Utilising fluorescence microscopy to visualise the dynamics and interactions of molecular chaperones and α-Synuclein.

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Utilising fluorescence microscopy to visualise the dynamics and interactions of molecular chaperones and α-Synuclein.

A thesis presented for the degree of

Doctor of Philosophy

by

Quill Bowden

Faculty of Medicine

Single Molecule Science

2015
In Parkinson’s Disease (PD), the protein α-synuclein and its early stage oligomers have been implicated as the primary cause of neuronal toxicity. Molecular chaperones play an important role in maintaining protein homeostasis in such diseases. The dynamic nature of the chaperone cycle and its interaction with the α-synuclein substrate makes it difficult to characterise by traditional biochemical techniques. Using a range of fluorescence microscopy approaches I show that α-synuclein exists in a concentration dependant dynamic equilibrium between monomeric and multimeric states, with rapid exchange of subunits between species. Dilution leads to rapid monomerisation and loss of structure followed by a slow recovery of higher order species. Chaperones from the Hsp40/Hsp70 machinery interact preferentially with the dissociated monomeric α-synuclein and delay the reassociation of subunits to higher order species implicating the multimer form as the potentially unknown native state of α-synuclein. Using single-molecule techniques I also studied the membrane binding properties of the larger α-synuclein oligomers associated with toxicity. This method allowed the visualisation of real-time of the perforation of lipid vesicles in the presence of α-synuclein oligomers and characterisation of the ability of chaperones to prevent this process. A greater understanding of the mechanism of chaperone mediated modulation of disease proteins and their preferential interactions will provide insights into the early stages of the progression of Parkinson’s disease.
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Abstract

α-Synuclein (αSyn) is an important protein in the pathogenesis of Parkinson’s disease (PD). The native and neurotoxic form of αSyn is currently under hot debate with new findings suggesting the presence of a stable multimer as the physiologically relevant form. Destabilisation of the multimer shifts the population to aggregation-prone monomers and subsequent soluble toxic intermediate species. The toxicity of these αSyn species derived from multimer destabilisation is thought to stem largely from their pathological association with membranes. Multimers however have been shown to play functional roles via membrane association. This area of study represents a new molecular pathway of native state to disease transitions of αSyn to be investigated; along with the potential role molecular chaperones can play in mediating these processes.

Using fluorescence lifetime imaging in this study it was demonstrated that αSyn multimers are highly dynamic with a rapid exchange between subunit populations. Fluctuation analysis indicates these αSyn populations to be comprised of a range of species from monomers through pentamers. In both these imaging techniques the αSyn multimer could be disrupted via dilution. This concentration dependence however appears to be reversible, with reformation of a multimeric form reoccurring over time. Interestingly, this reformation process can be delayed by the presence of molecular chaperones Hsc70 and its co-chaperone DNAJB1.

Intrinsic tyrosine fluorescence lifetime measurement reveals solvent quenching of native tyrosine in αSyn occurs upon dilution. This also indicates a concentration dependent loss of structure which is reversible (although not necessarily to its original structural form), as seen by the return of solvent shielding. Additionally, circular dichroism (CD) measurements show that the partial structure of the
multimer form present at higher concentrations is abolished by dilution and boiling. Assessment by CD shows no secondary structure is recovered, indicating reformation represents a potentially different conformation to the original structured multimer form. An aggregation assay using oligomer specific TPE-TPP dye further illustrates the time dependence of restructuring after dilution and highlights the significant changes increased temperature imparts on αSyn arrangements.

A single-molecule TIRF microscopy assay was developed to assess the membrane binding of αSyn monomers and oligomers. It was discovered that both species permeablise membranes via a similar rapid mechanism that leads to breakdown of the membrane structure. Incubation with chaperones was able to completely abolish this ability to disrupt the membrane, suggesting a role for Hsc70 and DNAJB1 in mediating these early stages of disease progression in PD.

αSyn multimers exist in a dynamic and delicate equilibrium which can be easily disturbed. The transition to association-prone monomers and downstream aggregate or oligomer forms of αSyn via destabilisation is a key mechanism responsible for toxicity in PD. Molecular chaperones play a pivotal role in mediating these early processes by interacting with monomers and oligomers and preventing membrane disruption, a process known to lead to cell death. Identifying the structure and dynamic elements of the αSyn multimer is an important step in understanding both native state molecular mechanisms and those that lead to disease progression.
Acknowledgements

A huge massive thank you to Till the most awesome supervisor for making my PhD an exciting adventure. Your love of science, ability to laugh even in the face of grant writing season and dedication is an inspiration. Thanks for your encouragement, open door and the creative freedom to pursue my random ideas. And for filling the cookie jar, a key factor in this thesis being completed.

To all the BMIF crew, but mostly to MacAttack who helped me turn every hair brained scheme into an imaging reality. You have gone above and beyond. I am so grateful for everything you have done for me. I hope that the entertainment you got from our microscopy sessions was worth the pain.

Thanks to Tobes and Anne Rich for your help with the CD measurements, analysis and falafels. Quyen and Wei for αSyn constructs and helpful discussions on preparation protocols.

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To my friends outside of the lab, I am sorry I have been a terrible friend for the past few years. Thank you for understanding all the missed occasions and cheap homemade gifts. They say friends are the family you choose for yourself and I am very lucky to have so many great people in my life.

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<td>Amp</td>
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<tr>
<td>αSyn</td>
<td>α-Synuclein</td>
</tr>
<tr>
<td>BS</td>
<td>Beam splitter</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>Cam</td>
<td>Chloramphenicol</td>
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<td>MWCO</td>
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<td>ONPG</td>
<td>Ortho-Nitrophenyl-β-galactoside</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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PC  Phosphatidylcholine
PDMS  Polydimethylsiloxane
PE  Phosphatidylethanolamine
PG  Phosphatidylglycerol
PH  Pinhole
PI  Phosphatidylinositol
PIE  Pulsed interleaved excitation
PLL-PEG  Poly-L-lysine poly(ethylene glycol)
PSF  Point spread function
ROS  Reactive Oxygen Species
SD  Standard deviation
SDS  Sodium dodecyl sulphate
SEC  Size-exclusion chromatography
smTIRF  Single-molecule total internal reflection fluorescence
SPAD  Single-photon avalanche photodiode detector
TPE-TPP  Tetraphenylethene triphenylphosphonium
UV  Ultraviolet

Amino acid abbreviations

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<td>Isoleucine</td>
<td>Xaa</td>
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<tr>
<td>Leu</td>
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<td>Leucine</td>
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Chapter 1 – Introduction

A caveat to improvements in modern medicine and increased life expectancy in the developed parts of the world is the growing population afflicted with diseases associated with aging. Particularly upsetting are diseases that cause decline in brain functions like memory loss and motor control, which have a huge impact both financially and emotionally on families worldwide. In Parkinson’s disease, as with most of these age related conditions, it is the decline in protein function which seems to be responsible for the onset of toxicity and disease. In the majority of patients with Parkinson’s the exact cause is unknown, with genetic mutations comprising only around 10% of Parkinson’s cases. The study of mutations which lead to more severe and earlier onset pathology has been used to provide a tractable way to study these diseases. Information gained through mutant studies can provide critical insight to the overall pathology in the majority of cases. This holds true for most neurodegenerative diseases, which are thought to stem from a common etiology. Understanding the early changes which shift a normal functioning protein onto a disease-causing pathway is one of the most critical pieces in the neurodegeneration puzzle.

In this research a biophysics approach was taken to try to understand the early changes occurring in protein populations which may initiate the progression of Parkinson’s disease and identify how key players mediate these processes. For a long time, biology and physics were two distinct entities co-existing with limited overlap in their application. Those days are now gone and interdisciplinary research is becoming increasingly prominent throughout the scientific community. The application of physics methodology to solving biological questions is flourishing, making significant contributions to our knowledge about a whole range of biological processes. Neurodegenerative pathways are made up of a range of different protein states and transitions which makes them difficult to characterise. In addition, the involvement of multi-component chaperone networks in preventing protein misfolding and aggregation in neurodegenerative pathways adds even more complexity. Fluorescence biophysical techniques allow
the dissection of these kinds of pathways to visualise individual steps and look at different processes at a molecular level.

In this introduction I will first provide a background to the key biological concepts of my research and then address how these were investigated outlining the rationale for using a range of biophysical techniques as well as the theoretical background to each of these approaches. I will present the systems that maintain protein homeostasis in the cell and will focus on chaperones as important factors which contribute to effective folding and maintenance of protein functionality. I will then review the mechanisms of chaperone function and the important roles that they play in neurodegeneration.

To put the medical significance of this work in context I will then describe different kinds of neurodegenerative diseases, what these have in common and how the shared features of these diseases could be critical to understanding the underlying disease mechanisms. More specifically I will discuss Parkinson’s disease and the Parkinson’s disease protein αSyn. With the functional form of αSyn currently being a controversial topic, I will consider both sides of this debate, summarise the current state of knowledge and identify the key open questions in the field.

I will then provide the basic background on the techniques that were used in this research. This will include a brief outline of fundamental concepts, in particular fluorescence and the principles of modern microscopy before describing each of the techniques used in this research. I will provide a rationale for choosing each technique, brief background to methodology and then outline the approaches used for analysis.

Finally I will summarise the aims of this research and why these questions are so important to the scientific community.
1.1 Proteostasis

To understand the initial changes that lead to neurodegeneration we must first think about the protein network as a whole. There are over 10 000 different proteins in mammalian cells. These proteins are structurally diverse and complex macromolecules and are involved in almost every essential biological process occurring within the human body. Proteins for the most part are highly dynamic, existing in flexible conformations which continuously fluctuate and are only just thermodynamically stable under physiological conditions. The stability and hence functionality of proteins can be quite easily disturbed and cells have therefore developed a number of elegant approaches to maintain homeostasis within their proteome (proteostasis).

Proteostasis is maintained through a complex network incorporating hundreds of proteins which work together in a highly organised manner (Figure 1-1). This network is composed of 332 chaperone genes as well as 30 components in the autophagy system and around 600 components in the ubiquitin-proteasome system (UPS). Molecular chaperones are the first to get involved, helping to fold nascent chain polypeptides during synthesis by interacting with exposed hydrophobic patches. This occurs while the rest of the protein is still being synthesised thereby preventing these exposed regions from unfavourable interactions which lead to aggregation. Chaperones also play an important role in other stages of a protein lifecycle by refolding proteins that may have become unfolded due to stress. When the chaperone refolding network is overwhelmed, or proteins are beyond rescue, the UPS and the autophagy systems come into play and remove aggregates or misfolded proteins which have been targeted for recycling and degradation. Together these systems successfully allow propagation, refolding and recycling of proteins to keep the cell functioning as it should.
Figure 1-1 The proteostasis network. This network encompasses molecular chaperones, the UPS and autophagy systems. Chaperones contribute by catalysing folding of newly synthesised nascent polypeptides, remodelling of misfolded or unfolded protein states and disaggregation of larger oligomers, aggregates or fibrils. Chaperones will also target certain aggregates or misfolded states for degradation. Protein degradation is mediated by the UPS and the autophagy system. Reproduced with permission from Hartl et al.²

It has become increasingly evident that age is an important factor in protein aggregation. Disorders of proteostasis deficiency are some of the most prevalent and debilitating conditions in the modern era. These include neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's Disease which result from a toxic gain-of-function and also include loss-of-function diseases such as cystic fibrosis⁷⁻⁹ which effects other regions of the body. Over time the different systems in the proteostasis machinery deteriorate or become overloaded and the ability to mediate protein misfolding and aggregation declines³,¹⁰. Additional stress factors such as chemical and UV exposure or disease causing mutations increase the pressure on the proteostasis system through higher proportions of misfolded or aggregated states or sequestration of vital cellular components into aggregates. This leads to a more rapid decline in proteostasis capability as shown by the earlier onset of diseases like Parkinson's when familial mutations are present¹¹. Understanding how to maintain proteostasis is a challenging yet pivotal field of
research. Because so many of these types of diseases are thought to stem from a common cause, understanding the molecular basis of disease and developing new treatments or biomarkers for detection could have a huge impact not just on one but many of these conditions. In this research the focus will be on the molecular chaperone component of the proteostasis network and how these proteins try to prevent the onset of neurodegeneration by catalysing protein folding, maintaining native state protein conformations as well as inhibiting the early stages of protein aggregation. In order to fully appreciate the role of proteostasis it is important to understand more about protein folding and the complexity of navigating the protein folding landscape.

1.1.1 Protein Folding and Unfolding

Thousands of proteins in mammalian cells are coded by mRNA, which is translated by the ribosome. The speed of protein synthesis can effect de novo folding of nascent peptide chains as they emerge and fold into their functional three dimensional polypeptides\textsuperscript{12}. As the amino acid chain emerges from the exit channel of the large ribosomal subunit long range interactions between protein domains are unable to occur, leaving the protein partially unfolded as the remainder of the sequence is being synthesised\textsuperscript{12}. In the crowded cellular environment where protein concentrations can reach up to 350 g L\textsuperscript{-1} the exposed hydrophobic patches within a nascent chain are extremely susceptible to aggregation\textsuperscript{13}. Protein folding has been shown to occur spontaneously however in vivo this would happen so slowly that the majority of proteins would never reach their functional end state\textsuperscript{5}. Small proteins of less than 100 amino acids can sometimes fold spontaneously on a biologically relevant timescale but large proteins or those with multiple domains would take minutes if not hours to fold without assistance. Anfinsen’s principle, established using ribonuclease, showed that all the required information to generate the folded native state of a protein is encoded within the polypeptide sequence\textsuperscript{14}. The assistance of other factors such as chaperones however is required to not only prevent non-native interactions
During the folding process but mainly to speed up protein folding to make it a biologically feasible procedure\textsuperscript{15}.

For the last 25 years, the energy landscape theory has provided a framework for thinking about protein folding\textsuperscript{16–18}. The conformational freedom of polypeptide chains means that entropy plays an extremely important role in protein folding. Figure 1-2 shows a protein as it explores the rugged funnel-shaped potential energy surface in an endeavour to find its native state, i.e. the conformation with the lowest free energy conformation\textsuperscript{19}. Protein folding can occur via an extremely vast number of conformational arrangements. It would take orders of magnitude longer than the age of the universe to sample all potential conformations, making it simply impossible during a normal folding pathway\textsuperscript{20}. To overcome this, discrete segments within a protein sequence will first independently sample a number of conformations to find the lowest energy state and then these can interact to drive the folding of the protein towards the lowest free energy conformation\textsuperscript{21}. As the number of native interactions increases the chain collapses and the potential conformations available become restricted, allowing the native state to be reached more rapidly.
Figure 1-2 Schematic of the free-energy landscape sampled during protein folding. The free-energy landscape is represented by a funnel-like surface which is explored by proteins as they progress towards their native state conformation. As proteins traverse the folding landscape they can become kinetically trapped in high-energy conformations and begin to accumulate. In order to successfully reach their native state conformation many proteins require the help of chaperones to overcome the kinetic barriers on the pathway to the native state. Reproduced with permission from Hartl et al.2

Intriguingly, there is also a large number of proteins that never acquire their native states during the initial folding process. Despite their lack of structure intrinsically disordered proteins (IDPs) can play critical roles in many cellular processes22 such as signalling and macromolecular recognition23. IDPs may also adopt their functional conformation upon interaction with binding partners and it is hypothesised that the code for folding of these proteins is only completed after this interaction24. The ability of IDPs to interact with multiple binding partners is thought to be derived from this property of adopting a conformation encoded in two or more partner proteins25. There appears to be some intrinsic property of
both IDPs and proteins which have not yet folded which are not fully understood\textsuperscript{26,27}.

Neurodegenerative disorders are often characterised by misfolding of disease proteins. During protein folding the free-energy protein folding landscape may be highly populated by folding intermediate states. These states can include on-pathway folding intermediates or misfolded and oligomeric species on path to forming aggregates and fibrils. Misfolded species are kinetically trapped and accumulation of these species can overwhelm proteostasis systems designed to help overcome the kinetic barriers of protein folding. The soluble oligomeric species on pathway to fibrillisation, for example, are directly linked with toxicity\textsuperscript{28}. These can be generated from a range of different proteins such as amyloid-β and αSyn which are associated with disease. Molecular chaperones are crucial to protein folding as they allow proteins to successfully navigate the folding-energy landscape, helping to overcome kinetic barriers which can trap proteins as potentially toxic or aggregation-prone folding intermediates. When chaperone functionality decreases intermediate states accumulate, which can eventually aggregate in a concentration dependent manner\textsuperscript{29}.

### 1.1.2 Molecular Chaperones and Heat-shock Proteins

Molecular chaperones are ubiquitously expressed throughout the body and are involved in all stages of a protein’s lifecycle including folding, unfolding, maintaining stability, correct formation of newly synthesised nascent polypeptides and membrane transport\textsuperscript{30}. Although essential in the heat-shock response, most chaperones are actually present under physiological conditions and are pivotal to normal cellular processes\textsuperscript{31}. The importance of chaperones in maintaining proteostasis is highlighted by their up-regulation in response to even the slightest change in physiological conditions such as an elevated temperature\textsuperscript{32}. Molecular chaperones often function in complex pathways which can include a range of co-chaperones, nucleotide exchange factors and other chaperone networks. They are implicated in a variety of diseases and disorders through over or under-expression
and have also been associated with pathogenesis in some disease states where they become entangled in aggregating protein complexes\(^33\).

Molecular chaperones were unknowingly first observed as “puffing” in response to heat-shock in fruit flies in the 1960's and in the mid 1970’s in *Drosophila*\(^34\). Around the same time as the heat-shock response was being observed in *Drosophila*, researchers in other fields were wondering about a missing component that seemed essential to protein folding. Just a few years before the discovery of the ‘puffing’ response the Anfinsen’s principle on the blueprint for spontaneous protein folding being contained in the amino acid sequence had been published; however subsequent attempts at purification of other proteins had resulted mostly in misfolding or aggregation\(^35,36\). Proteins just did not seem to fold on their own, the way that they should have. There was a critical factor missing. With some proteins being damaged and others being upregulated during heat-shock, the connection between protein folding and the protective nature of proteins induced during the heat-shock response was inevitable\(^37\).

**Heat-shock Proteins**

During times of stress there is an up-regulation of chaperone expression. Heat shock proteins (HSPs) are a major class of chaperones and occur as both constitutively expressed and stress inducible forms. HSPs are comprised of a number of families which are categorised based on their molecular weight. These families include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small heat-shock proteins (sHSPs)\(^33\). Similar structural and functional properties are well conserved within these families; however there is still a lot to divulge about the mechanisms and roles of many of these proteins. These types of chaperones typically function by either foldase or holdase activity. Holdases such as the sHSPs function in the absence of ATP and interact with partially folded intermediates, essentially “holding on” to substrates and maintaining them in a folding competent state\(^38\). This holdase activity will occur until an energy depleted environment is restored to normal and the substrate can be delivered to other “foldase” chaperones such as Hsp70 for active refolding\(^39\).
Heat shock proteins are found primarily in the cytosol, endoplasmic reticulum and mitochondria and are often localised to regions of protein instability and macromolecular crowding. Whilst individual families may primarily be involved in different processes, overall the chaperone network is interconnected with each family stepping in at different points along the aggregation process or under certain cellular conditions. Given the importance of the interconnected networks in chaperone function a brief outline of the different heat-shock families will be given in the context of how they interact with the Hsp70/40 chaperone cycle which is the focus of this research.

**Hsp100**

The Hsp100 family are members of a superfamily of hexameric ATPases which are associated with a range of cellular activities. This family has been found to be important for solubilisation and degradation and its function depends largely on the other co-factors or networks that it associates with. In the presence of the other chaperones, such as the Hsp70/40 systems, solubilisation and disaggregation occur. In bacteria, yeast and plants, the disaggregation machinery is extremely important for maintaining thermotolerance and resistance to stress. Proteins in these systems undergo far more severe fluctuations in stresses like temperature and ionic strength compared to eukaryotic proteins. In these systems Hsp100s disaggregate by extracting individual proteins from aggregates and passing them through their central pore. This occurs via a “bichaperone” system, where disaggregase activity is only observed in the presence of the Hsp70/40 machinery.

**Hsp90**

Hsp90 is an abundant chaperone that facilitates folding of a diverse range of substrate proteins. Hsp90 has more than a dozen co-factors and co-chaperones and these bind to their preferred conformations of the dimeric Hsp90. The specificity of co-factors or co-chaperones that bind to Hsp90 impart different functionality. Tetratricopeptide repeat (TPR) domain proteins such as HOP (Hsp70/90 organising protein) interact with the C-terminal EEVD motif of
Hsp90. Hsp70 also has an EEVD motif and it allows a linkage which seems to facilitate substrate transfer between the two chaperones\textsuperscript{53}.

**Hsp60**

Hsp60 is a mitochondrial chaperonin which is essential for transport and refolding of proteins between the cytoplasm and the mitochondrial matrix\textsuperscript{54}. The *E. coli* chaperonin GroEL is the most well-known and extensively studied of the chaperonins and its method of capturing its polypeptide substrates into an enclosed cavity with a capping cofactor GroES has been well characterised\textsuperscript{55,56}. These constitute a conserved class of large double-ring complexes of around 800 kDa with a central cavity\textsuperscript{57}. The Hsp60s perform a similar function to the Hsp70/40 system in different compartments of the cell\textsuperscript{58} and can function in conjunction to mitochondrial Hsp70\textsuperscript{59}.

**sHSPs**

The sHSP family are the first line of defence for cells to prevent aggregation\textsuperscript{60}. They are predominately holdases, interacting with partially unfolded proteins to prevent their aggregation. sHSPs, specifically Hsp27 and αB-crystallin have been shown to be extremely important in neuroprotection by maintaining substrate solubility until it can be actively refolded by the Hsp70/Hsp40 system\textsuperscript{61}. sHSPs function independently of ATP, so in ATP depleted systems they play a crucial role in stabilising proteins until the system is restored\textsuperscript{62}.

**Chemical chaperones**

There are also other kinds of chaperones, such as chemical or small molecule chaperones which perform similar functions as the molecular form\textsuperscript{63}. These hold promise as potential targets for therapeutic intervention however more detail on the exact mechanisms of chaperone function would make this a more feasible approach\textsuperscript{64}. These kinds of chaperones are outside the scope of this research but for more detail there is a number of interesting reviews on their development\textsuperscript{65–67}.

Overall it is the interconnectedness and diversity of substrate recognition in the chaperone network which is crucial to maintaining proteostasis. All of these
chaperones, as described have the ability to work in conjunction with the Hsp70/Hsp40 system in different ways. The Hsp70 and Hsp40 families were chosen for this research as they are critical to the early stages of protein unfolding and disturbances of native state proteins which occur at the onset of neurodegeneration.

1.1.3 The Hsp70/40 System

The Hsp70 family is comprised of 8 different human proteins, that include both stress-inducible and constitutively expressed forms\(^{68,69}\). There are specialised isoforms of Hsp70 which localise to the endoplasmic reticulum or mitochondria. Additionally, there are forms of Hsp70 which are expressed in different tissues throughout the body\(^{70}\). The cognate family members play predominately house-keeping roles such as folding of nascent chain polypeptides, assisting membrane translocation and uncoating of clathrin-coated vesicles\(^{71}\), whereas the stress inducible forms are more critical to protein refolding and preventing aggregation\(^{72,73}\). The nomenclature for the Hsp70, as with other chaperone families, can be extremely confusing. The most commonly used forms for research of heat-stress are the HSPA1 (Hsp70) and for understanding the basal function of chaperones, the HSPA8 (Hsc70) form. In this work, the Hsc70 protein was used as in the early stages of αSyn perturbations this chaperone would be present. However, it would be expected that Hsp70 and Hsc70 interactions with αSyn would be fundamentally the same due to the highly conserved nature of the Hsp70 family and the specificity of the interaction with αSyn binding sites.

There is a high level of sequence homology within the Hsp70 family across a range of species. Hsc70 is a 646 amino acid protein comprised of two domains, the N-terminal nucleotide binding (or ATPase\(^{74}\)) domain (NBD) from residues 1-381 and the substrate binding domain (SBD) from 382-646\(^{75,76}\). Both domains are critical for chaperone function. The ATPase domain region has an ATP binding cleft in the centre and is connected to the SBD by a hydrophobic linker. The SBD contains two components, the peptide binding domain consists of β-sheets and has
a peptide binding cleft; and the α-helical lid which is a smaller flexible domain able to open and close over the peptide binding cleft. An EEVD conserved motif is also at the end of this lid region and is important for both interactions with other chaperones and stimulation of substrate delivery or exchange\textsuperscript{53,77}.

Hsp70 family chaperones all have the same ATP-dependent binding and release cycle. The on/off rates are rapid in the ATP bound state and slow in the ADP bound state. This is primarily due to a conformational change, i.e. the closing of the α-helical lid over bound substrates which decreases the propensity of substrate exchange. Substrate proteins are bound through exposed hydrophobic amino acid side chains which interact with the SBD.

**Figure 1-3. The Hsp70 Reaction Cycle.** (1) Delivery of substrate to ATP-bound Hsp70 by Hsp40 (J-domain). (2) Hydrolysis of ATP to ADP, accelerated by Hsp40 induces closing of the α-helical lid and tighter binding of substrate by Hsp70. Hsp40 dissociation from Hsp70. (3) Dissociation of ADP is catalysed by nucleotide exchange factors (NEFs). (4) Opening of the α-helical lid is induced by ATP binding and results in release of the substrate. (5) Released substrate either folds to the native state (N), is transferred to downstream chaperones or is rebound to Hsp70. Adapted and reproduced with permission from Hartl \textit{et al}\textsuperscript{78}.

The regulation of substrate binding is controlled primarily by the interaction of nucleotide exchange factors (NEFs)\textsuperscript{79–81} and Hsp40 (J-domain proteins, in this
study DNAJB1). Hsp40 proteins recruit or deliver sequestered substrates to Hsp70, stimulate ATP hydrolysis and convert the Hsp70 to the ADP-bound form (Fig 1-3). In the ADP-bound form the helical lid is closed over the substrate binding cleft resulting in stable binding of the substrate protein⁷⁹. Dissociation of ADP is catalysed by NEFs leading to ATP binding, opening of the lid and substrate release. In the ATP-bound form the on off rates for the substrate is high⁸². Substrate proteins may be released in their native state, targeted to other proteins for further processing or rebound for an additional cycle⁷⁸. The interaction of Hsp70 with Hsp40 proteins and other co-factors facilitates diverse functionality and broad substrate specificity⁸³.

There are 41 putative family members of human J-domain proteins which can all interact with the same Hsp70⁸⁴. The Hsp40 proteins have a number of different domains, including the J domain which is responsible for substrate interactions and is highly variable, to allow for the promiscuity required to be able to target a whole range of substrates. There are also a range of other supportive proteins which help facilitate homeostasis including Hip, Hop and CHIP. These are all involved in the Hsp70 chaperone cycle with roles varying from binding and stabilising the ATP-bound state of Hsp70, facilitating substrate transfer between Hsp70 and Hsp90 to target proteins for lysosomal degradation and ubiquitination for targeting to the proteasome⁸⁵–⁸⁷. Overexpression or increased levels of Hsp40 or NEFs in terms of their stoichiometry can lead to prevention of substrate capture or reduced refolding capacity. This occurs due to the increases in frequency of release which does not provide adequate time for correct folding to occur⁸⁸.

1.1.4 Chaperone Networks in Neurodegeneration

The potential role of chaperones as mediators of neurodegenerative disorders was originally suggested based on studies in flies which develop Parkinson’s disease symptoms. These studies revealed that when αSyn is expressed in neurons in the presence of Hsc70, no neuronal loss is seen⁸⁹. These findings were some of the pioneering studies that lead to research in the area of
the effects of Hsc70 on αSyn aggregation and toxicity\textsuperscript{90}. In these studies it was also shown that αSyn inclusions were still evident when Hsc70 was co-expressed, revealing that the inclusions were not the neurotoxic form and that a soluble oligomer was the most likely culprit for cell death. The importance of chaperones in neurodegeneration was further supported by identification of members of various heat shock protein families in Lewy bodies, the amyloid protein aggregates primarily associated with PD\textsuperscript{91–93}. The Hsp70 system is inherently involved in the task of chaperoning neuronal disease proteins and sometimes gets caught up in the process.

1.2 Neurodegeneration, Parkinson’s Disease and α-Synuclein

1.2.1 Neurodegeneration

Protein conformational disorders such as Parkinson’s, Huntington’s and Alzheimer’s disease show regular patterns in terms of amyloid formation, common sequence features and the intermediate oligomeric form often being the main pathogenic species\textsuperscript{94}. The similar amyloid β-sheet fibril end point in these diseases is a reasonable indication of the common mechanism where a kinetically trapped intermediate form causes toxicity. Many reviews and scientific communications discuss the potential of a “common mechanism and common cure” \textsuperscript{95,96}. The main characteristic features observed in these diseases are oxidative damage and loss of cells. These are heavily associated with one of the greatest risk factors of neurodegenerative disease, aging\textsuperscript{97}. What is fundamentally missing is a universal insight into the underlying causes of neurodegeneration which can direct therapeutic design.

In Huntington’s disease the expansion of glutamine tracts above a critical number on a protein sequence leads to instability and aggregation\textsuperscript{98–100}. In motor neuron disease (MND), a variety of proteins have been identified as causing
disease, and pathology occurs in a range of ways depending on which aggregating protein is causing disease\textsuperscript{101-103}. Fascinatingly, other less commonly discussed neurological disorders like schizophrenia, bipolar disease and depression can also stem from the disruption of normal protein function\textsuperscript{104}. Misfolding diseases lead to symptoms such as tremors, memory loss, loss of motor control, confusion and other neurological symptoms\textsuperscript{105}. Protein aggregation leads to these symptoms by accumulating in regions of neuronal transport, preventing the passage of dopamine transfer, interfering with signal pathways and blocking normal processes\textsuperscript{106}.

### 1.2.2 Parkinson’s Disease

Parkinson’s disease (PD) is the second most prevalent neurological disorder worldwide, affecting around 6.5 million individuals. It is largely idiopathic, with up to 95% of cases having no specifically known cause. The genetic forms of PD are quite rare, and are either attributed to point mutations\textsuperscript{107-109} or duplications and triplications in the SNCA gene coding for the protein αSyn \textsuperscript{110}. The PARK, PINK and LRKK2 genes have also been shown to be linked to familial forms of PD\textsuperscript{111}. Lewy bodies, the amyloid protein aggregates which canonically have been the defining pathological hallmark of PD are comprised primarily of αSyn. Originally it was thought that Lewy bodies were the cause of PD, however current understanding implicates these aggregates as merely the innocent end product, potentially even playing a beneficial role by decreasing levels of the free soluble oligomeric forms\textsuperscript{112}. The search for the exact structural conformation of the cytotoxic species is now centred on understanding the different soluble oligomeric intermediate states of αSyn.

PD is caused by the degeneration of dopaminergic neurons in the substantia nigra in the basal ganglia. αSyn primarily localises in the pre-synaptic terminals of these affected neurons\textsuperscript{113}. These neuronal regions are responsible for vesicular trafficking of dopamine used in communication of the chemical signal pathways.
and/or blocking normal movement throughout the body. As no preventative treatments have been discovered, PD treatments involve restoration of depleted dopamine levels and ameliorating other symptoms\textsuperscript{114,115}. Surgical treatments such as deep brain simulation may also be used as an alternative or in addition to drug treatments\textsuperscript{116}. Stem cell therapy approaches are also under development\textsuperscript{117}, however in light of recent findings implicating a prion-like mechanism of disease spread these may not be the most promising as a long term cure\textsuperscript{118}.

1.2.3 α-Synuclein

αSyn is a 140 amino acid protein which is abundantly expressed, comprising up to 1% of all proteins found in neurons\textsuperscript{119}. The basic sequence of αSyn includes; an N-terminal domain (residues 1-65) which can adopt an α-helical conformation upon lipid interaction and is important for membrane binding; a hydrophobic non-Aβ component (NAC) (residues 66-95) which regulates self-assembly and fibrillisation, and a C-terminal domain (residues 96-140) which is largely unstructured\textsuperscript{120}. The N-terminal and NAC region contain a set of 7 imperfect repeats (KTKEGV) which encode amphipathic α-helices\textsuperscript{121}. Mutations in this region put structural limitations on the formation of α-helical arrangements leading to a higher likelihood to form β-sheets and a propensity to increased aggregation. Interestingly all but one familial mutation (A30P) identified thus far, cluster in this region\textsuperscript{122}.

αSyn Multimers and Controversy

Until recently αSyn was categorically defined as an intrinsically disordered monomer, after initial observations of recombinantly expressed natively unfolded protein \textit{in vitro}\textsuperscript{123}. Subsequently purification protocols used to obtain αSyn have included a range of chemical treatments, boiling or precipitation steps. As it was thought that the protein was unstructured, these steps were considered to have no effect on intrinsic properties of recombinant αSyn. Studies of the monomer have provided a wealth of knowledge on a broad range of properties. After the initial publications by the groups of Selkoe\textsuperscript{124} and Hoang\textsuperscript{125} identified the multimeric
form, an in-depth study encompassing a number of research groups sought to examine their methods, subsequently refuting the claims of the multimer\textsuperscript{126}. However, on closer inspection Lashuel and co-workers had many adaptations from the protocols originally used to obtain the multimer species. New evidence however continues to amass which indicates αSyn actually exists as a helically folded multimer which is resistant to aggregation\textsuperscript{124,125}. In order to obtain these αSyn forms, gentler purification protocols have been adopted. In this new paradigm αSyn multimers are proposed to be the physiological form which can become destabilised to initiate the formation of association-prone monomers and various downstream aggregates (Fig 1-4). All of the parameters defined by the monomer studies such as self-association, aggregation pathways and membrane associations can now be used, in conjunction with knowledge of the multimer, to start defining a clear image of the early transitions from native state αSyn to toxicity.

Figure 1-4 A simplified schematic representation of αSyn aggregation pathways. αSyn exists in an equilibrium of monomer and multimer species. As yet uncharacterised perturbations are thought to shift this equilibrium to a range of association-prone monomer forms. It is then possible for different association-prone monomers to generate a variety of aggregation pathways and end-products. The three major products of aggregation include amorphous aggregates, soluble oligomers (which includes globular and annular oligomers), and amyloid fibrils (which can occur in different morphologies). Cross-talk between these pathways and interconversion of species may occur in the cell. Adapted and reproduced with permission from Uversky\textsuperscript{127}.
αSyn multimers have now been observed in a range of samples including recombinant protein, neurons, red blood cell extracts, human brain tissues and in live cells\textsuperscript{125,128–130}. In addition, molecular modelling based on NMR, small angle X-ray scattering and residual dipolar couplings data has also revealed a multimeric species\textsuperscript{131}. An αSyn multimer represents a more thermodynamically favourable species inside cells with multimerisation leading to organisation into α-helices with polar residues solvent exposed and the burying of hydrophobic domains\textsuperscript{132}. For αSyn, which is present at high physiological concentrations, the burying of hydrophobic residues in a multimer assembly could be vital, as these are known to lead to aggregation. What remains to be characterised are the underlying dynamic properties of the multimer species, the interactions of its subunits and what disturbances shift this form into association-prone monomers and toxic oligomeric forms.
**Figure 1-5 Comparison of models for αSyn multimer structure.** (a) The structure of membrane bound monomeric αSyn, where the blue region is the N-terminus. This image was generated from the coordinates of a conformer calculated from NMR data retrieved from the PDB (accession number 1XQ8). (b) The tetrameric helix-turn-helix model proposed by Wang et al. This structure shows two amphipathic α-helices connected by a linker region. The C-terminal extension (residues 103-140) is proposed to be largely unstructured and has been omitted from this model. (c) Ensemble of states representing the multimer determined by NMR spectroscopy and molecular modelling as proposed by Gurry et al. (d) Model of self-association which occurs via hydrophobic contacts to form multimer structures. Proposed by Ullman et al. All figures are reproduced with permission.

**αSyn Association with Membranes**

Studies of the unstructured monomer have garnered an extensive amount of information on the membrane binding properties of the αSyn protein. As monomers likely represent the association-prone form derived from destabilisation of multimers, this provides information on a critical step in the
aggregation pathway. The disordered αSyn monomer undergoes conformational changes upon binding to lipids. This includes adoption of an α-helical conformation when associating with membranes, vesicles, micelles and other widely used mimics such as sodium dodecyl sulfate (SDS). The adopted structure can exist as either a broken-helix made up of two curved anti-parallel helices with a linker region, or an extended helix conformation and interacts with the membrane via a shallow insertion of a few N-terminal residues into the lipid bilayer. Monomer (and oligomer) membrane interactions can disrupt the membrane via either membrane thinning or pore-formation mechanisms.

The KTKEGV repeats in the N-terminal/NAC region are critical not only for membrane binding but are also key mediators of multimerisation. Mutations in these repeat regions cause a reduction in multimerisation and increase in neurotoxicity. In a model of aggregation on lipid membranes, free αSyn monomers slowly build up at a single nucleation point on a membrane, whereby accumulation of αSyn is localised to one region. Oligomers may also interact via this mechanism, where the monomer serves as a nucleation point to promote association in a defined region on the membrane. The soluble toxic oligomer species of αSyn often exists as annular structures which are able to permeabilise membranes, leading to cell death. It has been suggested that monomers can transiently interact with membranes, inducing a conformational shift to a structured form that can then associate into a multimer. If this interaction was disturbed or prolonged, it may lead to that monomer becoming a nucleation point.

It has been shown that αSyn can also be transmitted extracellularly and induce disease in healthy cells. A prion-like mode of infectivity has been suggested allowing αSyn aggregation to spread throughout the brain. Membrane disturbance by αSyn is thought to lead to the original externalisation of Syn from the cell.

Owing to the potential infective role of αSyn, understanding mechanisms behind the transition between multimer to monomer to disease seems more important.
than ever. In order to understand these mechanisms, it is essential to resolve the underlying processes at a molecular level.

### 1.2.5 Biophysics Methods to Solve Neurodegenerative Problems

As the need for temporal and spatial resolution of dynamic and complicated biological processes becomes more apparent, the fields of chaperone research and neurodegenerative research are turning towards biophysics to help illuminate the molecular mechanisms of these pathways. Biophysics along with more traditional biochemistry techniques provides the perfect complementary toolbox to resolve the underlying processes at each step of these problems. In many of these fluorescence methods the initial lengthy set-up and development stages of the technique are followed by rapid influx of novel insight and information on dynamics or molecular processes which were unattainable via traditional approaches.

Already chaperones have been extensively characterised using a range of techniques that look at fluctuations of populations\(^{143}\). Using single-molecule microscopy it was shown that there are both intra and inter-domain conformational changes that occur in the Hsc70 chaperone cycle. Parkinson’s disease and in particular αSyn is now being extensively studied using biophysics approaches \(^{144-146}\). This includes characterisation of the oligomerisation state of αSyn in cells\(^{147}\), on-surface aggregation of αSyn\(^{148}\), determining the molecular composition of oligomers\(^{149}\) and investigating the structure of membrane-bound forms of αSyn\(^ {150}\). Microfluidics in biomedical research\(^ {151}\) is also increasingly being adapted to answer question in research on neurodegenerative processes\(^ {152}\).

The benefits of fluorescence microscopy to capture these sorts of systems will be further outlined in Chapter 4 focused on characterising transient αSyn multimers and the effect of chaperones on oligomerisation pathways and in Chapter 5 focused on dissecting αSyn membrane association mechanisms. For a more comprehensive insight into these exciting experimental techniques,
biophysical approaches used to investigate αSyn are reviewed in Plotegher et al\textsuperscript{153} and for chaperone studies using biophysical techniques see Mashagi \textsuperscript{154}.

1.3 Fluorescence Microscopy

1.3.1 Overview of Fluorescence

Fluorescence microscopy is an invaluable tool which has provided access information on biological structures, interactions and dynamic processes\textsuperscript{155}. Advances in fluorescence microscopy has enabled the investigation of ensemble measurement and furthermore provided measurement down to single molecules.

“Figure has been removed due to copyright restrictions”

\textit{Figure 1-6 Perrin-Jablonski illustrating electronic transitions within a molecule.} Before the absorption of energy in the form of light (a photon), fluorophore molecule exists in an electronic arrangement known as the ground state. Upon excitation electrons in the fluorophore become excited and rise to a higher excited energy level. Fluorescence occurs when the excited electron transitions back to the ground state by emitting a photon via a radiative process. The fluorescence cycle occurs via three main steps being excitation, relaxation and emission. There are also other transitions possible between the vibrational energy states and singlet states as well as excursions into a different energy state known as the triplet state. Taken from Wegerhoff \textit{et al}\textsuperscript{156}.

The ground state of a fluorophore is represented by $S_0$. When a photon is absorbed by a molecule, there is a transition to a higher energy state (Fig 1-6, $S_1$). Excited singlet states also include “sub-levels” or vibrational energy levels and when a molecule absorbs a photon it is actually excited to a higher vibration energy level within the excited state. Before the transition back to the ground state the molecule relaxes to the lowest vibrational level (internal conversion). The transition from $S_1$ to $S_0$ is then associated with a loss of energy due to depopulation of the excited state by radiative (fluorescence) or non-radiative processes (quenching and relaxation)\textsuperscript{157}. During radiative relaxation (fluorescence) to the ground state there is an emission of a photon of lower energy\textsuperscript{158}. 

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Since fluorescence emission occurs primarily from the lowest $S_1$ vibrational state, the emission spectrum is independent of the excitation wavelength. Shifting the excitation wavelength alters the probability of excitation, and hence selection of an excitation wavelength close to the absorption maximum leads to higher emission intensity. The excited state may also be depopulated to the ground state via other pathways such as intersystem crossing, and quenching, for example via Förster resonance energy transfer (FRET) quenching. Intersystem crossing leads to spin conversion into a triplet state which is unfavourable, particularly in single-molecule imaging as it increases probability of photobleaching.

![Diagram of FRET](image)

**Figure 1-7 Schematic of the basic principle of Förster resonance energy transfer.** FRET is a non-radiative energy transfer between two fluorophores. It is distance dependent, with energy being transferred from a donor fluorophore (blue) to an acceptor fluorophore (red) by intermolecular long-range dipole-dipole coupling. FRET occurs typically within the intermolecular distance of 1-10 nm and can be used as a molecular ruler to determine distances between fluorophores in a range of fluorescence assays.

Instead of radiative relaxation to the ground state, a photon can also be transferred (absorbed) by another molecule. FRET occurs when a pair of fluorophores (donor and acceptor) are placed in close enough proximity (1-10 nm) such that photon emission from the donor can be used to excite the acceptor (Fig 1-7). This requires that the donor emission spectra overlaps with the acceptor excitation spectra (quantified by overlap integral $J(\lambda)$) and can be used to determine the distance between fluorophore dipoles$^{159}$ (Fig 1-8).
Fig. 1-8 Illustration of Stokes shift between excitation and emission spectra. This change in occurs due to the vibrational relaxation which occurs in the excited molecule singlet state prior to fluorescence emission. In order for FRET to occur there should be sufficient overlap between the emission spectra of the donor fluorophore and the excitation spectra of the acceptor fluorophore.

A critical experimental consideration in choice of fluorophores is therefore the overlap integral and quantum yield\(^{160}\). There is a plethora of synthetic dyes, fluorescent and photo-switchable proteins. All of which have characteristic profiles that absorb and emit energy at specific wavelengths, as well as have specific qualities which make them attractive to imaging.

Attractive properties of suitable fluorophores include a high molar extinction coefficient \((\varepsilon_{\text{abs}})\) and quantum yield \((\varphi)\). The photophysics of fluorophores must also be considered including how often the molecule visits the triplet state (blinking). There are measures that can help reduce these occurrences, such as the inclusion of an oxygen scavenger system and Trolox, a vitamin E derivative antioxidant, which prevents the formation of reactive oxygen species to help minimise photo-bleaching\(^{161}\). The size of the fluorophore is also an important consideration, with synthetic dyes being the preferred choice for dynamic or interaction studies due to their small size. Fluorescent proteins are larger, however they can be particularly beneficial for \textit{in vivo} studies where they are often expressed as fusion proteins to visualise processes in live cell experiments. Intrinsic fluorescence represents a unique ability to report on protein conformations and interactions without the addition of external probes. More on intrinsic fluorescence is provided in subsequent sections.
1.3.2 Principles of Microscopy

The objectives used for microscopy are essential for obtaining image clarity. The numerical aperture (NA) of a lens is a measure of the ability to gather light and resolve fine specimen detail. The width of the acceptance cone of the objective determines how much light contributes to the image formation (Fig 1-9). This is important for the resolution and contrast of the image. The higher the aperture the more oblique light rays can be collected. Immersion liquid reduces the refractive index mismatch. Immersion oils more closely match the refractive index of glass and while water is a worse match it better for imaging thick biological samples which are comprised mostly of water.

“Figure has been removed due to copyright restrictions”

Figure 1-9 Collection of light by objectives with different NA. The objective in a fluorescence microscope set-up has the important role of collecting as much of the fluorescence emission generated by the sample as possible. The width of the light acceptance cone determines how much light is able to be detected and used to build the image. The lower the NA, the less light is able to be collected. Taken from Rottenfusser et al\textsuperscript{162}.

Light is transmitted through a specimen and reconstituted on the image plane as a slightly blurred spot with diffraction rings; this is pattern is known as an Airy Disk. The Point Spread Function (PSF) is a 3D representation of the Airy Disk and is useful for determining the resolving power of a microscope and its different objectives. The size of the airy disk depends on wavelength and the NA of the objective\textsuperscript{163} (Fig 1-10).
The point spread function (PSF) and its effect on microscope imaging resolution. (a) The PSF is three-dimensional and is usually longer in the axial (z) dimension than in the lateral (xy) dimensions. (b) A single point-source of photons arrives at the detector as a broad intensity distribution, the size and shape of which is determined by the microscope’s PSF. When imaged in the focal plane, the bright central part of the PSF is called the Airy Disk. (c) When multiple photon sources are emitting simultaneously, e.g. fluorescent molecules on a surface, their respective PSFs overlap at the detector, becoming indistinguishable from each other, and generating a diffraction-limited image.

The resolution of an optical microscope is essentially a measure of the distance at which two points can be recognised as two distinctly separate entities. This is known as the Rayleigh criterion which stipulates points are resolvable if the maximum of one airy disc corresponds with the first minimum of the adjacent pattern. Resolution is limited by a number of factors including the sample to be studied, the magnification of the system, even the light waves themselves. Resolution is therefore said to be diffraction limited, due to the inherent physical nature of wave movement, obtaining the best resolution comes down to limiting the optical aberrations that can occur during the entire process; from sample preparation through to acquisition of images.

There is an ever increasing variety of imaging methods. These are often combined and adapted to suit the range of applications imaging is applied to in medical, biological, chemical or physics research. Advanced microscopy techniques are so varied that it is important to understand what is intended to be obtained from each experiment and tailor individual components such as fluorophores,
acquisition speed and excitation intensity, accordingly. An outline of the specific fluorescence methods chosen for this study is given in the following sections.

1.3.3 FCS, FCCS and PIE

Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) is a confocal technique which is commonly used to determine dynamic properties of freely diffusing particles. This type of measurement typically allows information on the weight and size of diffusing particles to be extracted by looking at the diffusion properties. FCS also allows the determination of information on molecular interactions such as binding. Typically, in an FCS measurement a high numerical aperture objective is used to focus the excitation beam to a diffraction limited spot of small volume. As molecules diffuse through the diffraction limited spot they emit a burst of photons. When molecules move in and out of the excitation volume it results in random fluctuations of fluorescence intensity, and from these fluctuations the time that the particle is within the excitation volume can be determined. Since, this volume is fixed this allows extraction of diffusion coefficients. FCS is a fundamental technique which in this study is combined with other microscopy techniques to provide information on dynamic protein interactions in solution.

The volume from which fluorescence is collected is defined by the PSF of the microscope and is known as the confocal volume. This is typically in the order of femtolitres and in the dilute solutions most commonly used in these measurements this equates to around one molecule in the detection volume at any time. The signal fluctuates around the average as molecules come in and out of the beam (Fig 1-11).
Figure 1-11 **An example intensity vs time schematic plot.** Over a given time period \((t)\) 6 molecules pass through the confocal volume giving rise to fluorescent signal. The intensity fluctuates as these molecules pass through the beam however this method allows the determination of an average fluorescent intensity by accounting for the variation around the mean intensity.

**Autocorrelation**

The autocorrelation function is applied in order to extract these “hidden” temporal fluctuations from the measured data. In order to determine the autocorrelation, the fluorescence intensity at time \(t\) is compared with itself at a later time point \((t + \tau)\). This time difference is often referred to as the lag time \((\tau)\) (Fig 1-12).

Figure 1-12 **Simplified image of how autocorrelation analysis is performed.** The fluorescence intensity of a signal at time \(t\) is compared with itself at later timepoints defined by \(t + \tau\). The time difference \(\tau\) is known as the lag time. Autocorrelation is therefore essentially just the correlation of a signal with itself at varying points in time.

The autocorrelation function is calculated by multiplying the intensity at time \(t\) with the intensity at a later lag time \((t + \tau)\). The average product of these two intensities is then divided by the square average intensity for each value of Tau squared. The autocorrelation function is given by:
\[ G(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t+\tau) \rangle}{\langle I \rangle^2} \]

Eq. (1)

Where \( G(\tau) \) is the autocorrelation function, \( \langle I \rangle \) is the average fluorescence intensity, \( \delta I(t) \) is the variance from the average at any point.

The intensity at time (t) is multiplied by the intensity at a later time point (t + \( \tau \)). The shorter the comparative time interval at which the signals are compared the higher the likelihood of correlation. As the lag time is increased (t + \( \tau + \tau \)), the likelihood decreases and there is a resultant decrease in the autocorrelation function. If the lag time is sufficiently long then the fluctuations will no longer originate from the same particles and an autocorrelation value of zero \( G(\tau)=0 \) will be obtained. The autocorrelation function allows non-random information (diffusion time) to be extracted from seemingly random intensity traces.

**Cross-correlation**

The principles of FCS were utilised in this work by performing a variant known as dual colour FCS, or fluorescence cross-correlation spectroscopy (FCCS). The principle is essentially the same, however the overlay is with two channels (two different fluorophores) instead of overlaying the molecule signal with itself (Fig 1-13). Two detectors are utilized in order to measure the differing spectral channels. A typical experiment would use a green emitting fluorophore and a red emitting fluorophore. Henceforth the subscripts \( g \) and \( r \) are used to denote the green and red channel respectively.
Cross-correlation is a measure of the correlation of two different signals (here represented as red and green) with each other at various points in time, as a function of the lag time $\tau$ relative to the other signal.

The normalized cross-correlation function is defined as follows:

$$G_{CC}(\tau) = \frac{\langle \delta I_g(t) \cdot \delta I_r(t + \tau) \rangle}{\langle I_g \rangle \langle I_r \rangle}$$

Eq. (2)

Where $G_{CC}(\tau)$ is the cross-correlation function, the intensity $\langle I \rangle$ and variance $\delta I(t)$ are as described in Eq.(1) however with green and red channels ($g$ and $r$).

Cross-correlation is essentially a measure of the synchronicity of fluorophores in two different channels. In this work fluorescence lifetime filtering (FLCS) was applied in order to eliminate spectral bleed-through in the cross correlation measurements, in this method each photon is weighted with a statistical filter function during the generation of individual autocorrelation functions\(^{168}\). These autocorrelation curves are then compared (Fig 1-14). This technique, used here for protein mixtures of labelled αSyn and Hsc70 in solution, provides information on chaperone binding and interactions at low concentrations.
**FCCS with Pulsed Interleaved Excitation**

FCCS with pulsed interleaved excitation (PIE) is a method which uses rapidly switching lasers for excitation of the donor and acceptor independently on the nanosecond timescale. This method concurrently allows the detection of lifetime, anisotropy, cross-correlation information as well as stoichiometry and FRET efficiency data\(^{169}\). The application of PIE increases the accuracy of single-molecule FRET by using the alternating lasers to determine the ratio of detection efficiencies between the donor and acceptor channels\(^{170}\), and by including only samples containing an active donor and acceptor in the analysis. Each emitted photon is characterized to its excitation pulse and these are separated into different channels (Fig 1-15). A burst search algorithm is used to distinguish fluorescence particles of interest from background via a threshold level. The all photons burst search (APBS) method\(^{171}\) requires a minimum number of photons in a sliding window of duration. Only bursts with a minimum number of photons are accepted.
Figure 1-15 Simplified image of single-molecule FRET using PIE. The left hand panel shows the fluorophore molecules present for each condition. The right-hand side indicates excitation pulse (dark) for donor or acceptor wavelengths and fluorescence decays which would be detected under each condition (light).

Single molecule PIE FRET measurements become possible due to the low sample concentration. Even subpopulations with different FRET values can be resolved within one sample, yielding information such as multiple conformational states of a molecular species. In order to interpret the data in terms of donor-acceptor distances, FRET efficiencies are calculated for each burst. The most straightforward way to approximate them is by calculating the fraction of acceptor photon counts $I_{FRET}$ out of the total photons:

$$E = \frac{I_{FRET}}{I_{FRET} + \gamma I_{DONOR}}$$

Eq.(3)

Where $I_{FRET}$ is the fraction of acceptor count, $I_{FRET} + I_{DONOR}$ is the total photon counts, and $\gamma$ is a correction factor for differences in quantum yield and detection efficiency between detectors. $\gamma$ is defined as:
\[
\gamma = \frac{\eta_A \phi_A}{\eta_D \phi_D}
\]
Eq.(4)

Where \( \phi \) and \( \eta \) are the quantum yield and detection efficiency respectively.

Furthermore, the stoichiometry ratio \( S_{PR} \) can be calculated by the ratio of photons collected after green excitation relative to the total number of collected photons. The stoichiometry of doubly labelled molecules can be defined by:

\[
S = \frac{I_{GG} + I_{GR}}{I_{GG} + I_{RG} + I_{RR}}
\]
Eq.(5)

Where \( I_{GG} \) is the intensity in the green channel after green excitation, \( I_{GR} \) is the intensity in the red channel after green excitation, and \( I_{RR} \) is the intensity in the red channel after red excitation. A value of \( S \approx 0.5 \) describes double-labelled single proteins or a stoichiometric interaction of a donor acceptor pair.

**1.3.4 Number and Brightness**

The number of photons collected at each pixel from a single diffusing particle during the pixel time is defined as the molecular brightness. Application of number and molecular brightness analysis (N&B) allows monomers, dimers, and higher-order aggregates at each pixel to be mapped, which can be used to study oligomerisation states of freely diffusing molecules. In this work this technique was applied to study \( \alpha \)Syn multimers and define subunit populations and dynamic exchange.

In the previous section it was shown that the intensity fluctuations are related to the diffusing particle through the beam. In FCS the beam is fixed, however N&B is a raster scanning technique. The laser beam integrates on a pixel by pixel basis for a fixed time, the photons from each pixel are collected and the process is repeated until the entire image is acquired (typically 50-100 frames).
Figure 1-16 Simplified schematic of the acquisition of data in raster scanning and how variance is used to prevent averaging out to mean intensities. When a large number of dim small particles are present these show small variance around the mean intensity (blue). When a few large bright particles are present there is a large variance around the mean intensity (red). These different particles will correspond to the same average intensity however the N&B method allows differentiation of oligomer populations by looking at the variance around the mean, not just the average intensity.

The analysis of acquired data measures the intensity fluctuations in each pixel due to molecular diffusion. The intensity variance in each pixel allows for distinguishing between a large numbers of dim molecules in the pixel compared with high order aggregates which display higher variance in the average signal (Fig 1-16).

\[
N = \frac{(<I> - \text{offset})^2}{\sigma^2 - \sigma_0^2}
\]

Eq.(6)

\[
B = \frac{\sigma^2 - \sigma_0^2}{<I> - \text{offset}}
\]

Eq.(7)

Where \(N\) is the apparent number of molecules, \(B\) is the intrinsic brightness of each molecule, \(<I>\) is the average signal intensity, the offset is a constant quantity characteristic of the detector settings, \(\sigma^2\) is the variance and \(\sigma_0^2\) is the readout.
variance of the detector. These values were adjusted to account for the use of pseudo-photon counting mode. 

1.3.5 FLIM/FRET

Fluorescence lifetime imaging microscopy (FLIM) combined with FRET is an excellent approach to study protein interactions which may result in conformational shifts or oligomeric rearrangements. This method was used to study αSyn multimers, to try to understand the dynamics and intermolecular exchanges occurring between subunits in donor and acceptor labelled populations. The read-out of FRET not only provides information on the dynamics of the interaction but also gives an idea of the intermolecular distances between fluorophores on each protein.

Figure 1-17 Lifetime decays in FLIM/FRET. 1/e is the time it takes for the fluorescence energy to decay to 33% of the original intensity.

Using FLIM/FRET molecular interactions can be measured by the change in the lifetime decay of the donor which is quenched by energy transfer (Fig 1-17). Phasor analysis is used to calculate the efficiency of the energy transfer between the donor and acceptor molecules. Quenching follows a trajectory from which the FRET efficiency can be calculated by looking at the ratios of the two lifetimes. Phasor analysis can be used for complex multi-component systems to give a more simple representation of the data (Fig 1-18). It provides a very intuitive analysis of
complex systems that by previous analysis would only have been in the realm of the “expert”, and therefore provides a much more approachable method for lifetime studies using FLIM/FRET and intrinsic fluorescence (described in the next section)\textsuperscript{174,175}.

![Figure 1-18 Schematic illustration of basic concepts represented in a phasor plot.](image)

Phasor analysis converts measurements in the time domain into frequency domain and is essentially a measure of the phase shift and modulation shift of the component lifetimes\textsuperscript{176}. Briefly, for measurements in the time-domain, a Fourier transformation of the decay curve at each pixel is performed and the resulting transforms are plotted as a 2D histogram, where X and Y represent the real and imaginary components\textsuperscript{177}. On the phasor plot all single exponential lifetimes must lie on the universal circle. A double exponential decay must be situated on a line between the lifetime of the two individual species with its position determined by the weighting of the two lifetimes ($\tau_1$ & $\tau_2$). The fluorescence lifetime decreases along the universal circle towards coordinate [0,1] where the lifetime value is 0 ns. Analysis is based on plotting $S$ against $G$ sine and cosine transforms of the phase domain as follows

$$S(\omega) = \frac{\int_0^\infty I(t)\sin(\omega t)dt}{\int_0^\infty I(t)dt}$$

Eq.(8)
\[ G(\omega) = \frac{\int_0^\infty I(t) \cos(\omega t) \, dt}{\int_0^\infty I(t) \, dt} \]

Eq.(9)

Where \( \omega = 2pf \) and \( f \) is the laser repetition rate, and \( I(t) \) is the fluorescence intensity at time \( t \).

The unquenched donor is measured in the absence of acceptor and its phasor position determined, and the background phasor position is determined separately. The unquenched donor and background obey the linear combination rule as described by Digman et al.\textsuperscript{175} All possible FRET efficiencies are described along a trajectory between unquenched donor and the background with the FRET efficiency being defined as

\[ E = 1 - \frac{\tau_{DA}}{\tau_D} \]

Eq.(10)

Where \( E \) is the FRET efficiency, \( \tau_{DA} \) is the lifetime of the donor with acceptor present and \( \tau_D \) is the lifetime of the donor only.

Lifetimes and FRET efficiency was also calculated using decay fitting to confirm the values obtained from phasor analysis.

For a single lifetime the decay is fitted to:

\[ I(t) = I_0 \exp^{-t/\tau} \]

Eq.(11)

Where \( I(t) \) is intensity at time \( t \), \( I_0 \) is intensity at time 0 and \( \tau \) is lifetime.

For more complex multi-exponential decays it is the sum of all the decays and is described:

\[ I_t = I_0 \sum_i \alpha_i \exp^{-t/\tau} \]

Eq.(12)
1.3.6 Intrinsic Fluorescence

Intrinsic protein fluorescence can be used for structural and physiochemical studies of proteins. The absorption and emission of proteins in the near-UV region are due to the presence of aromatic amino acid residues, namely tryptophan, tyrosine, and phenylalanine\textsuperscript{178,179}. In recent years there has been a revived interest in intrinsic protein fluorescence due to advances in excitation sources for these types of measurements\textsuperscript{180–182}. In biological applications the unique ability to visualise protein conformational changes without the addition of extrinsic probes, which have the potential for interference, is a significant advantage. There are many processes which lead to changes in the microenvironment of intrinsically fluorescent amino acids such as pH, temperature or concentration\textsuperscript{183,184}. In this work the conformation of αSyn multimers under different conditions were investigated using a native tyrosine residue located in a region proposed to undergo large conformational changes in destabilisation of the multimer (Fig 1-19).

![Figure 1-19 Cartoon of αSyn multimer conformation indicating location of tyrosine residues (red Y) on individual subunits utilised for intrinsic fluorescence studies. Adapted and reproduced with permission from Wang et al\textsuperscript{125}.](image)

Tryptophan is the most commonly studied of the intrinsically fluorescent amino acids as it has the highest quantum yield (~0.13) in aqueous solution at room temperature\textsuperscript{185}. In cases where tryptophan is not present, tyrosine and phenylalanine can also be used as intrinsic probes\textsuperscript{186}. It should be noted that these
alternate residues have much lower extinction coefficients than tryptophan, with phenylalanine only generally being used as an intrinsic marker in the absence of the other two amino acids. The application of this method has thus far been shown to be applicable to a variety of potential biological situations, albeit, in a very limited number of publications.

Solvent exposure leads to quenching of intrinsic fluorescence resulting in changes to lifetime and/or contributions of lifetimes. A single tryptophan alone can give rise to multi-exponential decays and proteins may contain a number of these aromatic amino residues, making the fitting of exponentials extremely difficult. Phasor analysis overcomes these problems allowing the changing lifetime components to be visualised simply through the movement of phasor points. The trajectory of the phasor point in response to a physical or chemical perturbation can be followed to provide insight into the processes under investigation. The use of phasor analysis with these intrinsic protein fluorescence lifetime studies in in vitro systems is a highly approachable method for conceptualising the observed changes in fluorescence lifetime measurements. The lifetime component for intrinsic measurements and phasor analysis here is the same as described previously for FLIM.

1.3.6 Single-Molecule TIRF

The power to view objects down to a nanometre scale and even study individual molecules has the potential to bridge many gaps in understanding biological process at a molecular level. Of particular interest for this work is the potential insight the technique can provide into the molecular mechanisms of αSyn membrane association and the ability of these interactions to cause cellular disruption. Single-molecule imaging provides a platform from which complex stoichiometry, rate kinetics and distinct pathways in cellular mechanisms can be defined.
Total internal reflection (TIRF) microscopy works via the basic principle of light refraction (Fig 1-20). At the interface of two mediums light is refracted, and the amount that is refracted depends on the incidence angle. At a critical angle light is refracted along the interface, and just beyond this critical angle light is totally internal reflected generating an evanescent field which is able to excite molecules captured to the surface up to around 200 nm. This eliminates a substantial amount of the background fluorescence of molecules outside of the range of the field. This kind of imaging is largely concentration dependent and typically requires nanomolar concentrations in order to visualise individual molecules. New developments in instrumentation, sample delivery and fluorophores are constantly being explored and hold promise for also allowing imaging at higher concentrations as well as improving signal to noise ratio for easy analysis\textsuperscript{189,190}.

“Figure has been removed due to copyright restrictions”

**Figure 1-20 Principle of total internal reflection and its application in TIRF illumination.**
Light travelling through two media of different refractive indices is refracted at the interface. The extent of refraction is dependent on the incident angle $\theta$ and at a certain incident angle light is totally internally reflected back into the higher-index medium (glass slide). This generates an evanescent field at the media interfaces which can penetrate a few hundred nanometres into the lower-index medium (aqueous medium). Surface-tethered fluorophores are excited and fluoresce, whereas those in the solution further from the evanescent field remain unexcited. Taken from Ross et al\textsuperscript{191}.

Single-molecule imaging allows various intermediates and populations to be studied within a single system. The detection of hundreds or thousands of molecules allows the construction of population histograms for different parameters such as time of interactions, intensity or ratios between molecules. By this method it is possible to determine population distributions which would be lost in ensemble averaging techniques.
1.4 Aims of This Study

In light of recent findings about the αSyn multimer, the shift from looking at results obtained from an unstructured monomer, to a highly ordered helix-turn-helix structure comprised of multiple subunits means there are many aspects such as structural changes and fluctuations, membrane binding and aggregation pathways that require all new understanding. The effect of mutations and conformational instabilities in certain aspects may have previously not been considered, given the model being worked on had no native structure. The validity of these studies will be substantially improved with the addition of advanced fluorescence microscopy and single-molecule imaging data showing the multimeric structure, its subunit dynamics and membrane binding interactions.

Membrane binding and disruption by αSyn represents a mechanism which seems to be critical to cellular toxicity. With the proposed roles of αSyn centred on functional membrane binding the question remains as to what shifts the functional interaction into a disease causing process.

There are three aims investigated in this work.

1. Characterise the monomer-multimer form

New research continually points to this being the functional form, but this remains controversial and a lot remains undefined about the properties of the multimer how its subunits interact and how it might then be shifted into a disease-causing protein.

2. Investigate how Hsc70/Hsp40 chaperones mediate αSyn

Molecular chaperones represent the cells main weapon to combat protein aggregation and neurodegeneration. By understanding how chaperones interplay with the multimer form and its perturbations it may provide insight into the early steps in disease progression.
3. Understand αSyn membrane associations

The underlying mechanisms of αSyn association which lead to membrane disruption are unknown and represent a critical gap in knowledge of how cell death occurs and how this may be mediated.
Chapter 2 – Methods

2.1 Media, Buffers and Other Reagents

2.1.1 Media

Luria Bertani (LB): 1% w/v Bacto-tryptone, 0.5% w/v yeast extract, 1% NaCl, 0.1% NaOH

LB Agar: 1% w/v Bacto-tryptone, 0.5% w/v yeast extract, 1% NaCl, 0.1% NaOH, 1.5% w/v agar

DYT: 1.8% w/v Bacto-tryptone, 0.9% w/v yeast extract, 1.8% NaCl, 0.1% NaOH

2.1.2 Buffers

All buffers were 0.22μm sterile filtered before use

BOG buffer: 100 mM Hepes pH 7.4, 150 mM NaCl, 10% Glycerol, 0.1% n-octyl-β-glucopyranoside

BOG wash buffer: 100 mM Hepes pH 7.4, 150 mM NaCl, 10% Glycerol, 0.1% n-octyl-β-glucopyranoside, 5 mM DTT

BOG elution buffer: 100 mM Hepes pH 7.4, 150 mM NaCl, 10% Glycerol, 0.1% n-octyl-β-glucopyranoside, 5 mM DTT, 15 mM Reduced L-Glutathione

BOG refolding buffer: 100 mM Hepes pH 7.4, 150 mM NaCl, 10% Glycerol, 0.1% n-octyl-β-glucopyranoside, 2 mM ATP, 5 mM MgCl₂, 50 mM KCl, 20 mM K₃PO₄

PreScission Protease cleavage buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT

A140C resuspension buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA

A140C purification buffer: 10 mM Tris-HCl pH 7.4

A140C elution buffer: 10 mM Tris-HCl pH 7.4, 1M NaCl

A140C oligomerisation buffer: 10 mM Hepes pH 7.4, 50 mM NaCl

A140C oligomerisation refolding buffer: 10 mM Hepes pH 7.4, 50 mM NaCl, 2 mM ATP, 5 mM MgCl₂, 50 mM KCl, 20 mM K₃PO₄

Hsc70 purification buffer: 50 mM Tris-HCl pH 7.5, 300 mM NaCl

Hsc70 wash buffer: 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 10 mM Imidazole

Hsc70 elution buffer: 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 80 mM Imidazole
**Hsc70 storage buffer:** 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.1 mM TCEP, 1 mM MgCl₂, 0.1 mM ATP, 50 mM KCl, 20% Glycerol

**DNAJB1 resuspension buffer:** 25 mM Hepes pH 8.0, 300 mM NaCl, 5% Glycerol, 5 mM β-mercaptoethanol

**DNAJB1 purification buffer:** 25 mM Hepes pH 8.0, 300 mM NaCl, 5% Glycerol, 40 mM Imidazole

**DNAJB1 elution buffer:** 25 mM Hepes pH 8.0, 300 mM NaCl, 5% Glycerol, 500 mM Imidazole

**DNAJB1 cleavage buffer:** 25 mM Hepes pH 8.0, 300 mM NaCl, 5% Glycerol

**DNAJB1 storage buffer:** 50 mM Tris-HCl pH 7.5, 150 mM KCl, 5% Glycerol

**GFP purification buffer:** 50 mM Tris-HCl pH 7.5, 300 mM NaCl

**GFP wash buffer:** 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 20 mM Imidazole

**GFP elution buffer:** 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 100 mM Imidazole

**SDS-PAGE running buffer:** 25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3

**2x sample buffer:** 62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% Glycerol, 0.01% Bromophenol blue, 700 mM DTT (aliquot and store at -20°C)

**β-Galactosidase unfolding buffer:** 25 mM HEPES pH 7.4, 5 mM MgCl₂, 50 mM KCl, 5 mM β-mercaptoethanol, 6 M Guanidine-HCl

**β-Galactosidase refolding buffer:** 25 mM HEPES pH 7.4, 5 mM MgCl₂, 50 mM KCl, 2 mM ATP, 10 mM DTT

**Biotinylation modification buffer:** 100 mM Na₃PO₄, 150 mM NaCl, pH 7.4

**BOG CD buffer:** 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% Glycerol, 0.1% n-octyl-β-glucopyranoside

**Blocking buffer:** 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 50 mM NaCl, 0.03% NaN₃, 0.025% Tween-20, 0.2 mg mL⁻¹ BSA

### 2.1.2 Other Reagents

**Antibodies**

Experiments involving surface capture of αSyn oligomers used a biotinylated polyclonal rabbit anti-amyloid αβ oligomer A11 antibody (Jomar Bioscience, SPC-506D-BI).
Cells and Plasmids

For cloning, preparation and storage of DNA *E. coli* Top 10 cells were used. Expression was performed in *E. coli* BL21 Rosetta pLysS or BL21 DE3 cells. All constructs were sequenced before use using Sanger sequencing performed at the Ramaciotti Centre UNSW following their standard protocol.

2.2 Molecular Biology

2.2.1 Transformation, Purification of Plasmid DNA and DNA Sequencing

Frozen competent cells were thawed on ice, DNA was then added with a 20 fold excess of cells by volume and gently mixed by tapping. This mixture was incubated on ice for 10 minutes, heat-shocked at 42°C for 90 seconds and returned to ice. DYT media was added and incubated at 37°C with shaking at 250 rpm for 45-60 minutes. The transformation mix was plated on relevant selective media until absorbed and incubated overnight at 37°C. Individual colonies were picked from the transformation plate and replated. From the restreaked plate a starter culture was inoculated for mini-prep of plasmid DNA. Plasmid DNA was purified from cell pellets using a Mini Prep Plasmid Kit (Bioline, BIO-52055) and construct identity was confirmed by DNA sequencing (Applied Biosystems, 4337455) with relevant primers.

2.3 Protein Expression, Purification and Labelling

2.3.1 α-Synuclein Multimer

2.3.1.1 Expression

αSyn was expressed as a glutathione S-transferase (GST) fusion protein using a pGEX-6P-1 Cys α-Syn plasmid which was a gift from Quyen Hoang (Indiana University). This plasmid has an additional 14 residues on the N-terminus (GPLGSPEFPGMKCK) which includes a cysteine for labelling. The plasmid was transformed into *E. coli* BL21 Rosetta pLysS cells and grown in LB media containing 100 ug mL⁻¹ ampicillin (Amp) and 34 ug mL⁻¹ chloramphenicol (Cam). LB media was inoculated with an overnight starter culture and grown at 37°C with 240 rpm shaking until the optical density (OD) reached 0.3. The culture was then
transferred to 20°C with shaking at 240 rpm until OD reached 0.4. Protein expression was induced using 1 mM IPTG and allowed to proceed for 16 hours at 20°C with shaking at 240 rpm. Cells were harvested by centrifugation in a Sorvall™ RC 6 Plus Centrifuge (ThermoFisher, 46915) using a SLA-3000 Super-lite® rotor (ThermoFisher, 07149) at 6000 rpm for 10 minutes at 4°C. Pellets were resuspended in ice-cold BOG buffer with the addition of protease inhibitor (Roche, 04693132001). Cell lysis was performed by probe sonication (Branson Sonifer, S-250A) for 4 minutes (cycles of 15 seconds on, 15 seconds off), and then cellular debris was pelleted by centrifugation at 14000 rpm using a SS-34 rotor (ThermoFisher, 28020 for 30 minutes at 4°C. The cell lysate supernatant was passed through a 0.22 µm filter before purification.

2.3.1.2 Purification

Cell lysate was loaded onto a GSTrap 4B 5 mL column (GE Healthcare, 28-4017-47) equilibrated in BOG wash buffer. The sample was loaded using a 50 mL Superloop (GE Healthcare, 59-7574-00) at 0.5 mL min⁻¹. After sample loading, the column was washed with 10 column volumes (CV) of BOG wash buffer at 0.6 mL min⁻¹ to remove unbound impurities. The GST-αSyn fusion protein was then eluted with 10 CV of BOG elution buffer at 0.6 mL min⁻¹. The fusion protein was buffer exchanged to remove free glutathione and then cleaved using 40 units (u) of Prescission protease per mL of bed volume of the GST column. The reaction was performed in PreScission Protease cleavage buffer with overnight incubation at 4°C with gentle rocking. The whole mixture was filtered through a 0.22 µm filter and then reapplied onto the GST column at 0.2 mL min⁻¹. The cleaved αSyn did not bind to the column and was collected in the flowthrough. Column wash and elution was performed as previously to elute cleaved GST, uncleaved GST-αSyn and PreScission Protease. Fractions corresponding to αSyn were identified by SDS-PAGE, pooled, buffer exchanged and concentrated using 3 kDa molecular weight cut-off (MWCO) centrifugal filters (Amicon, UFC900308) at 4000 x g until 500 µL volume was reached. αSyn was further purified by loading on a Superdex 200 10/300 GL size-exclusion column (SEC) (GE Healthcare, 17-5175-01) pre-equilibrated in BOG buffer at 0.2 mL min⁻¹ and then passed through the column at 0.25 mL min⁻¹. The purity of the αSyn was assessed by running on an SDS-page gel.
If required an additional round of GST and then SEC purification was used to polish αSyn to a final purity of >95%.

2.3.1.3 Labelling

αSyn was used unlabelled or labelled at the N-terminus with thiol reactive Alexa Fluor 488 C5-Maleimide (AF488-αSyn) or Alexa Fluor 568 C5-Maleimide (AF568-αSyn) fluorescent dye. Briefly, αSyn (1 mg mL⁻¹) was incubated with TCEP (1:20, mol/mol) for 30 minutes at room temperature with rocking, the dye was then added (1:15, mol/mol) while vortexing gently and the reaction was allowed to proceed for 1 hour at room temperature under argon with gentle rocking. Labelling reactions then were allowed to proceed overnight at 4°C with head over head rotation. The reaction was quenched using 0.2% β-mercaptoethanol and free dye was removed using Zeba™ columns (ThermoFisher, 89882) until no visible colour remained in the resin. The labelling yield was calculated from a UV-Vis scan (280-700 nm) performed on a Nanodrop spectrometer (ThermoScientific, ND1000 V3.8). Removal of free dye was confirmed using SDS-Page with fluorescence imaging on a Typhoon™ FLA-9000 imager (GE Healthcare, 28-9607-66). For all experiments using αSyn multimer samples referred to as concentrated were >55 µM and samples referred to as dilute were <13 µM. Dilution was always performed immediately prior to measurements.

2.3.2 α-Synuclein A140C

2.3.2.1 Expression

pT7-7-α-Syn A140C was transformed into E.coli BL21 DE3 cells and grown in LB media containing 100 µg mL⁻¹ Amp. LB media was inoculated with an overnight starter culture and grown at 37°C with shaking at 240 rpm until the OD reached 0.7. Protein expression was induced using 0.5 mM IPTG and allowed to proceed for 4 hours at 37°C with shaking at 240 rpm. Cells were harvested by centrifugation at 4000 rpm in a SLA-3000 rotor for 15 minutes at 4°C. Pellets were resuspended in A140C resuspension buffer with the addition of protease inhibitors. Cells were then lysed with the addition of 1 mg mL⁻¹ lysozyme, incubated on ice with stirring for 20 minutes and then 4 minutes probe sonication (cycles of 15 seconds on, 15
seconds off). Cellular debris was pelleted by centrifugation at 13000 rpm in a SS-
34 rotor for 30 minutes at 4°C.

2.3.2.2 Purification

An initial crude purification was performed using a two-step ammonium
sulfate precipitation at 0°C using a high salt ice-bath kept in a 4°C cool room. The
first step (30% saturation) was reached by very slowly adding solid ammonium
sulfate to the cell lysate with stirring. This mixture was incubated with stirring for
1 hour and then centrifuged at 13000 rpm in a SS-34 rotor for 30 minutes at 4°C.
The supernatant containing the αSyn was removed and the volume accurately
measured to calculate the mass of ammonium sulfate required for the second step.
For the second step solid ammonium sulfate was added as previously until 50%
saturation was reached. The sample was incubated for 1 hour with stirring and
centrifuged at 13000 rpm for 30 minutes at 4°C. The αSyn A140C pellet was
resuspended in A140C purification buffer and dialysed overnight with at least four
buffer changes to remove ammonium sulfate. The presence of αSyn in supernatant
or pellet at each step was assessed using SDS-PAGE. Samples were desalted using
Zeba™ columns prior to running on a gel to prevent the ammonium sulfate
interfering with staining.

αSyn A140C was loaded onto 4 x 1 mL DEAE anion exchange columns
connected in series (GE Healthcare, 17-5055-01) that were equilibrated in A140C
purification buffer. Elution using a linear gradient from 0-1 M NaCl was used for
initial characterisation of the elution profile, then a step gradient was used with
steps at 20, 30 and 100 mM NaCl to obtain a better resolution of protein peaks.
Fractions containing αSyn A140C were identified, pooled and concentrated for
polishing using SEC. A Superdex 200 10/300 column was equilibrated in A140C
purification buffer and a maximum of 500 µL of sample was loaded at a time at
0.25 mL min⁻¹. Purity of αSyn A140C was assessed using SDS-PAGE. If required
anion-exchange and SEC purification was repeated to obtain the required purity.
Pure αSyn A140C was either used for labelling (see next section) or buffer
exchanged into A140C oligomerisation buffer, concentrated to 250 µM, lyophilised
overnight and stored at -80°C.
2.3.2.3 Labelling

αSyn A140C was labelled at the engineered cysteine residue with thiol reactive Alexa Fluor C5-Maleimide 568. Briefly, αSyn A140C (1 mg mL\(^{-1}\)) was added to dye (1:3, mol/mol) while vortexing gently. The reaction was allowed to proceed for 4 hours at room temperature under argon with gentle rocking. The reaction was quenched using 0.2% β-mercaptoethanol. Sample was buffer exchanged into A140C purification buffer and free dye was removed using anion exchange chromatography on 4 x 1 mL DEAE columns (as described for purification). Fractions containing labelled protein and no free dye as visualised using SDS-PAGE were pooled, buffer exchanged into A140C oligomerisation buffer, concentrated to 100 µM, lyophilised and stored at -80°C. The labelling yield was calculated from a UV-Vis scan. Removal of free dye was confirmed using SDS-PAGE with fluorescence imaging.

2.3.2.4 Oligomer Preparation

αSyn A140C oligomers were prepared from lyophilised monomer stocks. Briefly, frozen dried αSyn A140C (labelled and unlabelled mixture) was resuspended in A140C oligomer buffer to final concentration of 1 mM and incubated for 18 hours with shaking at 1000 rpm and at 25°C. To remove large aggregates, the sample was spun down in a benchtop centrifuge at 14000 rpm for 30 minutes. The supernatant was carefully removed and loaded on a Superdex 200 10/300 SEC column equilibrated in A140C oligomer buffer to separate oligomer and monomer populations. Clear separation was achieved using a loading and flow rate of 0.25 mL min\(^{-1}\). Samples were either used immediately or snap frozen in liquid nitrogen and stored at -80°C.

2.3.3 Hsc70

2.3.3.1 Expression

The pProEX HTD plasmid with Hsc70 modified for site-specific labeling by adding a glycine and a cysteine residue to the C-terminus was transformed into E.coli BL21 DE3 cells and grown in LB media containing 100 ug mL\(^{-1}\) Amp. LB
media was inoculated with an overnight starter culture and grown at 37°C with 240 rpm until the OD reached 0.3. The culture was then transferred to 16°C with shaking at 240 rpm until the OD reached 0.4. Protein expression was induced using 0.1 mM IPTG and allowed to proceed overnight. Cells were harvested by centrifugation at 6000 rpm in a SLA-3000 rotor for 10 minutes. Pellets were resuspended in Hsc70 purification buffer with the addition of protease inhibitors. Cells were lysed by 4 minutes probe sonication (cycles of 15 seconds on, 15 seconds off). Cellular debris was pelleted by centrifugation at 14000 rpm in a SS-34 rotor for 30 minutes at 4°C. The cell lysate was then passed through a 0.22 µm filter before purification.

2.3.3.2 Purification

The N-terminal His-tagged Hsc70 was purified using gravity flow affinity chromatography. Ni-NTA beads were equilibrated in 5 CV of Hsc70 purification buffer. The beads were then added to the cell lysate and incubated with head over head rotation at 4°C for 2 hours. The bead lysate mixture was poured into a closed gravity flow column and the bed was allowed to settle. After the lysate had flowed through, the beads were washed with 8 CV of Hsc70 purification buffer. A further wash was performed with 8 CV of Hsc70 wash buffer. His-Hsc70 was eluted with 5 CV of Hsc70 elution buffer with the addition of 2 mM EDTA to the purified protein. Fractions containing Hsc70 were identified using SDS-PAGE. For removal of the His-tag, DTT was added to the sample to final concentration of 2 mM and TEV protease was added to a final concentration of 0.5 mg mL⁻¹. The reaction was allowed to digest for 5 hours at room temperature and then dialysed into Hsc70 purification buffer. To separate Hsc70 the cleavage mix was purified on Ni-NTA beads as earlier described, however in this step the Hsc70 protein was contained in the flowthrough. The TEV protease contains a His-tag and was therefore retained on the column along with the cleaved His-tag from Hsc70. Purity was confirmed by SDS-PAGE. Pure Hsc70 was then stored in Hsc70 storage buffer which contains TCEP, MgCl₂, ATP, KCl and glycerol. Samples were snap frozen in liquid nitrogen and stored at -80°C.
2.3.3.3 Labelling

Pure Hsc70 was labelled at the introduced cysteine residue at the C-terminus with thiol reactive Alexa Fluor C2-maleimide 647. Briefly, Hsc70 (1 mg mL⁻¹) was added to dye (1:7.5, mol/mol) while vortexing gently. The reaction was allowed to proceed for 80 minutes at room temperature under argon with gentle rocking. The reaction was quenched using 0.2% β-mercaptoethanol and free dye was removed using Zeba™ columns until no visible colour remained in the resin. The labelling yield was calculated from a UV-Vis Scan. Labelling reactions were performed on pure Hsc70 samples in storage buffer removed from -80°C immediately prior to labelling. Removal of free dye was confirmed using SDS-Page with fluorescence imaging.

2.3.4 HSP40 (DNAJB1)

2.3.4.1 Expression

pProEX HTb-DNAJB1 was a gift from Dr Ronald Melki (Institut des Neurosciences Paris Saclay, CNRS) and was transformed into E.coli BL21 DE3 RP cells and grown in LB media containing 100 ug mL⁻¹ Amp. LB media was inoculated with an overnight starter culture and grown at 37°C with shaking at 240 rpm until the OD reached 0.3. The culture was then transferred to 16°C with shaking at 240 rpm until OD reached 0.4. Protein expression was induced using 0.1 mM IPTG and allowed to proceed overnight. Cells were harvested by centrifugation at 6000 rpm in a SLA-3000 rotor for 10 minutes at 4°C. Pellets were resuspended in DNAJB1 purification buffer with the addition of protease inhibitors. Cells were lysed by addition of 0.1 mg mL⁻¹ lysozyme and 10 mM imidazole, incubated for 20 minutes with stirring and then 4 minutes probe sonication (cycles of 15 seconds on, 15 seconds off). Cellular debris was pelleted by centrifugation at 5000 rpm in a SS-34 rotor for 45 minutes at 4°C. The cell lysate was then passed through a 0.22 µm filter before purification.
2.3.4.2 Purification

The N-terminal His-tagged DNAJB1 was purified using a HisTrap HP 5 mL column (GE Healthcare, 17-5248-01) followed by SEC. The HisTrap column was equilibrated in DNAJB1 purification buffer. The cell lysate was then loaded using a 50 mL Superloop at 0.5 mL min\(^{-1}\). Unbound contaminants were washed away with 20 CV of DNAJB1 purification buffer. Bound His-DNAJB1 was eluted using a linear gradient from 40-500 mM Imidazole over 5 CV. Fractions containing eluted His-DNAJB1 were pooled and buffer exchanged into DNAJB1 cleavage buffer. Cleavage of the fusion protein was performed using TEV protease (2u TEV:20 ug fusion protein) for 6 hours at room temperature. To separate the cleaved DNAJB1, the cleavage mixture was purified on the HisTrap column as earlier described, however in this step the DNAJB1 protein was contained in the flow through and an isocratic elution of 500 mM imidazole was used to elute bound contaminants including the His-tagged TEV. The DNAJB1 fractions were pooled, concentrated and loaded onto a Superdex 200 10/300 column equilibrated in DNAJB1 storage buffer at 0.25 mL min\(^{-1}\) for final polishing. Purity was confirmed by SDS-PAGE.

2.3.4.3 Labelling

Pure DNAJB1 was labelled at the N-terminus by using an amine-reactive Alexa Fluor 488 SDP-ester. Briefly, DNAJB1 (1 mg mL\(^{-1}\)) was buffer exchanged into freshly made 100 mM sodium carbonate buffer pH 7.5. Buffer exchange was performed overnight at 4°C in a parafilm sealed container. Protein concentration was checked after dialysis and dye was added (1:10, mol/mol) while vortexing gently. The reaction was allowed to proceed for 3 hours at room temperature with gentle rocking. The reaction was then quenched for 1 hour at room temperature with ammonium sulfate (final concentration 2 mM). Free dye was removed using Zeba™ columns until no visible colour remained in the resin and the labelling yield was calculated from a UV-Vis scan. During removal of free dye protein was also buffer exchanged into DNAJB1 storage buffer. Removal of free dye was confirmed using SDS-PAGE with fluorescence imaging.
2.3.5 Preparation of aceGFP for Liposome Encapsulation

2.3.5.1 Expression

pRSFDuet-1-His-aceGFP obtained from Dr Lawrence Lee (Single Molecule Science, UNSW) was transformed into *E. coli* BL21 DE3 cells and grown in LB media containing 50 µg mL\(^{-1}\) kanamycin. LB media was inoculated with an overnight starter culture and grown at 37°C with shaking at 240 rpm until the OD reached 0.5. The culture was then transferred to 25°C with shaking at 240 rpm until the OD reached 0.6. Protein expression was induced using 0.3 mM IPTG and allowed to proceed overnight. Cells were harvested by centrifugation at 6000 rpm in a SLA-3000 rotor for 10 minutes at 4°C. Pellets were resuspended in GFP purification buffer with the addition of protease inhibitors. Cells were lysed by 4 minutes probe sonication (cycles of 15 seconds on, 15 seconds off). Cellular debris was pelleted by centrifugation at 14000 rpm in a SS-34 rotor for 30 minutes at 4°C. The cell lysate was then passed through a 0.22 µm filter before purification.

2.3.5.2 Purification

His-aceGFP was purified using gravity flow affinity chromatography. Ni-NTA beads were equilibrated in 5 CV of GFP wash buffer. The beads were then added to the cell lysate and incubated with head over head rotation at 4°C for 1 hour. The bead lysate mixture was poured into a closed gravity flow column and the bed was allowed to settle. After the lysate had flowed through, the beads were washed with 10 CV of GFP wash buffer. His-GFP was eluted with 5 CV of GFP elution buffer. Fractions containing GFP were identified by eye and the purity was determined using SDS-PAGE. GFP was then concentrated, snap frozen in liquid nitrogen and stored at -80°C.

2.4 General Biochemistry and Other Lab Techniques

2.4.1 SDS-PAGE

SDS-PAGE allows separation of proteins according to their size in polyacrylamide gel in the presence of SDS and an electric field. A mini-PROTEAN® Tetra vertical electrophoresis cell system (Bio-rad, 1658005) and Bio-rad pre-cast gels (Any kD, 4569035 or 12% gels, 4561045) were used in this work. Prior to
loading samples were mixed 1:1 (v/v) with 2x sample buffer and boiled for 15 minutes. All gels were run at 180 V. The molecular weight marker consisted of Precision Plus Protein™ Dual Colour Standards (Bio-rad, 1610374). This marker was used as it also contains three pink reference bands at 75, 25 and 2 kDa which are visible during fluorescent imaging. Bands were stained using SimplyBlue™ Safe Stain (ThermoFisher, LC6060) following the microwave protocol. Visualisation was either on an Epson scanner (EPSON, B11B223501), Gel Doc™ EZ System (Bio-rad, 1708270) or Typhoon™ FLA-9000 imager (GE Healthcare, 28-9607-66). SDS-PAGE was used in this study to confirm protein identity, purity, labelling and effective removal of free dye from labelling reactions.

2.4.2 Protein Concentrating, Buffer Exchange and Dialysis

Concentrating of all proteins was performed using Amicon centrifugal concentrator columns of appropriate molecular weight cut-off. Buffer exchange was performed by using either Zeba™ columns with buffer exchange protocol according to the manufacturer’s protocol or overnight dialysis using QuixSep Microdialysis cups (QuixSep, QS3-1510) and Cellu Sep Dialysis Membrane (Cellu Sep, 5015-19 3.5 kDa or 1205-76 12-14 kDa MWCO) with appropriate molecular weight cut-off for each protein.

2.4.3 Biotinylation of Antibodies

Biotinylation of antibodies was performed using the Sulfo ChromaLink™ Biotin reagent (Solulink, B-1007) with biotinylation modification buffer and Zeba™ columns. Biotinylation was performed following the “How to Biotinylate with Reproducible Results” Solulink guide and quantification of biotinylation was performed using EXCEL spreadsheet calculators, both provided on the Solulink website (www.solulink.com). Antibodies were biotinylated in order to allow capture to streptavidin coated coverslips for single-molecule assays.
2.4.4 Preparation of Liposomes

Liposomes with encapsulated dye were used to study the interaction of αSyn on membranes and the formation of membrane defects. All lipid stocks were dissolved in chloroform and stored in Teflon-lined glass vials at -80°C. A mixture of 99.495% DOPG, 0.5% Biotin-PE and 0.005% Atto647-DOPE was prepared in a glass test tube and the solvent evaporated with a gentle stream of nitrogen for 15 minutes. The lipid cake was then dried overnight in a vacuum desiccator protected from light. Lipids were rehydrated with the addition of A140C oligomerisation buffer (42°C) supplemented with either Alexa Fluor 488 free dye (20 µM) or GFP (20 µM) to final lipid concentration of 1 mg mL⁻¹. Trolox was also included in the buffer during the preparation process at 2 mM final concentration. Hydration proceeded for 1 hour at 42°C with vortexing every 15 minutes. The lipid mix was then subjected to 5 freeze-thaw cycles of liquid nitrogen and a 42°C water bath. An extruder apparatus (Avanti, 610000) with 200 nm pore-size membrane (Avanti, 610006) was assembled according to manufacturer's instructions and warmed to 42°C on a heating block. The lipid mix was then passed through the extruder a total of 35 times. Liposomes were stored under argon and protected from light at 4°C for up to 2 weeks after preparation.

2.4.5 Characterisation of Proteins

2.4.5.1 β-Galactosidase Refolding Assay

The β-galactosidase refolding assay was performed as outlined previously¹⁹² and was used to measure the functionality of recombinantly prepared chaperone proteins. Briefly, β-galactosidase was diluted 20 fold in 1 M glycylglycine pH 7.2 which helps maintain solubility during denaturation. This mixture was then diluted 10 fold in β-galactosidase unfolding buffer which contains 6 M guanidine-HCl to induce denaturation. A sample was taken before denaturation and instead diluted 10 fold in glycylglycine to act as a positive control of native β-galactosidase. Samples were incubated at 30°C for 30 minutes. To measure refolding of the denatured β-galactosidase by chaperones, the samples were diluted 124 fold in β-galactosidase refolding buffer supplemented with chaperones. Refolding buffer
was supplemented with Hsc70 alone, DNAJB1 alone, Hsc70 + DNAJB1 or labelled Hsc70-647 + DNAJB1-488. The assay was performed with Hsc70 concentration always at 1.6 µM and DNAJB1 at 3.2 µM. The final concentration of β-galactosidase was 68 nM. For the positive and negative controls refolding buffer was instead supplemented with 3.2 µM BSA. The chromogenic substrate ortho-Nitrophenyl-β-galactoside (ONPG) was included in the buffer at 0.8 mg mL⁻¹ as a read out for recovery of enzymatic activity of β-galactosidase. The reaction was allowed to proceed at 37°C and various time points were taken over a 2 hour period. At each time point the reaction was quenched immediately after removal from the reaction mixture using 0.5 M sodium carbonate. The absorbance of all time points was measured at 413 nm using an Omega FLUOstar plate reader (BMG Labtech, FLUOstar Omega) and the percent activity calculated in comparison to the values obtained for the native state positive control. At 413 nm there was a negligible amount of absorbance from the 488 fluorescent label conjugated to DNAJB1. BSA was used as a control to account for the additional molecular crowding/concentration effect in this assay and all other experiments where chaperones were studied in this work, including microscopy experiments.

### 2.4.5.2 TPE-TPP Oligomer Fluorescence Assay

The recently developed tetraphenylethene triphenylphosphonium (TPE-TPP) dye was used to perform oligomer fluorescence assays. This dye is an aggregation-induced emission luminogen which becomes fluorescent upon restriction of its intramolecular motions by interaction with oligomeric or fibrillar proteins. The TPE-TPP assay was performed by mixing 5 µM dye stock made in dimethyl sulfoxide (DMSO) with protein and allowing the reaction to proceed for 1 hour at room temperature with gentle rocking. Measurements were taken immediately after mixing of the dye with the protein (Time 0) and after the 1 hour incubation period. The reaction of the dye with proteins was fast such that the maximum fluorescence was already reached at Time 0 in positive control experiments. Insulin monomers or fibrils were used at 5 µM as negative and positive controls respectively. Fluorescence induced through complexation with oligomers was measured using a FluoroMax-4® spectrofluorometer (Horiba
Scientific) in a quartz sub-micro cuvette (Starna, 16.10-Q-10/Z15). Excitation was performed by a multi-wavelength LED with an excitation monochromator used to select 351 nm excitation with a 3 nm slit width. The emission monochromator scanned the emission between 300-650 nm with 1 nm intervals and 4 nm slit width. This assay was performed on monomer, multimer and oligomer forms of αSyn. More detail of these different experiments is described in the relevant results sections. Aggregation assays were not performed on labelled proteins due to the extremely low yields of high efficiency labelled αSyn. Labelled αSyn generated using this construct has previously been shown not to interfere with Congo Red aggregation assays (W.Wang, personal communication).

2.4.5.3 Circular Dichroism

Circular dichroism (CD) was performed to characterise the secondary structure of αSyn multimers and diluted monomers. CD spectra were recorded on a Chirascan™ Plus CD Spectrometer (Applied Photophysics) between 195-280 nm. For concentrated samples (>50 µM) a cuvette with an optical path-length of 0.1 mm was used. Diluted samples (<13 µM) were measured in a cuvette with an optical path-length of 0.5 mm. Spectra were recorded in 1 nm steps with a 10 second integration time. Spectra are represented as buffer corrected averages of 10 repeats. αSyn was buffer exchanged into BOG CD buffer for measurements. Tyrosine fluorescent lifetimes were recorded for each αSyn sample to confirm batch to batch reproducibility.
2.5 Microscopy and Cuvette-Based Fluorescence Measurements

All equations referred to in this section can be found in Chapter 1.3

2.5.1 FLIM/FRET

2.5.1.1 Experimental Procedure

Sample and Coverslip Preparation

Aliquots of AF488-αSyn and AF568-αSyn were freshly thawed for each FLIM measurement. For experiments involving chaperones aliquots of Hsc70 and DNAJB1 were also freshly thawed. Chaperones were used at 1.6 µM and 3.2 µM respectively. Dilution steps were carried out immediately prior to measurement in BOG buffer. For experiments involving chaperones dilution was carried out in BOG refolding buffer. Sterile optical quality glass bottom fluorodishes (World Precision Instruments, FD35-100) were used unaltered for these experiments. Each fluorodish was sealed with parafilm to avoid evaporation during measurements. For imaging, equal amounts of concentrated AF488-αSyn and AF568-αSyn were mixed together by gently pipetting up and down 10 times. Equilibration was allowed to proceed until changes in FRET efficiency reached a plateau, as determined experimentally to be ~1 hour. Mixed populations were then diluted in either the presence or absence of chaperones. Measurements were taken at regular intervals for both mixing and dilution steps.

Imaging Conditions

FLIM FRET measurements were performed using a time resolved, inverted confocal fluorescence microscope (Microtime200, PicoQuant GmbH). Excitation was via a single-photon fiber coupled pico-second-pulsed diode 473 nm laser (40 MHz repetition rate, 2 ms dwell time, 256 x 256 pixel array) using a 63x water objective (1.25 NA). Fluorescence emission was collected through a 510/42 Semrock BrightLine band pass emission filter onto a single-photon avalanche diode (SPAD) coupled to high speed timing electronics for time-correlated single-photon counting (TCSPC).
2.5.1.2 Analysis

Lifetime measurements were analysed using the phasor approach in SimFCS developed at the Laboratory for Fluorescence Dynamics (LFD) (UCI, Irvine, CA). Calibration was performed using Atto 488 free dye, which is known to have a mono-exponential lifetime (Atto-Tec, AD 488-41) and quenched lifetime measurements were referenced against a donor only sample in order to determine FRET efficiency. The quenching trajectory was determined by assigning the phasor position of the donor and background. The FRET efficiency was calculated for each condition from the change in lifetime compared to the donor only sample (Equation 10). Lifetimes were also calculated by exponential decay fitting using a non-linear least squares model, to confirm values obtained through phasor analysis (Equation 11 and 12). For more detail on these calculations refer to Chapter 1.3.5.
FLIM FRET between AF568-αSyn and Hsc70-AF647

These experiments were performed using the same protocol as described above with minor modifications. For FLIM experiments of FRET between αSyn and Hsc70, concentrated αSyn (58 µM) or dilute αSyn (13 µM) was mixed with Hsc70-AF647 (0.8 µM) and unlabelled DNAJB1 (1.6 µM) in BOG refolding buffer. A 20x refolding buffer was used to limit the dilution of concentrated αSyn at this step. Different to previous FLIM experiments, excitation was via a single-photon fiber coupled pico-second-pulsed diode 532 nm laser and fluorescence emission was collected through a 585/75 nm Semrock BrightLine band pass emission filter. The instrument response function was determined by measuring an Atto 565 free dye (Atto-Tec, AD 565-41) and AF568-αSyn alone was used for donor only phasor trajectory calibration.

2.5.2 N&B

2.5.2.1 Experimental Procedure

Sample and Coverslip Preparation

Aliquots of AF568-αSyn were freshly thawed for each N&B measurement. For experiments involving chaperones aliquots of Hsc70 and DNAJB1 were also freshly thawed. Chaperones were used at 1.6 µM and 3.2 µM respectively. Dilution steps were carried out immediately prior to measurement in BOG buffer. For experiments involving chaperones dilution was carried out in BOG refolding buffer. Sterile fluorodishes were used for these experiments. Each fluorodish was sealed with parafilm to avoid evaporation during measurements. For imaging, AF568-αSyn was used either concentrated or diluted in either the presence or absence of chaperones. Dilution was always performed immediately prior to imaging and samples were pipetted up and down gently 10 times. Measurements were taken at regular intervals after dilution.

Imaging Conditions

Raster scanned images were taken on a Fluoview FV1000 fluorescence inverted confocal microscope (Olympus) using a 100x oil UPLAN S Apochromatic objective (1.4 NA) and a 543 nm argon laser. 100 frames per measurement were
captured in pseudo-photon counting mode at 10 µs pixel dwell time in 256 x 256 pixel images.

2.5.2.2 Analysis

All data acquired were analysed using SimFCS (LFD, UCI, Irvine, CA). Background signal was included in at least one image for each dataset to adjust brightness measurements (Equation 6 and 7). A high pass filter algorithm (detrend filter) was applied to all images to correct for photobleaching, changes in background conditions, drift or other changes which could affect the variance. Free AF568 dye at a concentration of 1 µM was used to calibrate the monomer brightness value. Minor calibrations required to account for imaging in pseudo-photon counting were performed as outlined in Dalal et al.172.

2.5.3 Intrinsic Tyrosine Fluorescence

2.5.3.1 Experimental Procedure

Sample Preparation

Aliquots of unlabelled αSyn were freshly thawed for each intrinsic tyrosine fluorescence lifetime measurement. Dilution steps were carried out immediately prior to measurement in BOG buffer and samples were pipetted up and down gently 10 times. Measurements were taken at regular intervals after dilution. All measurements were performed at room temperature. To determine the fluorescence lifetime of thermally denatured protein, boiled samples were heated at 95°C for a minimum of 10 minutes in a Thermocycler (Bio-rad) prior to measurement.

Measurement Conditions

The intrinsic tyrosine fluorescence lifetime of unlabelled αSyn was measured using a Fluoromax-4 fluorimeter (Horiba) in a quartz sub-micro cuvette (Starna). Excitation was carried out using a 293 nm delta diode (Horiba) in Fluoromax-4C-TCSPC configuration with excitation polarisers out of the beam path and the diode
was pulsed at 16 MHz repetition rate. The emission monochromator was set to 315 nm with 10 nm slit width and the decay was measured until 10000 counts were reached in the peak channel. Ludox was used to measure the instrument response function.

### 2.5.3.2 Analysis

Data collected were converted to text files using DAS6 fluorescence decay analysis software (Horiba) and then analysed using TTTR Data Analysis software developed in the Biomedical Imaging Facility (BMIF), University of New South Wales. Lifetimes were calibrated to the measured instrument response function before applying a Fourier transform and representation in the form of a phasor plot (Equation 8 and 9). For a more detailed explanation of phasor plot representation and lifetime calculations refer to Chapter 1.3.5.

### 2.5.4 FCCS and PIE

#### 2.5.4.1 Experimental Procedure

**Sample and Coverslip Preparation**

Aliquots of AF568-αSyn, Hsc70-AF647 and unlabelled DNAJB1 were freshly thawed for each measurement. These experiments rely on a very low sample concentration to ensure that only single molecules diffuse through the confocal volume at any time therefore picomolar concentrations of the labelled proteins were used. Dilution was carried out in BOG refolding buffer.

Coverslips were cleaned as described for single-molecule TIRF experiments (Chapter 2.5.5). To prevent protein from sticking to the surface, passivation was performed overnight at 4°C with coverslips placed onto 1 mg mL$^{-1}$ BSA droplets on parafilm inside a home-made humidity chamber. Just prior to imaging coverslips were removed from the chamber and excess BSA was rinsed with buffer and then dried by blotting on its edge. The coverslips were used inside a 25 mm Chamlide chamber.
**Imaging Conditions**

These measurements were performed using a time resolved, inverted confocal fluorescence microscope (Microtime200, PicoQuant GmbH) and SymPho64 software. Excitation was carried out via pulse interleaved single-photon fiber coupled pico-second-pulsed diode 532 nm and 640 nm lasers (1 pulse of alternating laser line every 12.5 ns, each laser was operating at 40 MHz with overall duty cycle of 80 MHz sync rate) using a 63x water objective (1.25 NA). Fluorescence emission was collected using a 532/640 dichroic, a 649 beam-splitter and Semrock BrightLine single-band pass emission filters (585/40 nm for donor channel and 692/40 nm for acceptor channel) onto single-photon avalanche diodes (SPADs) coupled to high speed electronics for time-correlated single-photon counting (TCSPC). Photons for each measurement were collected over a 30 minute time period. Both burst analysis and cross-correlation analysis could be performed on a single data set obtained using these multi-parameter acquisition measurements.

![Diagram](image_url)

**Figure 2-2** Schematic of the microscope set-up with pulse-interleaved 532 and 640 nm lasers, a 532/640 dichroic mirror (DM), 100 µm pinhole (PH), 649 nm beam splitter (BS) and 585/40 nm emission filter for donor emission (EFD) and 692/40 nm emission filter for acceptor emission (EFA) and single-photon avalanche photodiode detectors (SPADs).
2.5.4.2 Analysis

Two-colour Cross-Correlation

Cross-correlation analysis was performed using the TTTR Data Analysis software (BMIF, UNSW). A lifetime weighted filter was applied to eliminate spectral bleed-through and remove false cross-correlation and detector dead time\textsuperscript{168}. For a more detailed description of auto-correlation and cross-correlation refer to Chapter 1.3.3.

Burst Analysis with PIE

Burst analysis of data acquired with PIE was performed using the PAM console, a stand-alone MATLAB GUI kindly provided by Professor Don Lamb (Ludwig Maximilians University of Munich, Fluorescence Applications in Biology Lab). The All Photon Burst Search Algorithm was used with 50 photons per burst and 5 photons per time window. A threshold was applied to intensity traces and only bursts above this threshold were analysed. For more detail on the formula and calculations used for this analysis refer to background of FCS with PIE in Chapter 1.3.3. Fractional contributions of the different component lifetimes were also calculated to determine the proportion of the molecules which were involved in FRET. For these calculations the fluorescence decay was fitted with exponential decay functions until the $\chi^2$ value was close to 1. The fractional intensity was then calculated from the weighted amplitude of each contributing lifetime.

2.5.5 Single-molecule TIRF

2.5.5.1 Experimental Procedure

Microfluidics and Coverslip Preparation

To perform single-molecule TIRF experiments a simple single channel microfluidics flow device was utilised for sample delivery. Devices with channel depth of 40 $\mu$m were fabricated from SylGard® 184 Silicone Elastomer Kit (Dow Corning) (hereafter referred to as PDMS (polydimethylsiloxane)) poured over a microfabricated wafer mould (Australian National Fabrication Facility, University of Queensland). The polymer was cured overnight at 70°C. Devices were cut to size
and holes for tubing were punched using a 1 mm Biopsy punch (ProSciTEch, T982-10). Inlet and outlet tubing with 0.38 mm internal diameter (Clay Adams, 427405) was cut to 5 cm and 10 cm respectively. Inlet tubing was cut on an angle to avoid trapping of air during sample delivery. A luer lock needle (BD Precision Glide, 301801) was filed flat and inserted into the outlet tubing to allow connection to the syringe pump. Before use the PDMS device was soaked in isopropanol followed by water for 10 minutes each to remove any fluorescent polymer that was not cross-linked and could otherwise leach into the solution inside the channel.

Round glass coverslips (25 mm diameter, Marienfeld Superior, 0117650) were cleaned by 30 minutes sonication in 100% ethanol, 30 minutes sonication in 1 M NaOH and then rinsed in 4 successive 1 L containers of 0.22 µm filtered Milli-Q water. In each rinse, coverslips were passed through the air water interface 10 times. Coverslips could be stored overnight in fresh Milli-Q or used straight away after being dried at 70°C. Microfluidic chambers were completed by attaching clean coverslips to the PDMS device. To remove organic surface contaminants and generate a negatively charged hydrophilic surface, PDMS was treated for 10 minutes in a plasma cleaner (Harrick Plasma). Treated PDMS was then immediately joined to a glass coverslip and allowed to bond for 1 hour at 70°C. Devices were assembled inside a 25 mm Chamlide CMB chamber (Live Cell Instruments, CM-B25-1).

Channels were passivated to prevent non-specific binding and to facilitate targeted surface capture of the molecule of interest. Passivation was performed by 30 minute incubation of poly-L-lysine poly(ethylene glycol) (PLL-PEG) spiked with 20% Biotin (SuSoS AG, PLL-g-PEG compound #4), rinsed with water, dried, then coated with 0.2 mg mL⁻¹ streptavidin prepared in Blocking buffer and incubated for 30 minutes. Once streptavidin was applied to the surface no air was allowed to enter the channel to avoid stripping off the surface layers. Channels were kept in buffer after the streptavidin incubation until ready to be used. Specific capture of molecules could then be achieved through the strong streptavidin-biotin interaction. For membrane interaction experiments this was facilitated through the inclusion of a biotinylated lipid in the lipid composition during liposome
extrusion and for surface tethered αSyn experiments this utilised a biotinylated antibody. Specific details of these experiments are outlined in the following sections.

**αSyn Oligomer Interactions with Surface Tethered Membranes**

**Sample Preparation**

Liposomes with encapsulated Alexa Fluor 488 dye and 647 lipid dye were prepared and stored at 4°C under argon for up to two weeks. Oligomers of αSyn A140C-AF568 were prepared, stored at 4°C and used within a week. Aliquots of Hsc70 and DNAJB1 (unlabelled) were freshly thawed and chaperones were used at 1 µM and 2 µM respectively. All dilutions were carried out in A140C oligomerisation refolding buffer with the inclusion of 2 mM Trolox. Freshly passivated microfluidic channels were used for these experiments. Liposomes were diluted to 0.5 mg mL\(^{-1}\) and captured directly to the streptavidin surface through interaction with biotinylated head groups. To capture liposomes at a surface density of ~500-1000 liposomes per filed of view, 1 μL of liposome solution was loaded into the inlet tubing at 20 μL min\(^{-1}\) and then quickly flushed through the channel with 250 μL of buffer at 250 μL min\(^{-1}\). Monomers and oligomers of αSyn A140C-AF568 at 1 µM (with respect to the monomer concentration) were flushed into the microfluidics channel (20 μL at 100 μL min\(^{-1}\)) to visualise their interactions with immobilised liposomes leading to membrane permeabilisation. For further experiments to determine if chaperones could alter the permeabilisation of liposome membranes in the presence of αSyn, oligomers were incubated with chaperones for 30 minutes prior to being added to channels containing tethered liposomes. Image acquisition was started just prior to the injection of αSyn.

**Imaging Conditions**

Samples were imaged using an inverted TIRF microscope (Till Photonics) with Zeiss α Plan-apochromat 100x oil objective (1.46 NA), 488, 561 and 640 nm laser lines, 525/50 and 585/40 Semrock BrightLine single-band pass emission filters and electron-multiplying charge-coupled device cameras (Andor). Emission signal was split onto the two cameras by a 561 nm long pass dichroic mirror. The
TIRF angle was adjusted to give an illumination depth of 200 nm. Images were acquired simultaneously using 50 ms exposure time at 6.24 FPS. To determine the location of the liposomes and avoid bleaching the encapsulated dye, focusing was performed using the 640 nm laser. A single image of the 647 lipid dye was also captured before and after the imaging series to confirm the location and presence of the liposomes. An image of 0.1 µm TetraSpeck™ Microspheres (LifeTechnologies, T-7279) was taken to align images captured on different cameras for analysis and account for chromatic aberrations.

Figure 2-3 Schematic of the Till Photonics microscope configuration used for smTIRF experiments with 4 wavelength lasers, acousto-optic tunable filter and a high numerical aperture objective. The TIRF angle was selected to produce the appropriate evanescent field depth in the microfluidic channel. Emission signal was split onto two CCD cameras using a 561 nm long-pass dichroic mirror. Appropriate emission filters were in place before the CCD cameras as outlined in the imaging conditions.

Chaperone Interactions with Surface Tethered αSyn Oligomers

Sample Preparation

Oligomers of αSyn A140C-AF568 were prepared, stored at 4°C and used within a week. Aliquots of Hsc70 and DNAJB1 (either labelled or unlabelled) were freshly thawed and chaperones were used at concentrations of 10 nM and 20 nM respectively. All dilutions were carried out in A140C oligomerisation refolding buffer with the inclusion of 2 mM Trolox. Freshly passivated microfluidic channels
were used for these experiments and oligomers were captured to the surface by coating the surface with a 1:1000 dilution of anti-αβ oligomer antibody. The antibody was loaded into the inlet tubing (5 μL at 20 μL min⁻¹) and then slowly passed through the channel with 25 μL of buffer at 5 μL min⁻¹. Oligomers at 300 nM (with respect to the monomer concentration) were flushed through the microfluidics channel (10 μL at 20 μL min⁻¹). These were allowed to absorb for 1 minute. At least 200 μl buffer was then flowed through the channel at 200 μL min⁻¹ to remove unbound oligomers. To further passivate the surface before the addition of chaperones, the channel was filled with 1 mg mL⁻¹ BSA and incubated for 5 minutes. Excess BSA was then removed from the channel by 40 μL of buffer at 20 μL min⁻¹. Chaperones were added to the system at 20 μL min⁻¹. Image acquisition was started just prior to the injection of chaperones.

**Imaging Conditions**

Samples were imaged using an inverted TIRF microscope (Till Photonics) with Zeiss α Plan-apochromat 100x oil objective (1.46 NA), 488, 561 and 640 nm laser lines, 525/50 and 585/40 Semrock BrightLine single-band pass emission filters and electron-multiplying charge-coupled device cameras (Andor). For 3 colour imaging, the emission signal was split onto the two cameras by a 561 nm long pass dichroic mirror. Images were acquired using 50 ms exposure time at the minimal cycle time (11.42 FPS) to capture the rapid kinetics of chaperone interactions. To minimise photo-bleaching a single frame of the 568 labelled oligomers was captured for every 10 frames of chaperone signal (Hsc70-AF647 and/or AF488-DNAJB1). Photobleaching movies of each individual protein component were taken at the same imaging conditions to provide corrections for photo-bleaching. An image of TetraSpeck™ Microspheres was taken to align images captured on different cameras for analysis.

**2.5.5.2 Analysis**

**αSyn Oligomer Interactions with Surface Tethered Membranes**

Fluorescence images were analysed using algorithms coded in MATLAB (The Mathworks)¹⁹⁴. Firstly, images were corrected for stage drift or movements during
acquisition. Gaussian flattening was used to correct for gradients in illumination. Channels were aligned by determining the shift between channels of the positions of reference molecules in different regions of image. The x/y-positions of immobilised fluorescent liposomes were determined in both channels using an algorithm that detects local maxima. Fluorescence intensity traces were calculated in both channels by integrating the fluorescence intensity in each frame of the fluorescence movie in regions of interest centred at each x/y-position corresponding to a fluorescent liposome. The arrival time of labelled molecules in solution that were injected into the flow channel was determined from the sudden increase in fluorescence background intensity in the red channel corresponding to AF568-labelled αSyn monomers or oligomers. Fluorescence traces showing signal loss in the green channel (indicative of the release of fluorescent dye due to membrane perturbations by αSyn) were sorted into four categories: (1) Single-step Fast Loss, (2) Multi-step Fast Loss, (3) Slow Loss and (4) Photo-bleaching.

**Chaperone Interactions with Surface Tethered αSyn Oligomers**

Fluorescence images were analysed using FIJI image analysis software and using algorithms coded in MATLAB (The Mathworks) (according to the protocol described above). The x/y-positions of immobilised αSyn oligomers were determined in the 568 channel. The arrival time of labelled chaperones was determined by the sudden increase in background fluorescence in the channel corresponding to labelled chaperones. Fluorescence traces were calculated at all positions corresponding to immobilised oligomers to extract traces of oligomer intensity (568 channel) and traces of chaperones (488 and 640 channels) co-localising at these locations. The oligomer intensity traces report on the disassembly of aggregates as a result of chaperone interactions while chaperone traces reveal the duration of chaperone interactions and the number of chaperones associated with the oligomer.
Chapter 3 – Preparing Functional Proteins for Microscopy

3.1 Introduction

In this project the main goals were to resolve the dynamics of the highly controversial αSyn multimer, to use αSyn as a known chaperone substrate to examine its interactions during the Hsp70 cycle and to investigate the permeabilisation of membranes upon αSyn binding. To achieve these goals a range of biochemical and biophysical techniques were applied to in vitro reconstituted model systems. An important consideration for this work was that there are 8 different Hsp70s and 41 different J-domain co-chaperones with varying roles expressed in different cell types. To investigate changes which occur from the native state of αSyn multimer to its less stable form, the chaperones which are present before the heat-shock response are the most relevant. The chaperones chosen for this study were heat shock cognate protein 70 (Hsc70) and DNAJB1. Hsc70 is predominantly present in the regions of the brain affected in Parkinson’s disease\textsuperscript{196} and as the constitutively expressed member of the Hsp70 family, would be present in the early stages of disease when aberrant protein aggregation starts to occur. DNAJB1 is one of the Hsp40 co-chaperones for the Hsc70 cognate protein and has been shown to function in mediation of αSyn aggregation\textsuperscript{197}.

Understanding the role chaperones play in protein misfolding and aggregation disorders requires their interactions with substrates to be studied at different points during the aggregation pathway. To investigate the initial stages of protein rearrangement and potential shifts towards toxicity recombinant αSyn multimers, produced through a gentle purification protocol\textsuperscript{125} were used. To understand the mechanisms of interaction with on-pathway intermediates an A140C mutant protein of αSyn was used. This protein was purified through a less gentle procedure, albeit still without boiling, which gave a higher yield required for making the oligomers. Oligomers prepared through this method were typically between 15-150mers and are hypothesised to represent the early stage soluble oligomers that are linked with toxicity\textsuperscript{198}.
Fluorophore choice is an important consideration for microscopy. In this study, the Alexa Fluor dye series was chosen for its high photo stability, good quantum yields and wide range of colours. The small size of the chemical fluorophores was also an important factor, as the size of some of the more popular fluorescent proteins such as mCherry and the GFP variants can be quite large\textsuperscript{199}. GFP for example is 26 kDa in size and when working with oligomerisation protocols and protein-protein interactions the addition of a molecule of this size is not optimal. This was an especially important consideration for αSyn multimers, as the dynamics and the regions which comprise the interaction interface for subunit exchange are currently unknown. A 26kDa tag added to a 14.5 kDa protein may have unknown effects on parameters like subunit associations that are yet to be characterised.

The use of thiol labelling through maleimide chemistry is well documented and has been successfully used on many cysteine mutants introduced into αSyn\textsuperscript{125,146,200}. The N-terminal additional residues including a cysteine in the αSyn multimer construct and the A140C mutation of αSyn for the oligomer preparation have been shown to have no effect on a range of αSyn properties such as fibrillisation and membrane associations. Thiol selective modification works by a thiol being added across the double bond of the maleimide to yield a thioester\textsuperscript{201}. Labelling via introduced cysteines works in either the absence of native cysteines (αSyn) or if the native cysteines are buried or inactive for labelling (Hsc70).

Another approach undertaken for labelling was through amine-groups and this was utilised for DNAJB1. This process can be specifically targeted to the N-terminus by using a buffer closer to neutral pH, as the pK\textsubscript{a} of the terminal amine is lower than that of the lysine ε-amino group. At neutral pH, the lysine residues which are present in the sequence will be protonated and therefore unreactive allowing a single fluorophore to be attached via a strong amide bond to the N-terminal primary amine\textsuperscript{202}. The sulfodicholorphenol (SDP) ester is the most hydrolytically stable of the amine-reactive dyes and therefore allows for more
consistent labelling reactions in water or physiological buffers typically used in protein purification.

In this chapter I present optimised ways of purifying and fluorescent labelling αSyn multimers, αSyn A140C monomers and oligomers, Hsc70 and DNAJB1 proteins. Once pure and labelled, protein assays to confirm chaperone functionality or to characterise αSyn oligomers were also performed. Together, these proteins make up the components required for characterising the dynamics of αSyn multimers and investigating how chaperones affect these processes (Chapter 4). Additionally these components were used to look at the permeabilisation ability of αSyn on membranes and how chaperone mediation can play a role in preventing membrane disruption (Chapter 5).

3.2 Production of α-Synuclein Monomer-Multimer

There are numerous protocols for the expression and purification of recombinant αSyn. Previously, αSyn has been described as an intrinsically disordered protein which can retain a small amount of α-helical content. Many of the purification protocols for αSyn involve the use of boiling steps in extracting the pure protein, whether that is recombinantly or from cells or tissue extracts. The more gentle protocol followed for producing the multimeric form of αSyn does not involve a boiling step as discussed in Chapter 1.2.3. There is a small amount (0.01%) of the detergent n-octyl-β-galactosidase (BOG) present in the buffers for this preparation, which has been shown to be below the critical micelle concentration. Wang et al. use a number of techniques to show that the multimers are present in the presence and absence of the BOG component. The detergent rather than inducing a structure that would not exist physiologically, functions to aide in maintaining the stability of the multimers present for the duration of experiments. Additional residues present on the N-terminus of this construct have been shown not to drive multimerisation (W. Wang, personal communication). Expression in E. coli BL21 Rosetta cells corrects for rare codons present in the αSyn sequence which can otherwise lead to stalling in the
translation process. These rare codons are particularly abundant in the N-terminus of αSyn.

Figure 3-1 Gel and FPLC chromatograms of αSyn expression and GST affinity purification. (a) Protein bands associated with expression (Lane 2), GST-αSyn lysate purification (Lane 3 & 4), crude SEC clean-up of GST-αSyn (Lane 5), cleaved GST and αSyn (Lane 6) and αSyn fractions after cleavage and separation using GST affinity chromatography (Lanes 7-12). (b) Elution profile of whole cell lysate purification of GST-αSyn showing flow-through peak and GST-αSyn peak eluted with 15 mM free-glutathione. (C) Elution profile showing separation of protease cleaved αSyn in the flow-through and GST eluted with 15 mM free-glutathione.

Expression of αSyn was achieved through IPTG induction of the tac promoter in the pGEX-6P-1 plasmid. In this construct αSyn is expressed as a fusion protein with GST which is located on the N-terminus. Sonication was used to lyse cells and affinity purification on an immobilised glutathione column was used to extract the GST-αSyn fusion from the cell lysate (Fig 3-1a Lanes 2-5). The GST-αSyn fusion (42 kDa) was then cleaved at the Precision protease cleavage site to yield αSyn with an additional 14-residues on the N-terminus, including a cysteine residue for labelling located two residues N-terminal of the start methionine. The additional residues have previously been shown to have no effect on αSyn normal behaviour\(^ {125}\). The cleaved GST (26 kDa) was removed from the αSyn (15.3 kDa) by again passing the solution through the GST resin. After cleavage αSyn passed through the column while uncleaved GST-αSyn fusion protein, GST alone and the Precision protease (26 kDa) all remained bound to the column (Fig 3-1b Lane 7-12). From initial steps around 85% pure αSyn protein (Fig 3-1) was obtained however labelling requires higher purity. αSyn was concentrated and subjected to size exclusion chromatography (Fig 3-2) to increase its purity. If required affinity chromatography and SEC were repeated until αSyn was pure enough for labelling. The total yield of αSyn was 0.2 mg per L of culture.
Figure 3-2 Gel and FPLC chromatogram of αSyn SEC purification. (a) Protein bands associated 
with the polishing step of αSyn using SEC. Pooled cleaved αSyn from GST affinity purification (Lane 2) 
was concentrated (Lane 3) and applied to a SEC column to remove further impurities from the 
αSyn fractions (Lanes 4-8). (b) Elution profile showing polishing of concentrated αSyn. Small 
impurities are removed with αSyn eluting in the main peak at elution volume 15 mL.

Optimal labelling conditions were tested to obtain the highest efficiency for 
this αSyn construct. The efficiency was improved by overnight reaction at 4°C and 
under the presence of argon. Overnight reactions at lower temperatures can 
improve labelling efficiency by slowing down the reaction and allowing more time 
for thiol conjugation to occur\textsuperscript{201}. The presence of the argon makes an oxygen free 
environment and prevents the re-oxidation of thiol groups allowing the maleimide 
chemistry reaction to occur more effectively. Free dye not incorporated onto the 
protein was removed using Zeba™ desalting columns with around 30% sample 
loss. Batch to batch variability was observed in the labelling efficiency (90-100%) 
however, it still produced a highly labelled protein of high purity (Fig 3-3).

Figure 3-3 Labelling of αSyn. (a) Labelling of >95% pure αSyn with Alexa Fluor 568 (Lane 2). 
Marker contains two fluorescent reference bands at 75 and 25 kDa (Lane 1). (b) Cartoon showing 
fluorescent label locations on putative αSyn tetramer subunits. Adapted from Wang et al\textsuperscript{125}
3.3 Production of α-Synuclein A140C Oligomers

The gentle purification method was critical for producing the multimer for characterisation studies of αSyn, however to produce the larger potentially toxic oligomers required for membrane binding studies, a different preparation protocol was adopted. This protocol uses ammonium sulfate precipitation followed by ion-exchange and SEC to produce αSyn A140C monomers. Monomers are then subjected to oligomerisation conditions and the large oligomers which form are separated from monomers using SEC. Previous studies have shown that the oligomers generated from this protocol are between 15-150 subunits and are annular soluble intermediates occurring naturally on-pathway during the fibrillisation process. Importantly for the biophysical approaches used in this work, these oligomers have also been shown to interact with negatively charged membranes and are stable upon dilution into the nanomolar range.

Figure 3-4 Gels and FPLC chromatogram of αSyn A140C monomer expression and anion exchange chromatography purification. (a) Protein bands associated with crude lysate ammonium sulfate precipitation purification of αSyn A140C. At 30% saturation impurities are removed in the supernatant (Lane 2) and αSyn is present in the supernatant (Lane 3), at 50% saturation αSyn is present in the pellet (Lane 4). (b) Protein bands associated with further purification of the αSyn pellet using anion exchange purification. Impurities eluted in the void volume (Lanes 2 & 3) and αSyn eluted at the 25 mM NaCl step (Lanes 4-6). Additional impurities were removed from the column at 35 and 100 mM NaCl (Lane 7). (c) Elution profile showing ammonium sulfate pelleted αSyn A140C purification using anion-exchange chromatography.

Expression of αSyn A140C was achieved through IPTG induction of the tac promoter in the pT7-7 plasmid. Again, an expression strain of E.coli (BL21 DE3 RP) which corrects for rare codons was used for optimal translation of αSyn. αSyn characteristically precipitates at 50% ammonium sulfate solution. A whole cell lysate was subjected to two rounds of ammonium sulfate precipitation. The initial 30% saturation precipitated a number of contaminants, which were pelleted (P).
by centrifugation and αSyn A140C remained in the supernatant (S). A 50% saturation resulted in αSyn A140C (14.5 kDa) precipitating out of solution and being contained in the pellet (Fig 3-4 a Lane 4). After resuspension the crudely purified αSyn A140C was loaded onto an anion-exchange column. Initially, a linear gradient was used to determine the salt concentration required to elute αSyn and to optimise the separation of the contaminant peaks. A step gradient of 25, 35 and 100 mM NaCl was then used for optimal separation (Fig 3-4c) which produced a high yield but required further purification (Fig 3-4b Lane 4&5). The sample was then polished using SEC (Fig 3-5b). If required an additional round of anion-exchange and SEC were performed to ensure the highest level of purity for labelling.

![Figure 3-5 Gel and FPLC chromatogram of αSyn A140C monomer SEC purification.](image)

(a) Protein bands associated with final pure αSyn from SEC. Fractions from anion-exchange were pooled (Lane 2) and concentrated (Lane 3) before loading on the column. High purity αSyn was eluted at 15 mL (Lanes 5-9). (b) Elution profile showing polishing step using SEC to obtain required purity of αSyn. αSyn eluted at its characteristic elution volume of 15 mL. * If required an additional round of anion exchange and then SEC was performed to produce >95% pure protein before labelling.

The optimal labelling conditions were determined using a time course as well as a ratio test of protein to dye. Given the high concentration of monomer required for producing the oligomers (1mM), finding the lowest amount of dye possible which gave a good labelling efficiency was critical. A 1:3 (mol:mol) ratio of protein to dye reacted for 4 hours was found to be optimal. Pure labelled monomers were successfully separated from free dye using a linear gradient in anion-exchange chromatography (Fig 3-6a,b). Unlabelled and labelled αSyn A140C was then concentrated and lyophilised for preparation of oligomers. The total yield of αSyn A140C was 9.92 mg per L culture.
Oligomers were made by subjecting a mix of labelled and unlabelled αSyn A140C to a fibrillisation protocol. Instead of allowing the process to proceed to fibrils, the preparation was stopped to obtain oligomers after 18 hours. Oligomer and monomer populations were then separated using SEC (Fig 3-6c), where large oligomers eluted in the void volume at 8 mL, corresponding to a molecular weight of around 2000 kDa and monomers eluted at 15 mL which is the characteristic elution volume for αSyn monomers.

**Figure 3-6 Gels and FPLC chromatogram of αSyn A140C monomer labelling and oligomer separation.** (a) Protein bands associated with fluorescent labelling of >95% pure αSyn A140C with Alexa Fluor 568 C5-maleimide. Marker contains two fluorescent reference bands at 75 and 25 kDa (Lane 1). Free dye eluted first (Lane 2), unlabelled αSyn A140C eluted with free dye (Lane 4) and labelled αSyn separated from free dye eluted last (Lanes 5 & 6). (b) Elution profile showing separation of free dye, unlabelled and labelled αSyn A140C monomer by anion-exchange. (c) Elution profile showing separation of αSyn A140C oligomer and monomers after oligomer preparation protocol by SEC. Oligomers eluted in the void volume around 8 mL and αSyn A140C monomers eluted at the characteristic elution volume on this column (15 mL).

### 3.4 Characterisation of αSyn Oligomers

There are a number of commonly used methods for testing the formation of amyloid fibrils in neurodegenerative diseases such as ThT and Congo Red assays. These are very efficient at measuring the formation of amyloid fibril, but what has been lacking until recently, is a way to measure the presence of intermediate oligomers before they reach the fibril stage. Exciting new studies in developing aggregation-induced emission (AIE) dyes has led to new assays to study these intermediates in aggregation pathways. These assays work by detection of oligomers through dyes which are non-fluorescent when molecularly dissolved but fluorescent when their intra-molecular motions become restricted through interaction with biomolecules. One such dye is tetrphenylethene tethered with triphenylphosphonium (TPE-TPP), which has been shown to be able to
differentiate monomeric, oligomeric and fibrillar species of αSyn as well as different conformations within these subsets.\textsuperscript{204,205}

Oligomers and monomers of αSyn A140C were taken directly from separation on a SEC column for the fluorescence assay. For this assay unlabelled αSyn A140C was used to avoid any interference from the Alexa Fluor dye fluorescence in the TPE-TPP emission spectra. A portion of monomer was also boiled for 15 minutes at 95°C, as previously it had been noted in our experiments that boiling causes significant changes to αSyn. Insulin monomers and fibrils were used as a negative and positive control respectively. After a 1 hour incubation with TPE-TPP the aggregation-induced fluorescence was measured with 321 nm excitation. Monomers of αSyn A140C show no fluorescence, whilst the oligomers show fluorescence intensity of around 200 000 CPS with its peak centred at 458 nm, which reflects the characteristic emission profile of this dye. Interestingly, after boiling the monomers exhibit the capability to interact with the dye and restrict its movement (blue). As there are no boiling steps in this preparation of αSyn A140C, this shows that boiling leads to a change in the intrinsic properties of αSyn, even when its monomers have been generated through a more standard preparation protocol.

\textbf{Figure 3-7 TPE-TPP fluorescence assay of αSyn A140C.} αSyn A140C oligomers are shown in black, αSyn A140C monomers are shown in red dashes. Monomers that have been subjected to boiling are shown in blue. An insulin negative control is shown as grey solid line and an insulin fibril positive control is shown as grey dashes. All protein samples were at the same concentration (5µM) and TPE-TPP concentration (15µM) was the same in every sample.
3.5 Production of Hsc70

Hsc70 was bacterially expressed with an N-terminal His₆-tag and additional GC residues on the C-terminus. Purification was performed using immobilised-metal affinity chromatography (IMAC) by gravity flow with Nickel-Nitrilotriacetic acid (Ni-NTA) beads. The imidazole rings in the His-tag sequence binds tightly with the nickel ions on the beads. Crude cell lysate was incubated with the Ni-NTA beads to allow efficient binding of tagged Hsc70 (70 kDa). Contaminant proteins were then removed by washing with a low concentration of imidazole to displace any low-affinity binding which can occur through dispersed histidine residues in non-tagged contaminant proteins. The more tightly bound His-Hsc70 was eluted using a high concentration of imidazole (Fig 3-8). After buffer exchange to eliminate excess imidazole, the His-tag was removed by cleavage at the TEV protease cleavage site. To separate the cleaved His and Hsc70 the cleavage mix was again incubated with Ni-NTA beads. With the His-tag removed, Hsc70 does not bind to the resin and was eluted in the flow through. This step allows removal of His, TEV and non-cleaved His-Hsc70 from the pure Hsc70 in a single step generating a highly pure product (Fig 3-8b). The total yield of purified Hsc70 was 25.2 mg per L culture. Hsc70 was then concentrated and labelled at the C-terminal engineered cysteine residue with Alexa Fluor 647 (Fig 3-8c). Excess dye was efficiently removed by use of desalting spin-columns to yield a highly pure, 100% labelled product (Fig 3-6b).

Figure 3-8 Gels of Hsc70 expression, purification and labelling. (a) Protein bands associated with His-Hsc70 expression (Lane 2), lysate purification using His-tag affinity chromatography (Lanes 3), pooled His-Hsc70 (Lane 4), concentrated His-Hsc70, His-Hsc70 (additional purification) before cleavage (Lanes 6-10). (b) Protein bands associated with pooled His-Hsc70 (Lane 2) and pure Hsc70 after cleavage and fluorescent labelling of >95% pure with Alexa Fluor 647 C2-Maleimide (Lane 3). Marker contains three fluorescent reference bands at 75, 25 and 2 kDa (Lane 1) (c) Cartoon of fluorescent label location on Hsc70 C-terminus.
3.6 Production of DNAJB1

Similar to Hsc70, DNAJB1 was also expressed as a His-tagged protein. The use of affinity tags in purification is extremely widespread as it allows a large increase in purity from crude lysate in a single step. His-tags are particularly popular due to their small size and the fact that they generally do not confer any functional changes to a protein. For this reason, many studies are performed without removal of the His-tag. Some studies also utilise the His-tag for specific capture in downstream experiments such as surface capture in microscopy. DNAJB1 was purified using a nickel charged affinity resin prepacked column to allow use of an automated system (FPLC). Crude lysate was loaded on the resin and most contaminant proteins either flowed through or were washed out using a low concentration of imidazole. A linear gradient of imidazole was used to elute His-DNAJB1 (40 kDa) (Fig 3-9). At this point His-DNAJB1 still contained some impurities (Fig 3-9a Lane 6). After buffer exchange the His-tag was cleaved off (Fig 3-9b) and the cleavage mix was run on a SEC column (Fig 3-10b) to separate out any further impurities. Pure DNAJB1 eluted around 13 mL and was then concentrated for labelling (Fig 3-10a Lane 4-7). The total yield of DNAJB1 was 10.6 mg per L of culture.

![Figure 3-9 Gels and FPLC chromatogram of DNAJB1 expression and His-tag affinity purification.](image)

(a) Protein bands associated with His-DNAJB1 before induction (Lane 2) and after induction and expression (Lane 3). Flow-through from affinity resin (Lane 4 &5) removed impurities and DNAJB1 was eluted using linear gradient of imidazole (Lane 6). (b) Protein band associated with cleavage mix of His, DNAJB1 and TEV protease. (c) Elution profile showing whole cell lysate purification of His-DNAJB1 using affinity chromatography and a linear gradient of imidazole.
Initially a number of methods to label DNAJB1 through its native cysteine residues were employed. There are three native cysteines present in its sequence, of which only one should be exposed for efficient labelling. Due to poor labelling of this cysteine in its native state, it was also attempted to label under either oxidative or reduced conditions. After many inconsistent results a new labelling strategy was employed. Labelling of DNAJB1 was successfully performed through specific targeting of the N-terminal primary amine. An Alexa Fluor 488 SDP ester was used as it is the most hydrolytically stable form of Alexa Fluor 488 for amine conjugation and produces stable carboxamide bonds between dye and protein. Amine reactive dyes react with non-protonated aliphatic amine groups either on the N-terminal amine or the ε-amino group of lysine residues. To specifically target the N-terminal amine for singly labelled DNAJB1, a more neutral pH was used. This results in protonation of lysine residues but not the N-terminal amine due to differences in the pKa. Before labelling DNAJB1 was buffer exchanged into sodium bicarbonate buffer to remove amines contained in the DNAJB1 storage buffer which interferes with labelling. Pilot labelling experiments were used to determine the optimal reaction time and temperature and free dye was removed using desalting spin columns as described previously in this chapter. The highly pure labelled product (Fig 3-11) had a labelling efficiency of 63%.

Figure 3-10 Gel and FPLC chromatogram of DNAJB1 polishing by SEC. (a) Protein bands associated with fractions of pure DNAJB1 after SEC (Lanes 2-9). (b) Elution profile showing concentrated DNAJB1 polishing step to remove contaminant TEV protease by SEC.
3.7 Functionality of Labelled Chaperones

Molecular chaperones are a pivotal part of our proteostasis machinery. They play a range of roles to maintain protein functionality and often work in complex networks of co-chaperones and other factors. In the Hsp70 (or Hsc70) cycle, the presence of a J-domain co-chaperone and a supply of ATP to drive the cycle are critical. With these minimal components the catalytic chaperone cycle can successfully be reconstituted in vitro. Here, a well characterised chaperone refolding substrate, β-galactosidase was used to confirm the refolding ability of recombinantly expressed Hsc70 and DNAJB1 and also to ensure the addition of a fluorescent label at either the N (Hsc70) or C (DNAJB1) terminus does not prevent chaperone function. The chromogenic substrate o-nitrophenyl-β-D-galactoside (ONPG) is recognised by β-galactosidase and when cleaved it yields galactose and o-nitrophenol which has a yellow colour. The amount of this yellow colour or more specifically its absorbance can be used as a read-out for the β-galactosidase enzyme activity. This assay can be used to study refolding activity of many different Hsp70 family members, including Hsp70, Hsc70 and the bacterial version DnaK, with similar activity observed across the family.

β-Galactosidase was unfolded by incubation with 6 M guanidine-HCl at 37°C. A time course of refolding was then measured as denatured β-galactosidase was
incubated with either BSA (negative control), Hsc70 alone, DNAJB1 alone, Hsc70 and DNAJB1 or labelled Hsc70-647 and DNAJB1-488. Refolding was measured through the absorbance of cleaved ONPG at 413 nm as β-galactosidase regained its enzymatic function. Absorbance was normalised to the absorbance of a positive control of native β-galactosidase with full enzymatic activity.

Hsc70 alone showed very little refolding with its absorbance just higher than the negative control. DNAJB1 alone was able to refold to around 40% of native activity. In the presence of both chaperones around 65% activity was recovered and slightly lower, with 57% when the chaperones were labelled. This shows that the chaperones expressed and purified are functional and able to actively refold denatured proteins. These values reflect the normal recovery of activity seen in other studies\textsuperscript{192}. The recovery of enzymatic activity in the presence of DNAJB1 alone was unexpected, as it does not traditionally refold substrates on its own.

![Figure 3-12 β-galactosidase refolding assay](image)

**Figure 3-12 β-galactosidase refolding assay.** β-Galactosidase was denatured and the refolding ability of chaperones was measured through a chromogenic substrate produced from β-galactosidase enzymatic cleavage. A positive control was measured using native β-galactosidase with 100% enzymatic activity. Absorbance measurements were normalised as a percentage of the positive control average absorbance. A negative control was measured using BSA, to confirm it was not simply the macromolecular crowding of extra protein in the system which promoted refolding.
3.8 Discussion

There are many different protocols for preparing recombinant proteins. Even the same proteins may be produced through a whole suite of different methods. A factor which is important to consider is the potentially different outcome, with reference to the intrinsic properties of the protein, which can results from different methods. The protocols and methods used in this project were carefully considered in order to best produce samples relevant to what was being studied. It will become evident in later chapters how critical some of these different approaches can be, especially in consideration of the monomer-multimer controversy surrounding αSyn.

The replication of the αSyn multimer recombinant purification protocol was a difficult one. Indeed other groups have attempted to reproduce this system, but have been unsuccessful\textsuperscript{126,207}. It was found that the overall yield, purity and function of the protein was improved by the expression and purification process being carried out as rapidly as possible. Over time (days) the protein degraded, leading to significant losses during subsequent purification steps. As discussed in Chapter 1.2.3 the multimer appears to be highly sensitive to concentration changes and temperature changes (namely boiling). Once these conditions were optimised, a highly pure multimeric αSyn was consistently produced.

Early labelling attempts on αSyn multimers also revealed small amounts of contaminant GST labelled very highly due to 6 cysteine residues in its sequence. Labelled GST in the αSyn sample would be indistinguishable from fluorescent αSyn and could therefore alter the results obtained. To increase the purity and yield of αSyn, steps additional to the published protocol were introduced. This included DTT included in purification steps to improve the purity and an increased pH during elution to improve the yield. As imaging techniques to be performed using this sample required high labelling efficiency, including subunit quantification which required every monomer to be labelled for accurate measurements, the labelling efficiency was also critical. Delays in purification lead to poor labelling efficiencies, as did trying to label freeze thawed aliquots. αSyn was therefore
produced from expression to snap freezing of labelled aliquots in no more than 5 days.

Optimisation of labelling conditions was a crucial procedure to allow for fluorescence measurements. Where reduction of disulphide bonds was used for labelling, TCEP was preferred over other reducing agents such as DTT or β-mercaptoethanol. As TCEP contains no thiols, it is not necessary to remove it before labelling reactions which improves the overall yield and processing time. The small amount of reactivity which can be observed between TCEP and maleimide dyes was overcome by adding an excess of dye in the labelling reaction. The separation of unlabelled and labelled proteins is a challenge that is faced in many protocols. In this work Zeba™ desalting columns were used in a number of proteins due to the simplicity of the technique. Although around 15-30% protein loss was often observed, this often amounted to a similar yield as techniques such as ion-exchange which required more steps and concentration of fractions.

A140C αSyn oligomers produced in this chapter were compared to those from the published report using the SEC elution profile from the same column used in the original papers. αSyn oligomers and monomers eluted at the exact same characteristic regions of 8 mL and 15 mL respectively, on the chromatogram as previously shown for oligomer-monomer separation. No additional methods were performed to assess αSyn structure; however the characteristic function of these oligomers is the ability to perforate membranes which was characterised in Chapter 5.

DNAJB1 is able to bind to target proteins and hold them to facilitate their interaction with Hsc70. One possible explanation for the higher than normal refolding of β-galactosidase in the presence of DNAJB1 in the chaperone refolding assay, is that DNAJB1 was keeping the substrate in a folding-competent state. This would allow for native refolding to occur over time by preventing initial unfavourable aggregation of denatured β-galactosidase. This would occur if
incubation of diluted β-galactosidase occurred at slightly reduced temperatures (compared to conditions of the assay) which can lead to spontaneous refolding.

The production of highly pure and functional proteins with high labelling efficiencies for biophysical and biochemical assays can be an arduous and time consuming task however it is critical to ensure that the samples to be studied in the subsequent chapters are an adequate in vitro representation of the physiological system being investigated.
Chapter 4 - Characterising Transient α-Synuclein Multimers and the Effect of Chaperones on Oligomerisation Pathways

4.1 Introduction

Until recently α-Syn was classified as an intrinsically disordered protein (IDP) that exists as an unstructured monomer. In 2011, two groups independently published evidence that α-Syn can exist as a multimeric form which is helically folded and resistant to aggregation. Its presence in this more structured and stable form was found in RBC, neurons and also in recombinant α-Syn expressed using a non-denaturing protocol\textsuperscript{124,125}. These results though very exciting, were highly controversial and the existence and relevance of the multimer form has been vigorously debated\textsuperscript{126,209}. There are numerous conflicting studies regarding the structure of α-Syn\textsuperscript{207} and one possible explanation for these differences is the use of various protocols for producing recombinant α-Syn. These could generate fundamental variations in samples of α-Syn being studied.

Early work focused on the characterisation of α-Syn as an unstructured monomer in solution and its interactions with biological membranes. Despite being classed as an IDP in its monomeric form α-Syn also exhibits a residual level of secondary structure\textsuperscript{93,210,211}, functionally relevant intermolecular interactions\textsuperscript{212} and has been shown to convert to an extended helix upon membrane binding or in the presence of lipids or lipid-mimics\textsuperscript{146,150,213–216}. The presence of conserved 11-mer repeats similar to that of apolipoproteins and other membrane binding proteins suggests membrane interaction to be an essential functional capability of α-Syn. These repeats have recently been shown to be critical in multimerisation\textsuperscript{129} of α-Syn suggesting that the multimer is linked to the functional properties. While the exact roles are not known, membrane-binding is thought to be important for α-Syn function and it has been shown to be important in processes such as vesicular transport and trafficking\textsuperscript{217–219}. Interestingly membrane interactions in the form of pore formation and membrane thinning have been proposed as potential modes of α-Syn cellular toxicity\textsuperscript{220,221} and generally occur in the presence of either the
monomer form or larger on-pathway oligomers. Thus, there seems to be a delicate balance between functionality and toxicity and currently what maintains this balance is an intriguing mystery.

A suite of biochemical techniques, cell toxicity assays, confocal microscopy and molecular dynamics simulations have identified conformationally diverse αSyn oligomer species\textsuperscript{124,125,132,145,220,222–224}. These diverse oligomeric species were primarily identified as intermediates in the aggregation pathway leading to cell death. In contrast, the more recently identified 60kDa multimeric species has been postulated to be a storage form of αSyn in the cytosol\textsuperscript{120}.

It is well known that chaperones modulate a variety of neurodegenerative disorders. They bind substrate proteins in their unfolded or disordered state where the propensity for unfavourable interactions is high due to exposure of hydrophobic residues and mediate refolding to the native state\textsuperscript{33}. The interaction of αSyn with chaperones has been extensively studied; however these studies have been performed using the intrinsically disordered monomer, oligomers or mature fibrils. The constitutively expressed Hsc70 chaperone and its J-domain co-chaperone DNAJB1 were specifically chosen in this study to investigate their interaction with monomeric and multimeric species produced using a non-denaturing protocol. These interactions are expected to occur during normal cell function and may be fundamental in shaping the monomer-multimer equilibrium of αSyn as well as preventing shifts into its potentially neurotoxic species.

The main aim of this chapter is to investigate the structure and dynamics of the αSyn multimer species. The effect of chaperones on these assemblies and their interactions was also investigated. Current models suggest a dynamic nature in the αSyn multimeric form and the existence of an easily disrupted equilibrium between monomer and multimer. Here I have utilised fluorescence microscopy techniques along with complementary biochemical approaches to assess the proposed dynamic and transient nature of αSyn multimers in real time.

\textsuperscript{*} In this thesis, the tetramer and other physiologically relevant multimeric species are referred to as "multimers" whereas toxic species are referred to as "oligomers".
Fluorescence methods such as fluorescence lifetime microscopy (FLIM) combined with Förster resonance energy transfer (FRET) can be used to analyse the dynamics of subunit exchange in intra- and inter-molecular protein interactions. By mixing αSyn multimer populations labelled with different colour fluorescent dyes, the exchange of subunits between multimers could be measured by FRET and used to investigate the stability of the multimer interaction upon dilution and the effect of chaperones on this process. Chaperones were found to interact primarily with the dissociated monomer and this interaction was consequently further characterised to determine the stoichiometry between αSyn and Hsc70 in the chaperone cycle. Number and molecular brightness analysis (N&B) is a fluorescence fluctuation method which can be applied in order to determine molecular aggregation or multimerisation in vivo and in vitro. The molecular brightness of highly labelled αSyn in multimer assemblies was determined to investigate the composition of multimer species in αSyn populations.

Shifts in secondary structure that occur upon dissociation of αSyn multimers upon dilution and subsequent aggregation were also studied using the intrinsic fluorescence of αSyn, circular dichroism and dye binding assays. Comparison of these properties in a sample before and after boiling suggests that boiling can lead to structural changes resulting in enhanced aggregation. Overall the insight gained through these techniques could help pinpoint some of the mechanisms which lead to transitions between monomer or multimer forms and potentially toxic oligomer intermediates.

4.2 Dynamic Subunit Exchange of α-Synuclein Populations

To investigate the presence of dynamic subunit exchange in αSyn multimers two solutions of αSyn labelled with different colour dyes were mixed at a concentration above the proposed “dissociation threshold” for multimer species. Subunit mixing was evaluated using FRET between molecules of Alexa Fluor 488 labelled αSyn (AF488- αSyn) as donor (D) and Alexa Fluor 568 labelled αSyn
(AF568- αSyn) as acceptor (A) molecules. FLIM measurements were used to measure quenching of the donor lifetime, which occurs due to energy transfer.

![Figure 4-1 Dynamic subunit exchange between αSyn molecules revealed by FRET. (a) Phasor plot showing change in FRET efficiency from initial mixing of αSyn populations (Time 0) to mean maximum FRET efficiency (Time 1 hour). (b) Phasor representation of changes in donor lifetime due to FRET at increasing time points during reassembly of αSyn molecules into higher order species. (c) Graph of the changes in FRET efficiency after mixing of αSyn populations with donor and acceptor labels (at a αSyn concentration of 65 μM as highlighted by the red background) and upon dilution to 13 μM. The grey timescale represents time points after dilution.](image)

Fig 4-1a shows a phasor plot of lifetime measurements of mixed donor and acceptor αSyn. (For greater detail on the construction of phasor plots refer to Chapter 1.3.5). The lifetime of the donor only αSyn is used as a reference point for calculating the FRET efficiency in the mixed samples. Upon initial mixing (Time 0) no FRET between the donor and acceptor molecules was observed. Measurements were then taken over time until no further change in lifetime was measured (Fig S1). The plateau occurred 1 hour after initial mixing with a FRET efficiency of 37.5% (±7) (mean value measured in 4 independent experiments). The occurrence of FRET over time indicates a dynamic exchange of subunits between multimers formed in concentrated αSyn solutions, whereby fluorophores attached to the N-termini are positioned within 1-10 nm of each other in the multimer. The shift from a low to higher FRET efficiency over time indicates the change in multimer composition from purely donor or purely acceptor to mixed species and/or conformational changes.
Previously it has been shown that dilution of αSyn multimer species leads to dissociation into monomers\textsuperscript{125}. Upon dilution of the mixed population from Fig 4-1a down to 13 µM, the αSyn fluorescence lifetime returns to the original unquenched lifetime indicating low FRET efficiency (Fig 4-1b). This loss of FRET can indicate either a lack of subunit interaction likely due to dissociation into monomers or a shift to a more loosely structured arrangement where subunits are not in close enough proximity to each other for energy transfer to occur. After the initial dilution, there is a reoccurrence of energy transfer, with FRET efficiency increasing over a time frame of 5 hours to a maximum mean FRET efficiency of 32.5% (±2.5). When these results (Fig 4-1 a & b) are plotted together (Fig 4-1 c) it shows an increase in FRET over an initial mixing period, followed by an abrupt loss of FRET efficiency which returns to almost the initial FRET value slowly over time. There appears to be a reversible concentration dependent dissociation or rearrangement of interacting αSyn subunits below the proposed concentration threshold for multimer association. Reassociation as shown by increasing FRET indicates structural rearrangements are occurring during this time to form a more closely interacting population. These experiments were performed at room temperature, which differed by several degrees between repeats of the experiment. Thus, differences in the rate of the re-occurrence of FRET after dilution between independent repeats were attributed to differences in temperature. Reassociation and increase in FRET signal occurred much more rapidly in the data sets where the room temperature was elevated between 4-10°C.

**Reoccurrence of FRET between α-Synuclein molecules after dilution is delayed by chaperones**

The dilution experiments were performed in the presence of chaperones from the Hsp70/Hsp40 system (Hsc70 and co-chaperone DNAJB1) to investigate the effect of these chaperones on the processes leading to the reoccurrence of FRET with a mean maximum FRET of 31% (±6). The comparison between the FRET in the absence and presence of chaperones was carried out on data sets recorded on the same day, such that effects of temperature would not be a factor between these two conditions. When chaperones are present, there is a longer lag.
time before re-association gives rise to higher FRET populations (Fig 4-2 a). This longer delay in the presence of chaperones is consistent in all data sets. For these measurements the ¼ transition time was calculated instead of the more commonly used ½ time to obtain an estimate of the onset of re-association. In the FRET recovery curves shown in Fig 4-2 b, the onset of FRET recovery is delayed by 24 minutes in the presence of chaperones. Importantly the presence of chaperones led to a delay in the reoccurrence of FRET populations across all data sets, albeit with differences in the extent of the delay, which ranged from ranged from 4-34 minutes. The species generated through dilution of the concentrated sample appears to interact with chaperones, and given the loss of FRET upon dilution, this species is most likely a monomeric form, in a partially unfolded state that represents a chaperone substrate.

The percentage of unquenched donor present in each sample was also calculated to determine the proportion of labelled αSyn molecules that were involved in FRET. In the absence of chaperones there was 6-10% unquenched donor present in the sample meaning that approximately 90-94% of molecules were involved in FRET. In the presence of chaperones there was 13-20% unquenched donor, i.e. around 80-87% of molecules were involved in FRET. The decrease in molecules involved in energy transfer is probably owing to monomers becoming incorporated into chaperone-substrate complexes and thus unable to form multimers.
Figure 4-2 Effect of Hsc70 and DNAJB1 on the recovery of FRET between αSyn molecules after dilution. (a) Phasor representation of changes in donor lifetime at increasing time points during recovery of FRET in the presence of chaperones. (b) FRET recovery curves measured after dilution to 13 μM in the absence and presence of chaperones. Individual data sets were fitted with sigmoidal functions and the transition quarter point was calculated to estimate the onset of FRET recovery.

4.3 Reversible Concentration Dependent Dissociation of α-Synuclein Multimers.

To obtain information about the oligomer composition of the interacting αSyn species, N&B analysis was applied to a time series of images. This method allows us to determine molecular number and oligomerisation in our sample by looking at the first and second moment of the fluorescence intensity distribution at each pixel. The fluorescent labelling and concentrations of αSyn used in these experiments were the same as above, however only a single label was required for these measurements. A representative histogram of molecular brightness (B) values shows a well-defined pixel distribution for concentrated αSyn-568 solution measurements. Peaks were fitted with Gaussian distributions to determine the background and mean values for B (Fig 4-3 a) where the background had a mean B value equal to 1 and the αSyn-568 sample had a mean B value equal to 2. An example 2D histogram of B values as a function of fluorescence intensity (Fig 4-3 b) and corresponding selection map (Fig 4-3 c) shows how data is represented. The focal plane was positioned above the coverslip surface in the solution containing freely diffusing AF568-αSyn molecules. The brightness distribution in the selection map was homogenous throughout the sample droplet indicating no aggregation or clustering as opposed to what may be seen in in vivo measurements. For in vitro measurements the background fluorescence level is taken by imaging on the edge of the sample droplet and determining the background brightness value, represented here by pink pixels. The brightness level of the sample is normalised to the background of 1 and the monomer brightness is calibrated using free dye in solution (monomer B value = 1.3). Coloured boxes indicate corresponding monomer through to higher orders species in solution, based on incremental (0.3) increases in brightness for each additional monomer subunit. These boxes highlight pixels with the appropriate average brightness and these are reciprocally mapped back to the selection map image.
Figure 4-3 Data representation in N&B. (a) Typical brightness histogram for AF568-αSyn in solution has a background peak centred at 1 (blue) and a broader higher brightness multimer peak (red). The B value distributions for the oligomeric forms cannot be separated from the monomeric distribution however the calculated weighted average can be determined. (b) Representative typical two-dimensional histogram of B values as a function of fluorescence intensity and associated selection map. The pixels associated with the brightness of each multimeric state are captured within each coloured box with monomers (red), dimers (green), trimers (blue) and tetramers (grey) also distinguished from background signal (pink). (c). Pixels are selected in histogram distribution and highlighted in the corresponding colour in the selection map. Background pixels (pink) can be separated from oligomer distribution (multicolour).

A representative data set of the concentrated αSyn and the dilution time course (Fig 4-4) shows a range of oligomer species present during different conditions in the absence and presence of chaperones. At 65 µM, αSyn is comprised of a range of species from monomers through pentamers. The centre of the 2D histogram is found within the blue and grey boxes, indicating a prevalence of trimers and tetramers. Dilution to 13 µM shifts the centre primarily within the red box, which corresponds to a prevalence of monomers and some dimers. At 1 hour after dilution to a concentration of 13 µM, multimer species are present in the sample, with the peak centre contained in the red, green and blue boxes, indicating a composition of monomer, dimer and trimers. This re-association into multimers appears to be delayed in the 1 hour after dilution when chaperones are present, as the composition is still centred at lower B values. After 3 hours at 13 µM the centre region is contained mainly in the blue box, indicating trimers as the dominant species in both the absence and presence of chaperones. B value versus fluorescence intensity histograms can provide only an approximate estimate of the range of pixel brightness values captured in an image.
Figure 4-4 2D B value versus fluorescence intensity histograms. A representative data set of concentrated αSyn and its dilution time course. The calculated weighted average was determined by fitting two Gaussian distributions (inset). Two-dimensional histogram of B values versus fluorescence intensity in the absence (a-d) and presence (e-h) of chaperones. The number of subunits is represented from red through to yellow boxes corresponding to monomers through to pentamers, respectively. All dilutions and timepoints were also performed in the presence of BSA to
check for non-specific effects of increased protein concentration on multimer association. No effect was observed.

To determine the mean value a brightness (B) distribution histogram (Fig. 4-4 inset) was fitted with Gaussian distributions for each condition. The average brightness values are represented in a bar graph with vertical error bars representing the standard error of the mean (SEM). The significance of differences between mean brightness values was determined using a multiple comparison unpaired t-tests. The average B value for the concentrated (65 µM) sample was 2.005±0.047. In the absence of chaperones upon dilution to 13 µM the average value was 1.182±0.059, which increased over time as seen at 1 hour where the average B value was 1.683±0.027 and at 3 hours it was 1.853±0.014 (Fig 4-5a). There was a significant difference between the mean average brightness value at most conditions (P<0.05) indicating a significant change in the αSyn sample composition upon dilution and over time. When compared individually, the mean brightness values for the concentrated and the dilution at the end of the time course (3 hours) were found to be not significant. When chaperones were included during dilution there was a similar pattern observed (Fig 4-5 c), except at 1 hour after dilution. When the averages were compared between the two data sets there was a significant difference (*P<0.05) in the average brightness value 1 hour after dilution with the average B value in the presence of chaperones only 1.313±0.009 compared to 1.683±0.028 in the absence of chaperones.

By determining the number of pixels present for each brightness level, a distribution of subunit composition was constructed in the absence and presence of chaperones (Fig 4-5 b and d, respectively). Concentrated αSyn is composed of around 36% trimer, 26% tetramer, 24% dimer and 4% monomer. Some pentamers (1.5%) were also present. Upon dilution, this composition shifts dramatically to approximately 60% monomer, 27% dimer, 10 % trimer and 2.5% tetramers. After 1 hour the diluted αSyn is composed of a more varied distribution of subunits, with 14%, 39%, 32%, 12% and 2.5% for monomer through to pentamers, respectively. At 3 hours after dilution, αSyn shows a shift to the higher order species with 8%, 32%, 38%, 18% and 4% for monomer-pentamers, respectively. It should be noted that the distribution of brightness values determined for a single fluorescent dye
molecule (centred on a brightness value of 0.3) is broad. Thus, the technique does not have the resolution to distinguish whether small amounts of pentamers were present in the sample or whether these are part of the tetramer peak. The N&B analysis reveals the transient nature of αSyn multimers and suggests that αSyn can reassemble into higher order species after initial dissociation upon dilution. In the presence of chaperones the 1 hour time point had a significantly lower mean average brightness value and shows a much higher proportion of monomer (46.5%) and dimer (40%) species present compared to the levels in the dilution without chaperones. The presence of chaperones appears to temporarily delay the reassembly of multimeric forms. Our hypothesis that is this delay occurs through the αSyn monomers being held in a chaperone-substrate complex that prevents reassembly.

Figure 4-5 Average brightness values and subunit composition of αSyn multimers. Average brightness values for different αSyn conditions in the absence (a) and presence (c) of chaperones. Means were calculated as outlined in Fig 4-3. Vertical bars represent the standard error of the mean calculated using unpaired t-test with Welch's correction. *P<0.05 (one-way ANOVA).
Distributions of subunit composition before and at several time points after dilution in the absence (b) or presence (d) of chaperones. Values were determined from the proportion of pixels in an image associated with each brightness value. Minimum 3 measurements were taken in at least 2 replicate experiments.

4.4 Chaperones Preferentially Interact with Monomers

FLIM-FRET measurements were utilised to determine which of the species within the monomer-multimer equilibrium were targeted for chaperone intervention. In these experiments AF568-αSyn was used as the donor molecule and Hsc70-AF647 was the acceptor molecule and the FRET efficiency was determined immediately after mixing and 1 hour after mixing. In the first set of experiments, Hsc70 (1.6 µM) and DNAJB1 (3.2 µM) were mixed with αSyn at high concentration (>55 µM) where αSyn is present in a dynamic equilibrium between multimeric states. Under these conditions no FRET was observed between AF568-αSyn and Hsc70-AF647 suggesting that the multimer is not a chaperone substrate and points to the multimer being a native state. In contrast, FRET was detected immediately between the monomeric AF-568-αSyn donor and the Hsc70-AF647 acceptor when chaperones were mixed with freshly diluted αSyn (13 µM) (Fig 4-6a). Multiple populations with FRET efficiencies ranging from 28-45% were present under these conditions indicating the presence of multiple conformations of the chaperone-substrate complex to produce different proximities of the donor and acceptor molecules on the two different proteins. Overall these results suggest that the monomer produced upon dilution is partially unfolded which would lead to exposure of hydrophobic regions in the αSyn structure and make the dissociated monomer a likely chaperone target.

To further investigate the interaction of monomeric AF568-αSyn with chaperones the samples were diluted to 1 nM. At these concentrations it was determined using single-molecule imaging and photobleaching analysis that αSyn was predominantly monomeric and did not assemble into higher order species even after an extended period of time. As outlined in Chapter 1.3.3, pulse interleaved excitation (PIE) uses rapidly alternating light sources to identify complexes consisting of molecules labelled with different fluorophores that diffuse simultaneously through a confocal volume. Using this method the stoichiometry of the αSyn and Hsc70 interaction can be determined along with the FRET efficiency.
of this interaction (Fig 4-6b). αSyn interacts with Hsc70 with a stoichiometry value of 0.62, which is indicative of an approximate ratio of 1:1. The FRET efficiency of this interaction was 26%, which is similar to the lower FRET efficiency population seen in the FLIM studies of these proteins. Two lifetime components were present in the sample (Table 4-1). The major component lifetime of 3.63 ns corresponded to the unquenched donor (no FRET) and was contributed by 91.6% of the population. The shorter lifetime of 1.457 ns corresponding to the species undergoing FRET between AF568-αSyn and Hsc70-AF647 was contributed by 8.3% of the population. This interaction was confirmed through fluorescence cross-correlation analysis which showed a positive cross-correlation curve calculated to correspond to 13% correlation. FRET and FCCS measurements by pulsed interleaving were also performed on samples containing ADP instead of ATP and in the presence of Hsc70 without DNAJB1. All samples showed similar stoichiometry, FRET efficiency and cross-correlation.

Figure 4-6 Interaction between AF568-αSyn and Hsc70-AF647 revealed through FRET and fluorescence cross-correlation. (a) Phasor plot showing difference in FRET efficiency in concentrated αSyn and diluted αSyn mixed with chaperones. (b) Stoichiometry versus FRET efficiency histogram derived from the interaction of AF568-αSyn and Hsc70-AF647 at nanomolar
concentrations. (c) Positive cross-correlation curve of αSyn and Hsc70 interaction. All dilutions and timepoints were also performed in the presence of BSA to account for non-specific effects of increased protein concentration on association. No effect was observed.

**Table 4-1 Table of Non-FRET and FRET populations.** Fractional contributions of the different component lifetimes present during interaction of αSyn-568 and Hsc70-647 in solution. Measurements were taken at nanomolar concentrations and percentages were derived from the weighted amplitude of each lifetime.

<table>
<thead>
<tr>
<th>Sample</th>
<th>α₁</th>
<th>τ₁</th>
<th>α₂</th>
<th>τ₂</th>
<th>Fractional Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSyn+Hsc70+ DNAJB1+ATP</td>
<td>81.5</td>
<td>3.63</td>
<td>18.48</td>
<td>1.457</td>
<td>91.6% Non-FRET 8.3% FRET</td>
</tr>
</tbody>
</table>

### 4.5 Characterisation of α-Synuclein Folded State

In order to measure αSyn conformational rearrangements, changes in the local environment of tyrosine residues induced by dilution were monitored. Tyrosine residues located on the inside of a folded protein are shielded from solution such that they exhibit a long fluorescence lifetime. Protein unfolding leads to exposure of these residues and a reduction in fluorescence lifetime as a result of solution quenching occur. The intrinsic fluorescence lifetimes of αSyn tyrosine under different conditions were visualised using phasor analysis; a representative experiment is shown in Fig 4-7 and the average phasor positions with error bars for all data sets are shown in Fig 4-8. The instrument response function (IRF) was measured using Ludox. In a concentrated αSyn sample (65 µM), the location of the phasor point (black) on the phasor plot (Fig 4-7a) corresponded to a longer fluorescence lifetime and was located inwards from the universal circle, indicating lifetime heterogeneity as expected for tyrosine residues in different environments. Dilution to 13 µM shifted the αSyn phasor point along the universal circle in the direction of shorter lifetimes, as well as outwards towards the circle. This decrease in the lifetime was most pronounced immediately upon dilution (red) and was then slowly reversed towards longer lifetimes over a number of hours (time course is represented by spectrum colours starting at red for Time 0). The phasor points with increasing time also moved inward from the universal circle. This corresponds to an increase in lifetime heterogeneity. When the diluted sample was measured after overnight incubation, the phasor point (grey) showed a longer
lifetime and higher degree of heterogeneity compared to the initial concentrated αSyn sample. Importantly the concentrated form and the reassociated form formed after overnight incubation were distinctly separate from other points.

Taken together these results are indicative of a folded state for the initial concentrated sample. This folded state is lost upon dilution when a conformational change exposes internal tyrosine residues to fluorescence quenching. The reoccurrence of longer lifetimes over time indicates a gradual rearrangement, which again shields the tyrosine from solvent exposure. From these results it cannot be determined if this reassembly of a structure that shields the region of the Y39 is the same conformation as observed in the initial concentrated sample.

![Intrinsic tyrosine fluorescence reveals concentration-dependent αSyn unfolding and reassembly.](image)

**Figure 4-7 Intrinsic tyrosine fluorescence reveals concentration-dependent αSyn unfolding and reassembly.** Concentrated αSyn is represented in black, dilution time course in multi-colour (starting at red) and overnight in grey. Colours corresponding to different times in the time course (0 – 4 h) are shown in the rainbow box on the right of the phasor. Representative data set of (a) αSyn concentrated, diluted and reassembly under normal conditions and (b) αSyn concentrated, diluted and diluted reassembled overnight after boiling. Measurements represent 3 independent repeats.

Previous studies have shown that boiling αSyn leads to a loss of structure. To investigate if the structural loss associated with dilution is similar to that incurred upon boiling, concentrated and diluted αSyn were boiled before measurement. The diluted boiled sample was also allowed to reassemble overnight (Fig 4-7b). The concentrated αSyn phasor point (black) after boiling has a lifetime close to [0,1] (0 ns) and is also more monodisperse than without boiling. Diluted αSyn after boiling similarly has a short lifetime but has a more heterogeneous lifetime distribution. Overnight the average lifetime of the boiled sample increased slightly, however the phasor point moves further into the circle indicating an increase in lifetime heterogeneity. These measurements suggest that
dilution and boiling induce a similar change in solvent exposure, however with the former being more reversible.

![Figure 4-8 Average phasor plot for tyrosine fluorescence experiments.](image)

**Figure 4-8 Average phasor plot for tyrosine fluorescence experiments.** Coloured points represent the average phasor location for each condition over all data sets. Colours corresponding to different times in the time course (0 – 4 h) are shown in the rainbow box on the right of the zoomed error phasor. Error bars represent SEM. Measurements were performed in triplicate and represent 3 independent repeats.

4.6 Secondary Structure and Dye-binding Properties of α-Synuclein Species

To further characterise the structural features that are perturbed during dilution and boiling of αSyn, changes in secondary structure were determined using circular dichroism (Fig 4-9). The concentrated (65 µM) form of αSyn exhibits characteristic of α-helical and unstructured regions as expected from previous studies\textsuperscript{125}. In the concentrated form αSyn has multiple peaks including a peak at 222 nm, which is characteristic of alpha-helical content. The peak at 205 nm is the convolution of a typical 208 nm helical peak and a 198 nm coiled-coil negative peak. The positive peak at 195 nm is also indicative of helical structure. There is a pronounced change in signal strength as well as a wavelength shift from 205 nm to below 200 nm in the diluted form, which indicates that it is largely unstructured. The CD spectrum of the diluted sample shown in Fig 4-9 is observed immediately and up to 24 hours after dilution (i.e. the spectra did not change over time). The boiled sample also shows a peak at around 200 nm indicative of a loss of structure. These results suggest that both dilution and boiling lead to structural perturbations compared to the concentrated form. In contrast to the boiled form,
the dilute form retains some helical content but not to the same extent as the concentrated αSyn. The reassembly process seen in the intrinsic tyrosine fluorescence studies is possibly due to tertiary and/or quaternary structural rearrangements, as no recovery of additional helical content is seen by CD.

An oligomerisation assay was performed utilising an aggregation-induced emission (AIE) dye which becomes fluorescent when the rotation of its bonds are restricted through complexation with biomolecules. Unlike other dyes used to study αSyn aggregation, TPE-TPP is able to detect intermediate species on the aggregation pathway as well as fibrillar forms of αSyn. The concentrated form of αSyn exhibited an intensity of around 580 000 counts per second (CPS) that did not change over time. The intensity of dilute αSyn was 120 000 CPS at initial dilution and increased to 465 000 CPS after 1 hour as it reassembled to multimeric species. As the dye detects oligomers, it is not surprising that the dilute species showed more fluorescence than the boiled sample after 1 hour (355 000 CPS) as boiling abolishes any secondary structure and slows or prevents any form of functional reassembly. The species in the boiled sample are likely the beginning of aggregates and these were also detected when the concentrated sample was boiled (740 000 CPS). The presence of a fluorescent signal for the diluted sample indicates the presence of some higher order species still exist after dilution, consistent with results from other methods. When these results with αSyn monomers are compared to the same assay using the αSyn A140C monomers and oligomers, the monomer has no fluorescence but boiling can generate the propensity for associations (Chapter 3, Fig 3-7).
Figure 4-9 Secondary structure and dye-binding assay of αSyn. (a) Circular dichroism (CD) spectra of αSyn concentrated (black), diluted (red) and boiled (blue). (b) TPE-TPP aggregation/oligomerisation-induced fluorescence emission assay of αSyn concentrated (65µM), diluted (13µM) and boiled with or without reassembly time. Insulin monomers were used as negative control and preformed insulin fibrils were used as a positive control.

4.7 Discussion

Toxicity and cell death in PD is now attributed to the presence of soluble toxic oligomers\textsuperscript{234}. These intermediates can prevent the formation of the more inert fibril forms\textsuperscript{235} and increase toxicity most likely through membrane disruption mechanisms\textsuperscript{203,236}. Protein aggregation generally occurs through complex networks of intermediates and pinpointing the functionally relevant forms has proven difficult in studies of αSyn. The controversy surrounding αSyn centres on the identity of the native state as a disordered monomer or a helically folded tetramer. A recent model for the tetrameric form postulates the presence of a dynamic equilibrium between monomers and tetramers, which can be shifted to induce aggregation prone species\textsuperscript{237}. Recent findings have given more weight to the idea of the tetramer being a physiologically relevant form. It has now been shown that the highly conserved KTKEGV repeat motifs in αSyn are critical for tetramerisation\textsuperscript{129}. Further, familial PD (fPD) mutations shift the tetramer to monomer ratio in favour of the monomer\textsuperscript{130}. These studies further highlight the importance of characterising the dynamic interactions and structural perturbations which occur within the tetrameric form. Understanding these mechanisms and identifying factors that modulate tetramer stability will help in determining whether the multimer acts as an inert reservoir to store excess αSyn or if it is indeed involved in the functions of αSyn.

The recovery of tetramers from the cell is highly dependent on protein concentration indicating that macromolecular crowding or other cellular components such as molecular chaperones may be essential in maintaining the tetrameric structure\textsuperscript{224,238}. Another factor that could play an important role in the stabilisation of the tetrameric form is post-translational modifications. With up to 85% of all human protein undergoing acetylation\textsuperscript{239} this modification along with other unknown factors could be critical for the stability of αSyn multimers. Investigation into the importance of N-terminal acetylation in αSyn
multimerisation has thus far yielded contradictory results with respect to the monomer-tetramer debate\textsuperscript{240–244}. Consistently however, N-terminal acetylation leads to an increase in helical content. In some cases stabilisation of the oligomeric form or decreases in aggregation propensity also occur \textsuperscript{145,240,245–247}. Recently \(\alpha\)Syn purified directly from human brain was shown to lose stability during the process of purification. It was suggested that a stabilising molecule such as a small lipid present inside neurons is lost during the purification procedure, but would normally be present in the cell\textsuperscript{128}. This area requires further investigation but there are interesting implications for the balance between monomeric and multimeric forms of \(\alpha\)Syn, how this is shifted and the functional relevance of these species.

In this chapter, the multimer species was produced using a gentle purification protocol described by Wang \textit{et al.} \textsuperscript{125}. The proposed subunit structure for the multimeric \(\alpha\)Syn species obtained with this protocol is an anti-parallel set of \(\alpha\)-helices\textsuperscript{125,248}. A structural model places the \(\alpha\)Syn subunit N-termini in close proximity to each other. Consistent with this structural model, it was demonstrated here using FRET that the proximity of the labelled N-terminus of \(\alpha\)Syn subunits was in the range required for energy transfer (1-10 nm). The presence of a FRET signal between subunits is indicative of interacting donor and acceptor \(\alpha\)Syn molecules, however does not provide information on the oligomeric state giving rise to these signals. The stability of the multimeric species is low as subunit exchange takes place readily upon mixing of two populations with different labels, whereby the molecules with different labels have re-equilibrated after around 1 hour.

Number and brightness analysis provided insight into the multimeric state of \(\alpha\)Syn and revealed a shift in the distribution of multimeric states under different conditions. The rapid transition from predominantly multimer to monomers followed by a slow reassembly of higher order species over time was unexpected. In the context of familial PD mutations high monomer prevalence over tetramers leads to decreases in solubility along with increases in neurotoxicity and cellular
inclusions. Here it was shown that multimers are capable of recovery to some extent and there seems to be an inherent property in αSyn to form multimers. A concentration-dependent effect on the rate of these transitions and subunit exchange is highly plausible, however was not investigated in this work as the primary interest was investigating the difference between concentrated and dilute samples within the range around the threshold level of 0.5 µM.

The techniques that are commonly used for studying protein unfolding rely on external dyes or extrinsic fluorescent probes. By using intrinsic fluorescence the innate fluctuations and changes within the protein are not perturbed and are more sensitive to their local environment. αSyn has four tyrosine (Y) residues (Y39, Y125, Y133, Y136), with the latter 3 contained within the proposed largely unstructured C-terminal extension region. The C-terminus of αSyn is thought to be unstructured within both the disordered monomer and the structured multimeric αSyn. Owing to this, in these studies it is assumed that changes in intrinsic fluorescence are primarily attributed to Y39, which is located within the proposed helix-turn-helix region in each subunit in the multimer form. This location has previously been shown to undergo large changes in local environment in tryptophan mutant studies. By utilising the intrinsic fluorescence of the tyrosine residues there is no unknown effects imparted through the amino acid substitution. Over time it is seen that the diluted sample is able to reassemble and left overnight it appears to be even more solvent protected than in the original concentrated form. Interestingly the region around Y39 in αSyn is also proposed as the binding site for interaction with chaperones from the Hsp70 family. Dilution and subsequent exposure of this region would represent a prime target for chaperone intervention. Clustering of mutations around this core binding region could also explain how the shift to the aggregation-prone form is generated, through either a loss of reassembly ability or interference with the chaperone binding site. Both of these situations would lead to an accumulation of unfavourable species.
In the concentrated sample, both disordered and helical content can be seen, although there is a less pronounced helical content than in previous findings\textsuperscript{125} it is probably due to a lower concentration being used in our studies. Upon dilution there is still some residual α-helical structure present. The complete absence of secondary structure in the boiled form shows the potential to shift the equilibrium between the structured multimer to unstructured monomers more permanently by harsher treatment of the protein. The oligomerisation assay using the new AIE oligomer detecting dyes shows that these same species which lack most secondary structure after reformation, can still be perturbed by boiling. Taken together these results suggest that structural perturbation due to disease-associated point mutations which induced monomerisation, stress or other factors which lead to destabilization of the tetramer induce the formation of a species that is perhaps less able to reassemble to a multimer form and therefore potentially more prone to aggregation.

The delay in FRET recovery resulting from subunit re-association when chaperones are present suggests a highly amenable target for chaperone intervention. The lag time differences associated with the presence of chaperones range from a few minutes to around half an hour. These delays are on the same time scale as the difference in subunit composition seen through brightness analysis. The predominant monomer species present at time 1 hour when chaperones are present points to a αSyn monomer-chaperone interaction that holds onto the substrate for long enough to slow down restructuring. This association was confirmed through fluorescence methods probing the interaction of αSyn with labelled Hsc70. These methods both show that there is a binding preference for the largely unstructured monomer generated through dissociation of subunits upon dilution and that these associations occur rapidly.

The idea that there is a transition from folded monomers of αSyn to a more association-prone monomer has been discussed in the past\textsuperscript{127}. Recently, this model was updated to include the transition from an equilibrium of folded monomer and tetramer to the association-prone monomer form\textsuperscript{237}. Here I propose a model which further builds on these ideas where αSyn exists as an equilibrium of highly
dynamic multimers which are easily disturbed through perturbations such as change of concentration, boiling or other stress. It suggests an intrinsic ability to reassemble the multimeric species, albeit with a slightly different conformation, appears to be retained in this partially unstructured monomer. The monomer generated through dilution is a highly favoured chaperone target which retains the capacity to form multimers if the protein is reconcentrated. Upon boiling however, the complete loss of any structure points to another type of monomer which is a more association-prone species and lacks the ability to reassemble into multimers. This pinpoints the importance of the idea of a subset of monomer species, which are partially structured with the ability to still function correctly, as opposed to those which become aggregation-prone and have the potential for toxicity. Over time this partially structured state is lost as even the monomers retaining residual structure can begin to have unfavourable associations which can lead to aggregation.

Figure 4-10 Proposed model for the αSyn monomer-multimer equilibrium and structural changes associated with perturbations. At sufficiently high concentrations, structured multimers are in equilibrium with structured monomers and there is a dynamic exchange of subunits between structured multimers. This equilibrium is easily shifted by perturbations including dilution, heat or potentially other stresses such as shearing forces or chemicals. In the perturbed state αSyn monomers exist in various levels of disorder. Boiled αSyn undergoes a complete loss of structure and over time forms unstructured assemblies on the pathway to aggregation. Monomers generated by dissociation of subunits upon dilution retain some helical structure and an innate ability to reassemble. These monomers can reassociate to form lower order oligomers. Over time these assemblies undergo conformational changes but do not reach the original multimer form. The presence of critical factors rescues these assemblies and allows them to attain their original multimer form.
Chapter 5 - α-Synuclein-Membrane Interactions

5.1 Introduction

Evidence in the previous chapter, as well as in an increasing number of recent publications suggests that the multimer form of αSyn is a physiologically relevant conformation\textsuperscript{129,130,237}. The 11-mer KTKEGV repeats critical for multimerisation are also extremely important for membrane binding\textsuperscript{122,129,214,255}. While the exact cellular roles of αSyn remain elusive, membrane-binding has been shown to be important for most of the proposed physiological αSyn functions. Interestingly, αSyn toxicity is also largely attributed to its membrane binding capability\textsuperscript{256}. Given the array of structural states of αSyn in the cell including monomers, multimers or soluble oligomers, it is plausible that these species play specific roles in αSyn functions or toxicity.

Membrane association is thought to help drive multimerisation\textsuperscript{237}, potentially through brief interactions that allow monomers to adopt the helically folded conformation required to form multimers. When multimers interact with membranes and then dissociate, small amounts of lipid often remain bound and may help stabilise the multimer complex in solution\textsuperscript{220}. Recent work with αSyn extracted from human brains confirmed that the removal of interacting lipids during purification leads to instability of the multimer\textsuperscript{128}. At synapses, αSyn multimers cluster in synaptic vesicles\textsuperscript{223,257,258} and may also function as a lipid carrying protein with the ability to remodel membranes\textsuperscript{259}. In fact many of the putative roles of αSyn may be mediated by a multimer form, and it has been suggested that αSyn multimers may function via an interlocking model between different multimer subunits\textsuperscript{223}. These roles require a functional ability to rearrange the membrane and if this process was to become exaggerated then it could cause significant membrane disruption. The interaction of αSyn multimers with membranes thus far reveals no membrane disturbing effect\textsuperscript{125,223}. Multimers can, however, readily dissociate to aggregation-prone monomers and subsequently
form oligomers, whereby the destructive properties of αSyn monomers and early stage oligomers are well documented²¹⁷,²⁶⁰.

Monomeric and oligomeric species of αSyn have been shown to permeabilise membranes via a proposed pore or channel like mechanism²³⁶,²⁶¹,²⁶². Pore formation allows uncontrolled diffusion of molecules across the membrane and can alter the membrane potential and ion gradient ultimately leading to cell death²⁶³. Alternatively a membrane thinning or remodelling mechanism has also been proposed¹³⁸,²⁶⁴. It has been suggested that the origin of αSyn toxicity is actually the mechanism of membrane association and not the species which causes it²⁶³. This rationale seems to fit with the observations that both monomeric and oligomeric αSyn can generate similar changes to lipid membranes. The exact mechanism which gives rise to membrane perforation is currently unknown, but it is one of the most critical factors in identifying the mode of αSyn cellular toxicity.

The power of single-molecule techniques to elucidate biological mechanisms is in the ability to capture and investigate intermediates of processes at the molecular level without the need for synchronisation. These techniques overcome the limitations of ensemble measurements which cannot resolve these intermediates as a result of ensemble averaging. Membrane permeabilisation studied at a single-liposome level can provide much more detail of processes which may all be occurring simultaneously²⁶⁵ and could potentially allow for identification of the interaction by which αSyn associates with membranes that leads to toxicity.

Chaperones are thought to play an important role in modulating processes that lead to αSyn toxicity and it has been hypothesised that when the chaperone network becomes overwhelmed then αSyn oligomers or monomers are able to induce membrane toxicity. However, there are currently no studies investigating the role chaperones play in mediating pathological αSyn membrane interactions, which represents a significant gap in our understanding.
The first section of this chapter focuses on the optimisation of conditions for single-molecule fluorescence microscopy assays designed to allow direct visualisation of the interactions between species captured on the surface of a glass cover slip and species in solution. This section includes an outline of surface passivation, coverslip cleaning and assembly of microfluidics devices.

The interactions of αSyn oligomers or monomers with immobilised liposomes is then explored using two colour co-localisation and single-molecule analysis. The perforation of the membrane is measured by monitoring the release of fluorescent dyes encapsulated in the liposomes whereby different mechanisms of membrane disruption can be distinguished by the kinetics of release. These processes are then investigated in the presence of chaperones to determine the ability of chaperones to modulate these processes.

5.2 Optimisation of Single-Molecule Approaches

For many single-molecule techniques the ability to specifically capture molecules onto surfaces at a defined density while preventing the non-specific adsorption of molecules in solution is critical. In smTIRF microscopy the spacing between molecules on the surface needs to be sufficient to allow detection of individual molecules on a coverslip as diffraction-limited objects. When the surface density becomes too high, the PSFs associated with single fluorophores overlap and cannot be resolved as individual molecules. Surface passivation is used in smTIRF to block non-specific interactions of labelled molecules with the glass surface. Proteins are immobilised through a specific interaction such as capture by an antibody on the surface such that the molecules remain functional and are tethered in a defined orientation. In this case the protein remains accessible for binding partners in solution. When proteins bind to glass through hydrophobic interactions they can denature, aggregate or become unstructured. Binding sites may also become inaccessible for their interaction with molecules in solution.
Figure 5-1 Surface passivation of coverslips for smTIRF. (a) Schematic of the surface chemistry utilised to prevent non-specific binding to coverslips during single-molecule microscopy. (b, c) TIRF microscopy images of molecules bound non-specifically on a cover slip exposed to a 10nM solution of αSyn-AF568 without (b) and with (c) surface passivation.

Surface passivation is achieved through multiple layers (Fig 5-1 a). First, the surface is coated with a copolymer composed of poly-L-lysine (PLL) and poly(ethylene glycol) (PEG), whereby 20% of the PEG chains contain a biotin group at their ends. This provides a polymer brush which coats the surface and prevents non-specific interactions of proteins. The incorporation of biotin provides the ability for the next layer to attach through the biotin-streptavidin interaction. Streptavidin is bound to the surface in a blocking buffer solution which also contains Tween-20 and BSA for further passivation. Finally, liposomes are captured on this surface via biotinylated lipids included in the lipid composition. Alternatively, a biotinylated antibody is used to capture proteins such as αSyn. At the concentrations used for smTIRF (around 1-10 nM) the difference in non-specific binding on glass (Fig 5-1 b) and on passivated glass (Fig 5-1 c) is substantial. While thousands of molecules bind to glass alone, after passivation only a few molecules absorb non-specifically.

In order to perform single-molecule experiments, a number of conditions need to be optimised. The accurate detection of single-molecules requires that coverslips and microfluidics devices used for imaging are free of fluorescent contaminants. Surface passivation is a very important factor in preparation of coverslips for single-molecule microscopy. Likewise, the cleaning method used before the surfaces are passivated can also significantly alter the level of background fluorescence. There are a number of different approaches for cleaning
coverslips for single-molecule TIRF. These include using solvents and strong bases, a mixture of sulfuric acid and hydrogen peroxide (piranha solution), sonication and plasma cleaning. Chemical, vibrational or ionic treatments each serve specific purposes, to remove particles and dust, breakdown organic material or generate charged surfaces. Often a combination of these approaches is utilised, whereby the optimal approach needs to be determined experimentally for the sample being studied.

The general protocol for surface cleaning used in this study included rinsing in 70% ethanol (to remove any dust or particles) followed by sonication in 100% EtOH. The coverslip was allowed to dry and then sonicated in 1 M NaOH, followed by thorough rinsing with ultrapure water. To optimise this process, the length of the sonication steps and the effect of an additional step using plasma cleaning on non-specific binding was investigated. Sonication was performed in each step for either 10 minutes or 30 minutes. After drying some coverslips were also treated in a plasma cleaner for 3 minutes (Fig 5-2). Coverslips were then passivated and exposed to solutions of αSyn-568 at a concentration of 1 nM.

Coverslips that had been cleaned with 10-minute rounds of sonication showed a large amount of non-specific binding (Fig 5-2, top-left), which was considerably reduced when plasma cleaning was used (Fig 5-2, top-right). Thus, sonication for 10 minutes rounds was deemed insufficient. Coverslips that had been subjected to 30-minute rounds of sonication showed even lower levels of non-specific absorption (Fig 5-2, bottom-left) and in this case plasma cleaning increased non-specific absorption (Fig 5-2, bottom-right). If the coverslip was already clean from the sonication, plasma cleaning would merely serve to negatively charge the glass, which can enhance non-specific adsorption of some proteins; this was apparently the case for αSyn. The 30 minute sonication method without plasma cleaning was determined as the best for this system.
Figure 5-2 Comparison of non-specific absorption of αSyn-AF568 on coverslips prepared by different cleaning protocols. It should be noted that non-specific adsorption on the surface treated with 30-minute sonication steps without plasma cleaning was only detected after 10 minutes of incubation with αSyn-AF568 whereas molecules were detected on the other surfaces immediately after adding the protein.

Next, the protocol for the assembly of microfluidics chambers was optimised, which relies on binding of glass coverslips with the PDMS device. Typically both components are plasma-treated to achieve a strong bond. As plasma cleaning of glass was found to increase non-specific binding of proteins, only the PDMS was treated in the plasma cleaner to generate a charged surface and promote the bonding between the chamber components. In early experiments the disassembly of these devices was a considerable problem. This was overcome through longer bonding time (1 hour at 70°C). PDMS devices were also found to leach fluorescent contaminants into the channel. This was attributed to uncross-linked PDMS polymer and this contaminant was removed by soaking the device in isopropanol prior to use. Separate microfluidics tubing was also used for experiments using either liposomes, purified protein or proteins produced in lysates to avoid cross-contamination.
5.3 α-Synuclein A140C Interactions with Membranes

αSyn has been shown to have lipid-binding properties, a feature which appears to be related to both the physiological function of αSyn as well as its mode of toxicity. This toxicity is thought to arise from pore formation or membrane thinning in localised regions around bound αSyn, however little is known about the actual mechanism. To investigate the membrane permeabilisation ability of αSyn, liposomes with encapsulated Alexa Fluor 488 dye were captured to the surface of a microfluidics channel. The binding of αSyn with membranes is most efficient in the presence of negatively charged lipids and depends on the curvature of the membrane. Liposomes were therefore made from a mixture of predominantly DOPG (negatively charged), with the inclusion of a small amount of biotinylated PE and Atto-647 labelled DOPE (for surface capture and membrane visualisation, respectively) (Fig 5-3). During extrusion liposomes were passed through a 200 nm pore. This diameter generates a curved membrane surface but is still large enough to encapsulate a sufficient number of dye molecules for monitoring the membrane permeabilisation process.

Figure 5-3 Schematic representation of single-molecule assay design to investigate the membrane permeabilisation ability of αSyn.
The principle of the assay is shown in Fig 5-3: Liposomes containing AF488 were captured on the surface of the glass cover slip and exposed to different species of αSyn-AF568. Image acquisition was initiated immediately prior to injecting αSyn-AF568 into the flow channel. The fluorescence intensity of the encapsulated dye (AF488) and AF568-labelled αSyn was captured onto separate cameras for analysis. The effect of different αSyn-AF568 species on membrane permeability was then determined by monitoring the loss of the fluorescence intensity of the encapsulated dye as a result of its release from the liposome upon membrane perforation.

First, the rate of photobleaching of the encapsulated dye was determined at the ensemble level by analysing the decay of the mean fluorescence intensity in the green channel. Photobleaching was measured multiple times in the absence of αSyn and the average bleaching curve is reasonably well described by a single exponential decay with a rate constant of 0.023 s^{-1} (Fig 5-4 a). Photobleaching was also measured whilst flushing 0.5 mg mL^{-1} BSA through the channel at the standard flow rate used for these experiments (100 μL min^{-1}) with similar results.

Next, the mean fluorescence profile in the red channel was used to determine the arrival time of αSyn-AF568. Solution exchange was rapid, whereby 80% of the maximum intensity was reached within ~5 seconds after arrival of the protein in the flow channel (Fig 5-4 b). Mean fluorescence decay curves for the loss of the encapsulated dye were measured in the presence of αSyn oligomers or αSyn monomers in the presence and absence of Hsc70 and DNAJB1 chaperones. In the presence of oligomers the mean fluorescence decay was multi-exponential with a fast component \(k = 0.1547\) s^{-1} and a slow component \(k = 0.0271\) s^{-1}. When oligomers were pre-incubated with chaperones for 30 minutes prior to addition to the liposome channel the fast component disappeared and only a single slow exponential decay \(k = 0.0264\) s^{-1} was observed (Fig 5-4 c). When monomers were added to liposomes, a fast \(k = 0.1890\) s^{-1} and a slow \(k = 0.0296\) s^{-1} component were also observed. In the presence of chaperones the loss of fluorescence was again slowed to a single slower decay rate \(k = 0.0268\) s^{-1} (Fig 5-4 d). From this bulk analysis it can be concluded that both oligomers and monomers of αSyn have
the ability to increase the rate of loss of fluorescence of the encapsulated AF488 dye. The slow process present in all data sets was attributed to photobleaching. The fast rates represent a process vastly different from photobleaching and was attributed to membrane perforation. Chaperones appear to have the ability to prevent this fast process in both the presence of oligomers and monomers.

In order to understand the cause of the fast decay rates in the presence of αSyn monomers and oligomers, a more in-depth analysis was performed at the

Figure 5-4 Ensemble fluorescence decays of AF488 dye encapsulated in liposomes and arrival curve of 568 labelled αSyn. (a) Average curve of 4 photobleaching traces measured in the absence of αSyn. (b) Representative data set of arrival curve of 568 labelled αSyn. (c, d) Loss of fluorescence of the encapsulated AF488 dye as a result of interactions with αSyn monomers (c) and oligomers (d) in the absence (dark) and presence (light) of chaperones. Concentrations used in relevant experiments were 1 µM αSyn, 1 µM Hsc70 and 2 µM DNAJB1. Experiments were also performed in the presence of 0.5 mg ml-1 BSA and only photobleaching traces were observed.
single-liposome level. Liposomes were detected as local maxima in the fluorescence image and the background-corrected fluorescence intensity trace was determined in both channels at each location corresponding to a liposome. Figure 5-5 shows the mean traces averaged across all liposomes for dye release (AF488) and αSyn binding (AF568). In contrast to the αSyn arrival trace shown in Fig 5-4b (which is dominated by the background fluorescence and reveals the fast kinetics of solution exchange), the αSyn binding trace reveals the kinetics of binding to the membrane. As the αSyn fluorescence (red) increases, the encapsulated dye fluorescence decreases (green). The association of αSyn with the liposome occurs with slower kinetics than the arrival of the αSyn in the channel. Monomers appear to associate very rapidly and then begin to bleach, or dissociate from the liposomes. Oligomers however continually build up on the immobilised liposomes.

![Graphs](image)

Figure 5-5 Background-corrected fluorescence intensity traces averaged across all liposomes in the field of view showing the loss of the encapsulated AF488 dye (green) and the binding of AF568 labelled αSyn monomers (a) or oligomers (b) (red).
Figure 5-6 Representative single-liposome traces of AF488 dye intensity showing single-step fast release.

Figure 5-7 Representative single-liposome traces of AF488 dye intensity showing multi-step fast release.
Figure 5-8 Representative single-liposome traces of AF488 dye intensity showing slow release.

Figure 5-9 Representative single-liposome traces of AF488 dye intensity showing photobleaching.
Loss of Encapsulated Dye Fluorescence Characterisation

The analysis of traces of individual liposomes revealed four distinct classes of fluorescence loss. Traces were categorised into: single-step fast, multiple-step fast, slow steps and photobleaching steps. Examples of the traces observed for each of these classes can be seen in Figures 5-6 through to 5-9, respectively. Traces for single-step fast and multiple-step fast liposomes showed large sudden drops in fluorescence. These kinds of traces were indicative of the abrupt loss of liposome content associated with membrane perforation and will be henceforth referred to as such.

In the presence of αSyn oligomers around half of the liposomes were perforated (23% single-step, 26% multiple-step) rapidly losing their encapsulated dye. The other half of the liposomes predominantly underwent photobleaching (4.5% slow steps, 46.5% photobleaching). When oligomers were pre-incubated with chaperones perforation of the membrane was completely abolished. There are some liposomes which undergo slow steps (10.5%); however the vast majority undergo photobleaching (89.5%). When αSyn monomers interacted with liposomes, around 40% of liposomes were perforated (16% single-step, 23.5% multiple-step). There were more slow-step traces in the presence of monomer than oligomer (11% compared to 4.5%) and 49.5% of liposomes underwent photobleaching loss of fluorescence. When monomers were pre-incubated with chaperones, the perforation of the membrane was again completely abolished with over 90% of traces classed as photobleaching and 9.8% as slow steps. The fast single and multiple-step classes of membrane perforation could represent an important mechanism in how αSyn causes cell death. Identification of different sub-populations of αSyn membrane binding is one of the benefits of single-molecule analysis. These classes would have been averaged out in ensemble experiments.
The loss of fluorescence through membrane perforation represents around half of all liposomes in the absence of chaperones. A closer look at the release of the dye content in the fast steps reveals that single and multiple-steps have similar content release kinetics (Fig 5-11). In the presence of monomers the distribution of content release times is well described by a single exponential decay function with a rate constant of $k \sim 0.1 \text{ s}^{-1}$ for both single and multi-step traces. When oligomers bound to the liposomes, similar distributions of content release times were observed, which could also be approximated with a single exponential decay with a rate constant of $k \sim 0.11-0.12 \text{ s}^{-1}$. Taken together these observations suggest that that dye release occurred via a similar process for liposomes exposed to monomeric and oligomeric αSyn and that this process was governed by a single rate-limiting step.
Figure 5-11 Encapsulated AF488 dye release time distributions determined for liposomes releasing dye in single or multiple fast steps in the presence of (a) monomers or (b) oligomers.

Figure 5-12 Intensity distribution of encapsulated AF488 dye in liposomes. (a) Histogram of background-corrected intensity values at the beginning of the experiment (mean value of the first three time points of the fluorescence intensity trace for each liposome). (b) Histogram the cubic root of the liposome intensities (same data as in panel (a)).
**Relationship between Class of Fluorescence Loss and Liposome Size**

With the presence of distinct classes of release within the system the next question that needed to be answered was whether certain classes of fluorescence loss corresponded to the size of the liposome. The content fluorescence intensity distribution of all liposomes in a field of view (Fig 5-12) was skewed to higher values as expected considering that the intensity value should be proportional to the liposome volume. Indeed, the distribution of the cubic root of the content dye intensity values of all liposomes (Fig 5-12) was reasonably well described with a Gaussian function, indicating that the radius of the liposome population was normally distributed.

Next, the intensity of a single AF488 molecule was determined using single molecule photobleaching. This calibration value was then used to estimate the number of dye molecules per liposome (obtained by dividing the content dye intensity of each liposome by the intensity of a single AF488 molecule). Analysis of the liposome intensities shown in Fig 5-12 revealed that liposomes contained on average 12 AF488 molecules (5 – 24 AF488 molecules for liposomes with an estimated diameter within one standard deviation of the mean). Assuming that the dye was incorporated at the same concentration as that of the solution used during liposome preparation, then the diameter of the liposomes would correspond to 120 ± 24 nm (mean ± SD), which was smaller than the pore diameter (200 nm) of the membrane used for extrusion. The discrepancy may be the result of the error associated with determining the single molecule intensity of AF488 and/or the result of partial quenching of the encapsulated dyes due to the high dye concentration.

The intensity of the encapsulated AF488 dye (which scales with the volume, i.e. the radius cubed) was related to the intensity of the AF647 lipid dye (which scales with the surface area, i.e. the radius squared) on a single-liposome level. As shown in Fig 5-13, the square root of the lipid dye intensity was strongly correlated to the cubic root of the content dye intensity a with Pearson’s correlation coefficient of 0.76 (p<0.00001). This analysis confirmed that the intensity of the content dye was related to the size of the liposomes.
Figure 5- 13 Correlation between the cubic root of the encapsulated AF488 dye intensity and the square root of the AF647 lipid dye intensity. Correlation was calculated using Pearson’s correlation coefficient = 0.76. p-value <0.00001.
Figure 5-14 Intensity distribution histograms of encapsulated AF488 dye in liposomes undergoing loss of fluorescence via processes with different kinetics (sorted into four different classes) in the presence of monomers (a) or oligomers (b).
The AF488 encapsulated dye intensity of each liposome within each class was also determined to ascertain if liposome size was related to its mode of dye loss. Liposomes exposed to αSyn monomers showed the following average number of dye molecules per liposome: 17.3 encapsulated molecules for fast/single step traces; 14.0 encapsulated molecules for fast/multiple step traces; 16.6 encapsulated molecules for slow traces and 7.5 encapsulated molecules for photobleaching traces. In the presence of oligomers the following average number of dye molecules per liposome were observed: 12.3 encapsulated molecules for fast/single step traces; 12.9 encapsulated molecules for fast/multiple step traces; 18.0 encapsulated molecules for slow traces and 8.1 encapsulated molecules for photobleaching traces. There was some variation amongst these values, however the lowest number of AF488 molecules per liposome were found in the photobleaching class in both monomer and oligomer samples. The fluorescence intensity in the photobleaching class suggested that they contained around half to one third of the number of AF488 molecules compared to the other classes. While αSyn still bound to these liposomes, no perforation occurred. This lack of activity could be due to factors such as the curvature of the liposome or insufficient surface area for formation of membrane disrupting complexes.

**Levels of αSyn Associated with Liposomes**

Next, the intensity of the signal in the αSyn-AF568 channel were analysed to determine the level of monomers and oligomers binding to the liposome membrane and to gain further insight into the mechanism of membrane perforation. αSyn monomers bound to liposomes to reach on average a maximum intensity of ~24500 per liposome (Fig 5-15 a), while oligomers reached on average a maximum intensity of ~28400 per liposome (Fig 5-15 b) during the course of the experiment. Interestingly, the average maximum intensity of αSyn species associated with each liposome was not diminished in the presence of chaperones (Fig 5-15 c, d), showing values of ~21000 and 31000 for monomers and oligomers, respectively. This observation suggests that the chaperones did not prevent binding.
To determine the level of αSyn binding required to induce membrane perforation in the samples without chaperones, the αSyn-AF568 intensity was determined for each trace in class 1 (fast, single step release of dye) at the precise frame before the content was released. The resulting histograms of αSyn-AF568 intensity at the time of release are shown in Fig 5-15 e and f for αSyn monomers and oligomers, respectively. The mean αSyn monomer intensity at the time of release was ~6200. In the presence of the oligomers the mean intensity was ~4000 when content was lost. Given the low incorporation of labelled αSyn into the oligomers, it is difficult to quantify the difference between monomers and oligomers or if a potential “build-up” of αSyn or threshold level is required to induce membrane perforation. However, it was apparent that binding of multiple monomers was required to induce membrane perforation. In contrast, the low level of αSyn signal associated with a liposome at the time of content release in the presence of oligomers suggested that one or a few oligomers with several AF568 labels each might be sufficient to disrupt the membrane. Further, it is clear that the αSyn continues to interact with the liposome even after the perforation has occurred.
Effect of Membrane Perforation on Lipid Integrity

To visualise the effect of αSyn on the membrane and try to understand the mechanism by which encapsulated dye loss occurs, the AF647 lipid dye incorporated into the membrane was imaged before and after the flow of αSyn samples through the channel. As only a single image was taken at the beginning and the end of the experiment, any changes in the lipid signal from before to after αSyn addition are due to changes in the amount of lipids and not photobleaching.
effects. In the control no loss of the lipid signal was observed, with the images recorded before and after αSyn addition showing approximately the same intensity at each location corresponding to a liposome (Fig 5-16 a). When oligomers were associated with liposomes, the image recorded after αSyn addition and the overlay image show a pronounced loss of liposome signal at a number of locations as well as a general blurring of the liposome signal (Fig 5-16 b). When oligomers were pre-incubated with chaperones the lipid signal remained undisturbed and liposomes had the same intensity before and after αSyn addition (Fig 5-16 c). The same was seen in the presence of monomers, with perhaps slightly less disturbance of the lipid signal than with oligomers (Fig 5-16 d). Again, chaperones prevented disruption of liposomes as apparent in the overlay image of all liposomes (Fig 5-16 e).

Photobleaching of the content dye did not disturb the integrity of the liposome, as can be seen by the absence of lipid loss in the chaperone incubated samples. The oligomer exhibited a robust ability to cause loss of membrane, although the monomers also induced a large amount of membrane loss. This lipid could occur via removal of individual lipids or via removal of greater portions of the membrane, e.g. as a result of pore formation. It could also involve a general destabilisation of the liposome, leading to complete fragmentation. The main difference between the oligomer and monomer populations in terms of their effect on content release is the presence of more fast single-steps in the oligomer sample. It could be speculated that the fast/single-step release is the result of a pore-formation or membrane fragmentation, leading to the greater loss of lipid from liposomes. The fast/multiple-step release may then perforate the membrane in a mechanism that is not quite as disruptive as the single-step process.
**Figure 5-16 Fluorescence images of the AF647 lipid dye captured at the beginning (before αSyn addition) and the end of the experiment (after membrane perforation and dye release).**

The lipid dye was included to verify that liposome remained on the surface (i.e. that the loss of the encapsulated dye was not due to the entire liposome simply detaching from the surface). Magenta images show the liposome dye signal present before addition of αSyn, cyan images show the liposome dye signal after αSyn had been flowed through the chamber. The overlay shows the intensity ratio between the before and after images whereby liposomes that have lost a considerable amount of lipid appear in magenta. The control (a) shows that under normal flow no lipid is lost and no molecules detach from the surface. In the presence of oligomers (b) the overlay shows a number of liposomes where the membrane has been disturbed. Chaperone incubation with the oligomers (c) prevents the loss of lipids as seen by the normal co-localisation in the overlay when chaperones are present. The same pattern is seen in the presence of monomers (d) where there is a loss of lipid which is prevented by the presence of chaperones (e).

![Graphs](image)

**Figure 5-17 Ratio of lipid intensity before and after exposure to αSyn.** In the absence of chaperones around half of the lipid was removed after αSyn interactions (a & c). When chaperones were present, little (b) or no (d) lipid was lost.

To quantify these changes to the membrane, the ratio of the lipid intensity in the images before and after αSyn addition was determined for each liposome in the
field of view. In the absence of chaperones around half of the lipid signal was lost after exposure to αSyn monomers or oligomers (Fig 5-17 a & c). When chaperones were added to the monomer (Fig 5-17 b), ~87% of the lipid signal remained. When chaperones were added to oligomers (Fig 5-17 d), the lipid signal remained unchanged. Chaperones therefore prevented the release of the encapsulated dye by a mechanism that also inhibited the loss of lipid from the liposome membrane.

A closer look at the lipid ratios present for each different class of content release showed that there were distinct differences between the classes and the potential mechanism of perforation. In the presence of monomers (Fig 5-17 a), the fast/single-step class lost 71.7% of its lipid signal, the fast/multiple-step class lost 58.7%, the slow step class lost 57.9% and the photobleaching class lost 42.6% of its original lipid intensity. When oligomers were present (Fig 5-17 b), the fast/single-step class lost 70.8%, the fast/multiple-step class lost 56%, the slow step class lost 53.5% and the photobleaching class lost 36.4% of its original lipid intensity. The fast/single-step in both monomers and oligomers showed over 70% of the original lipid intensity is lost. The fast/multiple-step and the slow/step classes have similar values around 50%, and this further points to the idea that the single-step and the multiple-step fast classes are representative of two different processes.
Figure 5-18 Distributions of intensity ratio of lipid dye before and after interaction with αSyn broken down into the individual classes of content release. Monomers (a) and oligomers (b) exhibit similar distributions of intensity ratios in the different classes.
5.5 Discussion

The structure-function relationship of αSyn remains mysterious, however the identification of a likely functional form in the multimer species provides a new framework for thinking about these relationships. In addition, the oligomer form being widely accepted as the neurotoxic species means that a picture of the molecular pathway between native state and disease is becoming clearer. Our model suggests that disturbance of the multimer equilibrium leads to aggregation-prone species but rescue of these forms can be achieved through increased concentration, lipid association and chaperone interactions. These association-prone monomers as well as oligomers represent distinct species however they appear to have quite similar effects on lipid membranes\textsuperscript{268}.

It has been hypothesised that membrane disruption may start in a seeding mechanism where a αSyn monomer binds and then protein continues to build up in a localised region\textsuperscript{263,269}. The two main methods proposed for membrane disruption by αSyn are pore-formation and membrane-thinning\textsuperscript{217}. In a seeding mechanism, as each new αSyn arrives it could remove lipids. This may manifest originally as membrane thinning but as the accumulation of αSyn continues it could lead to change in the membrane integrity and eventual pore formation\textsuperscript{270}. At high concentrations these processes may occur extremely rapidly. If the accumulation of monomers assembled into a similar arrangement as pre-formed oligomers on the membrane, this could be the explanation behind the similarity in membrane binding studies between the different αSyn species. It is possible that these mechanisms actually represent multiple steps in the same pathway. Physiologically both monomers and oligomers are likely present at the same time and may act in concert.

In this chapter recombinant αSyn oligomers which are stable at nanomolar dilutions and with a known membrane affinity were utilised\textsuperscript{271,272}. They are proposed to be an accurate in vitro representation of early stage protofibrillar αSyn oligomer species\textsuperscript{198}. Encapsulation of dye inside of liposomes, as well as
labelling of the lipid membrane and labelling αSyn allows all aspects of the interaction to be investigated.

The αSyn monomers (with a degree of labelling ~11%) and accumulate on the liposome before content release. While the precise number of molecules binding to the liposome could not be ascertained, this observation suggests that multiple monomers need to bind to disrupt the membrane. The intensity of αSyn on the liposome at the time that the encapsulated dye is lost represents only around 20% of the maximum level of αSyn accumulated suggesting that αSyn continues to accumulate on the liposome even after perforation has occurred.

There are four different classes of fluorescence loss traces for the encapsulated dye in the presence of αSyn oligomers and monomers. Rapid loss of encapsulated dye content in a single-step occurs in concert with removal of a large proportion of the lipid from the membrane. When the content is released in multiple steps, the membrane is still destabilised however not as much lipid is lost. A similar level of lipid loss to the multiple-step is also seen in the slow release step class. These classes may induce membrane perforation by the same mechanism however on different timescales.

The rates of perforation in the fast single-step and fast multiple-step also imply a similar mechanism, despite differences in lipid disruption. These rates support previous dye efflux assays on GUVs\textsuperscript{203} with similar times of membrane permeabilisation. One speculation for this mechanism is that pore-like structures can form by either a single-step or by a build-up in multiple-steps. When this occurs in a step-wise manner, rather than ripping out a whole portion in one step, it may not generate such a pronounced effect on the lipid structure. The multiple-step class along with the slow class, could represent accumulation of αSyn, building up at a single location, perhaps extracting lipids as it accumulates, until the membrane is perforated. The membrane may be thinned or locally destabilised as opposed to the immediate formation of a pores. Membrane thinning could represent the initial stages to pore-formation, and not a distinctly different
mechanism. Thinning of membranes has been linked with αSyn monomers, however since some monomers are always associated with oligomers during preparation, they may initiate this class of perforation in the oligomer sample. In this mechanism αSyn would slowly take individual lipids out of the bilayer and reshuffle them, incorporating them into the growing oligomer or aggregates. It has been shown that as lipids get bundled in with αSyn when the oligomer dissociates from the membrane it takes the lipids with it and this is thought to be the reason why a significant amount of lipid is found in Lewy bodies and αSyn aggregates.

The ability to cause perforation of membranes is completely abolished by a 30 minute incubation of αSyn with chaperones prior to addition to the liposomes. The lipid loss which is associated with the membrane disruption is also absent when chaperones are present. Chaperones most likely play a role in cells where they associate with the unstructured monomers or oligomers in solution prior to membrane interaction. The interaction itself is not prevented, but some property that generates lipid disturbance appears to be. Chaperones may play an important role in mediating the difference between the functional membrane binding role of αSyn and the toxic membrane associations which lead to cell death. There is currently not a lot known about αSyn membrane binding and the role that chaperones play in these interactions.

αSyn mutants have substantial variety in membrane binding ability and on aggregation properties. A53T, E46K and H50Q enhance fibril formation, A30P promotes more oligomers and prevents mature fibrils and G51D decreases aggregation and membrane binding. E46K and A53T have also been shown to flatten membrane curvature. The assay described in this work could be applied to the full range of αSyn mutants. This would provide information about different classes (potentially new unique ones attributed to mutations) and the amount of lipid lost for different mutants and would help build up a complete picture of the molecular mechanism using what is already known about the properties of the different mutants forms.
Chapter 6 – Conclusions

The aims of this study were to characterise the multimer form of αSyn, investigate how chaperones may play a critical role in mediating perturbations which shift the multimer equilibrium and understand the manner in which αSyn species generated through multimer disturbance associate with membranes and generate toxicity.

The recent discovery of the αSyn multimer has led to a paradigm shift in considering the molecular pathways of native state to disease transitions of αSyn. The multimer equilibrium appears to be highly dynamic and pinpointing the nature of the species involved, as well as how this can be disturbed requires the utilisation of methods which can resolve these kinds of interactions.

Within the new paradigm it is now thought that αSyn multimers represent the physiological form and that destabilisation leads to population of an association-prone monomer state and subsequent disease causing oligomers. A number of factors have been shown to be critical to maintaining the stability of the tetramer including the KTKEGV repeats, concentration-dependence and a unknown stabilising cofactor (thought to be small lipids). Missense mutations associated with PD also generate a shift of native multimers to monomers.

The physiological roles of αSyn are not clearly defined however a significant proportion of these involve membrane-binding properties. αSyn multimers have been shown to cluster synaptic vesicles and a shift from monomers to multimers has been identified in SNARE complex assembly. Multimers associate with membranes, however as yet no detrimental effects of this interaction have been found. In contrast αSyn monomers and soluble oligomers have the ability to destabilise membranes and induce cell permeabilisation and death.

In Chapter 4 the multimer of αSyn was investigated to provide insight into the underlying transitions occurring in the multimer equilibrium and determine whether chaperones could play a role in maintaining this form. It was found that
αSyn is comprised of a highly dynamic equilibrium which includes oligomer states from monomers through to tetramers. This composition of species has rapid subunit exchange, with a very close arrangement of N-termini of different subunits and can be pushed towards a predominantly monomer population through temperature or concentration changes. There appears to be an intrinsic affinity for αSyn to reassemble into a more compact oligomer state when its internal residues become exposed due to these perturbations. This is reflected by a presence of tertiary or quaternary structure; however the minimal level of secondary structure signifies a species which is at the tipping point for aggregation.

Chaperones were found to interact preferentially with the dissociated unstructured monomer generated through dilution. These interactions delayed the reformation of a multimeric assembly, most likely via capture of the monomer population in substrate chaperone complexes until they were ready to assemble. Chaperones did not show any direct interaction with the stable multimer, indicating a different monomer exists in a more structured form at equilibrium. The lack of interaction between the multimer and the chaperones suggests the multimer, when stable is a native state and does not require mediation by chaperone intervention.

In Chapter 5 the αSyn species that are generated through multimer destabilised (monomers and soluble oligomers) and their ability to disrupt membranes was examined. The mediation of these processes by chaperones was also investigated. The roles of αSyn seem to primarily correspond to membrane binding and multimers do not disrupt membranes, but rather seem to provide the functional interactions required for essential processes such as vesicle docking. In this system both monomers and oligomers specifically interacted with liposomes and caused membrane perforation in a rapid mechanism. The presence of αSyn on the liposomes led to widespread destabilisation and loss of lipids from the surface. This was able to occur through either a fast single-step or multi-step which, despite differences in membrane disruption levels, seem to be the same mechanism. Monomers and oligomers interacted with chaperones in this system,
and this interaction completely abolished the membrane permeabilisation ability of αSyn.

The shift from the multimer to these forms illuminates a pathway for the early stages of disease progression. Membrane interactions which can lead to permeabilisation and cell death seem to occur through a defined specific mechanism regardless of whether monomers or oligomers of αSyn are present. Physiologically both species would be present simultaneously, and probably interact to induce membrane instability. The mediation of membrane disruptive processes of αSyn by chaperones represents an exciting and relatively untouched area of research with an extensive capacity for therapeutic intervention. Some drugs have already been targeted to dissolving membrane bound αSyn oligomers, perhaps knowledge of the chaperone involvement in this pathway will help to guide these developments.

Recent work has suggested a possible cell to cell propagation of αSyn. A model of this system suggests that αSyn multimers would be released from cells during cell death. Outside the cytosol however multimers are at low concentration and this would initiate the transition to the association-prone monomer form. The aggregation-prone monomers with membrane binding properties are then free to interact with other healthy cells, propagating negative associations in a seeding like mechanism that spreads.

The pathways studied in this research including multimerisation, the steps that shift the equilibrium towards association-prone species, membrane binding of monomers and oligomers and the protective mechanisms of chaperones cover key areas which require more investigation as our understanding of the multimer and its physiological functions increases. Stabilisation of the multimer is a critical aspect with huge potential for targeted therapeutic intervention. Drugs that could maintain the stability of αSyn in a functional form would provide early intervention to potentially prevent PD rather than just ameliorate the symptoms.
6.1 Future Directions

Building on the information obtained through this work, there is a wide application of the techniques used in this study for further discovery. Already underway are experiments to screen the entire range of αSyn mutant oligomers and their interactions with membranes. This work will be made possible by a very recent collaboration with Dr Yann Gambin and Dr Emma Sierecki who have the potential to generate large numbers of protein mutants using cell-free systems. Preliminary results indicate significant differences between the rate kinetics of membrane perforation with different mutants and will report on the different toxicity involved with PD causing mutations. Liposomes encapsulating GFP instead of free dye have also been employed to investigate how encapsulated molecules of different size are liberated by membrane permeabilisation and will provide insight into the size of pore-like structures formed in permeabilisation.

The importance of the chaperone interaction in these processes has also initiated experiments to determine how chaperones associate with these oligomers and the kinetics of these interactions. I believe the more detail that can be extracted from these mechanisms the more clearly the big picture of the early stages in disease progression and the mode of toxicity will become.

Preliminary Results: Interactions between Chaperones and αSyn Oligomers

To further investigate the interactions between chaperones and αSyn that suppress the perforation of membranes, a similar single-molecule assay was designed and implemented. Labelled αSyn oligomers were captured to the surface using a biotinylated αβ-oligomer antibody (Fig 6-1) which specifically recognises an epitope present in on-pathway oligomeric species from a range of neurodegenerative proteins. Labelled chaperones were then injected into the flow channel to observe their interactions with the surface-attached oligomers using 2- and 3-colour TIRF microscopy.
Schematic representation of single-molecule assay designed to investigate chaperone interactions with αSyn oligomers.

These experiments were also performed under flow using a microfluidics device. αSyn oligomers were captured to the surface by flushing through the channel and then incubating in the system for approximately 5 minutes to achieve the desired surface density. Excess oligomers were flushed out of the system to remove fluorescence background and prevent interactions of chaperones with oligomers in solution instead of on the surface. An additional round of surface passivation was performed after oligomer capture, with 0.5mg mL$^{-1}$ BSA incubated in the chamber for 5 minutes to ensure there were no exposed hydrophobic regions left on the surface. As chaperones have a high affinity for hydrophobic regions, this additional step was found to greatly decrease the non-specific binding of chaperones to the surface. The BSA was flushed from the channel prior to addition of chaperones. A mixture of Hsc70-AF647 and DNAJB1-AF488 in oligomerisation refolding buffer was flushed through the microfluidics device (20 uL min$^{-1}$) and allowed to interact with surface-tethered αSyn-AF568 oligomers.
Figure 6-2 Averaged fluorescence images of surface tethered αSyn-AF568 oligomers and their interaction with Hsc70-AF647 and DNAJB1-AF488. Interactions were mostly transient and co-localisation occurred at various locations throughout the movie. Shown here is the maximum intensity projection of 2000 frames.

αSyn oligomers were successfully captured to the surface (Fig 6-2, left panel). Preliminary analysis showed around 10% co-localisation between Hsc70-AF647 and αSyn-AF568. The co-localisation of αSyn-AF568, Hsc70-AF647 and DNAJB1-AF488 was also observed. This surface assay requires further optimisation to yield higher proportion of interacting molecules. Ultimately this assay will provide information on the order of interaction of the chaperones with αSyn, the association time and the stoichiometry of these events.
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Supplementary Data

Figure S1. Graph of the Plateau in FRET efficiency reached after mixing of αSyn populations with donor and acceptor labels. Used to determine an endpoint for initial mixing in FLIM FRET of labelled αSyn populations (Chapter 4.2 Dynamic Subunit Exchange of αSyn Populations).