiment. The solid line is a phenomenological fit of a offset logarithm.]

Figure 3.11 shows that at short time scales the A\textsubscript{β} is bound to Cu\textsuperscript{2+}, and HSA is not able to compete. However, at longer timescales, the Cu\textsuperscript{2+} dissociates from the A\textsubscript{β}·Cu. Therefore, the K\textsubscript{d} of HSA with Cu\textsuperscript{2+} is lower than that of A\textsubscript{β} with Cu\textsuperscript{2+}. The plot of maximal A\textsubscript{β}·Cu formation shows that at 5 µM Cu\textsuperscript{2+}, only half the A\textsubscript{β} forms A\textsubscript{β}·Cu. For stoichiometric binding of Cu\textsuperscript{2+} to HSA, this value should tend to 1 rather than 0.5, as when the concentration of Cu\textsuperscript{2+} is above that of HSA all A\textsubscript{β} should form A\textsubscript{β}·Cu. Therefore, HSA must have multiple binding sites for copper, allowing it to soak up more than its equivalence in Cu\textsuperscript{2+}. The figure shows that the binding rate of HSA to Cu\textsuperscript{2+} is similar to that of A\textsubscript{β}.\\

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Given that the binding and removal of Cu$^{2+}$ are temporally well separated in figure 3.11, the maximal binding of Aβ to Cu$^{2+}$ may be determined by only considering the binding reactions, i.e.

\[
\begin{align*}
A\beta + \text{HSA} + \text{Cu}^{2+} & \xrightarrow{k_{A\beta \text{on}}^{\text{HSA}}} A\beta \cdot \text{Cu} + \text{HSA} \\
A\beta \cdot \text{Cu} + \text{HSA} & \xrightarrow{k_{A\beta \text{on}}^{\text{HSA}}} \text{HSA} \cdot \text{Cu} + A\beta
\end{align*}
\] (3.11)

This ignores the dissociation of Cu$^{2+}$ from the ligands and any interactions between them. The reaction was simulated in python using scipy’s Dormand-Prince method for solving the differential equations (see Appx. A.1) varying the ratio $k_{\text{on}}^{\text{HSA}}/k_{\text{on}}^{A\beta}$. The results in figure 3.12, based on the shape of the curves when compared to figure 3.11, suggests that the $k_{\text{on}}^{\text{HSA}} < k_{\text{on}}^{A\beta}$, at approximately $10^7 \text{M}^{-1} \text{s}^{-1}$ to $10^8 \text{M}^{-1} \text{s}^{-1}$. There are currently no literature values for comparison. It may be possible to further this methodology to obtain a more accurate value and determine the binding stoichiometry of Cu$^{2+}$ to HSA.

Figure 3.12: Simulation of the competition of Aβ & HSA for Cu$^{2+}$. 5 µM HSA was mixed with 50 nM Aβ, and various concentrations of Cu$^{2+}$ from equation 3.11. Proportion of Aβ bound to Cu$^{2+}$ after 1 s, varying the ratio of the association of Cu$^{2+}$ with HSA ($k_{\text{on}}^{\text{HSA}}$) to that of Aβ ($k_{\text{on}}^{A\beta} = 3 \times 10^8 \text{M}^{-1} \text{s}^{-1}$).
3.4.2 Glycine

When the competition experiment between HSA and Aβ for Cu²⁺ (see § 3.4.1) was repeated with glycine, fluorescence quenching by the Cu²⁺ was observed, but there was no fluorescence recovery. This suggests that the \( K_d \) of Cu²⁺ with glycine is much greater than a few micromolar, that ternary Aβ·Cu·Gly complexes can form, or that the solution reaches equilibrium within the dead time. The CSF concentration of glycine in one study was 11(2) µm \((n = 21)\)\(^{[263]}\).

![Figure 3.13: Competition for Cu²⁺ between Aβ and glycine. a) 500 nm CuCl₂ premixed with various concentrations of glycine to form complexes, which was then mixed using stopped flow with 60 nm Aβ₁₆LysHL488. b) 100 nm Aβ₁₆LysHL488 was premixed with 100 nm Cu²⁺, which was then mixed using stopped flow with various concentrations of glycine. 'Before' and 'After' represent the baselines of fluorescence of Aβ without Cu²⁺ or glycine, before and after the other measurements, respectively.](image)

To determine the effect on Aβ binding to Cu²⁺, 500 nm CuCl₂ premixed with various concentrations of glycine was then mixed using stopped flow with 60 nm Aβ₁₆LysHL488. Figure 3.13a shows that low concentrations of Cu²⁺ can still bind to Aβ, even with high concentrations of glycine.

To determine the effect of glycine on Aβ·Cu, 100 nm Aβ₁₆LysHL488 was premixed with 100 nm Cu²⁺, and then mixed using stopped flow with various concentrations of glycine. Figure 3.13b shows that tens of micromolar of glycine is unable to completely remove the Cu²⁺ from the Aβ·Cu complex.
3.5 Dissociation Kinetics of the Aβ·Cu Complex

To independently determine the $K_d$ from the underlying kinetics, both the association and dissociation rate constants are required. The dissociation rate constant will also determine the likely lifetime of the Aβ·Cu complex, and thus whether the complex has time to interact with other ligands in the synapse.

To determine the dissociation rate, the Aβ·Cu complex was preformed by stoichiometric mixing of labelled 100 nm Aβ and 100 nm CuCl$_2$. This equilibrium mixture of Aβ, Cu$^{2+}$ & Aβ·Cu was then mixed with various concentrations of a Cu$^{2+}$ binding ligand (HSA, EDTA, Ap$_{16}$, Ap$_{40}$) in much greater excess to quickly remove the unbound Cu$^{2+}$.

The model was expected to be

$$
Aβ·Cu + L \xrightarrow{K_{off}} Aβ + Cu^{2+} + L \xrightarrow{} Aβ + Cu·L,
$$

in which the dissociation of Cu$^{2+}$ from Aβ·Cu is irreversible, due to the excess of ligand. This model gives the differential equation

$$
\frac{d[Aβ·Cu]}{dt} = -k_{off}[Aβ·Cu],
$$

and thus the solution

$$
[Aβ] = [Aβ]_t - [Aβ·Cu]|_{t=0}e^{-k_{off}t},
$$

and is independent of ligand concentration. However, the results (such as in figure 3.14) show a linear dependence on ligand concentration, rather than no dependence. Therefore this simplistic model is incorrect and the ligand is interacting directly with the Aβ·Cu complex.

To incorporate the dissociation of the Aβ·Cu both directly and assisted by a Cu$^{2+}$
binding ligand, the model

\[
\begin{align*}
\text{A}^\beta \cdot \text{Cu} & \xrightarrow{k_{\text{off}}} \text{A}^\beta + \text{Cu}^{2+} \\
\text{A}^\beta \cdot \text{Cu} & \xrightarrow{k_{\text{Lo}}ff} \text{A}^\beta + \text{Cu} \cdot \text{L}
\end{align*}
\]  

(3.15)

was used, with a direct interaction between A\(^\beta\)·Cu and the ligand. This gives the differential equation

\[
\frac{d[\text{A}^\beta \cdot \text{Cu}]}{dt} = -k_{\text{off}}[\text{A}^\beta \cdot \text{Cu}] - k_{\text{Lo}}ff[\text{A}^\beta \cdot \text{Cu}][\text{L}]
\]  

(3.16)

and

\[
[\text{A}^\beta]_T = [\text{A}^\beta] + [\text{A}^\beta \cdot \text{Cu}]
\]  

(3.17)

gives the solution to be

\[
[\text{A}^\beta] = [\text{A}^\beta]_T - e^{-(k_{\text{off}} + k_{\text{Lo}}ff[L])t} [\text{A}^\beta \cdot \text{Cu}] \bigg|_{t=0}
\]  

(3.18)

to which the rate is linear with ligand concentration (\((k_{\text{off}} + k_{\text{Lo}}ff[L])\)).

The reverse reactions were not considered as the free copper concentration should be kept low by the competing Cu\(^{2+}\) ligand. The solution is a single exponential and plotting the apparent rate as a function of [L] may be used to obtain the off-rate \((k_{\text{off}}, \text{intercept})\) and removal rate constant via the ligand \((k_{\text{Lo}}ff, \text{gradient})\). However this model is unphysical as the copper ion ‘jumps’ from the A\(^\beta\)·Cu complex to the Cu·L complex. A more physical equivalent is

\[
\begin{align*}
\text{A}^\beta \cdot \text{Cu} & \xrightarrow{k_{\text{Lo}}ff} (\text{A}^\beta \cdot \text{Cu} \cdot \text{L})^* \\
\text{A}^\beta \cdot \text{Cu} & \xrightarrow{k_{\text{off}}} \text{A}^\beta + \text{Cu} \cdot \text{L} \\
\text{A}^\beta + \text{Cu}^{2+} & \xrightarrow{} \text{A}^\beta \cdot \text{Cu} + \text{L}
\end{align*}
\]  

(3.19)
with an explicit ternary complex forming. However, the significance of \( k_{\text{Loff}} \) is unclear, other than as a rate constant related to the removal of \( \text{Cu}^{2+} \) from \( \text{A}\beta \cdot \text{Cu} \).

Inserting the explicit ternary complex formation into the model, it becomes

\[
\begin{align*}
(A\beta \cdot \text{Cu} \cdot \text{L})^* & \xrightarrow{k_{\text{Ton}}} A\beta \cdot \text{Cu} + \text{L} \\
(A\beta \cdot \text{Cu} \cdot \text{L})^* & \xrightarrow{k_{\text{Toff}}} A\beta + \text{Cu} \cdot \text{L},
\end{align*}
\]

with the differential equations

\[
\begin{align*}
\frac{d[A\beta \cdot \text{Cu}]}{dt} &= -k_{\text{off}}[A\beta \cdot \text{Cu}] - k_{\text{Ton}}[\text{L}][A\beta \cdot \text{Cu}] , \\
\frac{d[(A\beta \cdot \text{Cu} \cdot \text{L})^*]}{dt} &= +k_{\text{Ton}}[\text{L}][A\beta \cdot \text{Cu}] - k_{\text{Toff}}[(A\beta \cdot \text{Cu} \cdot \text{L})^*] ,
\end{align*}
\]

which have the solutions

\[
\begin{align*}
[A\beta \cdot \text{Cu}] &= e^{-(k_{\text{off}}+k_{\text{Ton}}[\text{L}])t}[A\beta \cdot \text{Cu}]|_{t=0} , \\
[(A\beta \cdot \text{Cu} \cdot \text{L})^*] &= e^{-k_{\text{Toff}}t}[(A\beta \cdot \text{Cu} \cdot \text{L})^*]|_{t=0} \\
&- \frac{(e^{-(k_{\text{off}}+k_{\text{Ton}}[\text{L}])t} - e^{-k_{\text{Toff}}t})k_{\text{Ton}}[\text{L}][A\beta \cdot \text{Cu}]|_{t=0}}{k_{\text{off}} + k_{\text{Ton}}[\text{L}] - k_{\text{Toff}}} .
\end{align*}
\]

This gives the concentration of free \( A\beta \) to be

\[
[A\beta] = [A\beta]_T - [A\beta \cdot \text{Cu}] - [(A\beta \cdot \text{Cu} \cdot \text{L})^*] .
\]

The solution is therefore a double exponential, with rates \((k_{\text{off}} + k_{\text{Ton}}[\text{L}])\) and \(k_{\text{Toff}}\). Therefore the \( k_{\text{Loff}} \) in the previous model corresponds to the ternary complex formation rate. The amplitude of the \( k_{\text{Toff}} \) should increase, relative to the amplitude of the \((k_{\text{off}} + k_{\text{Ton}}[\text{L}])\) exponential. In the raw data, none of the other exponentials fitted this description. It is therefore likely that the \( k_{\text{Toff}} \) phase is too fast to observe and that the ternary complex is not highly populated, and thus very transient under these conditions. If it was the rate limiting step, there would be no dependence in
the apparent rate on ligand concentration.

![Graphs showing apparent rates for EDTA, HSA, and Aβ16](image)

Figure 3.14: Apparent removal rate of copper by various ligands from Aβ16LysHL488·Cu. The labelled Aβ16 and Cu²⁺ were premixed at 100 nM, before being mixed with twice the various concentrations of ligand on the graphs.

<table>
<thead>
<tr>
<th></th>
<th>EDTA</th>
<th>HSA</th>
<th>Aβ16</th>
<th>Aβ40</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAβ16·Cu</td>
<td>7.7(6)</td>
<td>0.157(3)</td>
<td>10.7(4)</td>
<td>0.185(2)</td>
</tr>
<tr>
<td>Aβ16·Cu</td>
<td>55(3)</td>
<td>0.42(2)</td>
<td>41(3)</td>
<td>0.52(1)</td>
</tr>
<tr>
<td>Aβ28·Cu</td>
<td>95(6)</td>
<td>0.29(3)</td>
<td>27(1)</td>
<td>0.75(6)</td>
</tr>
<tr>
<td>Aβ40·Cu</td>
<td>96(4)</td>
<td>0.46(2)</td>
<td>25(1)</td>
<td>0.611(8)</td>
</tr>
</tbody>
</table>

Table 3.4: Summary of fitted rates for the removal of Cu²⁺ from various Aβ·Cu’s by a ligand for figures 3.14, 3.15, 3.16 & 3.17. $k_{Ton}$ is the ternary complex formation rate constant of (Aβ·Cu·L) with units of $10^3$ M⁻¹ s⁻¹. $k_{off}$ is the dissociation rate constant of Aβ·Cu, with units of s⁻¹.
Figure 3.15: Apparent removal rate of copper by various ligands from murine Aβ28LysHL488·Cu. The labelled Aβ28 and Cu²⁺ were premixed at 100 nm, before being mixed twice with various concentrations of ligand on the graphs.

The dissociation rate and ternary complex formation was measured for labelled Aβ16·Cu (Fig. 3.14), labelled Aβ28·Cu (Fig. 3.15), labelled Aβ40·Cu (Fig. 3.16) and labelled murine mAβ16·Cu (Fig. 3.17). The ligands used were EDTA, HSA, unlabelled Aβ16, and unlabelled Aβ40. A summary of the results are shown in table 3.4. It was also found that the dissociation rates do not appear to be dependent on the concentration of Hepes, with 2 µm EDTA.

The human Aβ sequences have a mean dissociation rate constant of 0.6(2) s⁻¹ (0.51(8) s⁻¹ for Aβ16, 0.6(2) s⁻¹ for Aβ28, and 0.6(2) s⁻¹ for Aβ40). Using these dissociation rate constants with the association rate constants from table 3.3, gives the mean $K_d$ to be 1.2(4) nm for Aβ16, 1.2(4) nm for Aβ16, and 1.2(4) nm for Aβ40. This fits within the literature range of 10 pm to 100 nm.

The dissociation rate shows that the Aβ·Cu complex is relatively long lived, with a lifetime of 1.7(6) s (half-life of 1.2(4) s). This would give the Aβ·Cu complex time...
Figure 3.16: Apparent removal rate of copper by various ligands from 
\( \text{A}_{\beta 40} \text{Cys}_{20} \text{HL488} \cdot \text{Cu} \). The labelled \( \text{A}_{\beta 40} \) and \( \text{Cu}^{2+} \) were premixed at 100 nm, before being mixed with twice the various concentrations of ligand.

Figure 3.17: Apparent removal rate of copper by various ligands from 
\( \text{A}_{\beta 16} \text{LysHL488} \cdot \text{Cu} \). The labelled \( \text{A}_{\beta 16} \) and \( \text{Cu}^{2+} \) were premixed at 100 nm, before being mixed with twice the various concentrations of ligand.
to interact with other molecules and likely last between synaptic transmissions. It may therefore be possible to enhance the concentration of Aβ·Cu in the synapse, through repeated synaptic transmissions, as a form of short-term synaptic plasticity.

For comparison, murine Aβ has the mean dissociation rate of 0.17(2) s\(^{-1}\), giving a \(K_d\) of 280(40) pm. The \(K_d\) for murine Aβ with Cu\(^{2+}\) is therefore a factor of 4 stronger than human Aβ, with a lifetime of 5.9(7) s (half-life of 4.1(5) s). This suggests that the act of binding Cu\(^{2+}\) alone is not the initial step in the cause of Alzheimer’s disease, as muridae do not develop Alzheimer’s disease.

The ternary complex formation rate (\(k_{\text{Tot}}\)) varies somewhat between ligands and although similar for Aβ\(_{28}\) and Aβ\(_{40}\), is more different for Aβ\(_{16}\). This suggests that Aβ\(_{28}\) is a better model for studying Aβ·Cu interactions than Aβ\(_{16}\). Surprisingly, the strong copper chelator EDTA is less effective at removing the Cu\(^{2+}\) from Aβ·Cu than Aβ. Can this kind of experiment be used to measure and improve the efficacy and efficiency of finding drug candidates to interrupt Aβ·Cu interactions?

When the ligand used is Aβ itself, the ternary complex formation rate becomes the copper assisted dimerisation rate. The mean copper assisted dimerisation rate is 1.2(1) \(\times\) \(10^5\) M\(^{-1}\) s\(^{-1}\). This is three orders of magnitude faster than the literature value of approximately \(10^2\) M\(^{-1}\) s\(^{-1}\)\[^{264}\] for unassisted dimerisation, drastically reducing the concentration of Aβ required for dimerisation to occur. For comparison, the copper assisted dimerisation rate for murine-human Aβ\(_{16}\) is an order of magnitude slower than human-human Aβ\(_{16}\) dimerisation. This difference may be the reason as to why muridae are immune to Alzheimer’s disease.

### 3.6 Targeting Aβ·Cu with Clioquinol and L2-b

Clioquinol (CQ) and L2-b are two drug candidates (see Fig. 3.18), whose efficacy is thought to be derived from interfering with and removal of Aβ·Cu. CQ is a derivative of PBT2, which is currently in clinical trials. L2-b is a bi-functional ligand which also targets Aβ itself. Clioquinol promotes the degradation of metal-dependent Aβ oligomers to restore endocytosis and ameliorate Aβ toxicity\[^{265}\].

To test the efficacy of L-2b and CQ at removing Cu\(^{2+}\) from Aβ·Cu, the same
procedure as in section 3.5 was used. 100 nM labelled Aβ, was premixed with 100 nM CuCl₂ to form Aβ·Cu. This was then mixed with various concentrations of the drug candidates using stopped flow. However, unlike in section 3.5, there were two apparent phases to the fluorescence recovery. In the CQ experiments there was also a slow quenching phase (see §3.6.1), due to the solvent (either DMSO or ethanol) initially used to dissolve the CQ. This phase was ignored in all analysis.

The results in figure 3.19 show the two apparent removal rates of Cu²⁺ from Aβ·Cu. Both phases appear quicker with CQ and L2-b, than for HSA, EDTA or Aβ₁₆. This suggests that CQ and L2-b are more effective at removing Cu²⁺ from Aβ·Cu. The rate at which the rate of the kinetic phases increase in rate is different for the two phases. The rate of the faster phase is steeper than that of the slower phases. This suggests that the L2-b and CQ each act on Aβ·Cu under two different mechanisms.

To quantify the relative effectiveness of ligands, the mean apparent removal rate of all the phases at 10 µM ligand was chosen as a metric. This was calculated by taking the mean removal rate, weighted by the amplitudes of the individual phases, for different ligand concentrations. The mean removal rates were then fitted with a line (see Fig. 3.20), and finally interpolating to 10 µM Ligand concentration for comparison of the effectiveness of the ligands.

The data in table 3.5 shows that CQ appears to be the best at Cu²⁺ removal. The worst appears to be HSA, and is likely due to its bulky size (67 kDa) making it more difficult to bind to the Aβ·Cu complex. EDTA is also unexpectedly slow, given that it is a strong metal chelator and has a very low K_d. This is probably due
to the electrostatic interactions between Aβ·Cu and EDTA. EDTA has a charge of −4e, whereas the charge of Aβ alone is approximately −3e suggesting that when designing ligands to remove Aβ from Aβ·Cu, it may be best for them to be less negatively charged. It also shows that the binding affinity of ligands to Cu$^{2+}$ does not correlate with their ability to remove Cu$^{2+}$ from Aβ·Cu.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Mean Cu$^{2+}$ Removal Rate at 10 µm Ligand / s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ$^{16}$·Cu</td>
<td>1.28(6) 0.92(1) 0.84(1) 5.4(3) 2.3(1)</td>
</tr>
<tr>
<td>Aβ$^{28}$·Cu</td>
<td>1.60(7) 1.06(5) 0.92(4) 7.3(9) 3(1)</td>
</tr>
<tr>
<td>Aβ$^{40}$·Cu</td>
<td>2.6(2) 2.1(2) 5.1(3) 2.3(2)</td>
</tr>
</tbody>
</table>

Table 3.5: The effectiveness of ligands at extracting Cu$^{2+}$ from Aβ·Cu, and the approximate $K_d$ of the ligands binding to Cu$^{2+}$. The mean removal rate at 10 µm Ligand is calculated by fitting the mean rates of all the phases with a line (shown in figure 3.20) and interpolating to 10 µm ligand concentration.
Figure 3.19: The two apparent removal rates of Cu$^{2+}$ from labelled Aβ-Cu, by CQ and L2-b. 100 nm CuCl$_2$ premixed with 100 nm Aβ, to form Aβ-Cu, then mixed with various concentrations of CQ or L2-b.
Figure 3.20: Mean removal rate of Cu$^{2+}$ from 50 nm $\beta_{16}$-Cu, $\beta_{28}$-Cu or $\beta_{40}$-Cu by various ligands. Solid lines are a linear fit of the data.
3.6.1 The Effects of Ethanol and DMSO on Aβ

When measuring the removal rate of Cu\(^{2+}\) from Aβ by CQ, it was found that there was a slow phase, in which the labelled Aβ was re-quenching after its recovery from the removal of the Cu\(^{2+}\) (see Fig. 3.21). This slow phase is due to the DMSO or ethanol that the CQ had been initially dissolved in. In figure 3.21 the effect of ethanol and DMSO is visible, despite being only 0.52 v/v\% and 0.052 v/v\%, respectively. The quenching could be due to structural re-arrangement and quenching of the dye by the Aβ, possibly by the tyrosine. If the quenching is due to the tyrosine, it could be tested by using murine Aβ. Ethanol and DMSO are common solvents used in experiments with Aβ. However, even small v/v\% appear to affect Aβ experiments, especially in equilibrium measurements where the effects are not temporally separated. Therefore some caution needs to be taken when reviewing experiments in the literature, where the effects of residual solvents in the Aβ samples have not been considered.

It was found that with higher ethanol concentrations (≥15 v/v\%) there was an increase in fluorescence, shown in figure 3.22. It therefore may be possible to use labelled Aβ to study the folding of Aβ in different environments, for example, in fluorinated alcohols Aβ has been shown to take an α-helix structure\[^{56}\].

![Graphs showing removal of Cu\(^{2+}\) from Aβ by CQ in Ethanol and DMSO.](image-url)
3.7 Mechanism for the Dissociation and Interconversion of the Aβ·Cu Complexes

The experiments used to determine the removal rates of Cu\(^{2+}\) from Aβ·Cu for L2-b and CQ revealed that there appeared to be two phases, in which the ligands interact differently with the Aβ·Cu complex. Is the reason for this that L2-b and CQ interact differently from the other ligands, or that the increased reaction rates reveal a more complex underlying mechanism of Aβ·Cu? To test this, the removal of Cu\(^{2+}\) from Aβ·Cu by EDTA was repeated, increasing the concentration of EDTA across a wider range, from 2 µm to 2 mm.

The results in figures 3.23 & 3.24, for Aβ\(_{16}\) & Aβ\(_{40}\) both show that the rates and amplitudes for two phases can be obtained. This shows that the two phases are not exclusive to L2-b and CQ. Given the vastly different structures of the ligands, this suggests that two forms of the Aβ·Cu complex exist, component I ((Aβ·Cu)\(_{i}\)) and component II ((Aβ·Cu)\(_{ii}\)) each reacting with ligands very differently. The plot of the relative amplitudes shows that the relative amplitudes of the phases change with EDTA concentration. This shows the two components are able to directly interconvert

\[(Aβ·Cu)_{i} ↔ (Aβ·Cu)_{ii}, \quad (3.26)\]
rather than the Cu\(^{2+}\) dissociating and re-associating

\[
(A\beta\cdot Cu)_i \rightleftharpoons A\beta + Cu^{2+} \rightleftharpoons (A\beta\cdot Cu)_{ii}, \quad (3.27)
\]
as the less than millisecond binding of EDTA to Cu\(^{2+}\) would prevent re-association, and thus the relative amplitudes would be independent of concentration. The minimal symmetric reaction scheme that takes into account of the interconversion, the reactions of the complex with EDTA (the ligand L), and the spontaneous dissociation of the two components is

\[
A\beta + Cu^{2+} \quad \xrightarrow{k_{1\text{off}}} \quad (A\beta\cdot Cu)_i \quad \\quad \xrightarrow{k_{1\text{off}}} \quad (A\beta\cdot Cu)_{ii}
\]
\[
\xrightarrow{k_{2\text{off}}} \quad A\beta + Cu \cdot L \quad \xrightarrow{k_{2\text{off}}} \quad (A\beta\cdot Cu)_i \quad \xrightarrow{k_{2\text{off}}} \quad A\beta + Cu \cdot L. \quad (3.28)
\]
The association of free Cu\(^{2+}\) with the ligand is not included as this reaction does not contribute to any change of fluorescence. The formation of the ternary complex is omitted, as it is transient and does not appear well populated.

This model gives the differential equations

\[
\frac{d[(A\beta\cdot Cu)_i]}{dt} = -[(A\beta\cdot Cu)_i](k_{1\text{off}} + k_{1\text{off}}[L] + k_{1\text{off}}) + [(A\beta\cdot Cu)_{ii}]k_{2\to 1} \quad (3.29)
\]
\[
\frac{d[(A\beta\cdot Cu)_{ii}]}{dt} = -[(A\beta\cdot Cu)_{ii}](k_{2\text{off}} + k_{2\text{off}}[L] + k_{2\to 1}) + [(A\beta\cdot Cu)_i]k_{1\to 2} \quad (3.30)
\]
and

\[
[A\beta] = [A\beta]_T - [(A\beta\cdot Cu)_i] - [(A\beta\cdot Cu)_{ii}], \quad (3.31)
\]
from the conservation of \(A\beta\). If the experiments are performed under pseudo-first-order conditions, i.e. the ligand is in greater excess by at least a factor of 20, then

\[
\frac{d[L]}{dt} = 0. \quad (3.32)
\]
With this simplification, there are analytical solutions for the differential equations,
giving the concentrations of the two Aβ·Cu species as a function of time as

\[
[(Aβ·Cu)\] = \frac{1}{2\delta} \left[ e^{-\frac{1}{2}t(\alpha + \beta)} \left( (\beta + \gamma)[(Aβ·Cu)\] \right|_{t=0} - 2k_{2\rightarrow 1}[(Aβ·Cu)\] \right|_{t=0} + \right) + \\
+ e^{-\frac{1}{2}t(\alpha - \beta)} \left( (\beta - \gamma)[(Aβ·Cu)\] \right|_{t=0} + 2k_{2\rightarrow 1}[(Aβ·Cu)\] \right|_{t=0} + \right),
\]

(3.33)

\[
[(Aβ·Cu)\] = \frac{1}{2\delta} \left[ e^{-\frac{1}{2}t(\alpha + \beta)} \left( (\beta - \gamma)[(Aβ·Cu)\] \right|_{t=0} - 2k_{1\rightarrow 2}[(Aβ·Cu)\] \right|_{t=0} + \right) + \\
+ e^{-\frac{1}{2}t(\alpha - \beta)} \left( (\beta + \gamma)[(Aβ·Cu)\] \right|_{t=0} + 2k_{1\rightarrow 2}[(Aβ·Cu)\] \right|_{t=0} + \right),
\]

(3.34)

where

\[
\alpha = k_{1\rightarrow 2} + k_{2\rightarrow 1} + k_{1\text{off}} + k_{2\text{off}} + (k_{1\text{off}} + k_{2\text{off}})[L] ,
\]

(3.35)

\[
\beta = \left( k_{1\rightarrow 2}^2 + 2k_{1\rightarrow 2}(k_{2\rightarrow 1} + k_{1\text{off}} - k_{2\text{off}} + (k_{1\text{off}} - k_{2\text{off}})[L]) + (k_{2\rightarrow 1} - k_{1\text{off}} + k_{2\text{off}} - (k_{1\text{off}} - k_{2\text{off}})[L])^2 \right)^{\frac{1}{2}} ,
\]

(3.36)

\[
\gamma = k_{1\rightarrow 2} - k_{2\rightarrow 1} + k_{1\text{off}} - k_{2\text{off}} + (k_{1\text{off}} - k_{2\text{off}})[L] ,
\]

(3.37)

\[
\delta = \sqrt{-4(k_{2\rightarrow 1}(k_{1\text{off}} + k_{1\text{off}}[L]) + (k_{1\rightarrow 2} + k_{1\text{off}} + k_{1\text{off}}[L])(k_{2\text{off}} + k_{2\text{off}}[L])) + \alpha^2} .
\]

(3.38)

The free Aβ concentration can be deduced from

\[
[Aβ] = [Aβ]_T - [(Aβ·Cu)\] - [(Aβ·Cu)\] ,
\]

(3.39)

showing that the solutions are double exponential, with rates of \(\frac{1}{2}(\alpha \pm \beta)\).

The initial conditions used are

\[
[(Aβ·Cu)\] \right|_{t=0} = [Aβ]_T \frac{k_{2\rightarrow 1}}{k_{1\rightarrow 2} + k_{2\rightarrow 1}} ,
\]

(3.40)

and

\[
[(Aβ·Cu)\] \right|_{t=0} = [Aβ]_T \frac{k_{1\rightarrow 2}}{k_{1\rightarrow 2} + k_{2\rightarrow 1}} ,
\]

(3.41)

assuming that all Aβ and Cu^{2+} form complexes that do not dissociate, and thus that interconversion is the only mechanism of changing from one component to the other.
The two double exponentials to the solution for $[A\beta]$ therefore have amplitudes ($A_i$) and rates ($k_i$) of

$$A_1 = \frac{1}{2\delta} \left( (\beta + \gamma)[(A\beta \cdot Cu)_1]|_{t=0} - 2k_{2 \rightarrow 1}[((A\beta \cdot Cu)_n)|_{t=0}^+ \right) , \quad (3.42)$$

$$k_1 = \frac{\alpha + \beta}{2} , \quad (3.43)$$

$$A_2 = \frac{1}{2\delta} \left( (\beta - \gamma)[(A\beta \cdot Cu)_1]|_{t=0} + 2k_{2 \rightarrow 1}[((A\beta \cdot Cu)_n)|_{t=0}^+ \right) , \quad (3.44)$$

$$k_2 = \frac{\alpha - \beta}{2} , \quad (3.45)$$

where $A_1$ & $k_1$ are related to the fast phase and $A_2$ & $k_2$ are related to the slow phase.

Given that it is unlikely that both states quench the fluorophore by the same amount, a factor $\Gamma$ is introduced into the amplitude contribution from the $[(A\beta \cdot Cu)_n]$ species. This gives the amplitudes to be

$$A_1 = \frac{1}{2\delta} \left( (\beta + \gamma)[(A\beta \cdot Cu)_1]|_{t=0} - 2k_{2 \rightarrow 1}[((A\beta \cdot Cu)_n)|_{t=0}^+ \right) , \quad (3.46)$$

$$A_2 = \frac{1}{2\delta} \left( (\beta - \gamma)[(A\beta \cdot Cu)_1]|_{t=0} + 2k_{2 \rightarrow 1}[((A\beta \cdot Cu)_n)|_{t=0}^+ \right) , \quad (3.47)$$

i.e. when $\Gamma > 1$ the contribution from $(A\beta \cdot Cu)_n$ is greater. These amplitudes, along with the two rates give the three parameters

$$\frac{A_1}{A_1 + A_2} \quad k_1 \quad k_2$$

(3.48)

with which the data sets can be fitted. The use of the relative amplitude removes the experimental error in the differing concentrations of $A\beta$ in the preparation of the complexes. The amplitude and rate parameters are obtained from the data by individually fitting each fluorescence trace with a double exponential to obtain $A_1$, $k_1$, $A_2$, & $k_2$, from which the three parameters can be derived. These were fitted
using Origin 7. The free parameters obtained through fitting this interconverting model are

\[ k_{1\text{off}}, k_{1\text{Loff}}, k_{1\rightarrow2}, k_{2\text{off}}, k_{2\text{Loff}}, k_{2\rightarrow1}, \Gamma \]  

(3.49)

The model was fitted using Matlab 2013a, see appendix A.2.

The errors used to weight the three parameters are the errors derived from the parameters for fitting the fluorescence recovery curves. Propagating the uncertainty through the formulae for \( \frac{A_1}{A_1+A_2}, k_1, k_2, \) and \( \frac{A_1}{A_1+A_2} \), allows the error in the fitting parameters to be obtained to compare the fitted model with the data. Although theoretically it is possible to obtain analytical solutions to these, the solutions are long, in particular for \( \frac{A_1}{A_1+A_2}, k_1, k_2, k_{1\rightarrow2}, k_{2\text{off}}, k_{2\text{Loff}}, \) \( k_{2\rightarrow1}, \Gamma \). To simplify their acquisition and as they are only calculated once, instead they are obtained numerically. Using central finite difference methods to obtain the partial derivatives \( \frac{\partial f}{\partial x} \), where \( f \in \{ \frac{A_1}{A_1+A_2}, k_1, k_2, \frac{A_1}{A_1+A_2} \} \) and \( x \in \{ k_{1\text{off}}, k_{1\text{Loff}}, k_{1\rightarrow2}, k_{2\text{off}}, k_{2\text{Loff}}, k_{2\rightarrow1}, \Gamma \} \), to get the error in each \( f \) from

\[ \sigma_f = \sqrt{\sum_j \left( \frac{\partial f_i}{\partial x_j} \sigma_{x_j} \right)^2}, \]  

(3.50)
given that the \( x \)’s are assumed uncorrelated. The 1 \( \sigma \) uncertainty is shaded in the figures.

**High Ligand concentrations**

At very high ligand concentrations, where the sum of the bimolecular reaction rates is much faster than the interconversion rates, the variables \( \alpha, \beta, \gamma, \) and \( \delta \) reduce to

\[ \alpha = (k_{1\text{off}} + k_{2\text{off}})[L], \]  

(3.51)

\[ \beta = (k_{1\text{off}} - k_{2\text{off}})[L], \]  

(3.52)

\[ \gamma = (k_{1\text{Loff}} - k_{2\text{Loff}})[L], \]  

(3.53)

\[ \delta = \sqrt{-4k_{1\text{off}}k_{2\text{Loff}}[L]^2 + \alpha^2} = (k_{1\text{Loff}} - k_{2\text{off}})[L], \]  

(3.54)
and thus the rates become

\[ k_1 = \frac{\alpha + \beta}{2} = k_{1\text{off}}[L] \quad , \quad (3.55) \]
\[ k_2 = \frac{\alpha - \beta}{2} = k_{2\text{off}}[L] \quad . \quad (3.56) \]

The rates are linear with ligand concentrations, where the gradients are \( k_{1\text{off}} \), and \( k_{2\text{off}} \). For the amplitudes

\[ \beta + \gamma = 2(k_{1\text{off}} - k_{2\text{off}})[L] = \delta \quad , \quad (3.57) \]
\[ \beta - \gamma = 0 \quad , \quad (3.58) \]

thus

\[ A_1 = \frac{1}{2\delta} \left( 2(k_{1\text{off}} - k_{2\text{off}})[L][\langle A\beta \cdot \text{Cu} \rangle_i \bigg|_{t=0}] = [\langle A\beta \cdot \text{Cu} \rangle_i \bigg|_{t=0} \right) \quad , \quad (3.59) \]
\[ A_2 = \frac{1}{2\delta} \left( 2\Gamma(k_{1\text{off}} - k_{2\text{off}})[L][\langle A\beta \cdot \text{Cu} \rangle_u \bigg|_{t=0}] = \Gamma[\langle A\beta \cdot \text{Cu} \rangle_u \bigg|_{t=0} \right) \quad , \quad (3.60) \]

and therefore

\[ \frac{A_1}{A_1 + A_2} = \frac{[\langle A\beta \cdot \text{Cu} \rangle_i \bigg|_{t=0}]}{[\langle A\beta \cdot \text{Cu} \rangle_i \bigg|_{t=0} + \Gamma[\langle A\beta \cdot \text{Cu} \rangle_u \bigg|_{t=0}]} =: R_1 \quad , \quad (3.61) \]
\[ \frac{A_2}{A_1 + A_2} = \frac{\Gamma[\langle A\beta \cdot \text{Cu} \rangle_u \bigg|_{t=0}]}{[\langle A\beta \cdot \text{Cu} \rangle_i \bigg|_{t=0} + \Gamma[\langle A\beta \cdot \text{Cu} \rangle_u \bigg|_{t=0}]} =: R_2 \quad . \quad (3.62) \]

So the relative amplitudes at high ligand concentrations are relative to the proportions of the fluorescence recovery from the two species in equilibrium (\( R_1 \) and \( R_2 \)). Given that the fitted parameter \( \Gamma \) is known, the relative proportions of the concentration can be obtained from

\[ \frac{\Gamma R_1}{\Gamma R_1 + R_2} = \frac{[\langle A\beta \cdot \text{Cu} \rangle_i \bigg|_{t=0}]}{[\langle A\beta \cdot \text{Cu} \rangle_i \bigg|_{t=0} + \Gamma[\langle A\beta \cdot \text{Cu} \rangle_u \bigg|_{t=0}]} \quad , \quad (3.63) \]
\[ \frac{R_2}{\Gamma R_1 + R_2} = \frac{[\langle A\beta \cdot \text{Cu} \rangle_u \bigg|_{t=0}]}{[\langle A\beta \cdot \text{Cu} \rangle_i \bigg|_{t=0} + \Gamma[\langle A\beta \cdot \text{Cu} \rangle_u \bigg|_{t=0}]} \quad . \quad (3.64) \]

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This shows that the solutions reduce to the model without interconversion, i.e. \( k_{1 \rightarrow 2} = k_{2 \rightarrow 1} = 0 \), thus at high ligand concentrations \((A\beta \cdot Cu)_i\), and \((A\beta \cdot Cu)_ii\) act like independent species.

**Low Ligand concentrations**

Studying the reaction at very low ligand concentrations will allow for the derivation of the apparent dissociation rate. At low ligand concentrations, where the contributions from the bimolecular reaction rates are negligible, thus the variables \( \alpha \), \( \beta \), \( \gamma \), and \( \delta \) reduce to

\[
\alpha = k_{1 \rightarrow 2} + k_{2 \rightarrow 1} + k_{1\text{off}} + k_{2\text{off}}, \quad (3.65)
\]

\[
\beta = \sqrt{4k_{1 \rightarrow 2}k_{2 \rightarrow 1} + (k_{1 \rightarrow 2} - k_{2 \rightarrow 1} + k_{1\text{off}} - k_{2\text{off}})^2}, \quad (3.66)
\]

\[
\gamma = k_{1 \rightarrow 2} - k_{2 \rightarrow 1} + k_{1\text{off}} - k_{2\text{off}}, \quad (3.67)
\]

\[
\delta = \sqrt{-4(k_{2 \rightarrow 1}k_{1\text{off}} + k_{2\text{off}}(k_{1 \rightarrow 2} + k_{1\text{off}})) + \alpha^2}. \quad (3.68)
\]

The experimental data shows that the amplitude of the fast phase becomes very small, compared to that of the slow phase, so the rate of the slow phase approaches the apparent dissociation rate

\[
k_{\text{off}}^{\text{App}} = \frac{\alpha - \beta}{2} = \frac{1}{2}\left[k_{1 \rightarrow 2} + k_{2 \rightarrow 1} + k_{1\text{off}} + k_{2\text{off}} - \sqrt{4k_{1 \rightarrow 2}k_{2 \rightarrow 1} + (k_{1 \rightarrow 2} - k_{2 \rightarrow 1} + k_{1\text{off}} - k_{2\text{off}})^2}\right], \quad (3.69)
\]

which can be calculated from the fitted kinetic parameters.

The asymptotic apparent rate constant, at low ligand concentrations, can be calculated by evaluating

\[
k_{\text{loff}}^{\text{App}} \approx \lim_{[L] \to 0} \left( \frac{dk_2}{d[L]} \right) = \frac{1}{2}\left(k_{1\text{loff}} + k_{2\text{loff}} + \frac{(k_{1\text{loff}} - k_{2\text{loff}})(k_{1 \rightarrow 2} - k_{2 \rightarrow 1} + k_{1\text{off}} - k_{2\text{off}})}{\sqrt{(k_{1 \rightarrow 2} - k_{2 \rightarrow 1} + k_{1\text{off}} - k_{2\text{off}})^2 - 4k_{1 \rightarrow 2}k_{2 \rightarrow 1}}}\right). \quad (3.70)
\]

\[
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\]
Given that $k_{2\text{off}} \ll k_{1\text{off}}$ this reduces to

$$k_{\text{App}}^{\text{Lo}} = \frac{k_{1\text{off}}}{2} \left( 1 + \frac{k_{1 \rightarrow 2} - k_{2 \rightarrow 1} + k_{1\text{off}} - k_{2\text{off}}}{\sqrt{(k_{1 \rightarrow 2} - k_{2 \rightarrow 1} + k_{1\text{off}} - k_{2\text{off}})^2 + 4k_{1 \rightarrow 2}k_{2 \rightarrow 1}}} \right). \quad (3.72)$$

Results

The results for $A\beta_{16}$ (Fig. 3.23) and $A\beta_{40}$ (Fig. 3.24) show that the model appears to fit quite well. However, the fitting was unable to determine a value for $k_{2\text{off}}$. This is likely due to the spontaneous dissociation of the $(A\beta\cdot Cu)_n$ complex being too slow. The $(A\beta\cdot Cu)_n$ complex most probably dissociates by interconverting to $(A\beta\cdot Cu)_1$, after which the Cu$^{2+}$ dissociates. This simplifies the model slightly, with the mechanism becoming

$$A\beta + Cu^{2+} \rightarrow \frac{k_{1\text{off}}}{k_{2\text{off}}} (A\beta\cdot Cu)_1 \rightarrow A\beta + Cu \cdot L.$$

The amplitude data appears to deviate from the model at high ligand concentrations. This is likely to be an artifact due to the data fitting. When the rate of the first phases is approximately $100 \text{ s}^{-1}$, the phase is partially within the dead time of the stopped flow machine. Therefore the raw data no longer contains the whole of the phase, and at short timescales there is decreased signal to noise. The apparent rates appear to follow the model well, including the double sigmoidal shape in the second phase. The data for the first phase is relatively noisy at low ligand concentrations due to the small amplitude of the phase, making it harder to fit.

The model parameters, obtained through fitting, for the results are summarised in table 3.6. The phases in the figures do not correspond to $(A\beta\cdot Cu)_1$ or $(A\beta\cdot Cu)_n$, except at high EDTA concentrations.

The fitted parameters show that the interconversion is on the second timescale.
Figure 3.23: Apparent rates and relative amplitudes of the two major phases seen in the dissociation and removal by EDTA of Cu$^{2+}$ from Aβ$_{1-16}$LysHL488·Cu, in Hepes NaCl (pH 7.5), fitted with the model in 3.28 (solid line). Model parameters are shown in table 3.6.

Figure 3.24: Apparent rates and relative amplitudes of the two major phases seen in the dissociation and removal by EDTA of Cu$^{2+}$ from Aβ$_{40}$Cys$_{20}$HL488·Cu, in Hepes NaCl (pH 7.5), fitted with the model in 3.28 (solid line). Model parameters are shown in table 3.6.

The time scales of the interconversion process span the timescales of neurotransmission, therefore the process may be of physiological relevance in the synapse. It was thought that the two components were in fast equilibrium, according to NMR experiments. However, these experiments show this is not the case. The fast relaxation observed in the NMR experiments could be due to the interconversion of subspecies of (Aβ·Cu)$_i$ (such as the interchanging of the bound histidine), broadening the observed NMR peaks.

The apparent dissociation constant $k_{off}^{App}$ derived is within error of the means
Table 3.6: Summary of fitting parameters for the model (Eqn. 3.28) for the dissociation and removal by EDTA of Cu$^{2+}$ from Aβ·Cu.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aβ$_{16}$</th>
<th>Aβ$_{40}$</th>
<th>mAβ$_{16}$</th>
<th>Aβ$_{16}$</th>
<th>Aβ$_{40}$</th>
<th>mAβ$_{16}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>$k_{1\text{off}}$/s$^{-1}$</td>
<td>0.8(1)</td>
<td>1.04(6)</td>
<td>1.2(1)</td>
<td>4.3(2)</td>
<td>5.1(4)</td>
<td>9.1(6)</td>
</tr>
<tr>
<td>$k_{1\text{off}}$/10$^5$ M$^{-1}$ s$^{-1}$</td>
<td>1.3(1)</td>
<td>1.90(5)</td>
<td>1.1(1)</td>
<td>2.7(1)</td>
<td>3.4(2)</td>
<td>3.3(4)</td>
</tr>
<tr>
<td>$k_{1\rightarrow2}$/s$^{-1}$</td>
<td>0.9(2)</td>
<td>1.5(1)</td>
<td>1.8(2)</td>
<td>0.06(8)</td>
<td>0.2(1)</td>
<td>0.8(1)</td>
</tr>
<tr>
<td>$k_{2\text{off}}$/s$^{-1}$</td>
<td>Not Determinable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{2\text{off}}$/10$^3$ M$^{-1}$ s$^{-1}$</td>
<td>1.48(6)</td>
<td>1.6(1)</td>
<td>0.106(4)</td>
<td>3.1(4)</td>
<td>3.2(5)</td>
<td>0.099(4)</td>
</tr>
<tr>
<td>$k_{2\rightarrow1}$/s$^{-1}$</td>
<td>2.22(3)</td>
<td>2.53(7)</td>
<td>0.430(4)</td>
<td>1.29(6)</td>
<td>1.57(9)</td>
<td>0.534(3)</td>
</tr>
<tr>
<td>$\Gamma$</td>
<td>1.1(2)</td>
<td>0.89(7)</td>
<td>1.3(1)</td>
<td>4(5)</td>
<td>1.3(8)</td>
<td>1.9(2)</td>
</tr>
<tr>
<td>$k_{\text{App}}$/s$^{-1}$</td>
<td>0.54(7)</td>
<td>0.59(4)</td>
<td>0.15(2)</td>
<td>1.27(7)</td>
<td>1.47(9)</td>
<td>0.489(7)</td>
</tr>
</tbody>
</table>

Figure 3.23 3.24 3.25 3.27 3.28 3.26

obtained from the low ligand concentration experiments.

There is a two order of magnitude difference in the rate constants for the removal of Cu$^{2+}$ from the Aβ·Cu complexes. The (Aβ·Cu)$_{\text{ii}}$ complex is relatively inert, with removal via the (Aβ·Cu)$_{\text{ii}}$ complex starting to dominate at hundreds of micromolar to millimolar concentrations of EDTA, when the rate starts to straighten. Since EDTA reacts similarly as Aβ with the Aβ·Cu complex, and as the reaction rates were similar in the low ligand concentration experiments, this result suggests that copper assisted dimerisation would occur via (Aβ·Cu)$_{\text{ii}}$.

The values of the relative brightness of the two states $\Gamma$ are approximately 1. This suggests that the distances or interactions between the dye and the Cu$^{2+}$ are similar.

The experiment was similarly performed with murine Aβ$_{16}$ (see Fig. 3.25). The model fits the second phase's rates well, but not the other rates or amplitudes. This is because the second phase is large and slow, therefore its rate and amplitude are obtained with a very small uncertainty. The proportion of the two components.
Figure 3.25: Apparent rates and relative amplitudes of the two major phases seen in the dissociation and removal by EDTA of Cu$^{2+}$ from murine Aβ$\textsubscript{16}$LysHL488$\cdot$Cu, in Hepes NaCl (pH 7.5), fitted with the model in 3.28 (solid line). Model parameters are shown in table 3.6.

For murine Aβ$\cdot$Cu are significantly different to human Aβ$\cdot$Cu. The second phase is always much more dominant. The graph shows that the rates of the first phase have been underestimated, refitting the rates with a line gives the Cu$^{2+}$ removal rate constant for (Aβ$\cdot$Cu)$_{i}$ to be $2.9(2) \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, faster than for human Aβ. Comparatively, the equivalent rate constant for (Aβ$\cdot$Cu)$_{ii}$ is slightly slower. Therefore the order of magnitude difference in Cu$^{2+}$ assisted dimerisation rate constants, seen in section 3.5, does not come from different removal rate constants, but from the interconversion. This can be seen by calculating $k_{\text{App}}^{\text{Off}}/k_{1\text{Off}}^{\text{Off}}$. These are $0.59(5)$,
0.50(2) & 0.096(7), for Aβ16·Cu, Aβ40·Cu & mAβ16·Cu, respectively.

To check whether the model is valid and whether the inconsistencies are reduced when the relative proportions of the species are more equal, the experiment was repeated at pH 6.5. This is closer to the literature pKₐ of 6.3 for the murine Aβ·Cu complexes. Figure 3.26 shows that at a lower pH, the relative proportion of (Aβ·Cu)ₙ has increased, agreeing with the literature in that the pKₐ is less than 6.5. The model appears to fit the data. The model may not fit so well at pH 7.5 due to other species of Aβ·Cu forming, that are not accounted for in the model.

Figure 3.27: Apparent rates and relative amplitudes of the two major phases seen in the dissociation and removal by EDTA of Cu²⁺ from Aβ16LysHL488, in Hepes NaCl (pH 6.5), fitted with the model in 3.28 (solid line). Model parameters are shown in table 3.6.
Figure 3.28: Apparent rates and relative amplitudes of the two major phases seen in the dissociation and removal by EDTA of Cu$^{2+}$ from Aβ$\text{40}$Cys$_{20}$HL488, in Hepes NaCl (pH 6.5), fitted with the model in 3.28 (solid line). Model parameters are shown in table 3.6.

The experiments were repeated for Aβ$_{16}$ & Aβ$_{40}$ at pH 6.5, with the results shown in figures 3.27 & 3.28, respectively. These data sets do not fit will with the model. The amplitudes at high EDTA concentrations suggest that the pK$_a$ for Aβ$_{16}$ & Aβ$_{40}$ are greater than pH 7.5, as the relative amplitude has become more dominant. The relative amplitudes are expected to be equal in equilibrium when pK$_a$=pH. This may not be true if multiple pK$_a$’s exist around this range.

3.8 Direct Observation of Aβ·Cu Interconversion and the Binding Mechanism

With the interconversion of the two Aβ·Cu species and the dissociation mechanism obtained, along with the rate at which Aβ and Cu$^{2+}$ bind to form Aβ·Cu, this leaves the question of how does the binding mechanism relate to the interconversion and dissociation? Does it bind directly to form (Aβ·Cu)$_l$, or (Aβ·Cu)$_u$, or via a more complex mechanism that leads to these?

To directly probe the binding and interconversion of the Aβ·Cu complex, stopped flow with double mixing was used. Initially Aβ was reacted with Cu$^{2+}$ and then after a set delay with EDTA. Both the reactions of Aβ with Cu$^{2+}$ and then Aβ·Cu with EDTA were under pseudo-first-order conditions. The Cu$^{2+}$ concentration was chosen such that the binding reaction would complete within 2 ms, much
faster than the interconversion. The EDTA concentration was chosen such that its addition would ‘freeze’ the reaction and the fluorescence recovery amplitudes would be proportional to the concentration of the two components. The reaction rate with EDTA is used to determine which component is formed or if a new unknown intermediary species existed.

The binding of copper to the two components with interconversion can be described by

\[
A\beta + Cu^{2+} \xrightarrow{k_{on}} (A\beta \cdot Cu)^i \xrightarrow{k_{1 \to 2}} (A\beta \cdot Cu)^{ii}.
\] (3.74)

This assumes that the Cu\(^{2+}\) binds directly to form (A\(\beta\) \(\cdot\) Cu), without visible intermediates. The underlying model would be the same for direct binding to (A\(\beta\) \(\cdot\) Cu), as without data, which complex forms first cannot be determined. Since the binding of A\(\beta\) to Cu\(^{2+}\) is much faster than the interconversion, the model can be simplified to

\[
(A\beta \cdot Cu)^i \xrightarrow{k_{1 \to 2}} (A\beta \cdot Cu)^{ii},
\] (3.75)

with the initial conditions

\[
\frac{(A\beta \cdot Cu)^i}{(A\beta \cdot Cu)^i + (A\beta \cdot Cu)^{ii}}|_{t=0} = 1
\] (3.76)

and thus

\[
(A\beta \cdot Cu)^{ii}|_{t=0} = 0.
\] (3.77)

The model gives the differential equations

\[
\frac{d[(A\beta \cdot Cu)^i]}{dt} = -k_{1 \to 2}[(A\beta \cdot Cu)^i] + k_{2 \to 1}[(A\beta \cdot Cu)^{ii}],
\] (3.78)

\[
\frac{d[(A\beta \cdot Cu)^{ii}]}{dt} = +k_{1 \to 2}[(A\beta \cdot Cu)^i] - k_{2 \to 1}[(A\beta \cdot Cu)^{ii}].
\] (3.79)

which predict the change in concentration of the two components. The solutions to

\*It will be shown that (A\(\beta\) \(\cdot\) Cu)\(_i\) is formed initially and so this notation is used.

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these after applying the initial conditions are

\[
[(Aβ \cdot Cu)_i] = \frac{(k_{2 \to 1} + k_{1 \to 2} e^{-(k_{1 \to 2} + k_{2 \to 1})t}) [(A\beta \cdot Cu)_i]_{t=0}}{k_{1 \to 2} + k_{2 \to 1}}, \quad (3.80)
\]

\[
[(Aβ \cdot Cu)_n] = \frac{k_{1 \to 2}(1 - e^{-(k_{1 \to 2} + k_{2 \to 1})t}) [(A\beta \cdot Cu)_i]_{t=0}}{k_{1 \to 2} + k_{2 \to 1}}, \quad (3.81)
\]

so the relative amplitudes of the two components are

\[
\frac{[(A\beta \cdot Cu)_i]}{[(A\beta \cdot Cu)_i] + [(A\beta \cdot Cu)_n]} = \frac{k_{2 \to 1} + k_{1 \to 2} e^{-(k_{1 \to 2} + k_{2 \to 1})t}}{k_{1 \to 2} + k_{2 \to 1}}, \quad (3.82)
\]

\[
\frac{[(A\beta \cdot Cu)_n]}{[(A\beta \cdot Cu)_i] + [(A\beta \cdot Cu)_n]} = \frac{k_{1 \to 2}(1 - e^{-(k_{1 \to 2} + k_{2 \to 1})t})}{k_{1 \to 2} + k_{2 \to 1}}. \quad (3.83)
\]

The solutions show that the progress of the reactions in time are single exponentials, with the rate as the relaxation rate constant \((k_r = k_{1 \to 2} + k_{2 \to 1})\). Therefore the relaxation rate constant can be obtained by periodically measuring the relative amplitudes at different points in time. This allows the direct probing of the interconversion, for comparison and confirmation of the previous results.

When \(t \to \infty\), the solutions become

\[
R_1 := \frac{[(A\beta \cdot Cu)_i]}{[(A\beta \cdot Cu)_i] + [(A\beta \cdot Cu)_n]} = \frac{k_{2 \to 1}}{k_{1 \to 2} + k_{2 \to 1}}. \quad (3.84)
\]

\[
R_2 := \frac{[(A\beta \cdot Cu)_n]}{[(A\beta \cdot Cu)_i] + [(A\beta \cdot Cu)_n]} = \frac{k_{1 \to 2}}{k_{1 \to 2} + k_{2 \to 1}}. \quad (3.85)
\]

This allows the individual interconversion rates to be obtained from

\[
k_{1 \to 2} = R_2 \times k_r \quad , \quad (3.86)
\]

\[
k_{2 \to 1} = R_1 \times k_r \quad . \quad (3.87)
\]

It was found that there were two major phases with the reaction rates with EDTA corresponding to the rates found previously for \((A\beta \cdot Cu)_i\) and \((A\beta \cdot Cu)_n\). This suggests that any intermediary complex have lifetimes of much less than the minimum delay time (50 ms). The results in figure 3.29 show that initially \((A\beta \cdot Cu)_i\)
Figure 3.29: The relative change in amplitude of the two phases of freshly formed labelled $\alpha\beta\cdot\text{Cu}$ reacted with $1\text{ mm EDTA}$, after a set age time. The labelled $\alpha\beta\cdot\text{Cu}$ complex was formed by mixing $50\text{ nm}$ of each. The fitted lines are single exponential, to determine the relaxation rate $k_r$. Using the relative proportion at equilibrium, the interconversion rates $k_1\rightarrow k_2$ and $k_2\rightarrow k_1$ can be determined. See table 3.7 for the determined rates.

<table>
<thead>
<tr>
<th></th>
<th>$k_r/s^{-1}$</th>
<th>$k_{1\rightarrow2}/s^{-1}$</th>
<th>$k_{2\rightarrow1}/s^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha\beta_{16}\text{LysHL488}\cdot\text{Cu}$</td>
<td>3.6(1)</td>
<td>1.68(5)</td>
<td>1.96(5)</td>
</tr>
<tr>
<td>$\alpha\beta_{16}\text{LysHL488}\cdot\text{Cu}$ at pH 8.0</td>
<td>6.1(3)</td>
<td>4.9(3)</td>
<td>1.22(8)</td>
</tr>
<tr>
<td>$\alpha\beta_{40}\text{Cys}_{20}\text{HL488}\cdot\text{Cu}$</td>
<td>3.6(6)</td>
<td>1.6(3)</td>
<td>2.1(4)</td>
</tr>
<tr>
<td>Murine $\alpha\beta_{16}\text{LysHL488}\cdot\text{Cu}$</td>
<td>2.6(1)</td>
<td>1.98(8)</td>
<td>0.59(3)</td>
</tr>
</tbody>
</table>

Table 3.7: The relaxation $k_r$ and interconversion rates $k_{1\rightarrow2}$ and $k_{2\rightarrow1}$, obtained from fits in figure 3.29.
forms and that this then interconverts to \((A\beta\cdot Cu)_ii\). This appears to be true for both human and murine \(A\beta\). The completed mechanism for a single \(Cu^{2+}\) binding to \(A\beta\) is therefore

\[
A\beta + Cu^{2+} \xrightarrow{k_{on}} (A\beta\cdot Cu)_i \xrightarrow{k_{1\rightarrow2}} (A\beta\cdot Cu)_ii, \tag{3.88}
\]

with interactions with the \(A\beta\cdot Cu\) complex, such as dimerisation, via \((A\beta\cdot Cu)_i\).

When the experiment is performed in pH 8.0 Hepes NaCl, the \((A\beta\cdot Cu)_ii\) is dominant, therefore the \(pK_a\) is between pH 7.5 and pH 8.0. The \(pK_a\) can be determined using the Henderson-Hasselbalch relation, to be

\[
pK_a = pH - \lg\left(\frac{k_{1\rightarrow2}}{k_{2\rightarrow1}}\right), \tag{3.89}
\]

assuming that there are only two states available, one protonated \(((A\beta\cdot Cu)_i)\), and one not \(((A\beta\cdot Cu)_ii)\). These experiments and the previous set of EDTA experiments give the mean \(pK_a\) for human \(A\beta\) to be 7.6(2), and for murine \(A\beta\) to be 6.9(4), calculated from the data in table 3.8. The literature values for the \(pK_a\) are 7.8 & 6.2, calculated from the data in table 3.8. The literature values for the \(pK_a\) are 7.8 & 6.2,

<table>
<thead>
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<th>§3.7</th>
<th>§3.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.5</td>
<td>pH 7.5</td>
</tr>
<tr>
<td>A\beta_{16}LysHL488</td>
<td>7.8(6)</td>
</tr>
<tr>
<td>A\beta_{40}Cys_{20}HL488</td>
<td>7.4(2)</td>
</tr>
<tr>
<td>mA\beta_{16}LysHL488</td>
<td>6.32(5)</td>
</tr>
</tbody>
</table>

Table 3.8: Calculated \(pK_a\)'s for the \((A\beta\cdot Cu)_i\)-(A\beta\cdot Cu)_ii equilibrium. Calculated from the \(k_{1\rightarrow2}\)'s and \(k_{2\rightarrow1}\)'s from the experiments in sections 3.7 and 3.8, using equation 3.89.

for human and murine \(A\beta\), respectively. These are similar to the values obtained, however the individually obtained values are somewhat scattered. It would be best if the experiments in this section could be repeated with a shorter delay time for better accuracy and precision of higher pH values. Varying the pH would also show whether the relationship between the pH and \(\lg(k_{1\rightarrow2}/k_{2\rightarrow1})\) is flat, as predicted by
3.9 Multiple Copper Binding to Aβ

It was shown in section 3.2 that multiple Cu$^{2+}$ can bind to Aβ in equilibrium, with the second Cu$^{2+}$ binding at approximately 10 µM. In section 3.3.1 it was shown that the second Cu$^{2+}$ association rate constant was approximately $5 \times 10^5$ M$^{-1}$ s$^{-1}$. How does this fit into the single Cu$^{2+}$ binding model found so far?

To probe the binding of the second Cu$^{2+}$ binding to Aβ, double mixing was used to mix 50 nM labelled Aβ$_{1-48}$ with CuCl$_2$, under pseudo-first-order conditions. This mixture was then mixed with 2 mM EDTA, also under pseudo-first-order conditions. The resulting fluorescence recovery traces were globally fitted with multiple exponentials, sharing the rates across data sets.

The data in figure 3.30 shows that there are four species of Cu$^{2+}$ bound to Aβ. There are two new species III & IV, not seen in the previous measurements. These are therefore the Aβ with multiple Cu$^{2+}$ species. Species III appears at much shorter time scales than species IV. The relative amplitude of species III rises with (Aβ·Cu)$_{ii}$, and therefore is formed via (Aβ·Cu)$_{i}$. This can also be seen from figure 3.7, the linear dependence of the apparent association rate of the second phase with CuCl$_2$. If species III formed via (Aβ·Cu)$_{ii}$, the rate limiting step would be the interconversion, not the binding. Therefore, inserting the binding of Cu$^{2+}$ to (Aβ·Cu)$_{i}$ to form (Aβ·Cu$_2$)$_{iii}$ in the mechanism gives

$$
Aβ + Cu^{2+} \overset{k_{on}}{\underset{k_{off}}{\rightleftharpoons}} (Aβ·Cu)_i \overset{+Cu^{2+}}{\underset{k_{1\rightarrow 3}}{\rightarrow}} (Aβ·Cu_2)_{iii}.
$$

This leaves the dissociation of Cu$^{2+}$ from the (Aβ·Cu$_2$)$_{iii}$. For this there are two
Figure 3.30: Species distributions of multiple Cu$^{2+}$ binding to 50 nm Aβ$_{1-40}$HL488 in time.

possible mechanisms

\begin{align*}
(A\beta \cdot Cu)_i & \quad \rightarrow \quad (A\beta \cdot Cu)_i \\
(A\beta \cdot Cu)_ii & \quad \rightarrow \quad (A\beta \cdot Cu)_{2ii} \\
(A\beta \cdot Cu)_{2ii} & \quad \rightarrow \quad (A\beta \cdot Cu)_{2iii} \\
(A\beta \cdot Cu)_{iii} & \quad \rightarrow \quad (A\beta \cdot Cu)_{2ii}
\end{align*}

(3.91)

if it is assumed to reform as either (A\beta \cdot Cu)$_i$, or (A\beta \cdot Cu)$_{ii}$. However, there is also a fourth species. Assuming that there are no intermediary species, given that they are not seen in the data, there are therefore 9 different mechanisms of binding to the three existing species. This brings the number of possible mechanisms to 18.
Ignoring their dependence on $\text{Cu}^{2+}$, they are

Model 0

Model 1

Model 2

Model 3

Model 4

Model 5

Model 6

Model 7

Model 8

Model 9

Model 10

Model 11

(3.92)
To determine which model most accurately predicted the data, they were all fitted. The relative quenching of components I & II was set to 1, and for components III & IV was set to 1.3, as determined from figure 3.2. They were fitted by numerically solving the differential equations for each model, using the Runge-Kutta-Dormand-Prince method. These solutions are then fitted using least-squares regression, weighted by the errors obtained from fitting the raw data with multi-exponentials. The errors in the fit are obtained similarly as in section 3.7. The code used is shown in appendix A.3.

The fits for the models are shown in figure 3.31, with the fitted parameters shown in table 3.9. The table shows that for models 0 through 8, that $k_{1→2} > k_{2→1}$. This is contradictory to the previous results, which show that at equilibrium (Aβ-Cu) is
Figure 3.31: Part 1 of 3.
Figure 3.31: Part 2 of 3.
Figure 3.31: Part 3 of 3. Fitting of the models (Eqns. 3.92 & 3.93) to figure 3.30c.

<table>
<thead>
<tr>
<th>Model</th>
<th>0</th>
<th>1</th>
<th>2</th>
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<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{1\rightarrow 2}$</td>
<td>1.9(1)</td>
<td>1.9(1)</td>
<td>1.8(1)</td>
<td>2.0(3)</td>
<td>2.1(3)</td>
<td>2.0(3)</td>
<td>1.9(3)</td>
<td>2.0(3)</td>
<td>1.9(3)</td>
</tr>
<tr>
<td>$k_{2\rightarrow 1}$</td>
<td>0.9(1)</td>
<td>0.9(1)</td>
<td>0.8(1)</td>
<td>0.8(3)</td>
<td>1.1(3)</td>
<td>0.9(3)</td>
<td>1.0(3)</td>
<td>1.2(3)</td>
<td>1.0(3)</td>
</tr>
<tr>
<td>$k_{3\text{ on}}$</td>
<td>3.0(3)</td>
<td>3.0(3)</td>
<td>3.0(3)</td>
<td>3.1(6)</td>
<td>3.0(6)</td>
<td>3.2(7)</td>
<td>3.6(6)</td>
<td>3.6(7)</td>
<td>3.6(6)</td>
</tr>
<tr>
<td>$k_{3\text{ off}}$</td>
<td>1.7(2)</td>
<td>1.7(2)</td>
<td>1.8(2)</td>
<td>2.0(6)</td>
<td>1.9(6)</td>
<td>2.3(7)</td>
<td>2.2(7)</td>
<td>2.3(7)</td>
<td>2.3(7)</td>
</tr>
<tr>
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<td>0.23(1)</td>
<td>0.23(1)</td>
<td>0.26(5)</td>
<td>0.28(5)</td>
<td>0.25(5)</td>
<td>0.20(3)</td>
<td>0.20(3)</td>
<td>0.21(3)</td>
</tr>
<tr>
<td>$k_{4\text{ off}}$</td>
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<td>0.15(2)</td>
<td>0.15(2)</td>
<td>0.5(1)</td>
<td>0.5(1)</td>
<td>0.4(1)</td>
<td>0.28(6)</td>
<td>0.27(6)</td>
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</table>

<table>
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<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{1\rightarrow 2}$</td>
<td>1.5(2)</td>
<td>1.5(2)</td>
<td>1.5(2)</td>
<td>1.8(4)</td>
<td>1.8(4)</td>
<td>1.7(4)</td>
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<td>1.4(4)</td>
<td>1.4(4)</td>
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<tr>
<td>$k_{2\rightarrow 1}$</td>
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<td>2.3(3)</td>
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<td>2.8(7)</td>
<td>2.8(7)</td>
<td>2.6(6)</td>
<td>2.6(6)</td>
<td>2.7(6)</td>
</tr>
<tr>
<td>$k_{3\text{ on}}$</td>
<td>3.1(3)</td>
<td>3.1(3)</td>
<td>3.1(3)</td>
<td>3.1(6)</td>
<td>3.1(6)</td>
<td>3.2(7)</td>
<td>3.5(6)</td>
<td>3.6(6)</td>
<td>3.6(6)</td>
</tr>
<tr>
<td>$k_{3\text{ off}}$</td>
<td>1.7(2)</td>
<td>1.7(2)</td>
<td>1.8(2)</td>
<td>2.0(6)</td>
<td>2.0(6)</td>
<td>2.3(7)</td>
<td>2.1(6)</td>
<td>2.1(6)</td>
<td>2.3(6)</td>
</tr>
<tr>
<td>$k_{4\text{ on}}$</td>
<td>0.24(1)</td>
<td>0.24(1)</td>
<td>0.24(1)</td>
<td>0.23(4)</td>
<td>0.24(4)</td>
<td>0.23(4)</td>
<td>0.21(3)</td>
<td>0.21(3)</td>
<td>0.22(3)</td>
</tr>
<tr>
<td>$k_{4\text{ off}}$</td>
<td>0.17(2)</td>
<td>0.17(2)</td>
<td>0.16(2)</td>
<td>0.4(1)</td>
<td>0.4(1)</td>
<td>0.4(0)</td>
<td>0.30(7)</td>
<td>0.30(6)</td>
<td>0.31(7)</td>
</tr>
</tbody>
</table>

Table 3.9: Fitting parameters for figure 3.31. $k_{1\rightarrow 2}$, $k_{2\rightarrow 1}$, $k_{3\text{ off}}$ and $k_{4\text{ off}}$ are rate constants in s$^{-1}$. $k_{3\text{ on}}$ is a rate in s$^{-1}$. $k_{4\text{ on}}$ may be a rate or rate constant, in s$^{-1}$. 

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dominant at low $\text{Cu}^{2+}$ concentrations. Those models based upon

\[
\begin{align*}
(A\beta\cdot\text{Cu})_i & \quad \iff \quad (A\beta\cdot\text{Cu}_2)_{ii} \\
(A\beta\cdot\text{Cu})_{ii} & \quad \iff \quad (A\beta\cdot\text{Cu})_{iii} \\
\end{align*}
\]

which are models 9 though 17 are more correct. The rate constants for $k_{3\text{on}}$ and $k_{4\text{on}}$ for the models are $3.0 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ to $3.6 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ and $2.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ to $2.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$. $k_{3\text{on}}$ is similar to the rate constant obtained in section 3.3.1 (4.2(6) $\times 10^5 \text{ M}^{-1} \text{s}^{-1}$). However, the dissociation rate is different (1.7(2) s$^{-1}$ compared to 7.3(7) s$^{-1}$).

Of models 9 through 17, figure 3.31 shows that models 9 through 11 appear to have the best fit. In these models

\[
\begin{align*}
\text{I} & \quad \iff \quad \text{II} \quad \iff \quad \text{III} \quad \iff \quad \text{IV} \\
\end{align*}
\]

$(A\beta\cdot\text{Cu}_n)_{iv}$ is formed via $(A\beta\cdot\text{Cu})_i$. These models allow for the non zero amplitude of type IV to be fitted at short time scales.

To check these models and hypotheses, the copper was varied and the relative amplitudes measured at equilibrium. 25 nm $A\beta_{16}\text{LysHL488}$ was premixed with various concentrations of $\text{CuCl}_2$. This solution was then mixed with 4 mm EDTA, so that the recovery amplitudes would be proportional to the population. The obtained curves were globally fitted with a quintuple exponential, sharing the rates.

The results shown in figure 3.32 show that there were five exponentials visible, unlike in the previous experiment (see Fig 3.30) where there were four. It appears
that an extra slow phase has appeared. There is further inconsistency between the data figure 3.32 and the transient experiments in figure 3.30, in that the relative amplitudes of the time points at approximately 20 s do not correspond well to the equilibrium relative amplitudes. This may be due to the reaction being incomplete, due to the maximal time difference in the transient experiments being approximately 20 s, whereas the equilibrium experiments had approximately 600 s.

To compare the models in the transient experiments against the equilibrium, it was assumed that the both the formation of \((A\beta\cdot \text{Cu})_{\text{III}}\) and \((A\beta\cdot \text{Cu})_{\text{IV}}\) were copper binding processes and the reaction was modeled as before. The amplitudes at
1000 s were taken to be in equilibrium. These equilibrium relative amplitudes were then plotted over the equilibrium data obtained in figure 3.32, with the associated uncertainty from the fitting.

Figure 3.33 of the fitted models overlaying the data shows that none of the models, fit well. In particular models 9 through 11 cannot be correct. This is because with both III and IV form from I, therefore the models predict that $[\text{III}] \propto [\text{IV}]$. Therefore the relative proportion of III cannot decrease at high Cu$^{2+}$ concentrations, as in the data. It is likely that an extra copper binding term would need to be added to one of the models 9 through 11. This may then fit the small initial binding to Type IV, whilst allowing for it to further increase in proportion at higher Cu$^{2+}$ concentrations. However, all the models predict the association rate constant to Type IV to be approximately $2 \times 10^4 \text{m}^{-1} \text{s}^{-1}$. This slow rate constant suggests that the relevance of this species to the metal-Aβ hypothesis is questionable. That is unless a location in which both Aβ and Cu$^{2+}$ are freely available for extended periods of time can be found, unlike in a synapse.

![Figure 3.33: Part 1 of 2.](image-url)
Figure 3.33: Part 2 of 2. Equilibrium predictions of models 9 through 17 (Eqn. 3.93) with fitting parameters from table 3.9, overlaid onto the measurements from figure 3.32d.
3.10 Summary

In this chapter it has been shown that the fluorescence of labelled Aβ can be quenched, when Aβ binds to Cu²⁺. It was shown that the first Cu²⁺ binds to form component I and it can bind with at least three Cu²⁺ with sufficiently high Cu²⁺ concentrations (Approx. Kₐ’s: 1 nM, 9 µM & 50 µM). The first copper binding is near diffusion limited, with a low activation barrier. The lifetime of the Aβ·Cu complex is approximately 2 s. It was shown that at low Cu²⁺ concentrations two Aβ·Cu species exist, inter-converting on the second timescale. A second copper was found to bind at a rate of approximately 10⁵ M⁻¹ s⁻¹, to component I. Further Cu²⁺ binding species are apparent at higher Cu²⁺ concentrations.

Of the two Aβ·Cu species that are likely to form at the low transient Cu²⁺ concentrations in the synapse, (Aβ·Cu)i was shown to be more reactive. Copper assisted dimerisation via component I is three orders of magnitude faster than that without copper, and an order of magnitude faster than for murine Aβ. The mechanism of Cu²⁺ binding to Aβ and dimerisation can be summarised as

\[
\begin{align*}
\text{A}β + \text{Cu}^{2+} & \xrightarrow{k_{\text{on}}} \text{(A}β·\text{Cu)}_i \\
& \xleftarrow{k_{\text{off}}} \text{(A}β·\text{Cu)}_i \\
& \xrightarrow{k_3} \text{(A}β·\text{Cu})_{ii} \\
& \xrightarrow{k_3} \text{(A}β·\text{Cu})_{iii} \\
& \rightarrow \text{Oligomers}
\end{align*}
\]

It was found that compounds remove copper from Aβ·Cu is via (Aβ·Cu)i and the removal rate was uncorrelated to K_d. Using labelled Aβ and stopped-flow provides a method of measuring the efficacy of drug candidates, which could be used to better test the metal-Aβ hypothesis in vivo and develop new drug candidates.
Chapter 4

Interactions of Amyloid-β with Zn\(^{2+}\) and HSA

Having determined the kinetics of Cu\(^{2+}\) binding to Aβ, the focus is now moved towards Zn\(^{2+}\). Zn\(^{2+}\) like Cu\(^{2+}\) has also been implicated in Alzheimer’s disease and is released into the synaptic cleft during neurotransmission. Zn\(^{2+}\) is known to be a neurotransmitter and is co-released with glutamate, but its role in Alzheimer’s disease is still unclear.

The binding of Zn\(^{2+}\) to Aβ was first discovered in 1994\[76\], with an equilibrium dissociation constant (\(K_d\)) of approximately 100 nM. This estimate of the \(K_d\) has been revised to the range of 1 µM to 100 µM as the field has progressed. This range is above that for the CSF Zn\(^{2+}\) concentration (approximately 100 nM) and below that reached in the synaptic cleft (approximately 300 µM). Does the binding of Zn\(^{2+}\) to Aβ occur in the synaptic cleft lead to the oligomerisation of Aβ?

The carrier protein human serum albumin (HSA), although having been shown to bind metals, is also thought to bind Aβ with a \(K_d\) of approximately 5 µM\[166\]. HSA’s \(K_d\) with Aβ is similar to the concentration of HSA in the CSF (1 µM to 6 µM). The binding of HSA to Aβ oligomers is thought to inhibit the association of Aβ\[266\] and bind with \(K_d\)’s in the range of 1 nM to 100 nM at multiple binding sites\[267\].

In these experiments, the binding of HSA to Aβ is studied using fluorescence correlation spectroscopy (FCS). The aggregation of Aβ with Zn\(^{2+}\) is explored under
equilibrium conditions using FCS and total internal reflection (TIRF) microscopy. Finally, the kinetics of Zn\(^{2+}\) binding to A\(\beta\) and the formation of ternary A\(\beta\)·Cu·Zn species is studied by applying the quenching of labelled A\(\beta\) by Cu\(^{2+}\) using stopped-flow methodologies. Zn\(^{2+}\) itself does not quench the fluorophore complicating the determination of its kinetics. Ternary A\(\beta\)·Cu·Zn complexes have been shown to form in vivo, as well as both Zn\(^{2+}\) and Cu\(^{2+}\) being co-localised in amyloid plaques. However, this area is less explored. The kinetics of both the formation of mixed metal complexes and A\(\beta\)·Zn is unknown.

### 4.1 Binding to HSA

HSA is a common protein in both the blood and the brain, which has been shown to bind to A\(\beta\). However, it is unclear from the literature as to whether HSA binds to monomers\(^{[268]}\), oligomers, or both.

To study the binding of A\(\beta\) monomers to HSA, FCS was used to measure the mean diffusion time (\(\tau_D\)) with varying concentrations of HSA. 1.4 nM A\(\beta_{40}\)HL488 was mixed with varying HSA concentrations from 0.03 g l\(^{-1}\) to 30 g l\(^{-1}\) (0.432 \(\mu\)m to 432 \(\mu\)m).

The data in table 4.1 shows that the binding of A\(\beta\) to HSA is not in the 0.4 \(\mu\)m to 40 \(\mu\)m range as the \(\tau_D\) of HSA is approximately 0.37 ms (see §4.1.1), assuming that

<table>
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<th>HSA/g l(^{-1})</th>
<th>(\tau_D/\text{ms}^{-1}) Run 1</th>
<th>(\tau_D/\text{ms}^{-1}) Run 2</th>
<th>(G(0)) Run 1</th>
<th>(G(0)) Run 2</th>
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<td>0.171</td>
<td>3.05</td>
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<tr>
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<td>0.255</td>
<td>0.259</td>
<td>0.09</td>
<td>0.08</td>
</tr>
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</table>
the N-terminal dye label on Aβ does not affect the binding.

The fast diffusion without HSA is likely due to an average of the diffusion time of monomeric Aβ and free dye. The concentration which proportional to the inverse of amplitude of the correlation function ($G(0)$) is approximately 5 times lower at this point, compared to the other points. This suggests that Aβ may have adsorbed to the surfaces of the container. The increase in $\tau_D$ at 30 g l$^{-1}$ is likely due to the increase in viscosity or aggregation of HSA due to the high concentration.

As a point of reference the $\tau_D$ of AL488 (Alexa Fluor 488) in Tris NaCl was measured the previous day to be 130(3) µs with the same setup.

4.1.1 Mean Diffusion Time of BSA/HSA

To measure the $\tau_D$ of BSA so as to have a baseline for the previous experiment, BSA-AL488 was purchased. BSA-AL488 has on average six AL488 per BSA. The $\tau_D$ was measured using FCS using 10 nM BSA-AL488 in Hepes BSA. Hepes BSA was used to prevent the labelled BSA adsorbing to surfaces. This gave a $\tau_D = 0.618(4)$ ms which is larger than expected (range 0.4 ms to 0.5 ms). This is likely because the dyes are attached via linkers to BSA making the molecule appear larger, overestimating the $\tau_D$ of BSA.

Labelling HSA

To remedy this, HSA was labelled with HL488. HSA has one free cysteine on the surface of the protein and therefore it should be possible to singularly label it with a maleamide dye.

The labelling, purification and gel electrophoresis were performed by Christopher McDonald. The HL488 dye was dissolved in DMSO and the HSA in Hepes NaCl. 7.5 g l$^{-1}$ (110 µM) HSA was mixed at a concentration ratio of approximately 1 : 5 : 10 for HSA : TCEP : HL488, with and without TCEP. TCEP is a reducing agent that does not interfere with HL488, but breaks disulphide bridges. It is preferable not to add TCEP as HSA naturally contains some disulphide bonds which may also be broken. The dye, HSA and TCEP mixture was left for 2 h to react. The solution was then dialysed in PBS at 4°C overnight to remove some of the free dye. The
filter used for dialysis was 10 kDa. HSA is approximately 67 kDa, whereas HL488 is approximately 600 Da. The apparent intensity of the solution after dialysis was similar, as prior to dialysis. This suggests that the HSA blocked the pores in the dialysis membrane preventing the dye from diffusing out.

Figure 4.1: Labelling of HSA by maleimide HL488, with and without the reducing agent TCEP, prior to gel-filtration. The columns are 1) ladder, 2) 10 HSA, 3) HSA, 4) HSA, 5) blank, 6) HSA with TCEP, 7) HSA with TCEP, 8) HSA with TCEP, where 10, 5 & 2 are the relative concentrations of each lane.

To check whether the HSA had been labelled with HL488, gel electrophoresis was performed using an sodium dodecyl sulfate polyacrylamide gel. Figure 4.1 shows a fluorescence image of the gel and the gel stained with coomassie blue after fluorescence image was taken. The stained gel shows the position of the HSA. The fluorescence image shows that both with and without the TCEP the dye appears to be localised with the protein suggesting that it is labelled. There also appears to be ‘smaller’ labelled fragments further down the gel and some ‘larger’ aggregates nearer the top. This suggests that there are impurities in the HSA, or that it may have degraded during the labelling process.

After this, dye was further removed by using a tabletop gel-filtration chromatography column and different fractions collected.
Measuring the $\tau_D$ of HSA and Quenching by Cu$^{2+}$

The $\tau_D$ was measured by Martin Evans to be 0.357(5) ms, closer to the expected value. Free AL488 dye was measured to be 0.127(1) ms. Martin also showed that the dye is quenched when HSA binds to Cu$^{2+}$. However, the dye labelling occludes the Cu$^{2+}$ binding site in some way, increasing the apparent $K_d$ and prevents the kinetics of Cu$^{2+}$ with HSA from being determined directly using this construct. The only cysteine not in a disulfide bridge is residue 34[269] and the N-terminal region of HSA is thought to bind to Cu$^{2+}$[163]. However, the protein is folded, bringing the N-terminus and Cys$_{34}$ to within approximately 2 nm to 3 nm[269].

4.2 Aggregation of A$\beta$ and HSA with Zn$^{2+}$

To investigate the effect of Zn$^{2+}$ on A$\beta$ and HSA under near physiological conditions, FCS was used to measure the change in $\tau_D$ of labelled A$\beta$ in Hepes HSA or Hepes BSA with differing concentrations of Zn$^{2+}$.

Twice the concentration of the required final concentration of A$\beta$ and Zn$^{2+}$ were diluted separately in either Hepes HSA or Hepes BSA. These two solutions were then half and half mixed in an 8 well chambered cover glass (Lab-Tek). This is necessary to minimise localised high concentrations of Zn$^{2+}$ or A$\beta$ which rapidly aggregates. The wells were then covered and sealed with sellotape to reduce evaporation and left overnight at room temperature to reach equilibrium. The $\tau_D$ was measured using FCS over at least 2 min. Labelled particles on the cover glass were photobleached before the measurement was taken, otherwise there is an exponential decay in the baseline of the signal affecting the FCS curves.

Figure 4.2 and figure 4.3 show the results of the experiments with buffers including HSA and BSA respectively. For labelled A$\beta_{16}$ and A$\beta_{40}$ at low Zn$^{2+}$ concentrations there appears to be little change in $\tau_D$, whereas after a critical Zn$^{2+}$
Figure 4.2: Mean diffusion times of various mixtures of labelled and unlabelled Aβ at physiological concentrations (or ten times the concentration), with varying concentrations of Zn^{2+} in Hepes HSA. The parameters for the fitted lines are shown in table 4.2.
concentration the $\tau_D$ appears to increase logarithmically. To fit this phenomenon

$$\tau_D = a + \theta(z-c) \times m \ln\left(\frac{z}{c}\right)$$  \hspace{1cm} (4.1)$$

was used, where $z$ is the concentration of $\text{Zn}^{2+}$, $a$ is the monomer diffusion time, $c$ is the critical concentration, $m$ is the ‘gradient’, and $\theta$ is the Heaviside step function. The fitted parameters are given in table 4.2. The critical concentrations show that there is no significant dependence on the concentration of $\text{A}\beta$, and that the aggregation is thus limited by the $\text{Zn}^{2+}$ concentration. There appears to be no significant difference for HSA and BSA. The mean critical concentration is $40(20) \mu\text{m}$.

The similarity of the critical concentration for the N-terminally labelled and C-terminal lysine $\text{A}\beta$ suggests that N-terminal labelling has no effect on $\text{Zn}^{2+}$ binding. Thus the N-terminal amine group does not coordinate to $\text{Zn}^{2+}$. This in agreement with the proposed binding coordinations in the literature and opposite to what was
Table 4.2: Summary of critical concentrations \(c\), gradients \(m\) and initial diffusion times \(a\) in figures 4.2 & 4.3.

<table>
<thead>
<tr>
<th>Sample in Hepes HSA</th>
<th>(a/\mu s)</th>
<th>(m/\mu s)</th>
<th>(c/\mu m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A\beta_{16}) LysHL488</td>
<td>119(2)</td>
<td>23(4)</td>
<td>40(10)</td>
</tr>
<tr>
<td>(A\beta_{16}) LysHL488 (14 nm)</td>
<td>162(7)</td>
<td>40(10)</td>
<td>70(30)</td>
</tr>
<tr>
<td>(A\beta_{40}) HL488</td>
<td>218(1)</td>
<td>9(2)</td>
<td>60(20)</td>
</tr>
<tr>
<td>(A\beta_{40}) HL488 &amp; (A\beta_{42}) HL488</td>
<td>200(10)</td>
<td>40(10)</td>
<td>30(20)</td>
</tr>
<tr>
<td>(A\beta_{40}) HL488 &amp; (A\beta_{42})</td>
<td>190(10)</td>
<td>50(10)</td>
<td>40(20)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample in Hepes BSA</th>
<th>(a/\mu s)</th>
<th>(m/\mu s)</th>
<th>(c/\mu m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A\beta_{16}) LysHL488</td>
<td>156(1)</td>
<td>11(1)</td>
<td>70(10)</td>
</tr>
<tr>
<td>(A\beta_{40}) HL488</td>
<td>266(2)</td>
<td>50(4)</td>
<td>30(4)</td>
</tr>
<tr>
<td>(A\beta_{40}) HL488 (7.5 nm)</td>
<td>257(3)</td>
<td>45(5)</td>
<td>23(3)</td>
</tr>
<tr>
<td>(A\beta_{40}) HL488 &amp; (A\beta_{42}) HL488</td>
<td>265(4)</td>
<td>45(5)</td>
<td>32(8)</td>
</tr>
</tbody>
</table>

seen with \(Cu^{2+}\) binding.

\(A\beta_{42}\) which is known to be more prone to aggregation, does not appear to follow the trend of \(A\beta_{40}\) and displays a large variation in \(\tau_D\). This suggests that the behaviour is more stochastic with the formation of larger oligomers. The addition of \(A\beta_{40}\) to \(A\beta_{42}\) appears to reduce the variation of oligomer size, despite the increase in total \(A\beta\) concentration.

One possibility is that the \(Zn^{2+}\) is causing the HSA to aggregate. However, the literature also suggests that the HSA will not aggregate without heating\[^{270}\]. Another suggestion is that the \(A\beta\cdot Zn\) complexes are actually forming \(A\beta\cdot Zn\cdot HSA\) complexes, if the \(K_d\) of \(Zn^{2+}\) to \(A\beta\) is greater than that of \(A\beta\cdot Zn\) with HSA.

### 4.3 Kinetics of \(Zn^{2+}\) Binding to \(A\beta\)

In the previous chapter it was shown that the binding of \(Cu^{2+}\) to \(A\beta\) is nearly diffusion limited, which may be due to the electrostatic interactions between the
positive Cu$^{2+}$ ion and negative Aβ. Therefore the kinetics of binding between Zn$^{2+}$ and Aβ may be similar to that of Cu$^{2+}$ and Aβ. Determining the rate constants of Zn$^{2+}$ binding to Aβ will likewise allow for an assessment of the relative importance of Aβ’s interactions with Zn$^{2+}$ in the synaptic cleft.

The likely fast binding of Aβ to Zn$^{2+}$ and a high $K_d$ of approximately 10 µm prevents previous methodologies used to measure the $K_d$ from being applied to investigating the kinetics. Likewise, the method used to determine the Cu$^{2+}$ binding kinetics cannot be used directly because Zn$^{2+}$ does not quench labelled Aβ. Therefore, without a method of determining whether Aβ is bound to Zn$^{2+}$ indirect competition experiments must be used. There are two forms of competition experiments possible: a mixture of Aβ and a Zn$^{2+}$ indicator competing for Zn$^{2+}$, and Zn$^{2+}$ competing with Cu$^{2+}$ for labelled Aβ.

The problem with the Aβ competing against a zinc indicator for Zn$^{2+}$ is that the concentration of Aβ and zinc indicator must be in a 20 fold excess of the concentration of Zn$^{2+}$ for the experiment to be under pseudo-first-order conditions. Given that the literature $K_d$ is in the range of 1 µm to 100 µm, even stoichiometric concentrations of Aβ would cause rapid aggregation, more so with a 20 fold excess. Therefore the latter competition experiment was used.

If the model of Aβ binding to a mixture of Zn$^{2+}$ and Cu$^{2+}$ is taken to be

\[
A\beta + \text{Zn}^{2+} + \text{Cu}^{2+} \xrightarrow{k_z} A\beta \cdot \text{Zn} + \text{Cu}^{2+}, \quad (4.2)
\]

\[
A\beta + \text{Cu}^{2+} + \text{Zn}^{2+} \xrightarrow{k_c} A\beta \cdot \text{Cu} + \text{Zn}^{2+},
\]

and the experiment is under pseudo-first-order conditions, such that $[\text{Cu}^{2+}] \gg [A\beta] \ll [\text{Zn}^{2+}]$, then the solution for $A\beta \cdot \text{Cu}$ is of the form

\[
[A\beta \cdot \text{Cu}] = \frac{[A\beta]}{2\beta^2} \left( \frac{2\beta^2}{+\beta(k_c[\text{Cu}^{2+}] - k_z[\text{Zn}^{2+}] - k_{-z} - \beta)e^{-\frac{1}{2}(\alpha - \beta)}}{\beta(k_c[\text{Cu}^{2+}] - k_z[\text{Zn}^{2+}] - k_{-z} + \beta)e^{-\frac{1}{2}(\alpha + \beta)}} \right), \quad (4.3)
\]
where
\[ \alpha = k_c [\text{Cu}^{2+}] + k_z [\text{Zn}^{2+}] + k_z, \quad (4.4) \]
\[ \beta = \sqrt{\alpha^2 - 4 k_z k_c [\text{Cu}^{2+}]} , \quad (4.5) \]
and the initial conditions applied are
\[ [\text{A}^{\beta}\cdot\text{Zn}]_{t=0} = [\text{A}^{\beta}\cdot\text{Cu}]_{t=0} = 0. \quad (4.6) \]

Therefore the solution is a double exponential with the rates
\[ k = \frac{1}{2} (\alpha \pm \beta) \]
\[ = \frac{1}{2} \left( k_c [\text{Cu}^{2+}] + k_z [\text{Zn}^{2+}] + k_z \pm \sqrt{(k_c [\text{Cu}^{2+}] + k_z [\text{Zn}^{2+}] + k_z)^2 - 4 k_c [\text{Cu}^{2+}] k_z} \right) . \quad (4.8) \]

In this model there are many implicit assumptions in the model (Eqn. 4.2). The reaction of \( \text{A}^{\beta}\cdot\text{Cu} \longrightarrow \text{A}^{\beta} + \text{Cu}^{2+} \) is ignored as the rate is approximately 0.5 s\(^{-1}\), much slower than the observed binding rates (20 s\(^{-1}\) to 100 s\(^{-1}\)). The reaction \( \text{A}^{\beta}\cdot\text{Cu} + \text{Zn}^{2+} \longrightarrow \text{A}^{\beta}\cdot\text{Cu}\cdot\text{Zn} \) is ignored as the rate constant is \( 3 \times 10^3 \text{ m}^{-1} \text{s}^{-1} \) (see §4.4). At the highest \( \text{Zn}^{2+} \) concentration the rate is 0.6 s\(^{-1}\). The reaction \( \text{A}^{\beta}\cdot\text{Zn} + \text{Cu}^{2+} \longrightarrow \text{A}^{\beta}\cdot\text{Cu} \cdot \text{Zn} \) is ignored because in order for the reaction to have a rate of at least 10 s\(^{-1}\), a rate constant of \( 2 \times 10^7 \text{ m}^{-1} \text{s}^{-1} \) or greater is required. This is deemed unlikely given the \( \text{A}^{\beta}\cdot\text{Cu} + \text{Zn}^{2+} \longrightarrow \text{A}^{\beta}\cdot\text{Cu} \cdot \text{Zn} \) rate constant is \( 3 \times 10^3 \text{ m}^{-1} \text{s}^{-1} \) and \( \text{A}^{\beta}\cdot\text{Cu} + \text{Cu}^{2+} \longrightarrow \text{A}^{\beta}\cdot\text{Cu} \cdot \text{Cu} \) is \( 1 \times 10^5 \text{ m}^{-1} \text{s}^{-1} \). \( \text{A}^{\beta}\cdot\text{Cu} + \text{Cu}^{2+} \longrightarrow \text{A}^{\beta}\cdot\text{Cu} \cdot \text{Cu} \) is therefore also too slow to participate (0.05 s\(^{-1}\)). By the same reasoning the \( \text{A}^{\beta}\cdot\text{Zn} + \text{Zn}^{2+} \longrightarrow \text{A}^{\beta}\cdot\text{Zn}\cdot\text{Zn} \) is also ignored. This leaves the above model (Eqn. 4.2), the second simplest competition model.

To measure \( k \) predicted by the model, 500 nM CuCl\(_2\) was premixed with various concentrations of ZnCl\(_2\) which were then mixed with 25 nM \( \text{A}^{\beta}_{16}\text{LysHL488} \) using stopped-flow and the fluorescence quenching traces recorded. The traces were fitted with a double exponential from 2 ms to 0.1 s, without weights. The rate of the fastest
phase was then fitted with

\[
k = \frac{1}{2} \left( k_{\text{Cu}} + k_z [\text{Zn}^{2+}] + k_z K_d - \sqrt{(k_{\text{Cu}} + k_z [\text{Zn}^{2+}] + k_z K_d)^2 - 4k_{\text{Cu}}} \right), \tag{4.9}
\]

where the \(k_{\text{Cu}}\) is the association rate of 500 nM Cu\(^{2+}\) to A\(\beta\) under pseudo first order conditions \((k_{\text{Cu}} = k_c [\text{Cu}^{2+}])\) and \(K_d\) is the equilibrium dissociation constant of A\(\beta\) with Zn\(^{2+}\).

Figure 4.4: Apparent rate of Cu\(^{2+}\) binding to A\(\beta\) \((k)\) when competing against Zn\(^{2+}\). 25 nM A\(\beta\)\(_{1-40}\) was mixed using stopped flow with a solution of 500 nM Cu\(^{2+}\) and varying concentrations of Zn\(^{2+}\), in Hepes NaCl. The solid line is the fit from equation 4.9 with \(k_{\text{Cu}} = 160(20) \text{s}^{-1}\), \(k_z = 1.9(3) \times 10^6 \text{M}^{-1} \text{s}^{-1}\), and \(K_d = 58(9) \mu\text{M}\).

The fit in figure 4.4 gives \(k_{\text{Cu}}\) to be 160(20) s\(^{-1}\), \(k_z\) to be 1.9(3) \times 10^6 M\(^{-1}\) s\(^{-1}\) and the \(K_d\) to be 58(9) \(\mu\text{M}\). Therefore \(k_z\) is 110(20) s\(^{-1}\). \(k_{\text{Cu}}\) is within error of the expected value, i.e. 150 s\(^{-1}\) (see §3.3).

The \(K_d\) is within the expected range from the literature (1 \(\mu\text{M}\) to 100 \(\mu\text{M}\)). However, the value was obtained with 50 mM Hepes and 100 mM NaCl. This may increase the \(K_d\) by reducing \(k_z\) as with Cu\(^{2+}\) where it was a factor of approximately 2. The \(K_d\) is within error of the critical concentration for the onset of A\(\beta\) aggregation obtained in section 4.2. Given that the concentration of A\(\beta\) in the experiments was 1.4 nM, this suggests that the affinity of A\(\beta\) for A\(\beta\)\(\cdot\)Zn is nanomolar or less and thus that zinc assisted A\(\beta\) dimers are more stable than A\(\beta\)\(\cdot\)Zn alone. The binding of Zn\(^{2+}\) to oligomers or fibrils may be important for their growth.

It is surprising that \(k_z\) is approximately two orders of magnitude less than the
association rate constant of Aβ with Cu$^{2+}$. This suggests that Aβ may not be able to bind to Zn$^{2+}$ before it diffuses out of the synapse. The dissociation rate of Aβ·Zn suggests that the complex would have a lifetime of approximately 9 ms (half-life of 6 ms). For Zn$^{2+}$ assisted dimerisation to occur, the binding of free Aβ to Aβ·Zn without free Zn$^{2+}$ in the CSF would require a rate constant at least an order of magnitude above the limit by diffusion ($10^9$ M$^{-1}$s$^{-1}$), $1.1 \times 10^{10}$ M$^{-1}$s$^{-1}$ for 10 nm Aβ, which is highly unlikely. For comparison the dimerisation rate of Aβ with Aβ·Cu would need to be above $5 \times 10^7$ M$^{-1}$s$^{-1}$. This is also unlikely suggesting that dimerisation does not occur in the CSF, and that sustained high concentrations of metals or Aβ would be required. However, the relative parameters to find limits for the dimerisation on the synaptic membrane are unknown.

### 4.4 Kinetics of Mixed Metal Aβ·Cu·Zn Species

An extension to the amyloid-metal hypothesis seen in the literature is that mixed Aβ·Cu·Zn may be involved in the pathways of Alzheimer’s disease. To measure the associated parameters with this process, the displacement of Cu$^{2+}$ in Aβ·Cu by Zn$^{2+}$ is studied.

To measure the interactions of Zn$^{2+}$ with Aβ·Cu, 100 nM Aβ$_{16}$LysHL488 was premixed with 100 nM CuCl$_2$ to form Aβ·Cu. This solution was then mixed with varying concentrations of ZnCl$_2$. The recorded fluorescence recovery traces were fitted with a double exponential fit. A slow dominant phase with a rate of 0.47(3) s$^{-1}$ and a minor fast phase with rate of 5.2(2) s$^{-1}$ were observed. The slow rate corresponds to the rate of dissociation of Aβ·Cu. This suggests that the slow process is related to

$$A\beta \cdot Cu + Zn^{2+} \rightleftharpoons A\beta + Cu^{2+} + Zn^{2+} \rightleftharpoons A\beta \cdot Zn + Cu^{2+}, \quad (4.10)$$

which is unrelated to Zn$^{2+}$’s interaction with Aβ·Cu.

Figure 4.5 shows the apparent rate of the fast phase. The rate appears independ-
Figure 4.5: Apparent rate of the fast phase \((k)\) of the displacement of \(\text{Cu}^{2+}\) by varying concentrations of \(\text{Zn}^{2+}\) in 50 \(\text{nm A}^\beta\cdot\text{Cu}\), in Hepes NaCl. The solid line is the average 5.2(2) \(s^{-1}\), uncertainty not shown.

ent of \(\text{Zn}^{2+}\) concentration. Therefore, if the model of the experiment is

\[
\begin{align*}
\text{A}^\beta\cdot\text{Cu} + \text{Zn}^{2+} &\rightleftharpoons \text{A}^\beta\cdot\text{Cu}\cdot\text{Zn} \\
&\rightleftharpoons \text{A}^\beta\cdot\text{Zn} + \text{Cu}^{2+}
\end{align*}
\tag{4.11}
\]

this rate corresponds to the rate limiting step, i.e. the removal of \(\text{Cu}^{2+}\) from \(\text{A}^\beta\cdot\text{Cu}\cdot\text{Zn}\) and \(\text{A}^\beta\cdot\text{Cu}\cdot\text{Zn} \rightarrow \text{A}^\beta\cdot\text{Zn} + \text{Cu}^{2+}\). This rate constant is related to the lifetime of the \(\text{A}^\beta\cdot\text{Cu}\cdot\text{Zn}\) complex. However, the lifetime may be dominated by the removal of the \(\text{Zn}^{2+}\) instead. In this model, to determine the rate constant of \(\text{A}^\beta\cdot\text{Cu} + \text{Zn}^{2+} \rightarrow \text{A}^\beta\cdot\text{Cu}\cdot\text{Zn}\), it is required to be slow compared to the other steps, otherwise there will be little difference in the fluorescence change contributed by it. Theoretically, this rate could be slowed by reducing the concentration of \(\text{Zn}^{2+}\). However, this is impractical due to the small amplitude of the fast phase which would decrease further with a reduced \(\text{Zn}^{2+}\) concentration. Therefore an alternative approach is required.

For intramolecular processes such as dissociations, typically the Arrhenius pre-exponential factor is much larger than that of intermolecular processes. This is due to the relation between the pre-exponential factor and the ‘collision frequency’. Therefore, at sufficiently high temperatures, the rates of intermolecular processes may be slower than those of internal processes. By studying the underlying energetics of the process, it may be possible to extrapolate the association rate constant of
\[
A\beta\cdot\text{Cu} + Zn^{2+} \longrightarrow A\beta\cdot\text{Cu} \cdot \text{Zn} \text{ at } 25^\circ\text{C}.
\]

To test this hypothesis, 100 nm A\(_{16}\)LysHL488 was premixed with 100 nm CuCl\(_2\) to form A\(\beta\cdot\text{Cu}\). This was then mixed with 600 \(\mu\text{m}\) ZnCl\(_2\) varying the temperature of the reaction. The fluorescence recovery data was fitted with a multi-exponential, and the rate of the fastest phase was taken.

![Figure 4.6: Arrhenius plot of the apparent rate of the fastest phase (k) of 50 nm A\(_{16}\)LysHL488, with 300 \(\mu\text{m}\) ZnCl\(_2\). The solid line is a fit of equation 4.12, and the dotted line is an extension of the piecewise parts of equation 4.12. The intersection of the two lines is at 34(2) \(^\circ\text{C}\).](image)

Figure 4.6 shows that there appears to be a bend in the Arrhenius plot as the rate limiting step changes. To fit the data, two Arrhenius equations are used in a continuous piecewise manner, such that

\[
\ln(k) = \begin{cases} 
T^{-1} < \frac{R(c_1 - c_2)}{E_{a1} - E_{a2}}, & c_1 \frac{E_{a1}}{RT} \\
\text{otherwise}, & c_2 \frac{E_{a2}}{RT} 
\end{cases},
\]

where \(k\) is the apparent reaction rate, \(T\) is the temperature, \(R\) is the ideal gas constant, the \(c\)'s are the natural log of the Arrhenius pre-exponential factor of the two phases, and the \(E_{a}\)'s are the activation energies of the two phases. This shows that the transition temperatures between the two regimes is 34(2) \(^\circ\text{C}\), suggesting that for concentrations of ZnCl\(_2\) less than or equal to 300 \(\mu\text{m}\) and temperatures above 34(2) \(^\circ\text{C}\), the Zn\(^{2+}\) binding can be studied as it would be the dominant process. It should be noted that this transition temperature should change for different Zn\(^{2+}\)
concentrations.

To confirm that for temperatures near and above 34(2) °C the $A\beta \cdot Cu + Zn^{2+} \rightleftharpoons A\beta \cdot Cu \cdot Zn$ is the limiting process and to measure the binding rate constant, 100 nM $A\beta_{16}$LysHL488 was premixed with 100 nM CuCl$_2$ to form $A\beta \cdot Cu$. This was then mixed with different concentrations of ZnCl$_2$ (200 µM to 600 µM, twice the final concentration) at different temperatures (35 °C to 55 °C). The resulting fluorescence recovery data was fitted with a multi-exponential, and the fastest rate taken to be due to the association process (see Fig. 4.7a). The rates for each temperature point were taken and linearly fitted (see Fig. 4.7a). The gradient of the fit is taken as the $A\beta \cdot Cu + Zn^{2+} \rightleftharpoons A\beta \cdot Cu \cdot Zn$ association rate constant ($k_{on}$). The Arrhenius plot of $k_{on}$ in figure 4.7b was fitted with the Arrhenius equation. This fit can then be used to extrapolate $k_{on}$ at 25 °C, giving $3(1) \times 10^3 \text{m}^{-1} \text{s}^{-1}$. The error at 25 °C was calculated by shifting the data by $T^{-1} \rightarrow T^{-1} - (25 \degree C)^{-1}$ and refitting so as not to overestimate the error at this point.

The $3(1) \times 10^3 \text{m}^{-1} \text{s}^{-1}$ association rate constant is extremely slow, two orders of magnitude slower than the second Cu$^{2+}$ binding and thus $A\beta \cdot Cu \cdot Zn$ species are unlikely to form from $A\beta \cdot Cu$ in vivo.
4.5 Summary

In this chapter it has been shown that Zn$^{2+}$ may bind to A$\beta$ and may form mixed A$\beta$·Cu·Zn complexes. Dye labelled monomeric A$\beta$ has been shown not to bind to HSA.

At physiological concentrations of A$\beta$ and HSA there is a critical point of approximately 40(20) µM for the onset of aggregation with Zn$^{2+}$. This is well below the 300 µM Zn$^{2+}$ released during synaptic transmission. The critical point of aggregation under these conditions appears to depend only on Zn$^{2+}$ concentration and not on the concentration of A$\beta$ when the A$\beta$ concentrations are in the low nanomolar regime. The aggregates may or may not contain HSA. The critical point also provides an estimate for the $K_d$ of A$\beta$ binding to Zn$^{2+}$ or HSA·Zn.

The kinetics of A$\beta$ with Zn$^{2+}$ were determined to be

$$A\beta + Zn^{2+} \xrightarrow{1.9(3) \times 10^6 \text{M}^{-1} \text{s}^{-1}} 110 \text{s}^{-1} \xrightarrow{A\beta \cdot Zn}, \hspace{1cm} (4.13)$$

with $K_d$ of 58(9) µM, uncorrected for buffer effects. The association rate constant of Zn$^{2+}$ with A$\beta$ is two orders of magnitude lower than that for Cu$^{2+}$ and further from the diffusion limit, so it is harder to deduce the magnitude and possible relevance of A$\beta$ binding to Zn$^{2+}$ during synaptic transmission. The short lifetime of the A$\beta$·Zn complexes suggests that A$\beta$·Zn cannot dimerise in free solution with CSF concentrations of A$\beta$, without sustained high concentrations of Zn$^{2+}$.

The kinetics binding of Zn$^{2+}$ to A$\beta$·Cu is very slow,

$$A\beta \cdot Cu + Zn^{2+} \xrightarrow{3(1) \times 10^3 \text{M}^{-1} \text{s}^{-1}} A\beta \cdot Cu \cdot Zn, \hspace{1cm} (4.14)$$

but the complete mechanism is unclear. With a slow association rate constant of 3(1) × 10$^3$ M$^{-1}$ s$^{-1}$ mixed A$\beta$·Cu·Zn complexes are likely to be irrelevant in physiology or to the dimerisation of A$\beta$. 

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Chapter 5

Interactions of Amyloid-β with Model Lipid Membranes

Having determined some of the kinetics for Cu$^{2+}$ and Zn$^{2+}$ binding to Aβ as parameters to test the metal-amyloid hypothesis in the synapse, this leaves the effect of the synapse itself on the hypothesis. The C-terminal region of Aβ is known to bind to membranes, in particular to lipid rafts containing the ganglioside GM1. The effects of Aβ being bound to a membrane on the binding to Cu$^{2+}$ are unknown.

In the previous chapters, it was predicted that metal assisted Aβ dimerisation was unlikely to occur in solution for Cu$^{2+}$. This is because for the concentration of Aβ in the CSF a rate constant of $> 10^7$ M$^{-1}$ s$^{-1}$ was required for the rate of the reaction to be greater than the dissociation rate of Aβ·Cu. The measured rate was only $10^5$ M$^{-1}$ s$^{-1}$. This leaves the possibility that Aβ dimerisation occurs on the surface of synaptic membranes. To make an equivalent prediction for the dimerisation rate on membranes, surface density of Aβ, its metal assisted dimerisation rate constant and the kinetic parameters of Cu$^{2+}$ binding to membrane bound Aβ would be required.

Here the binding of Aβ to model lipid membranes is studied, leading to an order of magnitude estimate of the concentration on membranes. This is done by counting labelled Aβ observed through TIRF microscopy. The kinetics of Cu$^{2+}$ binding to Aβ bound to GM1 micelles are measured using stopped flow for comparison to the Aβ/Cu$^{2+}$ kinetics obtained in chapter 3.
5.1 Binding of Aβ to Sodium Dodecyl Sulfate Micelles

Sodium dodecyl sulfate (SDS) can be used as a membrane mimic due to its very polar head group and hydrophobic tail (see Fig. 5.1). In aqueous solutions they spontaneously form micelles when above the critical micelle concentration (CMC) of 8.2 mM$^{[271]}$. The micelles formed are approximately 60 units of SDS, however it may increases in strong electrolyte solutions$^{[272]}$.

To investigate the incorporation of Aβ$_{40}$ into SDS micelles, SDS was dissolved in PBS buffer at 20 mM to form micelles. The 20 mM SDS solution was diluted to the required concentration and mixed with 2.3 nM Aβ$_{40}$HL488. The mean diffusion time of the label was measured with FCS.

![Figure 5.1: Chemical structure of sodium dodecyl sulfate.](image)

![Figure 5.2: Mean diffusion time of 2.3 nM Aβ$_{40}$HL488 with varying concentrations of SDS. The data is fitted with a Hill function with, $A = -0.21(4)\, \text{ms}$, $K_d = 0.2(1)\, \text{mM}$, $c = 0.45(2)\, \text{ms}$, with $\alpha$ fixed at 1.](image)

Figure 5.2 shows the data fitted with a Hill function. The fit gives the $K_d$ to be in the low hundreds of micromolar (200(100) µm for SDS molecules, or equivalently 3(2) µm for SDS micelles). The mean diffusion time of the bound complex is 0.45(2) ms, which may be used to estimate the number of SDS units per micelle. If
the micelle is assumed to be spherical and the density ($\rho$) of the micelle and AÎ² are similar, then

$$m = \rho V , \quad V = \frac{4}{3} \pi r^3 , \quad R_H = \frac{k_B T}{6\pi \eta D} \quad \text{and} \quad \tau_D = \frac{\omega_0^2}{4D} , \quad (5.1)$$

where $V$ is the volume, $r$ is the radius of a sphere, $R_H \equiv r$ is the hydrodynamic radius, $T$ is the temperature, $\eta$ is the viscosity, $D$ is the diffusion constant and $\omega_0^2$ is the beam waist of the confocal volume, which can be used to show the that $\tau_D^3 \propto m$. From this the number of units ($n$) can be approximated from

$$\left( \frac{\tau_D AÎ²_{-\text{micelle}}}{\tau_D AÎ²} \right) = \frac{mAÎ² + mSDS \times n}{mAÎ²} . \quad (5.2)$$

Using the fitted diffusion with the micelle (0.45(2) ms) and the free AÎ²_{40} diffusion time (0.254 ms), this estimates the size to be approximately 70 SDS units, similar to that in the literature.

### 5.2 Binding of AÎ² to GM1 micelles

AÎ² is known to bind tightly to GM1 ganglioside, a lipid found in the plasma membrane of cells which is concentrated in lipid rafts. The binding of AÎ² to GM1 is thought to promote structural changes and oligomerisation.

GM1, like SDS, spontaneously forms micelles when the concentration is above the critical micelle concentration of approximately 1 µM. They have a hydrodynamic radius of 5.4 nm and are made up of 168(4) GM1 units (approximately 260 kDa). The C-terminal region of AÎ² has been shown to be the region binding to GM1 micelles leaving the N-terminal metal binding region exposed to the extracellular spaces, such as the synaptic cleft.

#### 5.2.1 Formation of Micelles

To make BODIPY(texas red) labelled micelles, GM1 and GM1 labelled with BODIPY are dissolved in methanol as stock solutions. The GM1 and labelled GM1 are mixed at a molar ratio of approximately 1 : 200 in methanol to allow for the lipids
to mix evenly. The methanol solution is left to evaporate to dryness. Aqueous buffer is added to the dried mixture of labelled and unlabelled GM1, such that the concentration is greater than 1 µM (620 µM, or 3.7 µM of micelles). This solution is then diluted to the required concentration. The 1 : 200 mixture of labelled to unlabelled lipids in the micelles should follow a Poisson distribution. Therefore the probability \( P(n) \) of number of dyes per micelle \( n \) is: \( P(0) = 43\% \), \( P(1) = 36\% \), \( P(2) = 15\% \), \( P(>2) = 5\% \), and thus the proportion of singularly labelled to multiply labelled micelles is 63\%. For unlabelled micelles the same steps were followed, but without adding dye labelled GM1.

To determine whether micelles were formed, the diffusion time of labelled micelles was measured in Tris NaCl buffer. \( \tau_D \) was measured to be 0.61(2) ms, significantly larger than for Aβ40 (approximately 0.25 ms). Using a similar calculation to that in section 5.2, this gives the number of units in a GM1 micelle to be 40(20). The literature value of the hydrodynamic radius of Aβ is approximately 0.9 nm[50]. This gives the GM1 micelles a radius of 2.2 nm, approximately half the literature value of 5.4 nm[147].

### 5.2.2 Binding of Aβ to GM1 Micelles

Aβ-GM1 micelle complexes were prepared at higher concentrations and diluted for the FCS measurement. This is because the binding of Aβ40 to Aβ-GM1 micelles complexes was too slow at low concentrations. 100 µM GM1 (667 nM micelles) was mixed with 500 nM Aβ40 HL488 and diluted by a factor of 200 for the FCS measurement in PBS.

The \( \tau_D \) of the micelles was measured to be approximately 0.51 ms, suggesting that binding had occurred. However, the literature states that the \( K_d \) is approximately 1 µM, so the complex should start to dissociate after dilution. Measuring the dissociation rate was attempted using FCS, but the concentration of fluorescence particles dropped over the course of 150 min, suggesting that upon dissociation the Aβ40 was adsorbing to the glass/plastic surface of the well.

In order to measure the kinetics, order of magnitude higher concentrations are required. However this would be too high for FCS measurements.
Anisotropy

$\text{A}_\beta^{40}\text{Cys}_{20}$HL488 was shown to bind micelles using fluorescence anisotropy. The anisotropy of 2 $\mu$m $\text{A}_\beta^{40}\text{Cys}_{20}$HL488 was measured with and without 2 $\mu$m GM1 micelles, using the fluorometer. The anisotropy increased from 0.04(1) to 0.193(2). This shows binding of the labelled $\text{A}_\beta$ to GM1 micelles. The binding occurred within 20 s, so the kinetics are too fast to be measured by the fluorometer, so require the change in anisotropy to be measured by stopped flow.

The fluorescence anisotropy ($A$) is defined as

$$A = \frac{r_0}{1 + \frac{\tau}{\phi}}$$  \hspace{1cm} (5.3)

where $r_0$ is the intrinsic anisotropy of the fluorophore, $\tau$ is the fluorescence lifetime of the fluorophore (4.1 ns), and $\phi$ is the rotational time constant. The Stokes-Einstein-Debye equation

$$\phi^{-1} = \frac{k_B T}{\eta V}$$  \hspace{1cm} (5.4)

relates $\phi$ to the temperature $T$ (298 K), the viscosity $\eta$ ($8.9 \times 10^{-4}$ kg m$^{-1}$ s$^{-2}$ for water), and the molecular volume $V$. Using the hydrodynamic radius of $\text{A}_\beta$ (0.9 nm) and its anisotropy (0.04), $r_0$ was determined to be 0.29. This gives the hydrodynamic radius of $\text{A}_\beta$·GM1 micelle to be 2.1 nm, approximately half the literature value.

5.2.3 Labelled Micelles are not Quenched by Cu$^{2+}$

To investigate the interactions of Cu$^{2+}$ with GM1 micelles, 100 $\mu$m of labelled micelles were mixed with 10 $\mu$m Cu$^{2+}$ and the fluorescence was measured before and after using the fluorometer. There was no apparent change in fluorescence.

As a possible positive control 1 $\mu$m unlabelled $\text{A}_\beta^{40}$ was mixed with 1 $\mu$m GM1 micelles and 10 $\mu$m Cu$^{2+}$ was added. There was no apparent change in fluorescence.

This suggests that Cu$^{2+}$ bound to $\text{A}_\beta$ on the micelle or Cu$^{2+}$ bound to the surface of the micelle is unable to interact with the dye on the lipid tail of the GM1 inside of the micelle. Therefore BODIPY labelled GM1 micelles with a label on the hydrophobic tail of GM1 cannot be used to investigate copper binding to micelles.
or unlabelled Aβ.

5.3 Binding of Aβ to Vesicles of GM1, Sphingomyelin and Cholesterol

To test the binding Aβ to a more physiologically relevant system of lipids, a mixture of GM1, Sphingomyelin and Cholesterol was used to create vesicles of approximately 100 nm in size. Varying the composition of the lipids in the system will vary the properties of the membrane that Aβ binds, such as curvature, surface tension and fluidity.

The vesicles were prepared such that the molar ratio of GM1:Cholesterol:Sphingomyelin is 1:2:1 with concentration of 140 µM:270 µM:140 µM in 1 mL of Tris NaCl. For labelled vesicles 0.8 % of the GM1 was replaced with GM1 Bodipy. Initially the lipids were dissolved in methanol to facilitate the mixing of the lipids and evaporated to dryness. The mixture of lipids was then hydrated in buffer, making large multilamellar vesicles. This solution was extruded through a 100 nm polycarbonate membrane filter (LipsoFast, Avestin) 15 times, to ensure that the solution used had passed through the membrane[274].

Figure 5.3 shows some of the vesicles (diffraction limited bright spots) on the glass cover slip. This shows that the vesicles have formed and have stuck to the surface.

Figure 5.4a shows that there are vesicles in solution using confocal microscopy. The bright spikes are the vesicles diffusing slowly through the confocal volume.

To test whether Aβ binds to the vesicles, FRET from the GM1 Bodipy in the vesicles to the Aβ40HLTR was measured. Figure 5.4b shows the fluorescence spectra of labelled vesicles with different ratios of Aβ40HLTR to GM1 Bodipy. The figure shows that there is a FRET peak at approximately 620 nm, showing that the Aβ is close to the GM1 Bodipy. The FRET peak increases up until the ratio of 4.54 after which there is a decrease in the FRET efficiency. This is likely due a high density of Aβ40HLTR binding to the vesicles allowing for Homo-FRET to occur. The
Figure 5.3: Labelled vesicles on a glass surface using TIRF microscopy. The colour denotes the pixel brightness of the CCD camera.

Figure 5.4: (a) Fluorescence trace of labelled vesicles diffusing through the confocal volume. (b) Emission spectrum of acceptor labelled $A\beta_{40}$ with donor labelled vesicles, with different ratios of $A\beta_{40}$HLTR to GM1 Bodipy. The sample is excited at 488 nm.
emission peak of GM1 Bodipy continues to decrease with the increased proportion of Aβ₄₀HLTR as expected.

5.4 Estimate of the Surface Density of Aβ

The number of Aβ molecules and the diffusion constant when bound to the synapse are important biophysical quantity for predicting whether dimerisation can occur on the membrane. Although the diffusion constant of Aβ monomers on some membranes has been measured, there has been no attempt to quantify the surface concentration of Aβ.

To obtain an order of magnitude estimate of the surface density of Aβ₄₀ on model membranes, two disparate forms of membranes were formed on a large glass bottomed dish (WillCo-Dish, GWSt-5040) and then TIRF microscopy was used to image the bottom of the well so that the single molecules could be counted.

The membranes were formed of POPC & POPG, or GM1, Sphingomyelin & Cholesterol. The POPC & POPG membrane was formed by dissolving POPG and POPC in chloroform and then mixing them in a glass vial such that 1 mg of each was in the vial in chloroform. This was left to evaporate to dryness. 1.3 ml of Hepes NaCl was added to the dried powder and left for a few hours. The white cloudy solution was sonicated for approximately 30 min until the solution had become clear. This was then pipetted into a glass bottomed dish and a further 4.3 ml Hepes NaCl was added. The solution was left at 5 °C overnight to allow the vesicles to collapse onto the surface. After this the solution was removed and the dish rinsed gently with Hepes NaCl buffer to the remove vesicles that had not collapsed. For the GM1, Sphingomyelin & Cholesterol membrane, 0.5 mg, 0.5 mg and 1 mg, were prepared respectively in methanol and left to evaporate to dryness. After that, the same procedure was followed.

For the TIRF measurements, 10 pm Aβ₄₀Cys₂₀HL488 was added into the glass bottomed dish such that the total volume was 6 ml. The solution was left for a few hours to reach equilibrium. 40 frames of 80 ms exposure time were taken using the EMCCD with a laser power of approximately 4.4 mW. The molecules were
counted in each frame by hand and fitted with exponentials as expected for the photobleaching processes.

As a possible control to see whether membranes had formed, the experiment was also performed on glass. Figure 5.5 shows that with only glass the surface has a very high coverage in which it is difficult to identify single molecules until most of the fluorophores have photobleached. For comparison, when the membrane has been prepared the number of molecules was significantly lower showing that the membranes had formed (see Fig. 5.6 & 5.7). However, it does not show the thickness of the membrane or the number of layers that may had formed. This also suggests that coating surfaces with a membrane could be used to reduce surface adsorption. In particular with POPC, as $\text{A}_\beta$ is not thought to bind to POPC membranes.

Figure 5.8 shows the number of spots on each of the two membranes as the fluorophores bleach in time. The bleaching of the spots on the POPC & POPG membrane appears more exponential than that with the GM1, Sphingomyelin & Cholesterol membrane. This suggests that on the GM1 containing membrane the $\text{A}_\beta$ molecules are more localised and possibly have formed oligomers. Extrapolating to frame 0 gives the number of spots to be 270 for POPC & POPG, and 280 for GM1, Sphingomyelin & cholesterol. This is surprisingly consistent for such differing membranes suggesting that the solution concentration may be a limiting factor. The area of frames is approximately 20 $\mu$m by 20 $\mu$m, giving a surface number density of approximately $7 \times 10^{11}$ m$^{-2}$ (11 fmoldm$^{-2}$). The bottom of the well is 40 mm in diameter giving the total number of molecules adsorbed to the surface as $3.5 \times 10^9$, whereas the total number of molecules in the dish is expected to be $3.6 \times 10^{10}$, so approximately 10 % are bound. The Langmuir isotherm

$$\theta = \frac{[P]}{K_d + [P]} \quad (5.5)$$

relates the fractional surface coverage $\theta$, to the solution concentration $[P]$ via the $K_d$. 

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Figure 5.5: Photobleaching of $\text{A}_\beta_{40}\text{Cys}_{20}\text{HL}488$ on glass. Frame one appears blank as it is overexposed due to the high surface density of $\text{A}_\beta_{40}\text{Cys}_{20}\text{HL}488$ on the glass. The colour denotes the pixel brightness of the CCD camera.
Figure 5.6: Photobleaching of $\text{A}_\beta_{40}\text{Cys}_{20}\text{HL488}$ on a membrane of GM1, Sphingomyelin and Cholesterol. The colour denotes the pixel brightness of the CCD camera.
Figure 5.7: Photobleaching of $\text{A}\beta_{40}\text{Cys}_{20}\text{HL488}$ on a membrane of POPC and POPG. The colour denotes the pixel brightness of the CCD camera.
Figure 5.8: Photobleaching of 10 pm Aβ40 Cys20 HL488 bound to a model lipid membrane (either POPC & POPG, or GM1, Sphingomyelin & Cholesterol). 400 µm² images were taken, using TIRF microscopy and the particles were counted manually. The membrane components were of an equal mass ratio. Fitting with a single exponential, with the error estimated by the square root of the particle number, gives approximately 270 & 280 molecules for the two membranes (POPC & POPG, or GM1, Sphingomyelin & Cholesterol, respectively). Therefore the surface density of Aβ is estimated to be approximately 1 pmol m⁻² (7 × 10¹¹ m⁻²).

As the proportion bound is relatively low, this suggests that \([P] \ll K_d\) and thus

\[
\theta \propto [P]. \tag{5.6}
\]

Given that the diameter of a synapse is approximately 300 nm, at 10 pm there are approximately 0.05/synapse. Therefore at physiological concentrations (1 nm to 10 nm) there are likely be approximately 5/synapse to 50/synapse. For comparison there are approximately up to 20 NMDA receptors per synapse and up to 200 AMPA receptors\(^{[275]}\).

### 5.5 Kinetics of Cu²⁺ Binding to Aβ on GM1 Micelles

The kinetics of Cu²⁺ binding to Aβ were measured in solution in chapter 3. However, Aβ may be bound to the synaptic termini when the Cu²⁺ is released rather than in solution which may affect its kinetics.

To investigate its kinetics methods similar to those in sections 3.3, 3.7 & 3.8 were used. Instead of labelled Aβ, Aβ₄₀ Cys₂₀ HL488 bound to unlabelled GM1 micelles was used, prepared as in section 5.2.1.
5.5.1 Association

To measure the association of Cu\(^{2+}\) to A\(\beta_{40}\)Cys\(_{20}\)HL488·GM1 micelles, 500 nM CuCl\(_2\) was mixed with 50 nM A\(\beta_{40}\)Cys\(_{20}\)HL488·GM1 micelles in Hepes NaCl, using stopped flow. The concentration of Hepes was varied such that the weak interactions between the Cu\(^{2+}\) and the Hepes in the buffer may be extrapolated away.

Figure 5.9: Determination of the Hepes independent association rate constant. 25 nM A\(\beta_{40}\)Cys\(_{20}\)HL488·GM1 micelles was reacted with 500 nM Cu\(^{2+}\) in various Hepes concentrations and 100 nM NaCl. The solid line is a fitted zero centred parabola \(k^{-1}_{\text{on(App)}} = A[H\text{epes}]^2 + C\), to the first six points. Extrapolating to zero give the Hepes independent association rate constant \(k_{\text{on}}\) to be 8(2)\(\times\)10\(^8\) M\(^{-1}\) s\(^{-1}\).

Figure 5.9 shows that the data appears to be parabolic in shape and is fitted with a zero centered parabola, similarly to as was found when A\(\beta\) was not bound to micelles. The first six points were fitted to give \(k_{\text{on}} = 8(2)\times10^8\text{M}^{-1}\text{s}^{-1}\) and \(A = 2.5(8)\text{mM}^{-2}\text{s}\). The \(k_{\text{on}}\) is not significantly different from the 5\(\times\)10\(^8\) M\(^{-1}\) s\(^{-1}\) obtained when A\(\beta\) is not bound to micelles.

5.5.2 Interconversion and Dissociation

To measure the rate of interconversion and dissociation of A\(\beta\)·Cu on GM1 micelles, 100 nM of A\(\beta_{40}\)Cys\(_{20}\)HL488·GM1 micelles was premixed with 100 nM CuCl\(_2\), which was then mixed with various concentrations of EDTA using stopped flow. The fluorescence recovery traces are then fitted with exponentials and the rates and relative amplitudes are fitted to the model in equation 3.28.
Figure 5.10: Apparent rates and relative amplitudes of the two major phases seen in the dissociation and removal by EDTA of Cu$^{2+}$ from Aβ$_{40}$Cys$_{20}$HL488·Cu on GM1 micelles, in Hepes NaCl at pH 7.5, fitted with the model in 3.28 (solid lines). Model parameters are shown in table 5.1

Figure 5.10 appears quite different from the figures in section 3.7. In particular at the highest EDTA concentrations, the rate of interaction with the fastest phase is an order of magnitude lower and this effect shifts the behaviour of the relative amplitude curves to higher EDTA concentrations. The fitted parameters in table 5.1 give the removal rate from (Aβ·Cu)$_i$ to be approximately two orders of magnitude slower when bound to micelles, whereas the removal rate from (Aβ·Cu)$_{ii}$ is slower but similar. This suggests that the Aβ·Cu complex on the surface may be protected from ligands in solution. This may be in part due to the very polar head group of GM1 being negatively charged, thus electrostatic interactions may repel the negatively charged EDTA and possibly other Cu$^{2+}$ binding ligands. As to whether there is a similarly large decrease in the rate of removal of Cu$^{2+}$ from Aβ·Cu when the ligand is also bound to the surface is an open question. This may have an effect on the possibility of metal assisted Aβ dimerisation on membranes.

The interconversion between (Aβ·Cu)$_i$ and (Aβ·Cu)$_{ii}$ on GM1 micelles is slower but similar to when Aβ is not bound to GM1 micelles. $k_{1\rightarrow2}$ and $k_{2\rightarrow1}$ gives the relative proportion of the two species to be 64 : 36 (Aβ·Cu)$_i$ to (Aβ·Cu)$_{ii}$, similar to that without micelles. One issue of the fit is the relative brightness ($\Gamma$) between the two species being 0.4(1) when it is expected to be close to 1. Refitting with $\Gamma$ fixed to 1 (see Fig. 5.11) gives the relative proportion of the two species to be 74 : 26, similar to the measurements without micelles. There are no other significant differences
### Table 5.1: Summary of fitting for the model (Eqn. 3.28) for the dissociation and removal by EDTA of Cu$^{2+}$ from Aβ$_{40}$Cys$_{20}$HL488·Cu on GM1 micelles.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aβ$_{40}$·GM1 Micelle</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{1\text{off}}$/s$^{-1}$</td>
<td>0.78(5)</td>
</tr>
<tr>
<td>$k_{1\text{loff}}$/M$^{-1}$s$^{-1}$</td>
<td>3.4(3) × 10$^3$</td>
</tr>
<tr>
<td>$k_{1\rightarrow 2}$/s$^{-1}$</td>
<td>0.52(9)</td>
</tr>
<tr>
<td>$k_{2\text{loff}}$/M$^{-1}$s$^{-1}$</td>
<td>70(50)</td>
</tr>
<tr>
<td>$k_{2\rightarrow 1}$/s$^{-1}$</td>
<td>0.91(6)</td>
</tr>
<tr>
<td>$\Gamma$</td>
<td>0.4(1)</td>
</tr>
<tr>
<td>$k_{\text{off}}^\text{App}$/s$^{-1}$</td>
<td>0.39(3)</td>
</tr>
</tbody>
</table>

Figure 5.10 and 5.11

5.5.3 Interconversion and Binding Pathway

To confirm the rate constants of interconversion, the experiments from section 3.8 were repeated for the Aβ$_{40}$Cys$_{20}$HL488·GM1 micelles construct. However, due to
the slower rate of Cu\(^{2+}\) removal from A\(\beta\)-Cu·GM1 micelles (see Fig. 5.10) a higher concentration of EDTA (4 mM) was used to remove the copper at a sufficiently fast rate.

To measure the rates of interconversion, 100 nM A\(\beta\)\(_{40}\)Cys\(_{20}\)HL488·GM1 micelles was mixed with 2 µM CuCl\(_2\) to form approximately 50 nM A\(\beta\)·Cu on GM1 micelles. After a chosen delay time this solution was mixed with 8 mM EDTA and the fluorescence recovery traces were recorded. All was performed in Hepes NaCl.

![Figure 5.12: The relative change in amplitude of the two phases of freshly formed 25 nM A\(\beta\)\(_{40}\)Cys\(_{20}\)HL488·Cu on GM1 micelles reacted with 4 mM EDTA, after a set age time. The fitted lines are single exponential with the rate of 1.3(1) s\(^{-1}\). Using the relative proportion at equilibrium gives interconversion rates to be \(k_{1 \rightarrow 2} = 0.74(6)\) s\(^{-1}\) and \(k_{2 \rightarrow 1} = 0.94(8)\) s\(^{-1}\) (assuming \(\Gamma = 1\)).

Fitting the data in figure 5.12 gives \(k_{1 \rightarrow 2} + k_{2 \rightarrow 1} = 1.3(1)\) s\(^{-1}\), similar to the sum of the rates in table 5.1. This confirms that the interconversion is indeed impeded when bound to the membrane and suggests that the A\(\beta\)·Cu complex spends more time in the more reactive component I state (A\(\beta\)·Cu).}

### 5.6 Summary

In this chapter it has been shown that A\(\beta\) binds to SDS micelles and GM1 micelles, as has been shown in the literature. It is therefore likely that A\(\beta\) is bound in the synaptic cleft to the membranes of the synaptic termini. An order of magnitude estimate surface density of A\(\beta\) in the synapse was made to be in the low 10's of A\(\beta\)
per synapse range.

The kinetics of the Aβ·Cu complex and its formation and dissociation on GM1 micelles is similar to that when not bound to micelles. The interconversion kinetics between the two components are a factor of two slower, suggesting that Aβ will spend more time in the more reactive (Aβ·Cu), form. The major difference in the kinetics is the removal rate constant of Cu$^{2+}$ from Aβ·Cu. With EDTA it is two orders of magnitude slower when Aβ·Cu is membrane bound, compared to when Aβ·Cu is free in solution. This suggests that membrane bound Aβ·Cu may be protected from Cu$^{2+}$ ligands in solution. Whether there is a similar protection against membrane bound ligands is unknown.
Chapter 6

Simulation of the Synapse during Neurotransmission

Having determined some of the kinetics of Cu$^{2+}$ and Zn$^{2+}$ binding to Aβ in solution and on membranes, some perspective is required for the magnitude of the parameters and whether the parameters allow metal ion binding to Aβ to may play a role in physiology or in Alzheimer’s disease. To gain this perspective a toy model of the synapse is created applying the mechanisms and parameters determined from the previous chapters.

Figure 6.1: Schematic of the diffusion of Cu$^{2+}$ into the synaptic cleft. The green discs with holes represent HSA. The red lines represent Aβ. The small blue circles represent Cu$^{2+}$.

A model of the synapse where the synapse appears two dimensional is used
(see Fig. 6.1). Cu\(^{2+}\) or Zn\(^{2+}\) is released into the centre of the synapse completely filling a cylinder of radius 20 nm with 30 µm Cu\(^{2+}\) or 300 µm Zn\(^{2+}\). These are close to the physiological concentrations of Cu\(^{2+}\) and Zn\(^{2+}\) thought to be released into the synapse. Physiological synaptic vesicles are thought to be approximately 40 nm in diameter\(^{[276]}\) with the distance between post- and pre-synaptic terminal at approximately 10 nm to 25 nm\(^{[277]}\). It is because of the above that the cylinder approximation is used. The metal ions diffuse with a diffusion coefficient (\(D\)) of \(D_{\text{Zn}^{2+}} = D_{\text{Cu}^{2+}} = 650 \text{nm}^2 \mu\text{s}^{-1}\). The toy model is then built up to include the interactions of 3 nM Aβ (\(D_{\text{Aβ}} = 304 \text{nm}^2 \mu\text{s}^{-1}\)) and 5 µM HSA (\(D_{\text{HSA}} = 61 \text{nm}^2 \mu\text{s}^{-1}\)\(^{[278]}\)). Finally the model is extended to include multiple pulses of metal ions into the synapse.

This may give insight into the role of Aβ in physiology and pathology of the brain’s 8(1) × 10\(^{10}\) neurons\(^{[279]}\) with approximately 7000 synapses each\(^{[280]}\).

### 6.1 Method

To simulate the diffusion of metal ions and Aβ in the synapse, Fick’s second law with constant diffusion coefficient

\[
\partial_t \varphi = D \nabla^2 \varphi
\]

is used, where \(\varphi\) is a scalar field of the concentration, \(t\) is the time and \(D\) is the diffusion coefficient. In cylindrical coordinates the operator

\[
\nabla^2 = \partial_r^2 + r^{-1} \partial_r + r^{-2} \partial_\theta^2 + \partial_z^2
\]

where \(r\) is the distance from the origin, \(\theta\) is the angle from a reference direction, and \(z\) is the distance above a reference plane. \(r, \theta\) and \(z\) are orthogonal. To simplify the calculation of the solutions to Fick’s law, it will be assumed there exists a rotational symmetry (perpendicular to \(z\)) and translational symmetry (along \(z\)) in \(\varphi\), that is

\[
\varphi(r, \theta, z) = \varphi(r, \theta + a, z + b) \forall a, b \in \mathbb{R}
\]
Therefore
\[
\partial_\theta \varphi = \lim_{\delta x \to 0} \frac{\varphi(r, \theta + \delta x, z) - \varphi(r, \theta, z)}{\delta x} = \lim_{\delta x \to 0} \frac{\varphi(r, \theta, z) - \varphi(r, \theta, z)}{\delta x} = 0 \quad (6.4)
\]
and thus higher order derivatives are also 0. Likewise \( \partial^2_\theta \varphi = 0 \). This simplifies the diffusion equation to be
\[
\partial_t \varphi = D \nabla^2 \varphi = D(\partial^2_r + r^{-1} \partial_r) \varphi \quad (6.5)
\]
which can be solved in one dimension to give the solutions for three dimensional space. To solve this equation numerically, the equation needs to be discretized. For the temporal term the Euler method is applied. For the spatial term the central finite difference methods are applied to \( \partial^2_r \) and \( \partial_r \) in a backwards manner. This gives
\[
\frac{\varphi_t^{t+\delta t} - \varphi_t^t}{\delta t} = D \left( \frac{\varphi_t^{t+\delta r} - 2 \varphi_t^{t+\delta t} + \varphi_t^{t-\delta r}}{(\delta r)^2} + \frac{\varphi_t^{t+\delta r} - \varphi_t^{t-\delta r}}{2r \delta r} \right) \quad . \quad (6.6)
\]
Equation 6.6 can be rearranged to give
\[
\varphi_t^t = \varphi_t^{t+\delta t} \frac{D \delta t}{\delta r} \left( \frac{1}{2r} - \frac{1}{\delta r} \right) + \varphi_t^{t+\delta t} \left( 1 + \frac{2D \delta t}{(\delta r)^2} \right) - \varphi_t^{t+\delta t} \frac{D \delta t}{\delta r} \left( \frac{1}{2r} + \frac{1}{\delta r} \right) \quad , \quad (6.7)
\]
which forms a set of equations for the next time step. In this function there is a pole at \( r = 0 \). Therefore the function needs to be moved off the axis by mapping \( r \mapsto r + \delta r/2 \) in the implementation, thus becoming
\[
\varphi_t^t = \varphi_t^{t+\delta t} \frac{D \delta t}{\delta r} \left( \frac{1}{2r + \delta r} - \frac{1}{\delta r} \right) + \varphi_t^{t+\delta t} \left( 1 + \frac{2D \delta t}{(\delta r)^2} \right) - \varphi_t^{t+\delta t} \frac{D \delta t}{r + \delta r} \left( \frac{1}{2r + \delta r} + \frac{1}{\delta r} \right) \quad . \quad (6.8)
\]
For the boundary conditions at the centre of the disc \( (r = 0) \), the boundary condition used is
\[
\varphi_0^t = \varphi_0^{t+\delta t} \left( 1 + \frac{2D \delta t}{(\delta r)^2} \right) - \varphi_0^{t+\delta t} \frac{D \delta t}{\delta r} \left( \frac{1}{2r + \delta r} + \frac{1}{\delta r} \right) \quad . \quad (6.9)
\]
At the edge of the disc \( (r = R) \), there are two main options. The concentration of the
ring outside of the disc \((R + \delta r)\) is set to 0, that is
\[
\varphi^t_R = \varphi^{t+\delta t}_{R-\delta r} \frac{D \delta t}{\delta r} \left( \frac{1}{2R + \delta r} - \frac{1}{\delta r} \right) + \varphi^{t+\delta t}_R \left( 1 + \frac{2D \delta t}{(\delta r)^2} \right).
\] (6.10)

This will cause the molecules to ‘leak’ off the edge of the simulation. Although this is suitable for metals diffusing from the centre, it is not suitable for a homogeneous concentration of \(A\beta\) as the \(A\beta\) will diffuse off the disc. To counteract this, the edge of the disc may be connected to itself. This can be done by mapping \(\varphi^{t+\delta t}_{R+\delta r} \rightarrow \varphi^{t+\delta t}_{R-\delta r}\), giving the boundary conditions to be
\[
\varphi^t_R = -\varphi^{t+\delta t}_{R-\delta r} \frac{2D \delta t}{(\delta r)^2} + \varphi^{t+\delta t}_R \left( 1 + \frac{2D \delta t}{(\delta r)^2} \right).
\] (6.11)

To incorporate reactions into the model, the rate of change of the concentration due to reactions \((R(\varphi))\) is added to the change due to diffusion, thus becoming
\[
\partial_t \varphi = D \nabla^2 \varphi + R(\varphi).
\] (6.12)

For example in the reaction
\[
A + B \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} C,
\] (6.13)

if the scalar fields of the concentrations of A, B & C, are \(\varphi\), \(\psi\) & \(\chi\), respectively. This gives the change in concentration due to reactions to be
\[
R(\varphi) = k_{\text{off}} \chi - k_{\text{on}} \varphi \circ \psi, \quad R(\psi) = k_{\text{off}} \chi - k_{\text{on}} \varphi \circ \psi \quad \& \quad R(\chi) = k_{\text{on}} \varphi \circ \psi - k_{\text{off}} \chi.
\] (6.14)

Hence with discretising these and applying the Euler method, the full set of equations to be solved are
\[
\begin{align*}
\varphi^t_r &= -\delta t(k_{\text{off}} \chi^t_r - k_{\text{on}} \varphi^t_r \psi^t_r) \\
&\quad + \varphi^{t+\delta t}_{r-\delta r} \frac{D \delta t}{\delta r} \left( \frac{1}{2r + \delta r} - \frac{1}{\delta r} \right) + \varphi^{t+\delta t}_r \left( 1 + \frac{2D \delta t}{(\delta r)^2} \right) - \varphi^{t+\delta t}_{r+\delta r} \frac{D \delta t}{\delta r} \left( \frac{1}{2r + \delta r} + \frac{1}{\delta r} \right) \\
\psi^t_r &= -\delta t(k_{\text{off}} \chi^t_r - k_{\text{on}} \varphi^t_r \psi^t_r)
\end{align*}
\] (6.15)
\[ + \psi^{t+\delta t}_{r+\delta r} \frac{D\delta t}{\delta r} \left( \frac{1}{2r + \delta r} - \frac{1}{\delta r} \right) + \psi^{t+\delta t}_{r} \left( 1 + \frac{2D\delta t}{(\delta r)^2} \right) - \psi^{t+\delta t}_{r+\delta r} \frac{D\delta t}{\delta r} \left( \frac{1}{2r + \delta r} + \frac{1}{\delta r} \right) \] (6.16)

\[ \chi^{t+\delta t}_{r+\delta r} = -\delta t(k_{on}\psi^{t}_{r,\chi} + k_{off}\chi^{t}_{r}) \]
\[ + \chi^{t+\delta t}_{r} \frac{D\delta t}{\delta r} \left( \frac{1}{2r + \delta r} - \frac{1}{\delta r} \right) + \chi^{t+\delta t}_{r+\delta r} \left( 1 + \frac{2D\delta t}{(\delta r)^2} \right) - \chi^{t+\delta t}_{r+\delta r} \frac{D\delta t}{\delta r} \left( \frac{1}{2r + \delta r} + \frac{1}{\delta r} \right) \] (6.17)

with the boundary conditions produced similarly.

In the reaction-diffusion equations (Eqn. 6.12 onwards) the reaction and diffusion parts may be treated separately in each time step simplifying the implementation, as the reaction only depends on the concentrations at particular \( r \) and the diffusion depends on all \( r \) but only for the concentration in one field. It can therefore be easily parallelized using OpenMP\(^*\).

The discretized solution for \( \phi \) is of the matrix form

\[ \phi^{t+\delta t} = M\phi^{t+1} + \delta tR(\phi, \psi, \chi), \] (6.18)

where \( M \) is a matrix of coefficients related to diffusion (similarly for \( \psi \) and \( \chi \)). This gives the procedural order to be: get the concentration fields, perform the reactions, and then calculate the concentrations at the next time step from diffusion. The concentrations of the next time step can be obtained from

\[ \phi^{t+1} = M^{-1}(\phi^{t+\delta t} + \delta tR(\phi, \psi, \chi)) \] (6.19)

However, equation 6.15 shows that \( M \) is tridiagonal, therefore solving for \( \phi^{t+1} \) may be done more efficiently than via matrix inversion. The gsl_linalg_solv_tridiag routine is used from the GSL\(^†\).

For the simulation, the time steps were chosen to be exponentially increasing after the second time step \( t_1 \) \( (t_0 = 0) \), calculated by

\[ t_{(i+1)} = t_1 \times 10^{\log(T/t_1)\times i/(N_t-1)} \] (6.20)

where \( T \) is the maximum time of the simulation, \( t_1 \) is the first non-zero time step, \( N_t \)

\(^*\)Open Multiprocessing API (2.5) [http://openmp.org].

\(^†\)GNU Scientific Library (1.16) [http://www.gnu.org/software/gsl/].
is the number of time steps, and \( i \) is an integer from 0 to \( N_t \). Exponentially increasing time steps allows the long timescale behaviour to be seen without exponentially increasing processing power. For the space steps linear spacing is used as it not possible to use exponentially increasing steps with the central finite difference method, as it requires \( \delta r \) to be constant across the simulation.

The simulation was written in C++, using the GSL and the OpenMP API, and compiled using GCC\(^*\) (see App. A.4).

Occasionally numerical error caused the concentration to become negative (at concentrations of approximately \( 10^{-11} \mu \text{m} \)). This causes the simulation to become very unstable. In simulations in which this occurred the negative concentrations were set to zero.

### 6.1.1 Multiple Pulses

To simulate the periodic nature of the release of neurotransmitter by neurons at each release, the concentration of metal ions at the centre (20 nm radius) of the simulation was reset to the initial concentration. However, this would cause a problem with the exponentially spaced time step. To counteract this when the system pulses, the exponential spacing of the time steps is reset, that is

\[
t_{i+1} = T_p \left\lfloor \frac{i}{N_s} \right\rfloor + t_1 \times 10^{\log(T_p/t_1) \times (i \mod N_s)/(N_s-1)} ,
\]

where \( T_p \) is the pulse period, \( N_s \) is the number of steps per pulse, and mod is the modulo operator. Then when

\[
i \mod N_s = 0
\]

the central 20 nm of the simulation is reset to the initial concentration of metal ions.

### 6.2 Diffusion of \( \text{Cu}^{2+} \) & \( \text{Zn}^{2+} \)

The rate of chemical reactions depends on the concentrations of the reactants. However, the concentration of metal ions released into the synapse decreases due

\*GNU Compiler Collection (4.9.1) https://gcc.gnu.org/.
not only to reactions but also due to diffusion. This effect cannot be simulated using stopped flow experiments. Simulating the diffusion of Cu²⁺ or Zn²⁺ in the synapse gives an indication of the concentration profile in time.

Figure 6.2: Diffusion of 30 µm Cu²⁺ and 300 µm Zn²⁺ in a simulated synapse. The contour lines correspond to the molar concentrations that are powers of 10.

Figure 6.2 shows that the concentration of Cu²⁺ drops below nanomolar concentrations ($K_d \approx 1 \text{ nm}$) after a few milliseconds. Whereas Zn²⁺ drops below 10’s of micromolar concentrations ($K_d \approx 60 \text{ µm}$) in less than 1 µs. Once the concentration of metal ions or Aβ drops below their respective $K_d$, the rate of binding becomes less than the rate of dissociation. However, this is insufficient to quantify the proportion of metal bound Aβ. For this, the association of Cu²⁺ or Zn²⁺ with Aβ in the simulation is required.
6.3 Reactions of Cu$^{2+}$ or Zn$^{2+}$ with Aβ

To simulate the binding and unbinding of metal ions to Aβ, 30 µM Cu$^{2+}$ or 300 µM Zn$^{2+}$ is released into a 20 nm cylinder in the centre with 3 nm Aβ, using the rate constants obtained in the previous chapters. Due to the unknown temporal separation of the release of Zn$^{2+}$ and Cu$^{2+}$ as part of synaptic transition, the limiting case will be considered in which both are independent.

6.3.1 Cu$^{2+}$ & Aβ

The model used for the reactions of Aβ binding to Cu$^{2+}$ is

\[
\text{Aβ} + \text{Cu}^{2+} \xrightarrow{k_{\text{on}}} \text{(Aβ·Cu)}_i \xrightleftharpoons[k_{\text{off}}]{k_1 \rightarrow 2} \text{(Aβ·Cu)}_{ii} \xrightarrow[k_{\text{on}}]{k_3 \text{ on}} \text{(Aβ·Cu)}_{iii} \xrightarrow[k_{\text{off}}]{+ \text{Cu}^{2+}} \text{(Aβ·Cu)}_{ii},
\]

where \( k_{\text{on}} = 3 \times 10^8 \text{M}^{-1}\text{s}^{-1}, \) \( k_{\text{off}} = 0.8 \text{s}^{-1}, \) \( k_{1 \rightarrow 2} = 0.9 \text{s}^{-1}, \) \( k_{2 \rightarrow 1} = 2.22 \text{s}^{-1}, \) \( k_{3 \text{ on}} = 4.2 \times 10^5 \text{M}^{-1}\text{s}^{-1}, \) and \( k_{3 \text{ off}} = 1.7 \text{s}^{-1}. \)

The simulation in figure 6.3 shows that during one pulse of Cu$^{2+}$ approximately 0.1% of the total Aβ forms Aβ·Cu, at timescales of 1 µs to 10 ms. Most of the Aβ·Cu is (Aβ·Cu)$_i$, with (Aβ·Cu)$_{ii}$ reaching approximately 0.01% at timescales of 0.3 ms to tens of millisecond. In equilibrium, the ratio of (Aβ·Cu)$_i$ to (Aβ·Cu)$_{ii}$ is approximately 70 : 30, but due to the diffusion of Cu$^{2+}$ out of the synapse in the simulation, the kinetics greatly favour the formation of only (Aβ·Cu)$_i$. In contrast (Aβ·Cu$_2$)$_{iii}$ only reaches tens of attomolar concentrations (approximately $10^{-6}$% of total Aβ), on millisecond timescales.

**Binding of Cu$^{2+}$ to Aβ·Cu**

To investigate the maximal amount of Cu$^{2+}$ binding to Aβ·Cu, the limiting case when all Aβ is already bound to copper forming the two species of Aβ·Cu (and
Figure 6.3: Part 1 of 2

(a) Reaction-diffusion of Cu$^{2+}$

(b) Reaction-diffusion of Aβ

(c) Reaction-diffusion of (Aβ-Cu)$_i$
Figure 6.3: Part 2 of 2. Simulation of the diffusion and reaction of 30 µm Cu²⁺ released from a 40 nm diameter cylinder into a reservoir of 3 nM Aβ, with the reactions shown in equation 6.23. The contours correspond to the molar concentrations that are powers of 10.

is prevented from dissociating), would give an estimate for the maximal possible formation of (Aβ·Cu₂)₃ in a single Cu²⁺ pulse. The model used is

\[
\begin{align*}
(Aβ·Cu)\_i & \quad k_{3\ on} \quad (Aβ·Cu₂)\_i \\
(Aβ·Cu)\_i & \quad k_{3\ off} \quad (Aβ·Cu₂)\_i
\end{align*}
\]
where $k_{1\rightarrow 2} = 0.9 \text{s}^{-1}$, $k_{2\rightarrow 1} = 2.22 \text{s}^{-1}$, $k_{3\text{on}} = 4.2 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, and $k_{3\text{off}} = 1.7 \text{s}^{-1}$.

The initial concentrations of Aβ·Cu are set to

$$[(\text{Aβ·Cu})_i]|_{t=0} = 3 \text{nm} \times \frac{k_{2\rightarrow 1}}{k_{1\rightarrow 2} + k_{2\rightarrow 1}}$$

$$[(\text{Aβ·Cu})_{ii}]|_{t=0} = 3 \text{nm} \times \frac{k_{1\rightarrow 2}}{k_{1\rightarrow 2} + k_{2\rightarrow 1}}$$

i.e. assuming that (Aβ·Cu)$_i$ and (Aβ·Cu)$_{ii}$ have reached equilibrium before the simulation had started.

Figure 6.4: Diffusion and reaction of 30 µm Cu$^{2+}$ released from a 40 nm diameter cylinder into a reservoir of 3 nm Aβ·Cu, with the concentrations split across (Aβ·Cu)$_i$ and (Aβ·Cu)$_{ii}$ as expected in equilibrium. The contour lines correspond to the molar concentrations that are powers of 10.

Figure 6.4 shows that in this limiting case (Aβ·Cu)$_{iii}$ reaches low of femtomolar concentrations, approximately $10^{-4}$% of total Aβ. Given the low number of Aβ molecules per synapse, this suggests that (Aβ·Cu)$_{iii}$ does not play a role in physiology.
The model used for the reactions of $\text{A}\beta$ binding to $\text{Zn}^{2+}$ is

$$
\text{A}\beta + \text{Zn}^{2+} \xrightarrow{1.9 \times 10^6 \text{ M}^{-1} \text{s}^{-1}} \xrightarrow{110 \text{ s}^{-1}} \text{A}\beta \cdot \text{Zn}.
$$

The results are shown in figure 6.5. At the timescale of milliseconds there is instability in the numerical solution and thus should be ignored. The figures show that $\text{A}\beta \cdot \text{Zn}$ reaches hundreds of attomolar concentrations across timescales of 0.5 $\mu$s to 30 ms (approximately $10^{-5}$ %). This suggests that it is unlikely that $\text{Zn}^{2+}$ binds to $\text{A}\beta$ during synaptic release. However, it may be possible for the $\text{A}\beta \cdot \text{Zn}$ to build up from repetitive release during transmissions.
Figure 6.5: Diffusion and reaction of 300 µM Zn$^{2+}$ released from a 40 nm diameter cylinder into a reservoir of 3 nm A$\beta$, based on equation 6.27. The contour lines correspond to the molar concentrations that are powers of 10.
6.3.3 Cu\textsuperscript{2+} & HSA

The binding of HSA to Cu\textsuperscript{2+} may have an effect on the levels of Cu\textsuperscript{2+} during neurotransmission, given that it is at micromolar concentrations in the CSF and binds quickly and strongly to Cu\textsuperscript{2+}.

To simulate the effect of 5 µm HSA on the diffusion of 30 µm Cu\textsuperscript{2+} after release, the model

\[
\text{HSA} + \text{Cu}^{2+} \xrightarrow{1 \times 10^8 \text{M}^{-1} \text{s}^{-1}} \text{HSA} \cdot \text{Cu}
\]  

was used. The dissociation of HSA \cdot Cu is ignored as it is likely to take longer than the timescales of the simulation. One caveat to this model is that free diffusion of HSA is assumed. However, the distance between the synaptic termini are approximately 10 nm to 25 nm\textsuperscript{277}, whereas the HSA is approximately 10 nm\textsuperscript{281}, so the diffusion is unlikely to be free.

Figure 6.6 shows that nanomolar concentrations of HSA \cdot Cu are formed, however it also attenuates the Cu\textsuperscript{2+} pulse. Without HSA, it drops below nanomolar after approximately 30 ms, whereas with HSA it drops after 3 ms. Due the high concentrations of HSA, this dominates the rate of the reaction once the concentration of Cu\textsuperscript{2+} is less than the concentration of HSA. This appears to cause an accelerated drop off in the concentration of Cu\textsuperscript{2+}, compared to Cu\textsuperscript{2+} diffusion without HSA (see Fig. 6.3a). If HSA can enter the synapse, the modulation of the profile of the Cu\textsuperscript{2+} concentration will likely affect the binding to Aβ, reducing the maximal concentrations of Aβ\cdot Cu reached.
Figure 6.6: Diffusion and reaction of 30 µm Cu\(^{2+}\) released from a 40 nm diameter cylinder into a reservoir of 5 µm HSA, based on equation 6.28. The contour lines correspond to the molar concentrations that are powers of 10.
6.3.4 Cu$^{2+}$, HSA & A$\beta$

To investigate the binding of A$\beta$ to Cu$^{2+}$ with the presence of HSA, the model

\[
\begin{align*}
A\beta + Cu^{2+} & \quad \overset{k_{\text{on}}}{\underset{k_{\text{off}}}{\rightleftharpoons}} \quad (A\beta \cdot Cu)_i \\
HSA + Cu^{2+} & \quad \overset{k_{\text{HSA}}}{\rightarrow} \quad HSA \cdot Cu
\end{align*}
\]

was used, where $k_{\text{on}} = 3 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, $k_{\text{off}} = 0.8 \text{s}^{-1}$, $k_{1 \rightarrow 2} = 0.9 \text{s}^{-1}$, $k_{2 \rightarrow 1} = 2.22 \text{s}^{-1}$, $k_{3 \text{ on}} = 4.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_{3 \text{ off}} = 1.7 \text{s}^{-1}$, and $k_{\text{HSA}} = 1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$.

Figure 6.7 shows that the inclusion of HSA into the model has little effect on the maximal concentration of $(A\beta \cdot Cu)_i$ and a factor of 60 drop for $(A\beta \cdot Cu)_ii$. However, there is a large effect on the temporal profile. The length of time that the concentration of $(A\beta \cdot Cu)_i$ is above picomolar is reduced by approximately two orders of magnitude.

![Figure 6.7: Part 1 of 2.](image-url)
6.4 Reaction-Diffusion of Aβ with Pulsed Cu\(^{2+}\) & Zn\(^{2+}\)

In the previous sections, the reaction-diffusion of Aβ with metal ions was studied for a single pulse of metal ions. However, in the brain, neurons can fire multiple times releasing metal ions into the synapse in quick succession.

To investigate the effect of repetitive release of metal ions into the synaptic cleft, the central 40 nm of the concentration of metal ions simulation was reset to the initial concentration, at a chosen frequency. The upper limit firing rate frequency of neurons is approximately 200 Hz\(^{[282]}\), so the range of 1 Hz to 100 Hz was simulated.

To obtain the mean concentration of Aβ-Metal ion complexes in the synapse of radius, the average concentration (of \(\phi\)) is

\[
\bar{\phi} = \sum_{i=0}^{R} \frac{\phi_i (r_i^2 - r_{i-1}^2)}{r_R^2},
\]

where \(\phi_i\) is the concentration from the simulation with radius \(r_i\), \(r_{i-1} = 0\), and where \(R\) (the maximal \(i\)) is such that \(r_R > 300\text{nm}\) & \(r_{R-1} < 300\text{nm}\). This is to account for the cylindrical geometry of the simulation.
6.4.1 Zn$^{2+}$

The reaction

$$A\beta + \text{Zn}^{2+} \xrightarrow{1.9 \times 10^6 \text{M}^{-1}\text{s}^{-1}} A\beta\cdot\text{Zn}$$

was simulated with multiple pulses of Zn$^{2+}$. Figure 6.8 shows the average concentration in the synapse after 10 s. The concentration appears to be monomial with frequency. However, at the highest frequency (100 s$^{-1}$) the concentration reaches low picomolar, 0.1 % of the total A$\beta$ concentration. This suggests that Zn$^{2+}$ binding to A$\beta$ is a relatively rare event. Figure 6.9 shows the temporal variation of A$\beta\cdot$Zn at different radii for different pulse frequencies. The figure shows that at this low frequency range, there is little increase in the maximum transient concentration of A$\beta\cdot$Zn. This is due to the 110 s$^{-1}$ dissociation rate constant of A$\beta\cdot$Zn. However, there is an increase in the minimum transient concentration of A$\beta\cdot$Zn after each pulse. For a sizeable build up of A$\beta\cdot$Zn between pulses, the pulse rate would need to be greater than the dissociation rate.

Figure 6.8: Mean concentration of A$\beta\cdot$Zn in the central 300 nm after 10 s at different pulse frequencies of 300 µM Zn$^{2+}$. The data points were empirically fitted with $f \rightarrow af^c$. In the fit $c$ was 1.208(3), and $a$ was 8.01(9) $\times 10^{-9}$ µM s$^c$. See figure 6.9 for time traces.
Figure 6.9: Concentration of A\(\beta\)-Zn for different pulse frequencies of 300 \(\mu\)m Zn\(^{2+}\), at different radii.
6.4.2 Cu$^{2+}$

The model used for the reactions of $\text{A} \beta$ binding to Cu$^{2+}$ is

$$
\begin{align*}
\text{A} \beta + \text{Cu}^{2+} & \overset{k_{\text{on}}}{\underset{k_{\text{off}}}{\rightleftharpoons}} (\text{A} \beta \cdot \text{Cu})_1 \\
& \overset{k_{3\text{ on}}}{\underset{k_{3\text{ off}}}{\longrightarrow}} (\text{A} \beta \cdot \text{Cu}_2)_m
\end{align*}
$$

where $k_{\text{on}} = 3 \times 10^8 \text{M}^{-1}\text{s}^{-1}$, $k_{\text{off}} = 0.8 \text{s}^{-1}$, $k_1\rightarrow2 = 0.9 \text{s}^{-1}$, $k_2\rightarrow1 = 2.22 \text{s}^{-1}$, $k_{3\text{ on}} = 4.2 \times 10^5 \text{M}^{-1}\text{s}^{-1}$, and $k_{3\text{ off}} = 1.7 \text{s}^{-1}$.

Figure 6.10 shows the average concentration in the synapse after 10 s. The concentrations appear to approximately follow the form of $f \mapsto \frac{c_f}{\sqrt{f}}$, for a frequency $f$. However, this form is an approximation, given that for the fitting $c_{(\text{A} \beta \cdot \text{Cu})_i} + c_{(\text{A} \beta \cdot \text{Cu})_ii} = 5 \text{nm}$, which is greater than the total concentration of $\text{A} \beta$ (3 nm). Across the frequency range simulated, there is a factor of slightly greater than 3 higher concentration of $(\text{A} \beta \cdot \text{Cu})_i$ than $(\text{A} \beta \cdot \text{Cu})_ii$. If all $\text{A} \beta$ is bound to Cu$^{2+}$, in equilibrium a factor of $2.47 = \frac{k_{2\rightarrow1}}{k_{1\rightarrow2}}$ is expected. Therefore repetitive neurotransmission may cause a decrease in the concentration of $(\text{A} \beta \cdot \text{Cu})_ii$, relative to $(\text{A} \beta \cdot \text{Cu})_i$.

The concentrations of $(\text{A} \beta \cdot \text{Cu})_i$ and $(\text{A} \beta \cdot \text{Cu})_ii$ obtained are significant, reaching $0.80 \text{nm}$ for $(\text{A} \beta \cdot \text{Cu})_i$ and $0.26 \text{nm}$ for $(\text{A} \beta \cdot \text{Cu})_ii$, a sizeable fraction of the total $\text{A} \beta$ concentration. This suggests that $\text{A} \beta$ may bind to Cu$^{2+}$ released during neurotransmission. The rate of Cu$^{2+}$ assisted $\text{A} \beta$ dimer formation would be maximal at equal proportions of $\text{A} \beta$ and $(\text{A} \beta \cdot \text{Cu})$.

After 10 s $(\text{A} \beta \cdot \text{Cu}_2)_m$ had reached concentrations of $240 \text{fm}$, suggesting that $(\text{A} \beta \cdot \text{Cu}_2)_m$ is unlikely to form under physiological conditions and thus lacks a role in physiology.
Figure 6.10: Mean concentration after 10 s of the central 300 nm at different pulse frequencies of Cu$^{2+}$. The data points were empirically fitted with $f \rightarrow \frac{cf}{a+f}$, where $f$ is the pulse frequency, $c$ is the concentration as $f \rightarrow \infty$, and $a$ is the frequency at which half are bound. The obtained parameters were $a = 360(20)$ s$^{-1} \& c = 3.7(1)$ nM for $(A\beta \cdot Cu)_i$, and $a = 407(5)$ s$^{-1} \& c = 1.32(1)$ nM for $(A\beta \cdot Cu)_{ii}$. See figures 6.11 & 6.12.
Figure 6.11: Concentration of $\left\langle A\beta \cdot Cu \right\rangle_i$ at different radii for different pulse frequencies of 30 $\mu$m Cu$^{2+}$.
Figure 6.12: Concentration of (Aβ·Cu)_{ii} at different radii for different pulse frequencies of 30 µm Cu^{2+}. The curves for the different radii are nearly superimposed.
6.5 Summary

In this chapter the reactions of Aβ with Cu\(^{2+}\) and Zn\(^{2+}\) have been simulated in a toy model of the synapse. This is to gain some perspective on the parameters obtained from the previous chapters and estimate the importance of reactions in physiology.

The results suggest that the binding of Zn\(^{2+}\) to Aβ in the synapse is minimal, from 10\(^{-3}\) % of the Aβ forming Aβ·Zn from a single pulse, to 10\(^{-1}\) % of Aβ when pulsing is at 100 Hz. Given the low probability of Aβ·Zn forming and its fast dissociation, this suggests that Aβ·Zn is unlikely to play a role in the oligomerisation of Aβ or in physiology.

The results for the binding of Cu\(^{2+}\) to Aβ show that a sizable proportion of Aβ may form Aβ·Cu, from 0.1 % during a single pulse to approximately 1/3 when pulsing is at 100 Hz. During repetitive releases of copper the ratio of (Aβ·Cu)_i to (Aβ·Cu)_ii is 75 : 25, with more (Aβ·Cu)_i than is expected in equilibrium. This suggests that Aβ·Cu may indeed form under physiological conditions, suggesting that the Aβ-metal hypothesis may still be valid as the cause of Alzheimer’s disease for Aβ with Cu\(^{2+}\).

The consequences of oligomerisation may be an increase in affinity for metal ions including Cu\(^{2+}\) and Zn\(^{2+}\). This in turn may cause the trapping and removal of Cu\(^{2+}\) and Zn\(^{2+}\) from the brain’s pool of metals. Trapping of Cu\(^{2+}\) by Aβ may produce reactive oxygen species close to the neuronal membranes damaging itself Aβ, proteins or lipids in the membrane. Oligomerisation of Aβ may slow the diffusion of Aβ on the membranes preventing any physiological roles\(^{[283]}\). Alternatively, oligomers may decrease the ability of proteases to break down Aβ, upsetting Aβ homeostasis.
Chapter 7

Conclusion

Chemical kinetics allows the temporal behaviour of chemical reactions to be predicted. The main aim of this thesis was to determine the kinetic rate constants of Aβ’s interactions with metal ions and elucidate their mechanisms which would allow for a closer examination of the amyloid-metal hypothesis of Alzheimer’s disease. This thesis describes both experimental and computational approaches to achieve this aim.

It was found that the binding of Aβ to Cu$^{2+}$ was nearly diffusion limited, with apparent dissociation on the second time scale, giving a low nanomolar $K_d$. The $K_d$ is within the expected range and is the first measurement deriving the $K_d$ from the underlying kinetics of the system. This combined with the toy simulations of the synapse suggest that Aβ·Cu is able to form under physiological conditions. However, measurements of the Cu$^{2+}$ assisted dimerisation rate constant suggests that dimerisation is unlikely to occur in the CSF. This also suggests that future research should focus on Aβ-metal-membrane interactions. The long lived formation Aβ·Cu also lends support to the hypothesis that redox cycling and reactive oxygen species (ROS) production may occur. However, the relevant kinetic parameters to determine the magnitude of this effect compared to basal levels are missing from the literature. Damage to Aβ by ROS may increase the stability of dimers, possibly by allowing covalent cross-linking.

The two components of Aβ·Cu were identified by their different reactivities, as seen in the literature where they were distinguished by their electron paramagnetic
resonance signatures. It was shown that they interconvert on the second timescale with the derived equilibrium population close to the literature values. Furthering this, (Aβ·Cu)\textsubscript{i} was found to be significantly more reactive with Cu\textsuperscript{2+} binding ligands than (Aβ·Cu)\textsubscript{ii}. The ability to measure the reactivity offers a new methodology for the optimisation of therapeutics and allowing the amyloid-metal hypothesis to be investigated in vivo.

The binding of Aβ to Zn\textsuperscript{2+} was found to be much lower than the diffusion limit unlike Aβ binding Cu\textsuperscript{2+} and the complex is very shorter lived. Although Zn\textsuperscript{2+} can cause Aβ at CSF concentrations to aggregate when the concentration of Zn\textsuperscript{2+} is greater than 40(20) µM, the simulations suggest that Zn\textsuperscript{2+} has little opportunity to do so under the transient Zn\textsuperscript{2+} concentrations in the synapse. Therefore the binding of Zn\textsuperscript{2+} to Aβ is unlikely to occur and is unlikely to be relevant in the initial oligomer formation or in physiology. However, it may have a role in the formation and growth of larger aggregates and fibrils.

Although Aβ bound to multiple metal ions have been observed the formation rate constant for Zn\textsuperscript{2+} or Cu\textsuperscript{2+} binding to Aβ·Cu are very low. This, along with the simulations suggest that it is unlikely that Aβ monomers could bind with more than one metal ion in the synapse and thus have little relevance in physiology or Alzheimer’s disease.

**Possible Role of Amyloid-β in Physiology**

Given the near diffusion limited binding of Cu\textsuperscript{2+} to Aβ’s N-terminal region, the membrane-binding properties of Aβ’s hydrophobic C-terminal region, and the ability of Aβ·Cu to react with ligands, the kinetics suggest that Aβ’s role in physiology may be to capture Cu\textsuperscript{2+} that has been released into the synaptic cleft and transport it to other membrane proteins (see Fig. 7.1). The fast binding of Aβ to Cu\textsuperscript{2+} as shown in the simulations, is required for Aβ to compete against other Cu\textsuperscript{2+} binding ligands and against the diffusion of Cu\textsuperscript{2+} out of the synaptic cleft after release. Membrane binding allows locally high surface concentrations of Aβ. It has been shown that diffusion in two dimensions can enhance reaction rates of ligands with
receptors$^{284}$. A mechanism as such may reduce the quantities of Cu$^{2+}$ required by neurons which would be beneficial given the toxicity of Cu$^{2+}$. The life-time for $\text{A}\beta\cdot\text{Cu}$ (on the second timescale) could allow time for interactions with other components on the membrane.

One candidate for the final destination of Cu$^{2+}$ is to modulate NMDA receptor response, which has a role in synaptic plasticity. NMDA receptors are known to be modulated by reactions involving Cu$^{2+}$ causing conformational change. The pathway by which Cu$^{2+}$ gets to NMDA receptors is currently unclear. However, it has been suggested that the it is via cellular prion protein (PrP$^c$) which complex with Cu$^{2+}$ and bind to NMDA receptors$^{285;286}$.

![Diagram of Cu$^{2+}$ and A$\beta$ interaction on neuronal synaptic membranes](image)

**Figure 7.1:** Hypothesis that A$\beta$’s role in physiology is to capture and transport Cu$^{2+}$ on neuronal synaptic membranes and transfer by some pathway to NMDA receptors (NMDAr).

Within this hypothesis, oligomers formed on the membrane would be problematic in that they may disrupt the membrane, slow the diffusion of A$\beta$$^{283}$, and bind more tightly to Cu$^{2+}$. This would prevent the movement of Cu$^{2+}$ on the synaptic membrane to its receptor causing synaptic dysfunction. A$\beta$ oligomers have been shown to interfere with PrP$^c$-Cu modulation of NMDA receptors$^{287}$. 

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Chapter 8

Further Work

The work in this thesis mainly focused on the kinetics studies of Aβ with Cu²⁺ and Zn²⁺. Below are listed some possible extensions to the work of this thesis.

**Measuring the Dissociation Rate of Aβ·Cu·Aβ**

In section 3.5 the copper assisted dimerisation rate constant of Aβ was measured. However, the corresponding dissociation rate could not be obtained. This is likely due to Aβ·Cu·Aβ not being populated under those conditions. It may be possible to increase the population in the Aβ·Cu·Aβ by increasing the concentration of Aβ.

**Zinc-Aβ Kinetics on GM1 Micelles**

As GM1 micelles are likely negatively charged, therefore Zn²⁺ may become loosely associated with them. This may enhance the binding rate constant of Zn²⁺ with Aβ when Aβ is bound to membranes. The Cu²⁺-Zn²⁺ competition experiment could be repeated to compare the binding rate constants of Zn²⁺ with Aβ when bound and not bound to GM1 micelles.

**Studying the Folding and Binding of Aβ on Membranes**

In section 3.6.1 it was shown that labelled Aβ was quenched on the time scale of a few Hertz when ethanol or DMSO was added. However, at these time scales with the very high concentrations of ethanol & DMSO added (given their quite different
structures) the quenching is unlikely due to ethanol or DMSO but instead may be due to folding of the Aβ. Therefore it may be possible to use the quenching to study the kinetics of folding on model membranes using stopped-flow as Aβ has been shown to adopt an α-helical conformation.

It was shown in section 5.2.2 that there is a measurable change in anisotropy when labelled Aβ binds to GM1. It may be possible to measure the binding kinetics of Aβ to GM1 micelles using stopped-flow anisotropy. This, along with the $K_d$ in the literature, may be used to help predict the surface density of Aβ in vivo.

**Measuring the Surface Concentration of Aβ on Living Cells**

In section 5.4, an estimate of the surface density of Aβ on cells was made using POP-C/POPG or GM1/Cholesterol/Sphingomyelin membranes. This could be extended to live cells to see if the density is still similar.

**Copper Assisted Dimerisation on a Membrane**

Although Cu$^{2+}$ binding was studied on GM1 micelles, it is an incompatible system to study the dimerisation of Aβ, due to its geometry and that high concentrations of Aβ disrupt micelles. It may be possible to study the dimerisation of labelled Aβ on membranes using TIRF microscopy under equilibrium conditions with Cu$^{2+}$ in solution. This could be extended to non-equilibrium conditions by using an electric field across a nanopipette to push pulses of Cu$^{2+}$ out at locally high concentrations near a membrane.

**Measuring Diffusion Within a Synapse**

In the simulation chapter (Ch. 6) it was assumed that diffusion within the synapse is free, with an apparent viscosity similar to that in water. However, the synaptic cleft is likely very crowded with surface bound proteins and receptors. It may be possible to measure the viscosity within a synapse by using fluorescent dye and STED-FCS to measure the diffusion coefficient. This may be useful in studies of neurotransmitter release.
Using labelled Aβ, the simulations suggest it may be possible to measure the concentration profile of Cu$^{2+}$ in the synapse, by measuring the quenching when Cu$^{2+}$ binds to Aβ by applying super-resolution techniques.

**Amyloid-β Peptides with Familial Alzheimer's disease Mutations**

There are a number of mutant variants of Aβ$^{[77–79]}$ to which the work in this thesis could be applied, in particular the Cu$^{2+}$ binding studies. Finding differences would lend support to the amyloid-metal hypothesis for Alzheimer’s disease and the mechanisms behind it.

**A Systematic Study of the Kinetics of Aβ with Fe$^{2+}$**

Although Fe$^{2+}$ is an important ion in physiology and is the other metal ion thought to be involved in the amyloid-metal hypothesis, the kinetics of Aβ with Fe$^{2+}$ were not studied. This is due to the fast oxidation of Fe$^{2+}$ to Fe$^{3+}$. It may be possible to study the binding of Fe$^{2+}$ to Aβ by using an reducing agent to prevent the formation of Fe$^{3+}$. Fe$^{2+}$ (like Cu$^{2+}$) is paramagnetic so should also directly quench fluorophore labelled Aβ and thus the experiments in this thesis could be repeated for Fe$^{2+}$.

**Studying the Kinetics of Copper Redox Cycling**

The quenching of dye labeled Aβ by Cu$^{2+}$ is due to the paramagnetic properties of Cu$^{2+}$. However, Cu$^{+}$ is not paramagnetic, therefore it should be possible to study the kinetics of the redox cycling of copper by physiologically relevant reducing and oxidizing agents. A kinetic understanding of the redox cycling of copper and iron would allow for a quantitative judgement on the magnitude of reactive oxygen species production, testing this hypothesis of Alzheimer’s disease.

With an understanding of the kinetics of the redox cycling of Aβ with copper, it may then be possible to prepare Cu$^{+}$ with reducing agents, whilst knowing the reaction rate of reducing agents with Aβ·Cu$^{2+}$. Then it may be possible to measure the kinetics of Aβ binding to Cu$^{+}$, in a similar way to with Zn$^{2+}$ (see §4.3), if the reaction rate of the reducing agent with Aβ·Cu$^{2+}$ is sufficiently slow.
It was shown that copper assisted dimerisation of Aβ was too slow to occur at CSF concentrations in solution. However in fibrils, Aβ has been found to be damaged which may form more stable oligomers. Mixing Aβ, Cu$^{2+}$, reducing agents, and oxidizing agents could be used to damage the Aβ in a systematic way. The experiments in section 3.5 could then be repeated to see if there is a substantial effect on the lifetime of the Aβ·Cu·Aβ complexes formed. The effect of production of redox species on tyrosine cross-linked dimers could also be studied.

**MPAC Optimisation**

In section 3.6, a method was described to measure the effectiveness of CQ and L2-b to remove Cu$^{2+}$ from Aβ·Cu. This method could be applied to discover and optimise drug candidates for the removal of Cu$^{2+}$ from Aβ·Cu, which could then be used to test the ‘metal-protein attenuating compounds’ (MPACs) treatment strategy.

**Extending the Methodology to Cu$^{2+}$ Binding Kinetics of Other Systems**

The quenching of a dye label when a protein or peptide binds Cu$^{2+}$ has not been exploited much in the literature. This system could also be used to find the kinetics of Cu$^{2+}$ binding to other proteins found in neurodegenerative diseases, such as PrP$^{c}$ (prion disease) and α-synuclein (Parkinson’s disease), but also to other Cu$^{2+}$ binding proteins in physiology, such as HSA.


[27] Inna Khlistunova, Jacek Biernat, Yipeng Wang, Marcus Pickhardt, Martin von Bergen, Zuzana Gasova, Eckhard Mandelkow, and Eva-Maria Mandelkow. Inducible expression of Tau repeat domain in cell models of tauopathy Aggregation is toxic to cells but can be reversed by inhibitor drugs. *Journal of Biological Chemistry*, 281(2):1205–1214, 2006.


[69] Natalia Salvadores, Mohammad Shahnavaz, Elio Scarpini, Fabrizio Tagliavini, and Claudio Soto. Detection of misfolded Aβ oligomers for sensitive


[137] Anja Schneider, Walter Schulz-Schaeffer, Tobias Hartmann, Jörg B Schulz, and Mika Simons. Cholesterol depletion reduces...


[233] Bruno Alies, Isabelle Sasaki, Olivier Proux, Stephanie Sayen, Emmanuel Guillon, Peter Faller, and Christelle Hureau. Zn impacts Cu coordination to amyloid-β, the Alzheimer’s peptide, but not the ROS production and the associated cell toxicity. *Chemical Communications*, 49(12):1214–1216, 2013.


[249] MCS-pci. ORTEC, 801 South Illinois Ave., Oak Ridge, TN 37831-0895 U.S.A.


Appendix A

Code Listings

A.1 Competition of Aβ and HSA for Cu$^{2+}$

Python 3 code to simulate the binding of Cu$^{2+}$ to a mixture of Aβ and HSA. The code was run using Python 3.4.3, with the libraries csv 1.0, matplotlib 1.4.3, numpy 1.9.2, and scipy 0.15.1. The code is used in section 3.4.1, to generate the data for figure 3.12.

A.1.1 ABHSAcompetition.py

```python
# HSA & AB Competing for Cu
import csv
import matplotlib.pyplot as plt
import numpy as np
import scipy as sp
from scipy.integrate import odeint

def leftab(HSA,AB,Cu,ka,kh):
    # Differential Equations
    def f(t,y):
        cu = y[0]
        ab = y[1]
        hsa = y[2]
        dcu = -ka *cu*ab -kh *cu*hsa
        dab = -ka *cu*ab
        dhsa = -kh *cu*hsa
        return [dcu,dab,dhsa]

    # Initial conditions
    y0 = [Cu,AB,HSA]
    t0 = 0
    t1 = 1
    # Solve DE's
    solver = sp.integrate.ode(f).set_integrator('dopri5')
    solver.set_initial_value(y0,t0).set_f_params()
    while solver.t < t1:
        solver.integrate(t1,step=True)
```

*Archive of source code: ABHSAcompetition.tar.xz*
A.2 Removal of Cu\textsuperscript{2+} by EDTA\textsuperscript{*}

Matlab code to fit the apparent amplitudes and rates of when Cu\textsuperscript{2+} is removed from A\textbeta \cdot Cu by a ligand (EDTA). The code is used in section 3.7.

The main.m should be run to perform the analysis, the other files contain helper functions. Lines 9 through 16 contain the initial fitting parameters. The variable file is a CSV (comma separated variable, tabs were used as the separating character) file containing the data. The columns should be [EDTA / \mu M, A\textsubscript{1}/(A\textsubscript{1}+A\textsubscript{2}), \sigma\textsubscript{A\textsubscript{1}/(A\textsubscript{1}+A\textsubscript{2})}, k\textsubscript{1}, \sigma\textsubscript{k\textsubscript{1}}, A\textsubscript{2}/(A\textsubscript{1}+A\textsubscript{2}), \sigma\textsubscript{A\textsubscript{2}/(A\textsubscript{1}+A\textsubscript{2})}, k\textsubscript{2}, \sigma\textsubscript{k\textsubscript{2}}]. Whether the fitting parameters are variable may be changed in the array tofit on line 49. The output is saved to the input file name with .fit.csv appended.

A.2.1 main.m

```matlab
%% Initialisation
clear; close all;
clc; % Clear Command Window
concat = @(xs)(reshape(xs,[],1));

%% Model Parameters
% Kinetic Parameters
HSA = 5E-6 # M
AB = 5E-9 # M
ka = 3E8 # M\textsuperscript{-1} s\textsuperscript{-1}
Cus = np.linspace(0,7.5,1000) * 1E-6 # [M]
khs = np.logspace(-1,1,7) * ka # [M\textsuperscript{-1} s\textsuperscript{-1}]
propAB = [[1-leftab(HSA,AB,c,ka,k)/AB for c in Cus] for k in khs]
fig = plt.figure()
for l in range(len(propAB)):
    plt.plot(Cus/1E-6,propAB[l])
plt.xlabel('[Cu] / uM')
plt.ylabel('[AB\cdot Cu]/AB] / M')
propAB = np.transpose(np.array([Cus]+propAB)).tolist()
with open('output.dat','w') as csvfile:
    wrtr = csv.writer(csvfile,delimiter='\t')
    wrtr.writerow(['#Cu'] + khs.tolist())
    wrtr.writerows(propAB)
plt.show(block=True)
```

---

\textsuperscript{*}Archive of source code: AB.Cu+EDTA.tar.xz
K1off = 0.5; % Hz
K1EDTA = 1E5; % Hz M^-1
K1to2 = 0.5; % Hz
K2EDTA = 1E3; % Hz M^-1
K2off = 0; % Hz
K2to1 = 1; % Hz
AB = 50E-9; % M
gamma = 1; % Correction Factor for brightnesses of quenched states

%% Fitting Data
file = '130408WeightedData.csv';
file = '131015-SimFormat.csv';
file = '131025-AB40Cys20HL488-VaryEDTA-50nMAB50nMCu.csv';
file = '131024-AB16LysHL488Rat-VaryEDTA-50nMAB50nMCu.csv';
file = '131030-AB40Cys20HL488-VaryEDTA-50nMAB50nMCu.csv';
file = '131203-AB16lysHL488r-pH6.5VaryEDTA-50nMAB50nMCu.csv';
file = '131205-AB16lysHL488r-pH6.5VaryEDTA-50nMAB50nMCu.csv';
file = '131204-AB40Cys20HL488pH6.5VaryEDTA-50nMAB50nMCu.csv';
file = '131129-AB40Cys20HL488Micelles-VaryEDTA-50nMABGM150nMCu.csv';
data = importdata(file);
data = data.data;
datax = data(:,1) * 1E-6; % [EDTA]: uM -> M
dataA1 = data(:,2); % A1/A
dataA1e = data(:,3); % A1/A error
dataK1 = data(:,4); % K1
ndataK1e = data(:,5); % K1 error
ndataA2 = data(:,6); % A2/A
ndataA2e = data(:,7); % A2/A error
ndataK2 = data(:,8); % K2
ndataK2e = data(:,9); % K2 error
dataconcaty = concat([dataA1 dataK1 dataK2 ]);
dataconcatye = concat([dataA1e dataK1e dataK2e]);

%% Perform Fit
fit = [K1off K1EDTA K1to2 K2off K2EDTA K2to1 gamma];
tofit = [true true true false true true true ];

% Fitting Parameters
opts = statset('nlinfit'); opts.Display = 'iter';
opts.MaxIter = 100000; opts.UseParallel = true;

%% Perform Fit
[fit(:,), residuals, jacobian, convB, MSE] ...
fit = nlinfitsome ...
(datax ..., % X points
dataconcaty ..., % Y points

W(A,t)(DoSolvi ... % Fitting Function
{abs(A(1)) ...) % K1off
(abs(A(2)) ...) % K1EDTA
(abs(A(3)) ...) % K1to2
(abs(A(4)) ...) % K2off
(abs(A(5)) ...) % K2EDTA
(abs(A(6)) ...) % K2to1
t ...) % EDTA
AB ...) % AB
gamma ...) % gamma
false ...) % Calculate Errors
)

, fit ..., % Fitting Parameters
tofit ..., % Fixed Parameters
, opts ..., % Options

, 'Weights', 1./dataconcatye.^2 ...
);

%% Calculate Errors
http://www.mathworks.co.uk/support/solutions/en/data/1-X92KR/ (16/07/13)
fit = zeros(size(fit));
ci = nlinparci(fit(tofit),residuals,'jacobian',jacobian); % 95% confidence intervals
t = tinv(1-0.05/2, length(dataconcaty)-length(datax));

fit(tofit)= transpose((ci(:,2)-ci(:,1)). / (2*t)); % Standard Error
residuals = reshape(residuals,[],3);

%% Get the values out of the fitted value array
[K1off, K1EDTA, K1to2, K2off, K2EDTA, K2to1, gamma ] = ... PatternMatch(abs(fit));
[K1offe, K1EDT Ae, K1to2e, K2offe, K2EDT Ae, K2to1e, gammae] = ... PatternMatch(abs(fit));

%% Calculate the Apparent Dissociation Rate
Appoff = 0.5*(K1to2 + K2to1 + K1off + K2off ... +(K1to2 - K2to1 + K1off - K2off)^2) ... ];
Appoff= @(A)(... 0.5*(A(1) + A(2) + A(3) + A(4) ... - sqrt(4*A(1)*A(2) ... + (A(1) - A(2) + A(3) - A(4))^2)) ... );
Appoff= Appofff([K1to2 K1to2 K1off K2off]);

CFD = CentralFiniteDifference ...

Appoffe = ... (K1to2e *cell2mat(CFD(1))).^2 ... + (K2to1e *cell2mat(CFD(2))).^2 ... + (K1offe *cell2mat(CFD(3))).^2 ... + (K2offe *cell2mat(CFD(4))).^2 ... ).^0.5;

%% Print Fitted Values
fprintf(['\%s\n', file], ... K1offe, K1offe, 100*K1offe /K1off);

%% Modelling the Data from Fitted parameters and get Errors for Plotting
numModelPoints = 300;
xPoints = Logspacelist(1E-6,2.5E-3,numModelPoints); %% EDTA
solveddata = DoSolving ( K1off, K1EDTA, K1to2 ... K2off, K2EDTA, K2to1 ... xPoints ... % EDTA

% Plot Data and Fits
warning('off', 'MATLAB:Axes:NegativeDataInLogAxis');
xPoints = transpose(xPoints);
fitA1 = solveddata(:,1); fitA1e = solveddata(:,4);
fitK1 = solveddata(:,5); fitK1e = solveddata(:,5);
fitK2 = solveddata(:,3); fitK2e = solveddata(:,6);
fitA2 = 1-fitA1; fitA2e = fitA1e;

scrsz = get(0,'ScreenSize');
% Plot
figure('Name','K1','Position',[1 scrsz(3)/2 scrsz(4)/2]);
hold all;
plot(xPoints,fitK1); % K1
errorbar(datax,dataK1,dataK1e,'.');
plot(xPoints,fitK1+fitK1e); % K1
plot(xPoints,fitK1-fitK1e); % K1
hold off;
legend('K1', 'K1 Data', 'Location', 'NorthWest');

% Plot
figure('Name','K2','Position',[scrsz(3)/2 1 scrsz(3)/2 scrsz(4)/2]);
hold all;
plot(xPoints,fitK2); % K2
errorbar(datax,dataK2,dataK2e,'.');
plot(xPoints,fitK2+fitK2e); % K2
plot(xPoints,fitK2-fitK2e); % K2
hold off;
legend('K2', 'K2 Data', 'Location', 'NorthWest');

% Plot
figure('Name','A_i/A','Position',[1 scrsz(4)/2 scrsz(3)/2 scrsz(4)/2]);
hold all;
semilogx(xPoints,fitA1); % A1/A
errorbar(datax,dataA1,dataA1e,'.');
semilogx(xPoints,fitA2); % A2/A
errorbar(datax,dataA2,dataA2e,'.');
semilogx(xPoints,fitA1+fitA1e); % A1/A
semilogx(xPoints,fitA1-fitA1e); % A1/A
semilogx(xPoints,fitA2+fitA2e); % A2/A
semilogx(xPoints,fitA2-fitA2e); % A2/A
hold off;
legend('A1/A', 'A1/A Data', 'A2/A', 'A2/A Data');

% Plot
figure('Name','K_i','Position',[1 scrsz(4)/4 scrsz(3)/2 scrsz(4)/2]);
loglog(xPoints,fitK1); % K1
hold all;
errorbar(datax,dataK1,dataK1e,'.');
loglog(xPoints,fitK2); % K2
errorbar(datax,dataK2,dataK2e,'.');
loglog(xPoints,fitK1+fitK1e); % K1
loglog(xPoints,fitK1-fitK1e); % K1
loglog(xPoints,fitK2+fitK2e); % K2
loglog(xPoints,fitK2-fitK2e); % K2
hold off;
legend('K1', 'K1 Data', 'K2', 'K2 Data', 'Location', 'NorthWest');

% Plot
figure('Name','A_i/A','Position',[scrsz(3)/2 scrsz(4)/4 scrsz(3)/2 scrsz(4)/2]);
hold all;
semilogx(xPoints,fitA1); % A1/A
errorbar(datax,dataA1,dataA1e,'.');
semilogx(xPoints,fitA2); % A2/A
errorbar(datax,dataA2,dataA2e,'.');
semilogx(xPoints,fitA1+fitA1e); % A1/A
semilogx(xPoints,fitA1-fitA1e); % A1/A
semilogx(xPoints,fitA2+fitA2e); % A2/A
semilogx(xPoints,fitA2-fitA2e); % A2/A
hold off;
legend('A1/A', 'A1/A Data', 'A2/A', 'A2/A Data', 'Location', 'West');

%% Save Simulated Data
outfile = strcat(file, '.fit.csv');
fid = fopen(outfile,'w');
fprintf(fid,'#%s
',file);
fprintf(fid,'#K1off 	=	%±g%g (%0.2g%%)
',K1off ,K1offe ,100 *K1offe /K1off );
fprintf(fid,'#K1EDTA	=	%±g%g (%0.2g%%)
',K1EDTA,K1EDTAe,100 *K1EDTAe/K1EDTA);
fprintf(fid,'#K1to2 	=	%±g%g (%0.2g%%)
',K1to2 ,K1to2e ,100 *K1to2e /K1to2 );
fprintf(fid,'#gamma 	=	%±g%g (%0.2g%%)
',gamma ,gammae ,100 *gammae /gamma );

The variable gamma overwrites the matlab function gamma.

### A.2.2 nlinfitsome.m

```matlab
function [varargout] = nlinfitsome(x,y,fun,beta0,fixed,varargin)
% "fixed" indicates which values of beta0 should not change
% Get separate arrays of coefficients to fix and to estimate
bfixed = beta0(fixed); beta0 = beta0(~fixed);
% Estimate only the non-fixed ones
varargout{1:max(1,nargout)} = nlinfit(x,y,@localfit,beta0,varargin{:});
% Re-create array combining fixed and estimated coefficients
b(~fixed) = varargout{1};
b(fixed) = bfixed;
varargout{1} = b;

% Nested function takes just the parameters to be estimated as inputs
% It inherits the following from the outer function:
% fixed = logical index for fixed elements
% bfixed = fixed values for these elements
% but its input is the
function y=localfit(beta,x)
b(fixed) = bfixed;
b(~fixed) = beta;
y = fun(b,x);
end
end
```

### A.2.3 DoSolving.m

```matlab
function out = DoSolving ( K1off, K1EDTA, K1to2 ... , K2off, K2EDTA, K2to1 ... , EDTA ... , AB ... % Initial Conditions , epsilon ... % Correction for different brightnesses , varargin ... % see varargs below ... , errors ... % Optional Arguments for Errors ... , K1offe, K1EDTAe, K1to2e ... , K2offe, K2EDTAe, K2to1e ... , epsilonse... }

% DOGSOLVING The solutions for the model:
% AB+Cu <---- (AB.Cu)_1 ----> AB + Cu.EDTA
% AB+Cu <---- (AB.Cu)_2 ----> AB + Cu.EDTA
if nargin<1 || varargin{1} == false)
errors = false;
umout = 3;
```
else
    errors = true; % varargin{1}
numout = 8;
K1offe = varargin{2};
K1EDTAe = varargin{3};
K1to2e = varargin{4};
K2offe = varargin{5};
K2EDTAe = varargin{6};
K2tole = varargin{7};
epsilone = varargin{8};
end

out = zeros ( length(EDTA) ... 
    numout ... 
);}

AC1at0 = AB *(K2to1/(K1to2+K2to1)); % AB.Cu Species 1
AC2at0 = AB *(K1to2/(K1to2+K2to1)); % AB.Cu Species 2

alpha = K1to2 + K2to1 + K1off + K2off + EDTA *(K1EDTA - K2EDTA);

beta = sqrt(K1to2^2 ... 
    +2*K1to2*(K1to2 + K1off - K2off + EDTA *(K1EDTA - K2EDTA)) ... 
    + (K2to1 - K1off - K2off - EDTA *(K1EDTA - K2EDTA)).^2);
gamma = K1to2 - K2to1 + K1off - K2off + EDTA *(K1EDTA - K2EDTA);
delta = sqrt(-4 *(K1to2 + K1off + EDTA*K1EDTA) ... 
    + (K1to2 + K1off + EDTA*K1EDTA).*(K2off + EDTA*K2EDTA)) + alpha.^2);

A1 = ((beta + gamma)*AC1at0 - 2*K2to1*AC2at0 ... 
    +((beta - gamma)*AC2at0 - 2*K1to2*AC1at0)* epsilon ... 
    )./(2*delta);
K1 = (alpha + beta)/2;
A2 = ((beta - gamma)*AC1at0 + 2*K2to1*AC2at0 ... 
    +((beta + gamma)*AC2at0 + 2*K1to2*AC1at0)* epsilon ... 
    )./(2*delta);
K2 = (alpha - beta)/2;

A = A1 + A2;
out(:,1) = A1./A;
out(:,2) = K1;
out(:,3) = K2;

if (errors)
    K1eAnalytical = sqrt(... 
        ( K1to2e ... 
            ^2.*(( K1to2 + K2to1 + K1off - K2off + EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
            + K2tole ... 
                ^2.*(( K1to2 + K2to1 - K1off - K2off - EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
                + K1offe ... 
                    ^2.*(( K1to2 - K2to1 + K1off - K2off + EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
                    + K2offe ... 
                        ^2.*(( K1to2 - K2to1 - K1off + K2off - EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
                        + EDTA*K1EDTAe, ... 
                            ^2.*(( K1to2 + K2to1 + K1off - K2off + EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
                            + EDTA*K2EDTAe, ... 
                                ^2.*(( K1to2 - K2to1 + K1off - K2off + EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
                                )./2;
    K2eAnalytical = sqrt(... 
        ( K1to2e ... 
            ^2.*(( K1to2 + K2to1 + K1off - K2off + EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
            + K2tole ... 
                ^2.*(( K1to2 + K2to1 - K1off - K2off - EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
                + K1offe ... 
                    ^2.*(( K1to2 - K2to1 + K1off - K2off + EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
                    + K2offe ... 
                        ^2.*(( K1to2 - K2to1 - K1off + K2off - EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
                        + EDTA*K1EDTAe, ... 
                            ^2.*(( K1to2 + K2to1 + K1off - K2off + EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
                            + EDTA*K2EDTAe, ... 
                                ^2.*(( K1to2 - K2to1 + K1off - K2off + EDTA*(K1EDTA - K2EDTA))./(beta)).^-2 ... 
                                )./2;
    CFD = CentralFiniteDifference(#(A)(DoSolving(A(1) ... % K1off 
        , A(2) ... % K1EDTA 
        , A(3) ... % K1to2 
        , A(4) ... % K2off 

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A.2.4 CentralFiniteDifference.m

```matlab
function out = CentralFiniteDifference( function ... % relative StepSize 
    , stepSize ... % Minimum StepSize 
    , varargin) 
%Central Finite Difference 
%Avarargin the point arround the function 
%returns cells use cell2mat() to get matrix 
varargin = cell2mat(varargin); 
[f] = cell(length(varargin)); 
out = cell(length(varargin)); 
for arg = 1:length(varargin) 
    for j = 1:length(js) 
        tmp = varargin; 
        if abs(tmp(arg)*stepSize) > minStepSize 
            tmp(arg) = tmp(arg) + js(j)*stepSize; 
            else 
                tmp(arg) = tmp(arg) + js(j)*stepSize; 
        end 
    end 
    f{j} = {Function(tmp)}; 
end 

if abs(tmp(arg)*stepSize) > minStepSize 
    diff = diff ./ (tmp(arg)*stepSize); 
```
A.2.5 PatternMatch.m

```matlab
function [a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,p,q,r,s,t,u,v,w,x,y,z] ... = PatternMatch(in)
%PATTEMATCH Pattern match a list into variables
% If number of output variables is greater than input list,
% values set to NaN
numvalues = 27-length(in);
in=[in zeros([1 numvalues])];
in(numvalues:27) = NaN;
a=in(1) ;b=in(2) ;c=in(3) ;d=in(4) ;e=in(5) ;f=in(6) ;g=in(7) ;h=in(8) ;
i=in(9) ;j=in(10);k=in(11);l=in(12);m=in(13);n=in(14);o=in(15);p=in(16);
qu=in(17);r=in(18);s=in(19);t=in(20);u=in(21);v=in(22);w=in(23);x=in(24);
y=in(25);z=in(26);
end
```

A.2.6 Logspacelist.m

```matlab
function [ point ] = Logspacelist( max, min, numpoints)
%LOGSPACE Summary of this function goes here
% Detailed explanation goes here
point = zeros(1, numpoints);
for n = drange(1:numpoints)
    point(n) = 10^((n-1)/(numpoints-1)*log10(max/min)+log10(min));
end
end
```

A.3 Multiple Binding of Cu$^{2+}$ to Aβ$^*$

Matlab code to fit the relative populations of the different phases for different age times of when ([Aβ⊗Cu$^{2+}$]⊗EDTA) (main.m), and code to take the fitting parameters from main.m and generate the relative equilibrium populations of Cu$^{2+}$ bound Aβ species and plot them over equivalent experimental data (main2.m). The code is used in section 3.9. The follow files are not included in the listing below as they appear in section A.2: Logspacelist.m (§A.2.6), nlinfitsome.m (§A.2.2), PatternMatch.m (§A.2.5), and CentralFiniteDifference.m (§A.2.4).

*Archive of source code: AB+Cu+EDTA.tar.xz*
main.m  The initial parameters for fitting are lines 8 through 13, with lines 14 through 17 as the relative quenching of the different amplitudes. Whether the fitting parameters are variable is declared in the array tofit (line 26). The variable AB is the concentration of Aβ (line 18), and Cu is the concentration of Cu$^{2+}$ (line 30). Line 22 selects which models to fit from model2dot.m. The experimental data should be in a CSV file with columns [Age, Time / s, $A_1/A$, $A_2/A$, $A_3/A$, $A_4/A$, $A_5/A$], where $A = A_1 + A_2 + A_3 + A_4 + A_5$. The output parameters are saved to the input file name with .fit., the model number and .csv appended.

main2.m  It takes the base name (without .fit.modelnum.csv) file outputted from main.m in the variable fittedfilebase with the concentration of Cu$^{2+}$ to calculate rate constants and experimental data in file (line 9) in the format [Cu / µM, $A_1/A$, $A_2/A$, $A_3/A$, $A_4/A$, $A_5/A$], where $A = A_1 + A_2 + A_3 + A_4 + A_5$. Line 16 selects which models to fit from model2dot.m. The output is saved to the base name with .fit.modelnum.csv.fit.varyCu.csv appended.

A.3.1 main.m

```matlab
%% Initialisation
clear; % close all;
clec; % Clear Command Window
concat = @(xs)(reshape(xs,[],1));

%% Model Parameters
% Kinetic Parameters
k12 = 1.1; % Hz
k21 = 3.1; % Hz
k3on = 10; % Hz
k3off = 4; % Hz
k4on = 0.01; % Hz
k4off = 0.25; % Hz
rA1 = 1;
rA2 = 1;
rA3 = 1.3;
rA4 = 1.3;
AB = 50E-9; % M
initial = AB*[1 0 0 0]; % [M M M M]
model = 2; % model number in model2dot.m
for model = 0:17
    %% Initial Parameters
    fit = [k12 ,k21 ,k3on ,k3off,k4on ,k4off,rA1 ,rA2 ,rA3 ,rA4 ];
tofit = [true ,true ,true ,true ,true ,true ,false,false,false,false];
    %% Fitting Data
    file = '140324-AB16LysHL488-VaryTime-10uMCu100nMAB2mMEDTA.csv';Cu=10E-6;%M
    % file = '140325-AB16LysHL488-VaryTime-5uMCu100nMAB2mMEDTA.csv';Cu=5E-6;%M
    % file = '140325-AB16LysHL488-VaryTime-2uMCu100nMAB2mMEDTA.csv';Cu=2E-6;%M
    % file = '140409-AB16LysHL488-VaryTime-2uMCu100nMAB2mMEDTA.csv';Cu=2E-6;%M
    data = importdata(file);
```
data = data.data;
data1 = data(:,1);
dataA1 = data(:,2);
dataA1e = data(:,3);
dataA2 = data(:,4);
dataA2e = data(:,5);
dataA3 = data(:,6);
dataA3e = data(:,7);
dataA4 = data(:,8);
dataA4e = data(:,9);
dataconcaty = concat([dataA1 dataA2 dataA3 dataA4 ]);%
dataconcatye = concat([dataA1e dataA2e dataA3e dataA4e]);

%% Perform Fit
% Fitting Parameters
opts = statset('nlinfit'); opts.Display = 'iter' ;
 opts.MaxIter = 200 ; opts.UseParallel = true;

% Perform Fit
[fit(:), residuals, jacobian, convB, MSE] ... = nlinfit( ... % X points
, dataconcaty ... % Y points
, @(A,t)(concat(model2 ... % Fitting Function
(model ........
, abs(A(1)) ... % k12
, abs(A(2)) ... % k21
, abs(A(3)) ... % k3on
, abs(A(4)) ... % k3off
, abs(A(5)) ... % k4on
, abs(A(6)) ... % k4off
, abs(A(7)) ... % rA1
, abs(A(8)) ... % rA2
, abs(A(9)) ... % rA3
, abs(A(10))... % rA4
, t ... % time
, Cu ... % Copper
, initial ... % Starting Conditions
, false ... % errors
)) ... ...
,
,t ... % time
,
, Cu ... % Copper
, initial ... % Starting Conditions
, false ... % errors

);% Get the values out of the fitted value array
[k12 ,k21 ,k3on ,k3off ,k4on ,k4off ,rA1 ,rA2 ,rA3 ,rA4 ] = ...
PatternMatch(abs(fit ));
[k12e,k21e,k3one,k3offe,k4one,k4offe,rA1e,rA2e,rA3e,rA4e] = ...
PatternMatch(abs(fit ));

%% Print Fitted Values
fprintf('

file
');
fprintf('k12 	=	%±g%g (%0.2g%%)
',k12 ,k12e ,100 *k12e/k12 );
fprintf('k21 	=	%±g%g (%0.2g%%)
',k21 ,k21e ,100 *k21e/k21 );
fprintf('k3on 	=	%±g%g (%0.2g%%)
',k3on ,k3one ,100 *k3one/k3on );
fprintf('k3off 	=	%±g%g (%0.2g%%)
',k3off ,k3offe ,100 *k3offe/k3off);
fprintf('k4on 	=	%±g%g (%0.2g%%)
',k4on ,k4one ,100 *k4one/k4on );
fprintf('k4off 	=	%±g%g (%0.2g%%)
',k4off ,k4offe ,100 *k4offe/k4off);
fprintf('rA1 	=	%±g%g (%0.2g%%)
',rA1 ,rA1e ,100 *rA1e/rA1 );

% Calculate Errors
% http://www.mathworks.co.uk/support/solutions/en/data/1-X92KR/ (16/07/13)
fite = zeros(size(fit ));
ci = mlpri(fit(fit ),residuals,'jacobian',jacobian);% 95% confidence intervals
t = tinv(1-0.05/2,length(dataconcaty)-length(datat));% Standard Error
residuals = reshape(residuals,[],3);

%% Get the values out of the fitted value array
[k12 ,k21 ,k3on ,k3off ,k4on ,k4off ,rA1 ,rA2 ,rA3 ,rA4 ] = ...
PatternMatch(abs(fit ));
[k12e,k21e,k3one,k3offe,k4one,k4offe,rA1e,rA2e,rA3e,rA4e] = ...
PatternMatch(abs(fit ));

%% Print Fitted Values
fprintf('

file
');
fprintf('k12 	=	%±g%g (%0.2g%%)
',k12 ,k12e ,100 *k12e/k12 );
fprintf('k21 	=	%±g%g (%0.2g%%)
',k21 ,k21e ,100 *k21e/k21 );
fprintf('k3on 	=	%±g%g (%0.2g%%)
',k3on ,k3one ,100 *k3one/k3on );
fprintf('k3off 	=	%±g%g (%0.2g%%)
',k3off ,k3offe ,100 *k3offe/k3off);
fprintf('k4on 	=	%±g%g (%0.2g%%)
',k4on ,k4one ,100 *k4one/k4on );
fprintf('k4off 	=	%±g%g (%0.2g%%)
',k4off ,k4offe ,100 *k4offe/k4off);
fprintf('rA1 	=	%±g%g (%0.2g%%)
',rA1 ,rA1e ,100 *rA1e/rA1 );
fprintf('rA2 \t=\t%±g\%g (%0.2g\%)
',rA2 ,rA2e ,100 *rA2e/rA2 );
fprintf('rA3 \t=\t%±g\%g (%0.2g\%)
',rA3 ,rA3e ,100 *rA3e/rA3 );
fprintf('rA4 \t=\t%±g\%g (%0.2g\%)
',rA4 ,rA4e ,100 *rA4e/rA4 );

\% Modelling the Data from Fitted parameters and get Errors for Plotting
numModelPoints = 300;
xPoints = transpose(Logspacelist(100,1E-3,numModelPoints)); \% time
solveddata = model2 ... % Fitting Function
( model ... 
, k12 , k21 , k3on , k3off , k4on , k4off ... 
, rA1 , rA2 , rA3 , ... 
, xPoints ... \% time 
, Cu ... \% Copper 
, initial ... \% Initial Conditions 
, true ... \% errors 
, k12e, k21e, k3one, k3offe, k4one, k4offe ... 
, rA1e, rA2e, rA3e, rA4e ... 
);

\% Plot Data and Fits
warning('off','MATLAB:Axes:NegativeDataInLogAxis');

fitA1 = solveddata(:,1);
fitA2 = solveddata(:,2);
fitA3 = solveddata(:,3);
fitA4 = solveddata(:,4);
fitA1e = solveddata(:,5);
fitA2e = solveddata(:,6);
fitA3e = solveddata(:,7);
fitA4e = solveddata(:,8);

scrsz = get(0,'ScreenSize');

figure ('Name',sprintf('Ai/A (Cu = %0.2g M)',Cu));
semilogx(xPoints,fitA1);
hold all;
semilogx(xPoints,fitA1+fitA1e);
semilogx(xPoints,fitA1-fitA1e);
errorbar(datat,dataA1,dataA1e,'.');
semilogx(xPoints,fitA2);
semilogx(xPoints,fitA2+fitA2e);
semilogx(xPoints,fitA2-fitA2e);
errorbar(datat,dataA2,dataA2e,'.');
semilogx(xPoints,fitA3);
semilogx(xPoints,fitA3+fitA3e);
semilogx(xPoints,fitA3-fitA3e);
errorbar(datat,dataA3,dataA3e,'.');
semilogx(xPoints,fitA4);
semilogx(xPoints,fitA4+fitA4e);
semilogx(xPoints,fitA4-fitA4e);
errorbar(datat,dataA4,dataA4e,'.');
hold off;

outfile = strcat(file, '.fit.', num2str(model), '.csv');
fid = fopen (outfile, 'w');
fprintf(fid,'%s
',file);
fprintf(fid,'#k12 \t=\t%±g\%g (%0.2g\%)
',k12 ,k12e ,100 *k12e/k12 );
fprintf(fid,'#k21 \t=\t%±g\%g (%0.2g\%)
',k21 ,k21e ,100 *k21e/k21 );
fprintf(fid,'#k3on \t=\t%±g\%g (%0.2g\%)
',k3on ,k3one ,100 *k3one/k3on );
fprintf(fid,'#k3off \t=\t%±g\%g (%0.2g\%)
',k3off ,k3offe ,100 *k3offe/k3off );
fprintf(fid,'#k4on \t=\t%±g\%g (%0.2g\%)
',k4on ,k4one ,100 *k4one/k4on );
fprintf(fid,'#k4off \t=\t%±g\%g (%0.2g\%)
',k4off ,k4offe ,100 *k4offe/k4off );
fprintf(fid,'#rA1 \t=\t%±g\%g (%0.2g\%)
',rA1 ,rA1e ,100 *rA1e/rA1 );
fprintf(fid,'#rA2 \t=\t%±g\%g (%0.2g\%)
',rA2 ,rA2e ,100 *rA2e/rA2 );
fprintf(fid,'#rA3 \t=\t%±g\%g (%0.2g\%)
',rA3 ,rA3e ,100 *rA3e/rA3 );
fprintf(fid,'#rA4 \t=\t%±g\%g (%0.2g\%)
',rA4 ,rA4e ,100 *rA4e/rA4 );
fclose (fid);
dlmwrite(outfile ... 
,[xPoints fitA1 fitA2 fitA3 fitA4 fitA1e fitA2e fitA3e fitA4e] ... 244
A.3.2 main2.m

%% Initialisation
clear; % close all;
cle; % Clear Command Window
concat = @(xs)(reshape(xs,[],1));

%% Model Parameters
file = '130128-ABCu+2mMEDTA-VaryCu-4phaseanalysis.csv';

file = '140429-AB16LysHL488-VaryCu-25nMAB-2mMEDTA.csv';

fittedfilebase = '140324-AB16LysHL488-VaryTime-10uMCu100nMAB2mMEDTA.csv'; Cu = 10E-6;

AB = 50E-9; % M
initial = AB*[1 0 0 0]; % [M M M M]

for model = 9:17
    % Get Fitted Parameters
    fittedfile = strcat(fittedfilebase, '.fit.', num2str(model), '.csv');
    [params, paramse] = importfile(fittedfile);
    [k12, k21, k3on, k3off, k4on, k4off, rA1, rA2, rA3, rA4] = ...
    PatternMatch(transpose(params));
    [k12e, k21e, k3one, k3offe, k4one, k4offe, rA1e, rA2e, rA3e, rA4e] = ...
    PatternMatch(transpose(paramse));
    k3on = k3on / Cu; k3one = k3one / Cu;
    k4on = k4on / Cu; k4one = k4one / Cu;

    %% Do modelling
    numpoints = 50;
    npoints = 50;
    tPoints = transpose(Logspacelist(1000,1E-3,npoints)); %% time
    fitA1 = []; fitA2 = []; fitA3 = []; fitA4 = [];
    fitA1e = []; fitA2e = []; fitA3e = []; fitA4e = [];
    outx = Logspacelist(200E-6, 0.05E-6,numpoints);
    parfor i = 1:numpoints
        fprintf('%d
', i);
        Copper = outx(i);
        solveddata = model2 ... % Fitting Function
        (model + 100 ...% +100 for models with explicit Cu
        , k12, k21, k3on, k3off, k4on, k4off ...
        , rA1, rA2, rA3, rA4 ...
        , tPoints ...% time
        , Copper ...% Copper
        , initial ...% Starting Conditions
        , true ...% Errors
        , k12e, k21e, k3one, k3offe, k4one, k4offe ...
        , rA1e, rA2e, rA3e, rA4e ...
        );

        fitA1 = [fitA1, solveddata(:,1)];%#ok< *AGROW>% n
        fitA2 = [fitA2, solveddata(:,2)];%#ok< *AGROW>% npoints-1,1);
        fitA3 = [fitA3, solveddata(:,3)];%#ok< *AGROW>% npoints-1,3);
        fitA4 = [fitA4, solveddata(:,4)];%#ok< *AGROW>% npoints-1,4);
        fitA1e = [fitA1e, solveddata(:,5)];%#ok< *AGROW>% npoints-1,1);
        fitA2e = [fitA2e, solveddata(:,6)];%#ok< *AGROW>% npoints-1,2);
        fitA3e = [fitA3e, solveddata(:,7)];%#ok< *AGROW>% npoints-1,3);
        fitA4e = [fitA4e, solveddata(:,8)];%#ok< *AGROW>% npoints-1,4);
    end

figure1 = figure;
colormap(’winter’);
axes1 = axes(...
    ’Parent’,figure1 ...)
    ’YScale’, ’log’, ’YMinorTick’, ’on’...
data = importdata(file);
data = data.data;
dataCu = data(:,1)./1E6; % uM -> M
dataA1 = data(:,2);
dataA2 = data(:,3);
dataA3 = data(:,4);
dataA4 = data(:,5);
dataA5 = data(:,6);

figure2 = figure('name','t=1000');
axes1 = axes('Parent',figure2,'XScale','log','XMinorTick','on');
xlim(axes1,[5e-07 5e-5]);
semilogx(outx,fitA1(length(outx),:));
hold all;
semilogx(outx,fitA2(length(outx),:));
semilogx(outx,fitA3(length(outx),:));
semilogx(outx,fitA4(length(outx),:));
semilogx(dataCu,dataA1,'.');
semilogx(dataCu,dataA2,'.');
semilogx(dataCu,dataA3,'.');
semilogx(dataCu,dataA4+dataA5,'.');
semilogx(outx,fitA1(length(outx),:)+fitA1e(length(outx),:));
semilogx(outx,fitA2(length(outx),:)+fitA2e(length(outx),:));
semilogx(outx,fitA3(length(outx),:)+fitA3e(length(outx),:));
semilogx(outx,fitA4(length(outx),:)+fitA4e(length(outx),:));
hold off;

outfile = strcat(fittedfile, '.fit.varyCu.csv');
fid = fopen(outfile,'w');
');
close (fid);
dlmwrite(outfile,...
   {transpose(outx) transpose(fitA1(length(outx),:)) transpose(fitA2(length(outx),:))
    transpose(fitA3(length(outx),:)) transpose(fitA4(length(outx),:))
    transpose(fitA1e(length(outx),:)) transpose(fitA2e(length(outx),:))
    transpose(fitA3e(length(outx),:)) transpose(fitA4e(length(outx),:))},
   'delimiter','	','-append');
end
A.3.3 importfile.m

```matlab
function [VarName3,VarName4] = importfile(filename, startRow, endRow)
% Auto-generated by MATLAB on 2014/09/05 16:49:57

delimiter = {'	','(','±'};
if nargin<=2
    startRow = 3;
    endRow = 12;
end
formatSpec = 'sfileID = fopen(filename,'r');dataArray = textscan(fileID, formatSpec, endRow(1)-startRow(1)+1, 'Delimiter', delimiter, 'HeaderLines', startRow(1)-1, 'ReturnOnError', false);for block=2:length(startRow)rewind(fileID);dataArrayBlock = textscan(fileID, formatSpec, endRow(block)-startRow(block)+1, 'Delimiter', delimiter, 'HeaderLines', startRow(block)-1, 'ReturnOnError', false);for col=1:length(dataArray)dataArraycol = [dataArraycol;dataArrayBlockcol];endendfclose(fileID);VarName3 = dataArray(:,1);VarName4 = dataArray(:,2);
```

A.3.4 model2.m

```matlab
function out = model2 ...
( modelnum ...
, k12 , k21 , k3on , k3off , k4on , k4off ...
, rA1 , rA2 , rA3 , rA4 ...
, time ...
, Cu ...
, initial ...
, varargin ...
, errors ...
... % Optional Arguments for Errors ...
... , k12e, k21e, k3one, k3offe, k4one, k4offe ...
... , rA1e, rA2e, rA3e, rA4e ...
)
if (nargin < 1 || varargin{1} == false)
    errors = false;
else
    errors = true; % varargin{1}
k12e = varargin{2};
k21e = varargin{3};
k3one = varargin{4};
k3offe = varargin{5};
k4one = varargin{6};
k4offe = varargin{7};
rA1e = varargin{8};
rA2e = varargin{9};
rA3e = varargin{10};
rA4e = varargin{11};
end
odeoptions = odeset( 'Reltol',1e-6 ...
, 'Abstol',1e-30 ...]
[T,Y] = ode45(@(t,A)(model2dot ...
, modelnum ...
, k12, k21, k3on, k3off, k4on, k4off ...
, Cu ...
, A(1), A(2), A(3), A(4) ... %A's ...

... , [0; time] ... % Put in Initial time point ...
, initial ...
, odeoptions ... % Simulation Options ...
]
out = Y(2:length(T),:); % Remove in Initial time point
out = out .* repmat([rA1, rA2, rA3, rA4], size(out,1),1); % Rel Brightness
out = out ./ total;
```

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if (errors)
    CFD = CentralFiniteDifference(@(A)(model2 ... % Fitting Function
    ( modelnum ... % k12
        A(1) ... % k12
        A(2) ... % k21
        A(3) ... % k3n
        A(4) ... % k3f
        A(5) ... % k4n
        A(6) ... % k4f
        A(7) ... % rA1
        A(8) ... % rA2
        A(9) ... % rA3
        A(10) ... % rA4
        time ... % time
        Cu ... % Copper
    , initial ... % Starting Conditions
    false ... % errors
    ) ... ...
    , 1E-6 ... % relative StepSize
    , 1E-8 ... % Minimum StepSize
    , k12, k21, k3n, k3f, k4n, k4f ...
    , rA1, rA2, rA3, rA4 ...
    ));
errs = ...
    ( (k12e *cell2mat(CFD(1))).^2 ...
    + (k21e *cell2mat(CFD(2))).^2 ...
    + (k3one *cell2mat(CFD(3))).^2 ...
    + (k3offe *cell2mat(CFD(4))).^2 ...
    + (k4one *cell2mat(CFD(5))).^2 ...
    + (k4offe *cell2mat(CFD(6))).^2 ...
    + (rA1e *cell2mat(CFD(7))).^2 ...
    + (rA2e *cell2mat(CFD(7))).^2 ...
    + (rA3e *cell2mat(CFD(7))).^2 ...
    + (rA4e *cell2mat(CFD(7))).^2 ...
    ).^0.5;
out(:,5) = errs(:,1); % A1/A error
out(:,6) = errs(:,2); % A2/A error
out(:,7) = errs(:,3); % A3/A error
out(:,8) = errs(:,4); % A4/A error
end
end

A.3.5  model2dot.m

function dy = model2dot ...
    ( model ... ...
        k12, k21, k3n, k3f, k4n, k4f ...
        Cu ...
        A1, A2, A3, A4 ...
    )
    dy = zeros(4,1);
switch(model)
case 0
dy(1) = - A1*k12 + A2*k21 - A1*k3n + A3*k3f - A1*k4n + A4*k4f;
end
dy(2) = + A1*k12 - A2*k21;
dy(3) = + A1*k3n - A3*k3f;
dy(4) = + A1*k4n - A4*k4f;
case 1
dy(1) = - A1*k12 + A2*k21 - A1*k3n + A3*k3f - A1*k4n + A4*k4f;
dy(2) = + A1*k12 - A2*k21 + A4*k4f;
dy(3) = + A1*k3n - A3*k3f;
dy(4) = + A1*k4n - A4*k4f;
end
case 2
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} + A_3 k_{3f} - A_1 k_{4n} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} + A_4 k_{4f} ; \]
\[ dy(4) = + A_1 k_{4n} - A_4 k_{4f} ; \]
\[ case 3 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} + A_3 k_{3f} + A_4 k_{4f} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} - A_2 k_{4n} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} ; \]
\[ dy(4) = + A_2 k_{4n} - A_4 k_{4f} ; \]
\[ case 4 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} + A_3 k_{3f} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} - A_2 k_{4n} + A_4 k_{4f} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} ; \]
\[ dy(4) = + A_2 k_{4n} - A_4 k_{4f} ; \]
\[ case 5 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} + A_3 k_{3f} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} - A_2 k_{4n} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} + A_4 k_{4f} ; \]
\[ dy(4) = + A_2 k_{4n} - A_4 k_{4f} ; \]
\[ case 6 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} + A_3 k_{3f} + A_4 k_{4f} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} - A_3 k_{4n} ; \]
\[ dy(4) = + A_3 k_{4n} - A_4 k_{4f} ; \]
\[ case 7 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} + A_3 k_{3f} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} + A_4 k_{4f} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} - A_3 k_{4n} ; \]
\[ dy(4) = + A_3 k_{4n} - A_4 k_{4f} ; \]
\[ case 8 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} + A_3 k_{3f} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} + A_3 k_{3f} + A_4 k_{4f} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} - A_3 k_{4n} ; \]
\[ dy(4) = + A_3 k_{4n} - A_4 k_{4f} ; \]
\[ case 9 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} + A_4 k_{4f} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} + A_3 k_{3f} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} ; \]
\[ dy(4) = + A_1 k_{4n} - A_4 k_{4f} ; \]
\[ case 10 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} + A_4 k_{4f} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} + A_3 k_{3f} + A_4 k_{4f} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} ; \]
\[ dy(4) = + A_1 k_{4n} - A_4 k_{4f} ; \]
\[ case 11 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} + A_3 k_{3f} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} + A_4 k_{4f} ; \]
\[ dy(4) = + A_2 k_{4n} - A_4 k_{4f} ; \]
\[ case 12 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} + A_4 k_{4f} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} + A_3 k_{3f} - A_2 k_{4n} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} ; \]
\[ dy(4) = + A_2 k_{4n} - A_4 k_{4f} ; \]
\[ case 13 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} + A_3 k_{3f} - A_2 k_{4n} + A_4 k_{4f} ; \]
\[ dy(3) = + A_1 k_{3n} - A_3 k_{3f} ; \]
\[ dy(4) = + A_2 k_{4n} - A_4 k_{4f} ; \]
\[ case 14 \]
\[ dy(1) = - A_1 k_{12} + A_2 k_{21} - A_1 k_{3n} ; \]
\[ dy(2) = + A_1 k_{12} - A_2 k_{21} + A_3 k_{3f} - A_2 k_{4n} ; \]
\[ dy(3) = + A1*k3n - A3*k3f + A4*k4f; \]
\[ dy(4) = + A2*k4n - A4*k4f; \]

**case 15**

\[ dy(1) = - A1*k12 + A2*k21 - A1*k3n + A4*k4f; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f; \]
\[ dy(3) = + A1*k3n - A3*k3f - A5*k4n; \]
\[ dy(4) = + A3*k4n - A4*k4f; \]

**case 16**

\[ dy(1) = - A1*k12 + A2*k21 - A1*k3n; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f; \]
\[ dy(3) = + A1*k3n - A3*k3f - A3*k4n; \]
\[ dy(4) = + A3*k4n - A4*k4f; \]

**case 17**

\[ dy(1) = - A1*k12 + A2*k21 - A1*k3n; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f + A4*k4f; \]
\[ dy(3) = + A1*k3n - A3*k3f - A3*k4n; \]
\[ dy(4) = + A3*k4n - A4*k4f; \]

**case 109**

\[ dy(1) = - A1*k12 + A2*k21 - A1*Cu*k3n - A1*Cu*k4n + A4*k4f; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f; \]
\[ dy(3) = + A1*Cu*k3n - A3*k3f; \]
\[ dy(4) = + A1*Cu*k4n - A4*k4f; \]

**case 110**

\[ dy(1) = - A1*k12 + A2*k21 - A1*Cu*k3n - A1*Cu*k4n; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f + A4*k4f; \]
\[ dy(3) = + A1*Cu*k3n - A3*k3f; \]
\[ dy(4) = + A1*Cu*k4n - A4*k4f; \]

**case 111**

\[ dy(1) = - A1*k12 + A2*k21 - A1*Cu*k3n - A1*Cu*k4n; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f; \]
\[ dy(3) = + A1*Cu*k3n - A3*k3f + A4*k4f; \]
\[ dy(4) = + A1*Cu*k4n - A4*k4f; \]

**case 112**

\[ dy(1) = - A1*k12 + A2*k21 - A1*Cu*k3n + A4*k4f; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f - A2*Cu*k4n; \]
\[ dy(3) = + A1*Cu*k3n - A3*k3f; \]
\[ dy(4) = + A2*Cu*k4n - A4*k4f; \]

**case 113**

\[ dy(1) = - A1*k12 + A2*k21 - A1*Cu*k3n; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f - A2*Cu*k4n + A4*k4f; \]
\[ dy(3) = + A1*Cu*k3n - A3*k3f; \]
\[ dy(4) = + A2*Cu*k4n - A4*k4f; \]

**case 114**

\[ dy(1) = - A1*k12 + A2*k21 - A1*Cu*k3n; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f; \]
\[ dy(3) = + A1*Cu*k3n - A3*k3f + A4*k4f; \]
\[ dy(4) = + A2*Cu*k4n - A4*k4f; \]

**case 115**

\[ dy(1) = - A1*k12 + A2*k21 - A1*Cu*k3n + A4*k4f; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f; \]
\[ dy(3) = + A1*Cu*k3n - A3*k3f - A3*Cu*k4n; \]
\[ dy(4) = + A3*Cu*k4n - A4*k4f; \]

**case 116**

\[ dy(1) = - A1*k12 + A2*k21 - A1*Cu*k3n; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f; \]
\[ dy(3) = + A1*Cu*k3n - A3*k3f - A3*Cu*k4n + A4*k4f; \]
\[ dy(4) = + A3*Cu*k4n - A4*k4f; \]

**case 117**

\[ dy(1) = - A1*k12 + A2*k21 - A1*Cu*k3n; \]
\[ dy(2) = + A1*k12 - A2*k21 + A3*k3f; \]
\[ dy(3) = + A1*Cu*k3n - A3*k3f - A3*Cu*k4n + A4*k4f; \]
\[ dy(4) = + A3*Cu*k4n - A4*k4f; \]
A.4 Simulation of Cu$^{2+}$, Zn$^{2+}$ and Aβ in the Synapse*

C++ code to simulate the reaction and diffusion of metals and Aβ in the synapse. The code may be compiled using the make file.

The experiments are listed in experiments.h, and may be selected on line 9 of main.cpp. For the pulsed diffusion simulations timeSteps = 200000 was used, and the pulseRate was set to \{1, 2, 5, 10, 20, 50, 100\}. The code is currently setup to perform the single release simulations.

getr.hs (§A.4.10) is haskell code that allows for the extraction of different radii from the output files produced by the C++ program.

A.4.1 makefile

```plaintext
BIN = Diff
GPP = g++ -Wall -Wextra -W -std=c++0x -g #-fprofile-arcs #-ftest-coverage
OPTI = -O2 -fopenmp #-ffast-math #-mfpmath=sse #-funroll-loops
CFLAGS = `pkg-config --cflags gsl`
LIBS = `pkg-config --libs gsl`
FLAGS =

all: Diff
Diff: main.o gslvec.o diftrimat.o diftrimatsolve.o
    $(GPP) $^ -o $(BIN) $(FLAGS) $(LIBS) $(OPTI)
main.o: main.cpp experiments.h gslvec.h diftrimat.h
    $(GPP) -c $< -o $@ $(COMPFLAGS) $(CFLAGS) $(OPTI)
gslvec.o: gslvec.cpp gslvec.h
    $(GPP) -c $< -o $@ $(COMPFLAGS) $(CFLAGS) $(OPTI)
diftrimat.o: diftrimat.cpp diftrimat.h gslvec.h
    $(GPP) -c $< -o $@ $(COMPFLAGS) $(CFLAGS) $(OPTI)
diftrimatsolve.o: diftrimatsolve.cpp diftrimat.h gslvec.h
    $(GPP) -c $< -o $@ $(COMPFLAGS) $(CFLAGS) $(OPTI)
clean: cleanish
    rm -f $(BIN)
cleanish:
    rm -f *.o
    rm -f *.dat
    rm -f *.aux
    rm -f *.log
    rm -f *.sty
    rm -f *.eps
```

*Archive of source code: Diffusion.tar.xz
A.4.2 main.cpp

```cpp
#include <cmath>
#include <iostream>
#include <omp.h>
#include "diftrimat.h"
#include "gslvec.h"

#include "experiments.h"
using namespace std;

int main() {
  //Simulation Parameters
  const int numRPoints = 2000000,
          timeSteps = 2000;
  const double maxR = 400000, // in nanometers
               dr = maxR / numRPoints; //dr = maxR / numRPoints;
  const double maxT = 1e7, // in microseconds
               t1 = 0.001; // initial time point
  const double pulseRate = 1e6/maxT, // in Hz
              pulsePeriod = 1e6/pulseRate, // in microseconds
              numPulses = (maxT-t1)/pulsePeriod;
  const int stepsPerPulse = timeSteps/numPulses;

  // Get Array of solution components under initial conditions
  auto l = makel(numRPoints, dr);

  //pragma omp parallel for
  for(size_t t = 0; t < timeSteps; ++t){
    auto &a = l[t];
    *a.fout << "\# numRPoints: " << numRPoints
       << "", timeSteps: " << timeSteps
       << "", maxR: " << maxR
       << "", maxT: " << maxT
       << "", pulseRate: " << pulseRate
       << "\n;"
    *a.fout << "\n";
  }

  // Simulation
  double lastt = 0;
  for (int t=0; t != timeSteps; ++t){
    //formula for next exponentially increasing time step
    const double thist = (t / stepsPerPulse) * pulsePeriod
       + pow(10, ((double)(t%stepsPerPulse))/(stepsPerPulse-1)+log10(pulsePeriod/t1)) * t1);
    const double dt = thist-lastt;

    // Pulse
    if (t%stepsPerPulse == 0)
      pulse(1,dr);

    // Reaction
    #pragma omp parallel for
    for (int i = 0; i < numRPoints; ++i)
```

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reaction(l, i, dt);

// Diffusion
#pragma omp parallel for
for(size_t i = 0; i < l.size(); i++){
    auto &a = l[i];
    a.rDiff(a.D, dr, dt);
}

// Save to File
#pragma omp parallel for
for(size_t i = 0; i < l.size(); ++i){
    auto &a = l[i];
    diftrimatsolve(a.rDiff, a.r, a.outr);
    for (double i=1; i < numRPoints; i*=1.1){
        const double r = double(i) * dr;
        >> a.fout << thist << '	'
        << double(r) << '	'
        << a.outr.get(i)<< '
';
    }
    >> a.fout << '\n'; // Newline for gnuplot
}

// Set last outr to incr
#pragma omp parallel for
for(size_t i = 0; i < l.size(); ++i){
    auto &a = l[i];
    swap(a.r, a.outr);
}

// Set this time to previous
lastt=thist;

cout << '\r' << "Done: " << (double)t / timeSteps *100 << "% "; // <<
flush
}
cout << endl;
for(size_t i = 0; i < l.size(); ++i){
    auto &a = l[i];
    delete a.fout; // Close files
}
return 0;

A.4.3 experiments.h

#ifndef __Experiments__
#define __Experiments__

#include <array>
#include <fstream>
#include <iostream>
#include <memory>
#include <iostream>
#include <memory>
using namespace std;

#include "gslvec.h"

struct T {
    std::ofstream *fout; // Output file
diftrimat rDiff; // Tridiagonal matrix
const double D; // Diffusion constant in nm^2 /us
gslvec r; // Vector of r points
gslvec outr; // Tmp output vector of r points
};

const double Dzn = 650; //Diffusion Coefficient in (nm)^2 /us
const double Dcu = 650;
const double DAB = 304;  // martin
const double DHSAA = 61;  // http://bionumbers.hms.harvard.edu/bionumber.aspx?id=100612&ver=6

const double ABCukon = 3.0e8 * 1e-12;  // in (uM us)^-1
const double ABCukoff = 0.8 * 1e-6;  // in us^-1
const double ABCu12 = 0.9 * 1e-6;  // in us^-1
const double ABCu21 = 2.22 * 1e-6;  // in us^-1
const double ABCu13 = 4.2e5 * 1e-12;  // in (uM us)^-1
const double ABCu32 = 1.7 * 1e-6;  // in us^-1

const double ABZnkoff = 110 * 1e-6;  // in us^-1
const double ABZnkon = 1.9e6 * 1e-12;  // in (uM us)^-1
const double HSACukon = 1.0e8 * 1e-12;  // in (uM us)^-1

inline double defpos (double in){
    return (in<0)?0:in;
}

#define EXPT 1  // Diffusion of Zn and Cu

std::array<T,2> makel(const int nr, const double dr){
    gslvec rZn(nr), rCu(nr);
    // Initial conditions
    for (int i = 0; i <nr; ++i){// vesicle diameter = 40nm
        const double r = i * dr;
        rZn.set( i, ((20>r)? 300: 0) ); // concentration in uM
        rCu.set( i, ((20>r)? 30 : 0) ); // concentration in uM
    }
    return std::array<T,2>{ T{
        new std::ofstream("1-Zn.dat"), diftrimat(nr), Dzn, rZn, gslvec(nr)
    }, T{
        new std::ofstream("1-Cu.dat"), diftrimat(nr), Dcu, rCu, gslvec(nr)
    }};
}

inline void reaction (std::array<T,2> & l , const size_t i , const double dt){
    const double Cu = l[0].r.get(i) , AB = l[1].r.get(i)
    , ABCu1 = l[2].r.get(i) , ABCu2 = l[3].r.get(i)
    , ABCu3 = l[4].r.get(i);
    dCu = -ABCukon *AB*Cu + ABCukoff *ABCu1
         + -ABCu13 *ABCu1*Cu + ABCu32 *ABCu3;
}

#elif EXPT == 2  // Reaction of Cu with AB

std::array<T,5> makel(const int nr, const double dr){
    gslvec rCu(nr), rAB(nr), rABCu1(nr), rABCu2(nr),rABCu3(nr);
    // Initial conditions
    for (int i = 0; i <nr; ++i){// vesicle diameter = 40nm
        const double r = i * dr;
        rCu.set( i, ((20>r)? 30 : 0) ); // concentration in uM
        rAB.set( i, 0.003); // concentration in uM
        rABCu1.set( i, 0); // concentration in uM
        rABCu2.set( i, 0); // concentration in uM
        rABCu3.set( i, 0); // concentration in uM
    }
    return std::array<T,5>{ T{
        new std::ofstream("2-Cu.dat") , diftrimat(nr), Dzn, rZn, gslvec(nr)
    }, T{
        new std::ofstream("2-AB.dat") , diftrimat(nr), DAB, rCu , gslvec(nr)
    }, T{
        new std::ofstream("2-ABCu1.dat") , diftrimat(nr), DAB, rABCu1 , gslvec(nr)
    }, T{
        new std::ofstream("2-ABCu2.dat") , diftrimat(nr), DAB, rABCu2 , gslvec(nr)
    }, T{
        new std::ofstream("2-ABCu3.dat") , diftrimat(nr), DAB, rABCu3 , gslvec(nr)
    }};
}

inline void reaction (std::array<T,5> & l , const size_t i , const double dt){
    const double Cu = l[0].r.get(i) , AB = l[1].r.get(i)
    , ABCu1 = l[2].r.get(i) , ABCu2 = l[3].r.get(i)
    , ABCu3 = l[4].r.get(i);
    dCu = -ABCukon *AB*Cu + ABCukoff *ABCu1
         + -ABCu13 *ABCu1*Cu + ABCu32 *ABCu3;
    dAB = -ABCukon*AB + ABCukoff*ABCu1
         + -ABCu13*ABCu1 + ABCu32*ABCu3;
    dABCu1 = ABCukon*AB*Cu - ABCukoff*ABCu1;
const double dABCu2 = ABCu12 *ABCu1 - ABCu21 *ABCu2 + ABCu32 *ABCu3;
const double dABCu3 = ABCu13 *ABCu1 *Cu - ABCu32 *ABCu3;
}  

inline void pulse (std::array<T,5> & l, const double Cu)
#endif

inline void reaction (std::array<T,3> & l, const size_t i, const double dt)
{ std::ofstream * fout = new std::ofstream("3-AB.dat");
  fout->precision(8);
  return std::array<T,3>{
    T{ new std::ofstream("3-ABZn.dat") , diftrimat(nr), DAB, rABZn, gslvec(nr) }
    , T{ new std::ofstream("3-ABZn.dat") , diftrimat(nr), DAB, rABZn, gslvec(nr) }
    },

inline void pulse (std::array<T,3> & l, const double Cu)
#endif

inline void reaction (std::array<T,4> & l, const size_t i, const double dt)
{ std::ofstream * fout = new std::ofstream("4-Cu.dat") , diftrimat(nr), Dcu, rCu , gslvec(nr) 
  , T{ new std::ofstream("4-ABCu1.dat") , diftrimat(nr), DAB, rABCu1, gslvec(nr) }
  , T{ new std::ofstream("4-ABCu1.dat") , diftrimat(nr), DAB, rABCu1, gslvec(nr) }
}
inline void reaction (std::array<T,4> & l , const size_t i , const double dt){
    const double Cu = l[0].r.get(i) , ABCu1 = l[1].r.get(i) , ABCu2 = l[2].r.get(i) , ABCu3 = l[3].r.get(i);
    double dCu = -ABCu13 *ABCu1*Cu + ABCu32 *ABCu3 + -ABCu12 *ABCu1 + ABCu21 *ABCu2;
    double dABCu1 = ABCu12 *ABCu1 - ABCu21 *ABCu2 + ABCu32 *ABCu3;
    double dABCu2 = ABCu13 *ABCu1*Cu - ABCu32 *ABCu3;
    double dABCu3 = -ABCu13 *ABCu1*Cu + ABCu32 *ABCu3;
    l[0].r.set(i,Cu + dt * dCu );
    l[1].r.set(i,ABCu1 + dt * dABCu1);
    l[2].r.set(i,ABCu2 + dt * dABCu2);
    l[3].r.set(i,ABCu3 + dt * dABCu3);
}

inline void pulse (std::array<T,4> &, const double){}

#elif EXPT==5 // AB + Cu + HSA
std::array<T,7> makel(const int nr, const double dr){
    gslvec rCu(nr),rAB(nr),rABCu1(nr),rABCu2(nr),rABCu3(nr),rHSA(nr),rHSACu(nr);
    // Initial conditions
    for (int i = 0; i <nr; ++i){// vesicle diameter = 40nm
        const double r = i * dr;
        rCu .set( i, ((20>r)? 30 : 0) ); // concentration in uM
        rAB .set( i, 0.003); // concentration in uM
        rABCu1.set( i, 0); // concentration in uM
        rABCu2.set( i, 0); // concentration in uM
        rABCu3.set( i, 0); // concentration in uM
        rHSA .set( i, 5); // concentration in uM
        rHSACu.set( i, 0); // concentration in uM
    }
    return std::array<T,7> {
        T{new std::ofstream("5-Cu.dat") , diftrimat(nr), Dcu, rCu , gslvec(nr)}
        , T{new std::ofstream("5-AB.dat") , diftrimat(nr), DAB, rAB , gslvec(nr)}
        , T{new std::ofstream("5-ABCu1.dat") , diftrimat(nr), DABCu1, gslvec(nr)}
        , T{new std::ofstream("5-ABCu2.dat") , diftrimat(nr), DABCu2, gslvec(nr)}
        , T{new std::ofstream("5-ABCu3.dat") , diftrimat(nr), DABCu3, gslvec(nr)}
        , T{new std::ofstream("5-HSA.dat") , diftrimat(nr), DHSA, rHSA , gslvec(nr)}
        , T{new std::ofstream("5-HSACu.dat") , diftrimat(nr), DHSA, rHSACu, gslvec(nr)}
    };
}

inline void reaction (std::array<T,7> & l , const size_t i , const double dt){
    const double Cu = l[0].r.get(i) , AB = l[1].r.get(i) , ABCu1 = l[2].r.get(i) , ABCu2 = l[3].r.get(i) , ABCu3 = l[4].r.get(i) , HSA = l[5].r.get(i) , HSACu = l[6].r.get(i);
    double dCu = -ABCukon *AB*Cu + ABCukoff *ABCu1 + -ABCu13 *ABCu1*Cu + ABCu32 *ABCu3 + -ABCu12 *ABCu1 + ABCu21 *ABCu2 + ABCu13 *ABCu1*Cu - ABCu32 *ABCu3;
    double dAB = -ABCukon *AB*Cu + ABCukoff *ABCu1;
    double dABCu1 = ABCukon *AB*Cu - ABCukoff *ABCu1 + ABCu12 *ABCu1 + ABCu21 *ABCu2 + ABCu32 *ABCu3;
    double dABCu2 = ABCu13 *ABCu1*Cu - ABCu32 *ABCu3;
    double dABCu3 = ABCu13 *ABCu1*Cu - ABCu32 *ABCu3;
    l[0].r.set(i,Cu + dt * dCu );
    l[1].r.set(i,AB + dt * dAB);
    l[2].r.set(i,ABCu1 + dt * dABCu1);
    l[3].r.set(i,ABCu2 + dt * dABCu2);
    l[4].r.set(i,ABCu3 + dt * dABCu3);
    l[5].r.set(i,HSA + dt * dHSA);
    l[6].r.set(i,HSACu + dt * dHSACu);
}
const double dABCu2 = ABCu12 * ABCu1 - ABCu21 * ABCu2 + ABCu32 * ABCu3;
const double dABCu3 = ABCu13 * ABCu1 - ABCu32 * ABCu3;
const double dHSA = -HSACukon * HSA * Cu;
const double dHSACu = +HSACukon * HSA * Cu;

l[0].r.set(i, defpos(Cu + dt * dCu));
l[1].r.set(i, defpos(AB + dt * dAB));
l[2].r.set(i, defpos(ABCu1 + dt * dABCu1));
l[3].r.set(i, defpos(ABCu2 + dt * dABCu2));
l[4].r.set(i, defpos(ABCu3 + dt * dABCu3));
l[5].r.set(i, defpos(HSA + dt * dHSA));
l[6].r.set(i, defpos(HSACu + dt * dHSACu));
}

inline void pulse (std::array<T,7> & l, const double dt){

#elif EXPT == 6 // HSA + Cu
std::array<T,3> makel(const int nr, const double dr){

gslvec rCu(nr), rHSA(nr), rHSACu(nr);

// Initial conditions
for (int i = 0; i < nr; ++i){// vesicle diameter = 40nm
    const double r = i * dr;
    rCu.set(i, ((20 > r)? 30 : 0)); // concentration in uM
    rHSA.set(i, 5); // concentration in uM
    rHSACu.set(i, 0); // concentration in uM
}
return std::array<T,3>{
    T{new std::ofstream("6-Cu.dat"), diftrimat(nr), Dcu, rCu, gslvec(nr)}
    T{new std::ofstream("6-HSA.dat"), diftrimat(nr), DHSA, rHSA, gslvec(nr)}
    T{new std::ofstream("6-HSACu.dat"), diftrimat(nr), DHSA, rHSACu, gslvec(nr)}
};
}

inline void reaction (std::array<T,3> & l, const size_t i, const double dt){
    const double Cu = l[0].r.get(i), HSA = l[1].r.get(i);
    dCu = dHSA = dHSACu = -dCu;
l[0].r.set(i, defpos(Cu + dt * dCu));
l[1].r.set(i, defpos(HSA + dt * dHSA));
l[2].r.set(i, defpos(HSACu + dt * dHSACu));
}

inline void pulse (std::array<T,3> & l, const double dt){

#elif EXPT == 7
std::array<T,2> makel(const int nr, const double dr){

gslvec rZn(nr), rCu(nr);

// Initial conditions
for (int i = 0; i < nr; ++i){// vesicle diameter = 40nm
    const double r = i * dr;
    rZn.set(i, ((20 > r)? 300 : 0)); // concentration in uM
    rCu.set(i, ((20 > r)? 30 : 0)); // concentration in uM
}
return std::array<T,2>{
    T{new std::ofstream("7-Zn.dat"), diftrimat(nr), Dzn, rZn, gslvec(nr)}
    T{new std::ofstream("7-Cu.dat"), diftrimat(nr), Dcu, rCu, gslvec(nr)}
};
}

inline void reaction (std::array<T,2> & l, const size_t i, const double dt){
    const double Cu = l[0].r.get(i), HSA = l[1].r.get(i);
    dCu = dHSA = dCu = dCu;
l[0].r.set(i, defpos(Cu + dt * dCu));
l[1].r.set(i, defpos(HSACu + dt * dHSACu));
}

inline void pulse (std::array<T,2> & l, const size_t i, const double dt){
    for (int i = 0; i * dr < 300; ++i){ // vesicle diameter = 40nm
        l[0].r.set(i, 300); // concentration in uM
        l[1].r.set(i, 30); // concentration in uM
    }
}
#elif EXPT == 8 // pulse AB + Cu

std::array<T,5> makel(const int nr, const double dr){
    gslvec rCu(nr), rAB(nr), rABCu1(nr), rABCu2(nr), rABCu3(nr);

    // Initial conditions
    for (int i = 0; i < nr; ++i){// vesicle diameter = 40nm
        const double r = i * dr;
        rCu.set(i, ((20 > r)? 30 : 0)); // concentration in uM
        rAB.set(i, 0.003); // concentration in uM
        rABCu1.set(i, 0); // concentration in uM
        rABCu2.set(i, 0); // concentration in uM
        rABCu3.set(i, 0); // concentration in uM
    }
    return std::array<T,5>{
        T{{new std::ofstream("8-Cu.dat") , diftrimat(nr), Dcu, rCu , gslvec(nr)}},
        T{{new std::ofstream("8-AB.dat") , diftrimat(nr), DAB, rAB , gslvec(nr)}},
        T{{new std::ofstream("8-ABCu1.dat"), diftrimat(nr), DAB, rABCu1, gslvec(nr)}},
        T{{new std::ofstream("8-ABCu2.dat"), diftrimat(nr), DAB, rABCu2, gslvec(nr)}},
        T{{new std::ofstream("8-ABCu3.dat"), diftrimat(nr), DAB, rABCu3, gslvec(nr)}},
    };
}

inline void reaction (std::array<T,5> & l , const size_t i , const double dt){
    const double Cu = l[0].r.get(i) , AB = l[1].r.get(i),
    ABCu1 = l[2].r.get(i), ABCu2 = l[3].r.get(i),
    ABCu3 = l[4].r.get(i);
    const double dCu = -ABCukon * AB * Cu + ABCukoff * ABCu1
    + -ABCu13 * ABCu1 * Cu + ABCu32 * ABCu3;
    const double dAB = -ABCukon * AB * Cu + ABCukoff * ABCu1;
    const double dABCu1 = ABCukon * AB * Cu - ABCukoff * ABCu1
    + -ABCu13 * ABCu1 * Cu
    + -ABCu12 * ABCu1 + ABCu21 * ABCu2;
    const double dABCu2 = ABCu12 * ABCu1 - ABCu21 * ABCu2
    + ABCu32 * ABCu3;
    const double dABCu3 = ABCu13 * ABCu1 + Cu - ABCu32 * ABCu3;
    1[0].r.set(i,Cu + dt * dCu);
    1[1].r.set(i,AB + dt * dAB);
    1[2].r.set(i,ABCu1 + dt * dABCu1);
    1[3].r.set(i,ABCu2 + dt * dABCu2);
    1[4].r.set(i,ABCu3 + dt * dABCu3);
}

inline void pulse (std::array<T,5> & l, const double dr){
    for (int i = 0; i * dr < 20; ++i){// vesicle diameter = 40nm
        l[0].r.set(i, 30); // concentration in uM
    }
}
#else
#error "No experiment (EXPT) defined"
#endif
#endif

A.4.4 gslvec.h

#ifndef __gslvec__
#define __gslvec__

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#include <gsl/gsl_vector.h>

class diftrimat;

class gslvec {
public:
gslvec(size_t n);
gslvec(gslvec& &);
gslvec(const gslvec &);
gslvec& operator=(gslvec &);
gslvec();
gslvec& operator<<(gslvec & a);
inline double get(const size_t & i){return gsl_vector_get(vec, i);} 
inline void set(const size_t & i, const double & num){gsl_vector_set(vec, i, num );}
friend void diftrimatsolve(diftrimat &, gslvec & in, gslvec & out);
private:
gsl_vector *vec;
};
#endif // __gslvec__

A.4.5 gslvec.cpp

#include <algorithm>
#include "gslvec.h"
using namespace std;
gslvec::gslvec(size_t n){
vec = gsl_vector_calloc(n);
}
gslvec::gslvec(gslvec & & from){
vec = from.vec;
from.vec = 0;
}
gslvec & gslvec::operator=(gslvec & & from){
swap(vec, from.vec);
return *this;
}
gslvec::gslvec(const gslvec & from):
gslvec(vec->size)
{gsl_vector_memcpy(vec, from.vec);
}
gslvec::gslvec(){
if (vec != 0)
gsl_vector_free(vec);
}
gslvec & gslvec::operator<<= (gslvec & b){
gsl_vector_free(vec);
vec = b.vec;
b.vec = 0;
return *this;
}

A.4.6 diftrimat.h

#ifndef __diftrimat__
define __diftrimat__
```cpp
#include "gslvec.h"
#include "diftrimatsolve.h"

class diftrimat{
public:
    diftrimat(size_t n);
    diftrimat(size_t n, double D, double dr, double dt);
    void operator()(double D, double dr, double dt);
    friend void diftrimatsolve(diftrimat&, gslvec& in, gslvec& out);
private:
    const size_t Size;
    gslvec diag;
    gslvec upper;
    gslvec lower;
};

#endif //__diftrimat__

A.4.7 diftrimat.cpp

#include "diftrimat.h"
#include <cmath>
using namespace std;
diftrimat::diftrimat(size_t n): Size(n), diag(n), upper(n-1), lower(n-1){}
diftrimat::diftrimat(size_t n, double D, double dr, double dt)
    : diftrimat(n){
    operator()(D, dr, dt);
}

void diftrimat::operator()(double D, double dr, double dt){
    const double diagelm = 1+2*dt*D/(dr*dr);
    const double dr2 = dr * dr;
    for(size_t i = 0; i != Size-1; ++i){
        diag.set(i, diagelm);
        lower.set(i, dt*D*(1/(2*(i+1.5))-1)/dr2);
        upper.set(i, dt*D*(-1/(2*(i+0.5))-1)/dr2);
    }
    diag.set(Size-1, diagelm);
    lower.set(Size-2,-2*dt*D/dr2);
}

A.4.8 diftrimatsolve.h

#ifndef __diftrimatsolve__
#define __diftrimatsolve__

#include "gslvec.h"
#include "diftrimat.h"

void diftrimatsolve(diftrimat&, gslvec& in, gslvec& out);

#endif

A.4.9 diftrimatsolve.cpp

260
#include "diftrimat.h"

#include<gsl/gsl_linalg.h>

#include "diftrimat.h"
#include "gslvec.h"

void diftrimatsolve(diftrimat &trimat, gslvec &in, gslvec &out){
    const int err =
    gsl_linalg_solve_tridiag(
        trimat.diag.vec,
        trimat.upper.vec,
        trimat.lower.vec,
        in.vec,
        out.vec
    );
    if (err != 0)
        throw err;
    else
        return;
}

A.4.10 getr.hs

{-# LANGUAGE ScopedTypeVariables #-}
import Control.Applicative
import Data.List (elemIndex, transpose)
import Data.List.Split (splitOn)
import Data.Maybe
import Data.Traversable
import Control.Monad
import Prelude
import System.Environment (getArgs)

main :: IO()
main = do
    file <- head <$> getArgs :: IO (String)
    radii <- map read . tail <$> getArgs :: IO ([Double])
    contents <- filter (/= []) . map (map read) . map words . splitOn ""
                      . lines <$> readFile file :: IO ([[Double]])
    let times = map head . head . transpose $ contents
    let rs = map (!!1) $ contents
    let wantrs = catMaybes . map (flip find' rs) $ radii
    let getsrs = [flip (!!1)] <<< map snd wantrs
    let vals = map (map (!!2)) $ foreach getsrs $ transpose contents
    let ys = transpose $ times : vals
    writeFile (file ++ ".r.dat") . (++) "#time ".unlines . map (unwords . map show) $ map fst wantrs : ys

find' :: Ord a => a -> [a] -> Maybe (a, Int)
find' _ [] = Nothing
find' x xs = sequenceT (e,join n)
where
e = listToMaybe . dropWhile (<x) $ xs
     n = flip elemIndex xs <$> e
sequenceT :: (Maybe a, Maybe b) -> Maybe (a,b)
sequenceT (Just a, Just b) = Just (a,b)
sequenceT _ = Nothing
foreach :: [[a]->b] -> [a] -> [b]
foreach [] = []
foreach (f:fs) a = f a : foreach fs a
Appendix B

Other Information

This thesis∗ was written using \LaTeX{} using many \LaTeX{} packages (see source in footnote). The graphs where generated using gnuplot. GNU Parallel was use to parallelize the compilation of the gnuplot graphs, Zsh was used to locate files via globbing, GNU Core Utils were used to edit strings in pipes.

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The raw data for each figure† may be accessed from within the pdf version of the thesis by clicking the images. This requires a pdf reader that is capable of accessing embeded files. When multiple files are attached to single image, they are bound in a single file using tar, and then compressed using xz compression. The files can be extracted using GNU Tar via ‘tar -xJf filename.tar.xz’, or equivalent utility.

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Figure 2.8

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