Characterising the early aggregation events of human lysozyme using single-molecule microscopy

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September 2018
Declaration

This dissertation is a summary of research carried out under the supervision of Dr. Janet Kumita and Prof. Sir Christopher Dobson in the Department of Chemistry of the University of Cambridge, between October 2014 and September 2018. The work described in the dissertation is my own and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and acknowledgements. It has not, either in part, or as whole, been submitted for a degree, diploma, or other qualification at any other university. The length of this dissertation (approx. 40,000 words) does not exceed the word limit set by the Degree Committee (60,000 words).

Ana Bernardo Gancedo

September 2018
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This thesis is dedicated to the two incredible women that made me the person I am today

Esta tesis está dedicada a las dos mujeres de mi vida, mi abuela y mi madre,

Julia Nieto Navas y Teresa Gancedo Nieto
Abstract

Human lysozyme (HuL) is a widely characterised protein whose mutational variants misfold, forming amyloid fibrils that are associated with a rare systemic amyloidosis. Given that a number of proteins, including lysozyme, can misfold and give rise to disorders such as Alzheimer’s disease, Parkinson’s disease, and type II diabetes, it is vital to understand the mechanistic features by which this process occurs, in order to attempt to cure or regulate these diseases. Work on lysozyme and other globular proteins has revealed that they require a process of global or partial unfolding to initiate protein aggregation, which is different from the natively unfolded proteins known as intrinsically disordered proteins (IDPs). Although the processes of folding and fibril formation for HuL have been well studied, the details of the early events leading to aggregation have proven difficult to study. Recent advances in site-specific labelling of HuL have made it possible to introduce Alexa fluorophores into the I59T variant of HuL without perturbing the process of in vitro fibril formation as compared to the unlabelled protein. Using this fluorescently labelled protein, we have used single-molecule fluorescence microscopy techniques including two-colour coincidence detection (TCCD) and fluorescence resonance energy transfer (FRET) to determine the oligomer population distributions present during the aggregation of HuL. Our data showed that HuL populates disordered small low-FRET oligomers (<10 monomers) which in turn can undergo a conformational change to form more stable and structured high-FRET oligomers over the course of the aggregation. The second type of oligomers can then grow to form amyloid fibrils. We fitted our data from the early stages of the aggregation reaction to an early-time kinetic model, in which the monomer concentration can be assumed constant during the selected period of time. Using this model, we calculated the different rate constants associated with the microscopic steps along the aggregation pathway that we were able to monitor using SM techniques. In addition, the interactions between HuL oligomers and the extracellular chaperone clusterin were investigated. Previous studies have shown that clusterin inhibits I59T HuL aggregation by interacting with species formed early in the aggregation pathway. Here, we show that clusterin enhances the population of small high-FRET oligomers, trapping them and preventing their growth into larger aggregates over the time-course of our analysis. These results allow us to directly compare the mechanism of protein aggregation in a globular system versus that of IDPs, providing potential insight into targeting these pathways for therapeutic interventions.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>5-carboxyfluorescein N-succinimidyl ester</td>
<td>F-NHS</td>
</tr>
<tr>
<td>8-Anilino-1-naphthalenesulfonic acid</td>
<td>ANS</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>A488</td>
</tr>
<tr>
<td>Alexa Fluor 647</td>
<td>A647</td>
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<tr>
<td>Amino acid analysis</td>
<td>AAA</td>
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<td>Amyloid A</td>
<td>AA</td>
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<tr>
<td>Amyloid light chain</td>
<td>AL</td>
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<tr>
<td>Association quotient</td>
<td>Q</td>
</tr>
<tr>
<td>Atomic force microscopy</td>
<td>AFM</td>
</tr>
<tr>
<td>Avalanche photodiode detector</td>
<td>ADP</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>BSA</td>
</tr>
<tr>
<td>Circular dichroism</td>
<td>CD</td>
</tr>
<tr>
<td>Differential scanning calorimetry</td>
<td>DSC</td>
</tr>
<tr>
<td>Dynamic light scattering</td>
<td>DLS</td>
</tr>
<tr>
<td>Equation</td>
<td>Eq</td>
</tr>
<tr>
<td>Excited states</td>
<td>S₁, S₂</td>
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<tr>
<td>Fluorescence resonance energy transfer</td>
<td>FRET</td>
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<tr>
<td>Ground state</td>
<td>S₀</td>
</tr>
<tr>
<td>Guanidine thiocyanate</td>
<td>GdnSCN</td>
</tr>
<tr>
<td>Heat shock protein 70</td>
<td>Hsp70</td>
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<tr>
<td>Heat shock protein 90</td>
<td>Hsp90</td>
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<tr>
<td>Hen egg-white lysozyme</td>
<td>HEWL</td>
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<tr>
<td>Human lysozyme</td>
<td>HuL</td>
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<tr>
<td>Immunoglobulin</td>
<td>Ig</td>
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<tr>
<td>Intrinsically disordered proteins</td>
<td>IDPs</td>
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<tr>
<td>Mass spectrometry</td>
<td>MS</td>
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<tr>
<td>Melting temperature</td>
<td>Tₘ</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>MW</td>
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<tr>
<td>Molecular weight cut-off</td>
<td>MWCO</td>
</tr>
<tr>
<td>N-hydroxysuccinimide</td>
<td>NHS</td>
</tr>
<tr>
<td>Nuclear magnetic resonance</td>
<td>NMR</td>
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<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Pentameric formyl thiophene acetic acid</td>
<td>pFTAA</td>
</tr>
<tr>
<td>Quantum yield</td>
<td>φ</td>
</tr>
<tr>
<td>Resonance energy transfer</td>
<td>RET</td>
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<tr>
<td>Single-molecule</td>
<td>SM</td>
</tr>
<tr>
<td><em>Sulfolobus solfataricus</em> acylphosphatase</td>
<td>Sso AcP</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
<td>SDS-PAGE</td>
</tr>
<tr>
<td>Thioflavin T</td>
<td>ThT</td>
</tr>
<tr>
<td>Total internal reflection fluorescence microscopy</td>
<td>TIRFM</td>
</tr>
<tr>
<td>Transmission electron microscopy</td>
<td>TEM</td>
</tr>
<tr>
<td>Two-colour coincidence detection</td>
<td>TCCD</td>
</tr>
<tr>
<td>Wild-type</td>
<td>WT</td>
</tr>
<tr>
<td>α2-macroglobulin</td>
<td>α2M</td>
</tr>
<tr>
<td>α-synuclein</td>
<td>αS</td>
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Chapter 1: Introduction

1.1. Protein aggregation and disease

1.1.1. Misfolding diseases and their connection to protein aggregation

A substantial number of commonly known medical conditions such as Alzheimer’s and Parkinson’s diseases are characterised by the presence of insoluble amyloid fibrils and their deposition in the brain, but also in different parts of the body [1]. These disorders are identified as ‘deposition diseases’, and can be grouped into three main categories; neurodegenerative diseases which affect neuronal cells, non-neuropathic systemic diseases that involved multiple organs and localised amyloidosis where aggregates are observed in a single organ [2]. Some of the more common diseases and the proteins associated with aggregation are listed in Table 1. The interest in these diseases has exponentially increased as the number of affected individuals is rapidly rising [3, 4]. The ageing population possess a higher probability of suffering from one of these deposition diseases, likely because a decrease in the efficiency of the mechanisms that maintain proteins in their functional states occurs with age [5].
Table 1. Human diseases associated with protein aggregation. Adapted from reference [2].

<table>
<thead>
<tr>
<th>Disease</th>
<th>Aggregating protein or peptide</th>
<th>Number of residues</th>
<th>Native structure of protein or peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neurodegenerative disorders</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>Amyloid-β peptide</td>
<td>37-43</td>
<td>Intrinsically disordered (ID)</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis</td>
<td>Superoxide dismutase 1 and TDP-43</td>
<td>153 and 4114</td>
<td>β-sheet and Ig-like</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>Huntingtin fragments</td>
<td>Variable</td>
<td>Mostly ID</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>α-synuclein</td>
<td>140</td>
<td>ID</td>
</tr>
<tr>
<td><strong>Non-neuropathic systemic amyloidosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amyloid light chain (AL) amyloidosis</td>
<td>Immunoglobulin (Ig) light chains or its fragments</td>
<td>~ 90</td>
<td>β-sheet and Ig-like</td>
</tr>
<tr>
<td>Amyloid A (AA) amyloidosis</td>
<td>Serum amyloid A1 protein fragments</td>
<td>76-104</td>
<td>α-helical and unknown fold</td>
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<td>Hemodialysis-related amyloidosis</td>
<td>β2-microglobulin</td>
<td>99</td>
<td>β-sheet and Ig-like</td>
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<td>Lysozyme amyloidosis</td>
<td>Lysozyme variants</td>
<td>130</td>
<td>α-helical and β-sheet</td>
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<td>Senile systemic amyloidosis</td>
<td>Wild-type transthyretin</td>
<td>127</td>
<td>β-sheet</td>
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<td><strong>Non-neuropathic localised amyloidosis</strong></td>
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<td></td>
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<td>Cataracts</td>
<td>γ-Crystallins</td>
<td>Variable</td>
<td>All β- and γ-crystallin-like</td>
</tr>
<tr>
<td>Injection-localised amyloidosis</td>
<td>Insulin</td>
<td>21 and 30</td>
<td>α-helical and insulin-like</td>
</tr>
<tr>
<td>Type II diabetes</td>
<td>Amylin</td>
<td>37</td>
<td>ID</td>
</tr>
</tbody>
</table>
1.1.2. Protein misfolding and the misfolding landscape

In recent years, a great effort has been placed on characterising the free-energy landscape of proteins in order to understand their folding and misfolding processes [6, 7]. The energy landscape is a statistical description of the potential surface of a protein, from the unfolded state to the folded native-state, the latter representing a minimum in a rugged, funnel-like landscape that includes several folding intermediates with different structures. More recently, this concept has been expanded to include the energy landscape of intermolecular interactions leading to aggregates and other possible states [8]. Figure 1.1 shows an energy landscape diagram of a protein where folding processes are represented in purple and aggregation processes are in pink. The folding processes involve intramolecular contacts within a protein; initially, the unfolded ensemble represents a wide population of high energy states that can be present in a multitude of conformations, which sample more compact structures via intramolecular interactions, leading to folding intermediates, partially-folded states and finally, the native-state [9]. The aggregation pathway involves intermolecular contacts between protein molecules, such as partially-folded states which can lead to oligomers but also amorphous aggregates and amyloid fibrils, which are thermodynamically stable and correspond to energy minima on this pathway [7, 8, 10, 11]; therefore, given that they are less thermodynamically favourable, the folding events leading to the native-state are kinetically driven [12].
Figure 1.1. Energy landscapes of protein folding and aggregation. The purple region on the left represents the conformational states that are populated during folding processes in order to reach the native-state. These conformations are formed by intramolecular interactions. The pink region on the right represents the conformational states present in the aggregation processes, where the interactions are intermolecular, and the amyloid fibrils represent the more thermodynamically stable state. Adapted with permission from reference [9].

1.1.3. Amyloid fibrils

Amyloid fibrils are proteinaceous deposits which can be formed from a diverse range of proteins, independent of their native structure or primary sequence. Amyloid fibrils display a number of common attributes including birefringence when binding Congo red dye and increased fluorescence upon binding to Thioflavin-T. Structurally, they possess a high degree of β-sheet content with a common cross-β structure and their morphology appears as long and unbranched strands, up to microns in length with a diameter of a few nanometres [13, 14]. These amyloid fibrils can be negatively stained, i.e. the background can be stained with a dye
such as uranyl acetate, and observed by electron microscopy (Figure 1.2). Although these structures were first related to a range of disease-associated proteins, amyloid fibrils are now described as an alternative state that may be accessible to all proteins [1, 5, 15].

**Figure 1.2. Structural features of amyloid fibrils.** a) Solid-state NMR-based structural model of protein fibrils with an interstrand spacing of 4.7 Å and an intersheet spacing of 10 Å (adapted with permission from reference [16]). b) Solid-state NMR atomic resolution structure of amyloid fibrils (centre) and a close up image (right). An electron microscopy image is shown on the left (reproduced with permission from reference [17]).

1.1.4. **Mechanisms of protein fibril formation**

Whilst protein folding is highly dependent on the amino acid sequence, protein aggregation is thought to have its origins in the polypeptide chain behaviour, which makes the protein act as a typical polymer with the main chain amide-groups forming crucial intermolecular hydrogen bonds [18]. This process is dependent on environmental conditions such as pH, temperature, and the presence of other molecules. Independent of the solution conditions, fibril formation can be generalised as having a lag phase where nucleation takes place, an elongation process representing the rapid growth of the fibrils, and a final plateau where mature amyloid fibrils are formed (Figure 1.3, a) [19]. Recent research has developed a more detailed analysis of the kinetic processes involved during amyloid fibril formation [20-24]. The analyses of precisely controlled aggregation experiments are able to identify the events occurring during the formation of the amyloid fibrils; whether this is through primary nucleation, where aggregate
formation is dependent on the monomer concentration, or through secondary events, such as monomer-independent fragmentation or monomer-dependent secondary nucleation (i.e. nucleation of new aggregates is catalysed by available sites on existing fibrils) (Figure 1.3, b-e) [20, 25, 26]. This kinetic analysis approach has shed light on the aggregation process of a number of systems, including amyloid-β [25, 27], α-synuclein [28, 29], the yeast prion protein Ure2 [30], tau [31-33], and hen egg-white lysozyme (HEWL) [34], and it has been used to identify how small molecules [35-38] and biomolecules such as chaperones and antibodies [39-41] can modulate these events. Although an incredibly powerful tool, this analysis generally requires specific in vitro aggregation conditions, in particular the use of quiescent conditions, which limits its application as these are not experimentally amenable to all protein systems.
Figure 1.3. Schematic of the different species and processes generally involved in protein fibril formation. a) Different phases along the fibril forming pathway as followed by ThT fluorescence, which is proportional to the fraction of fibrils. The lag phase, elongation and plateau or equilibrium phase are shown in blue, orange and green, respectively. Below, the typical species found during these phases are shown in the same colours. Monomers are shown as circles, oligomers are shown as four circles assembled together and fibrils are shown as a series of rounded squares. Note that the proportion of oligomers is always low regardless of the phase. This is generally the case in the literature [8, 29-31, 42-44]. b), c), d) and e) Schematics of the defined kinetic processes occurring during protein fibril formation. b) Primary nucleation and its corresponding rate of formation ($k_n$). c) Elongation ($k_+$) and dissociation ($k_{off}$). d) Fragmentation ($k_-$). e) Monomer-dependent secondary nucleation ($k_2$). Modified from reference [26].
1.2. Human lysozyme fibril formation and its connection to disease

1.2.1. Human lysozyme and systemic amyloidosis

Discovered by Alexander Fleming in 1922, human lysozyme (HuL) is an antibacterial enzyme that functions by degrading bacterial cell walls and is found in a number of human secretions including tears, saliva and human milk [45]. Human lysozyme belongs to the group of lysozymes defined as chicken or conventional type (c-type) lysozymes [46]. Among the c-type lysozymes, HEWL has been especially useful in the study of enzymatic mechanisms after its structure was defined by X-ray crystallography [47]. Human lysozyme is a globular 130-residue protein which contains four disulphide bridges and two domains: the α-domain (including residues 1-38, and 86-130), which contains four α-helices (A, B, C, and D), and the β-domain (residues 39-85), which mostly contains β-sheet structure [47-49]. The X-ray structure of wild-type human lysozyme (WT HuL) is shown in Figure 1.4.
Human lysozyme was first linked to disease in 1993, when the I56T and D67H point mutations were discovered to be the cause of hereditary systemic amyloidosis [50]. The patients showed large amounts of fibril deposits in their visceral organs including the liver, kidneys and spleen (Figure 1.5) [51]. Further analysis of the *ex vivo* deposits showed that the fibrils consisted solely of variant protein and that these deposits shared common features of amyloid fibrils [48, 50, 51]. Although the cause of lysozyme fibril formation *in vivo* remains unknown, it is likely related to two main reasons: first, the amyloidogenic variants show an increased ability to fluctuate to the transient intermediate state (section 1.2.2), which allows these variants to more readily nucleate and aggregate over time. Second, the amyloidogenic variants are likely
escaping the protein quality control mechanisms in patients somehow, since only amyloidogenic variants are found in the masses of fibrils. These mechanisms are responsible for clearing unfolded proteins from the body and do not do so in these patients, whereas these variants are cleared by healthy organisms such as yeast, the human cell line HEK293, and *Drosophila melanogaster* [63-66].

![Deposition of variant lysozyme in a patient suffering from systemic amyloidosis](image)

**Figure 1.5. Deposition of variant lysozyme in a patient suffering from systemic amyloidosis.** Scintigraphic images of post-mortem fibril deposits found in several visceral organs (kidneys, spleen and liver) of an affected individual (a) compared to a normal patient (b). Adapted with permission from reference [51].

After the discovery of the I56T and the D67H variants, five other naturally occurring disease-related mutations (Y54N, F57I, F57I/T70N, W64R, L102S and T70N/W112R) were identified, along with two variants (T70N and W112R), which are not disease-associated on their own (Figure 1.4) [52-57]. In order to understand the consequences of the mutations on the folding and misfolding processes of human lysozyme, previous studies characterised the I56T and D67H variants by using different biophysical techniques and showed that these variants possess lower native-state stabilities compared to WT HuL [58, 59]. X-ray crystallography and NMR
analysis have shown that the mutations introduce structural changes to the interface region between the α- and β-domains [60]. The unfolding of the two amyloidogenic variants occurs in a less cooperative process than WT HuL and this results in partly unfolded intermediate states being significantly populated, in which the β-domain and the C-helix are significantly unfolded whereas the rest of the protein maintains the native-like conformation [48]. This reduction in global cooperativity is not unique to the amyloidogenic variants as WT HuL and the non-disease related T70N variant can also populate the partially-unfolded intermediates under destabilising conditions [58, 61, 62]. Though much research has been done on the naturally occurring HuL variants, the main limitation to their study is their low expression yield in recombinant protein expression systems. This has been extensively studied in the yeast, Pichia pastoris, which demonstrated that there is a relationship between the expression yield of different variants of human lysozyme and their native-state stability, with the less stable variants causing an upregulation in the quality control mechanisms within Pichia pastoris and resulting in the degradation of these proteins [63, 64]. Interestingly, this stringent quality control mechanism, resulting in lower expression levels of amyloidogenic variants, was also identified when these proteins were over-expressed in Drosophila melanogaster and the mammalian cell line, HEK293 [65, 66]. To alleviate the issues with protein expression, a non-natural variant (I59T) is used for many structural studies as this variant was found to possess the amyloidogenic properties displayed by the naturally occurring I56T variant, albeit showing a native-state stability which lies between that of the latter and WT HuL [67]. The higher native-state stability results in a higher expression yield than the amyloidogenic variants and therefore makes I59T HuL an ideal model for the study of lysozyme amyloidosis [63, 67].

1.2.2. The mechanisms of unfolding and fibril formation of human lysozyme

Since being linked to systemic amyloidosis, the unfolding and aggregation of amyloidogenic variants of HuL have been extensively investigated [48, 68, 69]. Like other globular proteins, human lysozyme locally unfolds to initiate aggregation [70]. The presence of an unfolding intermediate was initially characterised using hydrogen-deuterium exchange mass spectrometry (HDX) experiments by Canet and co-workers in 2002 [58]. HDX allows for two types of processes to be observed: type 1 (EX1), which occurs when the rate of exchange of deuterium for hydrogen of the transient partially-unfolded intermediate is faster than its closing rate to the folded native-state; and type 2 (EX2), which occurs when the closing rate is faster than the exchange rate [71, 72]. Under EX1 conditions, and using physiologically relevant
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conditions, EX2 was detected for both WT HuL and the variants whereas EX1, which corresponds to the presence of a transient partially-unfolded intermediate, was only detected in the case of the variants, D67H and I56T (Figure 1.6, a). Recently the ability of the D67H variant to form this transient partially-unfolded intermediate was shown to be reduced in the presence of a camelid nanobody, cAb-HuL6, resulting in the inhibition of fibril formation [73]. This study therefore showed that the formation of this intermediate is a crucial step for lysozyme fibril formation [73] (Figure 1.6, b). This transient intermediate is extremely lowly populated; however, under acidic conditions it is possible to populate a molten globular state to a high enough percentage to use 2D NMR techniques to explore its structural attributes. In 2010, Dhulesia and co-workers used a range of biophysical techniques including differential scanning calorimetry and NMR to demonstrate that, under amyloid forming conditions, the intermediate species of the I59T and I56T variants appear to be an ensemble of interconverting conformers with varying degrees of denaturation [74]. Most recently, the intermolecular interactions between the partially-unfolded intermediate species of the I59T variant, which are believed to give rise to fibril formation, were studied using paramagnetic relaxation enhancement (PRE) measured by NMR [75]. This study demonstrated that the initial interactions leading to fibril formation most likely occur between residues within the unfolded β-domain [75].

Figure 1.6. Unfolding dynamics of HuL as detected by HDX experiments. a) Electrospray mass spectra of WT and D67H HuL after exposure to HDX conditions (37 °C, pH 8.0). For D67H HuL, local unfolding events give rise to the peaks coloured in yellow (EX1) whereas the peaks coloured in red arise from EX2. The peaks in green and black are the control samples recorded for time zero and time infinity, respectively. Reproduced with permission from
b) Electrospray mass spectra of D67H lysozyme in the presence of an equimolar amount of the camelid nanobody, cAb-HuL6. The local unfolding events shown in (a) for D67H HuL (yellow peaks) are not detected in the presence of the nanobody. The peaks coloured in green arise from EX2. The peak in black was observed from a control sample recorded after complete hydrogen–deuterium exchange. Adapted with permission from reference [73].

In addition to the formation of a partially-unfolded intermediate, previous studies have shown that as native-state stability decreases for HuL variants, faster rates of aggregation are achieved [76]. However, native-state stability is not the only factor determining the aggregation dynamics as the location of the mutations also plays a crucial role in determining the rate of the population of the intermediate state which in turn will favour amyloid formation [77]. Mutations located in the β-domain, which is the region predominantly unfolded when the transient intermediate is formed, have a higher ability to populate the intermediate than those situated in the α-domain [77]. Interestingly, as shown in Figure 1.4, all the naturally occurring mutations happen to be located in the β-domain or at the interface between the two domains.

In addition to the presence of the transient intermediate, in vitro fibril formation of HuL shows a notable seeding effect [62], which suggests that the aggregation follows a nucleation-dependent mechanism (Figure 1.7). In parallel to the study of the aggregation mechanism, its inhibition has also been investigated extensively. A series of camelid antibodies was shown to prevent the formation of these partially-unfolded species by restoring the global stability of different HuL variants and hence stopping aggregation [73, 78], and the extracellular chaperones clusterin, haptoglobin and α₂-macroglobulin (α₂M) have been shown to inhibit lysozyme amyloid formation by interacting with pre-fibrillar species in the early stages of the aggregation process [69, 79].
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Figure 1.7. Proposed mechanism of aggregation of HuL. The monomeric native-state can partially unfold to populate a transient intermediate that results from the reduction in cooperativity shown by human lysozyme variants in combination with decreased native stability. This intermediate is also in equilibrium with the unfolded state. The unfolded β-sheet domains of the intermediates are likely the first point of contact leading to the formation of oligomeric-like species (which have not yet been structurally characterised in the literature) before forming amyloid fibrils. Modified from reference [69].
As mentioned above, globular proteins must undergo local and/or global unfolding before they can start forming amyloid fibrils. One of such proteins is wild-type β2-microglobulin, which is related to dialysis-related amyloidosis. β2-microglobulin, like human lysozyme, requires the population of an intermediate state, in which aggregation-prone sequences are exposed, in order to form fibrils. This kinetically trapped intermediate displays native-like secondary structure and has been shown to interact with pre-formed fibrils and promote elongation [201]. This intermediate also shows increased conformational dynamics which allow for the formation of a rarely populated species that, under certain conditions, enables the formation of oligomeric species [202]. This ability of wild-type β2-microglobulin to form amyloid fibrils is believed to be catalysed by the presence of the truncated variant ΔN6, in which β2-microglobulin is truncated at Lys6, which is present in ex vivo amyloid deposits in significant proportions (~26%), and can both assemble into oligomers leading to fibril formation and increase the ability of the wild-type protein to aggregate into amyloid fibrils in a prion-like manner [203]. A wide variety of single amino acid mutations have been proved to change β2-microglobulin’s propensity to aggregate by changing its conformational dynamics in a similar way to lysozyme mutants [202, 204, 205]. Using mass spectrometry and analytical ultracentrifugation, Smith and co-workers were able to show that β2-microglobulin forms small oligomers (dimers to tetramers) in the early stages if fibril formation [206]. These oligomers then undergo conformational changes to form more stable, less dynamic structures in order to form fibrils [206, 207]. In addition, the naturally occurring D76N mutant of β2-microglobulin is related to a rare form of familial systemic amyloidosis and is found only as the full-length protein in the amyloid deposits in patients, which are found in visceral organs, whereas the deposits of wild-type protein are found mostly in bones and ligaments. This mutation causes a destabilisation of the protein structure that enhances its ability to populate the amyloid-prone intermediate [205, 208].

Transthyretin is a homotetrameric protein that is associated with senile systemic amyloidosis, familial amyloid polyneuropathy, and familial amyloid cardiomyopathy. This tetrameric protein can dissociate into unstable monomers that then undergo oligomerisation by intermolecular interactions and finally form fibrils [209]. The dissociation of the tetramer is believed to be generally caused by genetic disposition, i.e. mutations [210, 211]. This belief is reinforced by transthyretin mutants being found in the aggregate deposits in patients [212]. Regarding the mechanism of transthyretin aggregation, recent research showed that small oligomers (5-10 monomers) are formed in the early stages of fibril formation [213]. Research
on these small oligomers showed that they are more cytotoxic than fibrils when added to human neuroblastoma cells [214], which is in agreement with previous research by Reixach and co-workers where they showed that either monomeric transthyretin or very small oligomers formed in the early stages of aggregation were the major cytotoxic species towards human neuroblastoma cells [215]. These studies are also in agreement with research on the same cell line by Sörgjerd and co-workers, even though the latter found that the early oligomers were formed by an average of 20-30 monomers [216]. These oligomeric non-fibrillar aggregates have also been related to the early stages of the disease in vivo for human patients [217, 218] and mice [218, 219].

The tetrameric form of transthyretin can be stabilised by clusterin under acidic conditions, inhibiting its dissociation into monomers, and in turn fibril formation [220]. In addition, Clusterin co-localises with non-fibrillar and fibrillar transthyretin in ex vivo deposits [212], its expression is increased in transgenic mice for human transthyretin mutant V30M, and its expression and secretion are enhanced in human neuroblastoma cells [221]. In addition, the same study showed that clusterin binds extracellular oligomeric aggregates and inhibits fibril formation, probably through the stabilisation of said early aggregates [221]. In 2015, Greene and co-workers showed that clusterin indeed interacts with monomeric and oligomeric transthyretin under acid stress in vitro, stabilising both species and preventing fibril formation [209]. Figure 1.8 shows the mechanism of aggregation of transthyretin and how it can modulated by clusterin, as proposed in reference [209].
The pharmacological solution for transthyretin aggregation in patients has been proved by different approaches. These approaches are: the modulation of the heat shock response via regulation of extracellular chaperones, such as Hsp70 and clusterin, which was investigated to show that they are involved in the clearance of non-folded transthyretin in vivo [212, 221, 222]; small molecules, amongst which doxycycline was approved by the European Medicine Agency to be administered in the treatment of familial amyloid cardiomyopathy (EU/3/12/955) [223]; and humanised antibodies, which have been found to prevent amyloid formation and deposition in mice [224]. The modulation of the aggregation pathway and stabilisation of the transthyretin tetramer has also been approached by the use of small molecules such as epigallocatechin gallate (EGCG), which is obtained from green tea, and curcumin [225, 226]; both of which inhibit transthyretin aggregation by increasing the stability of the tetrameric conformation and modulating intermediary species, such as preformed pre-fibrillar aggregates, as well as the formation of non-toxic oligomers in the presence of EGCG [225, 226, 227]. Furthermore,
curcumin has been found to disaggregate fibrils in mice [228]. In addition to these natural compounds, a combination therapy using doxycycline and tauroursodeoxy-cholic acid (TUDCA) has been shown to significantly reduce transthyretin deposition and toxic aggregates, and clinical trials for this therapy are currently underway in Italy and the USA (www.clinicaltrials.gov). Other small molecules and more intrusive therapies, such as liver transplantation, are currently being trialled in patients [229].

1.3. Oligomers and their importance in the aggregation pathways

Oligomers are pre-fibrillar species that are present along the aggregation pathway before the formation of amyloid fibrils [80]. Although these species are lowly populated, many recent studies have found ways to enrich or produce stable oligomers (similar to those found on the fibril forming pathway) [81-84], thereby allowing for these species to be better characterised using biophysical techniques. Interestingly, oligomers from different protein systems share common biophysical attributes such as high levels of exposed hydrophobicity and a spherical or sometimes pore-like appearance [81, 85, 86] (Figure 1.9). The structural similarities between oligomers from different protein systems was confirmed when the binding of an antibody, raised specifically to bind to amyloid-β oligomers, was found to recognise oligomers formed by other proteins, successively reducing their toxicity towards cells [87]. This work hence demonstrated that the structure and the associated mechanism of pathogenesis for oligomers was most likely shared by all protein systems included in that study, independent of primary sequence. Oligomers have been found to have a level of cytotoxicity analogous or higher to that of amyloid fibrils of disease-related peptides and proteins [18, 87-89] and it is widely believed in the literature that the reason for their high toxicity lies in their exposed hydrophobic surfaces [87, 89-91]. The potential to prevent the formation of these toxic oligomers or to regulate their toxicity, in the context of disease, therefore makes these species a very attractive field of research. In fact, the literature shows numerous studies where the inhibition of oligomer toxicity is being modulated by using different chaperones [41, 69, 79, 91-95], antibodies [40, 73, 78, 96, 97] and small molecules [35, 36, 98].
Oligomers share common biophysical properties. a) ANS fluorescence suggests that oligomers are more hydrophobic than amyloid fibrils (reproduced with permission from [86]). b) α-Synuclein (αS) oligomers show a pore-like structure (reproduced with permission from [81]). c) Oligomers are usually small in size. In the picture, αS oligomers show a broad band around 1050 KDa (<10 monomers) (reproduced with permission from [81]). d) Oligomers and annular protofibrils as characterised by AFM and electron micrographs (reproduced with permission from [85]). e) αS oligomers are more toxic to cells than fibrils as measured by the effect on the rate of cytoplasmatic reactive oxygen species (ROS) production (reproduced with permission from [81]).

Although much progress in understanding oligomer formation and their role in disease has been reported, these pre-fibrillar species are formed, often transiently, at small percentages in regards to initial monomer concentration and in highly heterogeneous environments and hence are challenging to characterise. Indeed, as mentioned in the section above, the oligomeric species formed in the aggregation pathway of HuL have not yet been characterised and it will be the objective of this thesis to shed light on their properties.
1.4. Single-molecule fluorescence

Unlike ensemble techniques where the data represents the average behaviour of a group of molecules, single-molecule (SM) techniques allow for individual molecules to be observed, thereby deconvoluting their properties and allowing insight to be gained on different species within a large population. This is very important when studying biological systems, where often, populations are heterogeneous and some species are lowly populated and therefore their study requires this high degree of sensitivity. A wide variety of fluorescence methods have been developed in recent years [42, 99-104]. In this section, the fundamentals of fluorescence will be covered before addressing the application of SM fluorescence microscopy to studying oligomeric species formed during protein aggregation.

1.4.1. Fundamentals of fluorescence

Fluorescence consists of the rapid emission of a photon caused by the relaxation of an electron going from an excited singlet state to the ground-state orbital [105]. This emission of a photon can be illustrated by the Jablonski diagram (Figure 1.10) as described by Aleksander Jabonski in 1933 [106]. In brief, following light absorption, a particular molecule (commonly known as fluorophore) can get excited to a higher vibrational level of the excited states (S₁ or S₂) from which it rapidly relaxes to the lowest vibrational level of the first excited state (S₁). The excitation of the fluorophore occurs in a timescale of $10^{-15}$ seconds (s). The process of internal relaxation occurs within $10^{-12}$ s and it is usually known as internal conversion or vibrational relaxation. The return to the ground-state ($S₀$) or the process of fluorescence usually occurs within $10^{-8}$ s and it is followed by its own internal conversion until a thermal stability is reached.

As can be observed in the Jablonski diagram, the energy of emission is always lower than the energy of absorption i.e. fluorescence emission occurs at longer wavelengths. The difference between the wavelength of absorption and that of emission is called Stokes shift [107]. This emission of a photon is observable by a detector and this is the basis for fluorescence techniques.
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1.4.2. Fluorophores

Fluorophores are organic molecules that are able to absorb and emit light. If a fluorophore is attached to a protein, information can be gathered that may help characterise the protein e.g. its location, quantity or structure. The number of emitted photons relative to the number of absorbed photons is known as quantum yield ($\phi$). The brightness of a fluorophore is proportional to the product of its quantum yield and its extinction coefficient i.e. the higher the quantum yield of a fluorophore, the brighter it is. The extinction coefficient is given by the capacity of the fluorophore to absorb light at a given wavelength and can be calculated using...

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**Figure 1.10. Fundamentals of fluorescence.** a) A representative Jablonski diagram. The fluorophore gets excited from the ground state ($S_0$) to a higher vibrational level of an excited state ($S_2$). Internal conversion to the lowest vibrational level of the first excited state ($S_1$) follows before the emission of a photon (fluorescence) and the return to $S_0$. The associated timescales for each of the processes have been included. b) Due to internal conversion, fluorescence emission will always appear at higher wavelengths than that of excitation. The difference between those two wavelengths is known as Stokes shift. Modified from references [108, 109].
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the Lambert-Beer law, in which the absorbance of light equals the extinction coefficient multiplied by the concentration of the fluorophore and the distance travelled by the light. A fluorophore with a large extinction coefficient will therefore need a lower concentration to absorb the same number of photons as a fluorophore with a small extinction coefficient. The lifetime of a fluorophore can be described as the average time the fluorophore remains in the excited state before going back to the ground-state [108, 109]. Hence, the ideal fluorophore will be one that has high quantum yield (number of photons emitted per photons absorbed), extinction coefficient (number of photons absorbed at a given concentration) and lifetime (time available to gather data from its emission before the fluorophore relaxes back to the ground-state) as well as a large Stokes shift so the fluorescence emission can be separated from the excitation light.

1.4.3. Alexa fluorophores

In this thesis, the fluorophores Alexa488 (A488) and Alexa647 (A647) were chosen for all the SM fluorescence experiments and their molecular structures are shown in Figure 1.11 (a) and (b). A488 is excited with blue light while A647 is a red light excited dye, and they have maxima of excitation/emission of 494/519 and 651/672 nm, respectively. The reason for this choice of fluorophores was based on their wide use in the literature and their superior properties such as high extinction coefficients, quantum yields and good photostability and lower self-quenching [110]. Quenching can be described as the loss of fluorescence signal due to interactions of the fluorophore with its short-range molecular environment e.g. the same fluorophore if there is more than one label per monomer. In all experiments within this thesis, only one fluorophore will be incorporated per protein molecule and therefore this last property is less relevant than the others. Finally, A488 and A647 make a good FRET pair as the emission of A488 overlaps well with the excitation spectrum of A647 (Figure 1.11, c). The process of FRET will be explained in Section 1.5.3.
1.5. Fluorescence microscopy and its application in SM fluorescence experiments

Fluorescence microscopy refers to different fluorescence methods in which a microscope is used to illuminate and then observe the emitted light from a sample, which is filtered using the Stokes shift phenomenon to allow for its separation from the excitation light.
1.5.1. **Confocal microscopy**

Confocal microscopy is a form of fluorescence microscopy in which the volume of the observed sample is minimised whilst also minimising the out-of-focus light from the image [111, 112]. The latter is achieved by using a pinhole, which is a small circular hole, and a series of dichroic mirrors and filters in order to filter the light beam that will reach the detector, therefore only allowing the observation of the relevant wavelengths (Figure 1.12). The detectors used in the experiments described in this thesis are avalanche photodiode (APD) detectors, which are able to detect photons at a nanosecond resolution [113]. Using sub-nanomolar concentrations in addition to a small sample volume (~1 μm diameter, ~0.5 fL) allows for the excitation of only one fluorophore, on average, at any given time [114]. This technique uses a bright single point source of excitation, generally a laser, and a sequential scanning method which allows for the separation of different fluorophores by optimising for the appropriate laser wavelength, and hence eliminating the risk of false positive signals [115]. For this thesis, TCCD and FRET are the most relevant confocal microscopy fluorescence methods and are explained below.

![Figure 1.12. Schematic of a confocal microscope set-up. a) The sample is placed on a cover slide and is excited with a laser. The fluorescence emission is then passed through a series of](image)

**Figure 1.12. Schematic of a confocal microscope set-up.** a) The sample is placed on a cover slide and is excited with a laser. The fluorescence emission is then passed through a series of...
lenses, filters and a pinhole to remove out-of-focus light before it is detected by an avalanche photodiode (APD) detector. b) Side-view of the confocal volume. Note that the excitation is restricted to a small volume (~1 μm diameter, ~0.5 fL). Figure modified from the PhD thesis of Dr. Mathew Horrocks.

1.5.2. Two-colour coincidence detection (TCCD)

Two-colour coincidence detection (TCCD) is a SM fluorescence technique that uses confocal microscopy to detect coincident events by exciting the sample with two lasers, which provide excitation for two different fluorophores. TCCD is very useful for the study of protein aggregation as it is a highly sensitive technique that allows for the monomers, for which only one burst of fluorescence is observed, to be analysed separately from the oligomers, for which coincident bursts from both fluorophores are observed (Figure 1.13) [116, 117]; hence this technique has been extensively used to detect and characterise the oligomeric species formed during fibril formation of a range of protein systems, including the SH3 domain from bovine phosphatidylinositol-3’-kinase (PI3–SH3) [118], amyloid-β (1-40) [119], and the polymerisation of neuroserpin [120]. In addition, a study of the interactions of amyloid-β (1-40) oligomers with the extracellular chaperone, clusterin, demonstrated that this chaperone sequesters the oligomers, thus inhibiting fibril formation [41]. TCCD has also been used to detect molecular complexes [121], even at femtomolar concentrations [100], to study the turnover rate of an enzyme [122, 123], to determine gene activity by counting mRNA molecules [124] and to quantify the binding of an antibody to a protein [125].

In order to apply this technique to follow protein aggregation, the protein of interest needs to be singly-labelled with one of two different fluorophores whose emission can be distinctly separated from one another. The photons emitted by the fluorophores are detected by two independent detectors (one for each fluorophore) and only bursts that show higher photon counts than an applied threshold will be accounted for. In addition, the ratio of the brightness from coincident bursts in each of the channels correlates to the stoichiometric ratio in which the dyes are present in the detected complex. In order to analyse the data in an accurate and reliable manner, a method was developed in 2006 in the Klenerman Group (University of Cambridge, UK) to account for random coincident events which are caused by non-associated molecules that simultaneously pass through the confocal volume by chance [100].
Figure 1.13. Schematic of the intensity signal collected during TCCD measurements. Oligomeric species (represented with blue and red spheres) will show coincident bursts in both the blue and the red channels whilst monomers (represented by only one sphere) will only show a burst in the channel corresponding to their respective fluorophore (either blue or red). The green dashed lines represent the threshold applied for the analysis.

After applying the appropriate threshold and accounting for the “chance” events, the coincident events can be quantified and analysed. This number can then be used to calculate the association quotient (Q), which is the fraction of molecules formed by two or more different fluorophores as described before [118] using the following equation:

\[
Q = \frac{r_S}{r_B + r_R - r_S} = \frac{r_C - r_E}{r_B + r_R - r_C + r_E} \quad \text{(Eq. 1.1)}
\]

where \(r_B\) and \(r_R\) are the burst rates detected in the blue and red channels respectively, and \(r_S\) is the significant coincident event rate which is calculated as the subtraction of the expected rate of “chance” events (\(r_E\)) from the total coincident events rate (\(r_C\)) [118]. The threshold is chosen so that it is higher than the background photon count rate, and minimises the rate of chance events while maximising that of real coincidence events [126]. In practice, this means selecting for a threshold that maximises the association quotient (Q). Although being easy to use when all of the species are the same e.g. DNA duplex, this method for choosing a threshold quite often provides artificially high thresholds when applied to aggregates since there will be some species that are very bright and the Q is maximised when only these are selected. For this reason, in this thesis, the threshold was manually selected to maximise the signal-to-noise ratio.
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for each of the dyes and then applied consistently to all the time points throughout the time-course.

1.5.3. **Fluorescence or Förster resonance energy transfer (FRET)**

The process of resonance energy transfer (RET) or fluorescence resonance energy transfer (FRET) occurs when the emission spectrum of a fluorophore (donor) overlaps with the excitation spectrum of another fluorophore (acceptor). FRET results when the excitation of the donor is followed by a transfer of energy to the acceptor [127, 128] (Figure 1.14). Experimentally, this means only using one wavelength to excite the sample. However, for this phenomenon to occur, the donor and acceptor fluorophores must be within 10 nm in proximity to each other as the transfer of energy occurs because the donor and acceptor are coupled by non-radiative dipole-dipole interactions. The efficiency of the energy transfer is inversely proportional to the sixth power of the distance between the molecules. In addition to the distance between the fluorophores, the efficiency of the energy transfer depends on the dielectric environment, and on the orientation and properties of the dyes. Such properties include the overlap of the emission spectrum of the donor with the excitation spectrum of the acceptor, and the lifetime and quantum yield of the donor, i.e. the longer the lifetime of the donor, the longer the time available to transfer energy to the acceptor, albeit giving more time for the fluorescence of the donor itself. The Förster radius or distance refers to the distance from the donor dye to the acceptor dye at which the efficiency of the energy transfer is 50% and is typically 1-10 nm, which is comparable to the diameter of a standard protein in its monomeric state [129]. The Förster radius for the FRET pair formed by A488 and A647 is 56 Å (as calculated from spectroscopic data by ThermoFisher in The Molecular Probes® Handbook). This is important for the work within this thesis because only interacting monomers, e.g. oligomers formed during the early stages of fibril formation, will be close enough for the fluorophores on the proteins to transfer energy successfully and hence be detected. SM FRET has been successfully used in the literature to characterise the structure and population distributions of the oligomeric species of different systems [29-31, 33, 42-44] as well as to study their interactions with different biomolecules [40, 90, 91, 130]. The use of SM FRET to observe and analyse the interactions between oligomers and biomolecules, in this case chaperones, will be covered in more depth in Chapter 5.
**Figure 1.1. Description of SM FRET.** a) Jablonski diagram adapted to show FRET. The energy transferred from the donor excites the acceptor to an excited state which then returns to its ground state as it emits a photon. b) Schematic of the intensity signal collected during SM FRET measurements. Oligomeric species that go through the confocal volume (represented with blue and red spheres) show coincident bursts in both the donor (blue) and acceptor (red) channels whilst monomers (represented by only one sphere) are only detectable in the donor channel since only the laser corresponding to the donor is used as the excitation source. The green dashed lines represent the applied threshold.

In this thesis, the data from SM FRET was analysed by a well-established method that has been explained in detail before [29, 42-44]. In brief, the FRET efficiency for each oligomer was calculated using the following equation

$$E_{\text{FRET}} = \frac{I_A}{\gamma \times I_{\text{DA}} + I_A} \quad \text{(Eq. 1.2)}$$

where $I_A$ is the intensity of the acceptor, $I_{\text{DA}}$ is the intensity of the donor in the presence of the acceptor, and $\gamma$ is a correction factor that can be defined as
\[ \gamma = \frac{\phi_A \times \eta_A}{\phi_D \times \eta_D} \] (Eq. 1.3)

where \( \phi_A \) and \( \phi_D \) are the quantum yields of the acceptor and donor, respectively, and \( \eta_A \) and \( \eta_D \) are the efficiencies for detection of the acceptor and donor photons of the instrument, respectively. The apparent size of the oligomer was calculated using the following expression:

\[ \text{Size}_{\text{apparent}} = 2 \times \frac{I_{DA} + \frac{I_A}{\gamma}}{I_{D\_monomer}} \] (Eq. 1.4)

where \( I_{D\_monomer} \) corresponds to the average intensity of donor monomers. Following the analysis from previous studies, species with an apparent size of more than 150-mers were excluded from the analysis as the goal was to study only pre-fibrillar species [42, 43].

In summary, whereas FRET registers the interaction of two fluorophores in the same complex and hence these must be spatially close enough to present an appropriate spectral overlap, TCCD registers events of simultaneous emission by the two fluorophores; and therefore allows the fluorophores to be placed anywhere in the molecule. In practice, the high sensitivity of TCCD is extremely useful to quantify species and calculate stoichiometries of complexes. On the other hand, SM FRET provides extremely useful information about the structure and size for individual complexes as well as for an entire population. Although the size of an oligomer cannot be directly determined from SM FRET data, an estimation of its apparent size can be made and, although not entirely accurate in part because of the different ways that an oligomer can pass through the confocal volume and also the stochastic nature of single molecule excitation and emission, it can be useful to compare the relative sizes of different oligomer populations. In addition, data from TCCD and SM FRET can be mathematically modelled to provide further insights into the association and dissociation rates for complexes such as DNA [100] and into the mechanism of protein aggregation through the calculation of a number of rate constants [29, 42]. This use of SM FRET data will be further explored in Chapter 4.
1.5.4. Combining microfluidics with confocal microscopy to use single-molecule fluorescence for oligomer characterisation

As established above, using SM microscopy techniques allows for individual molecules to be observed and therefore their unique characteristics can be studied. This is particularly useful for studying oligomeric populations as they are usually present in low proportions and have a highly heterogeneous nature. The coupling of SM microscopy with microfluidics is currently widely applied as the latter allows for the sample to be observed as it flows through a microfluidic device, substantially decreasing the time required to collect sufficient events for the data to be significant as well as decreasing the background noise [131, 132]. Although it would seem tempting to increase the flow rate to minimise time, speed up the collection process and maximise the number of events, previous research showed that doing so can actually decrease the brightness of the individual bursts until they are no longer detectable [133]. This method of fast flow microfluidics coupled with SM fluorescence, optimised in reference [43], has been successfully used in the literature [29, 33, 43, 44, 130]. In this thesis, the experimental parameters optimised by that study [43] are used to maximise the reliability of the events detected while minimising collection time. For this reason, a flow rate of 0.61 cm s\(^{-1}\) was chosen for all the SM microscopy experiments. The instrumental set-up used for this work is shown in Figure 1.15. In brief, the sample is flowed through a channel in a microfluidic device, which is 25 μm in height, 100 μm in width and 1 cm in length. The microfluidic devices used in this thesis were provided by Dr. Chris Taylor. The sample is then excited with a laser, corresponding to the wavelength of the fluorophore, which has previously been filtered and focused using a series of filters and mirrors to reject unwanted signals. The light emitted by the sample is then collected by two different APD detectors. This instrument was described in detail in references [121, 125].
1.5.5. Following HuL aggregation with extrinsic dyes

The confocal set-up described above (Figure 1.15) can also be used to follow protein complexes using extrinsic dyes and label-free protein. This label-free method allows for the number of aggregates as well as their intensity to be tracked, providing insights into the mechanistic properties of fibril formation [134]. In this thesis, Thioflavin T (ThT) and pentameric formyl thiophene acetic acid (pFTAA) were used as the extrinsic dyes (Figure 1.16). The amyloid-binding dye, ThT, is typically used as a probe to follow amyloid fibril formation as in the presence of fibrils it becomes highly fluorescent [135-137]. While pFTAA is also an amyloid-binding dye, it has been shown to bind amyloid-β aggregates earlier than ThT making it a great alternative to detect pre-fibrillar species as well as fibrils [138, 139]. This method is also used in this thesis as a comparison to the experiments using labelled lysozyme in Chapters 3 and 4.
1.5.6. Limitations of TCCD and SM FRET

Although both TCCD and SM FRET offer a robust method for the study of protein complexes, samples need to be diluted to concentrations in the picomolar (pM) range. This requires the experimentalist to manually dilute each sample by 5-6 orders of magnitude, whilst using multiple eppendorf tubes and pipette tips, which can result in human error. In addition, this process of dilution is time-sensitive and could alter the population in the sample if the stability of some of the species is poor. The use of automated devices has been proved successful before [140], but their use in this thesis has not been possible due to the very specific conditions under which HuL undergoes fibril formation.

1.6. Thesis aims

Given the broad knowledge about the general mechanisms of the folding and unfolding of human lysozyme, it is of great interest to characterise its aggregation process at a molecular level in order to understand the intermolecular interactions involved in the early processes of fibril formation, as well as to gain insights into the structure of the species present during different stages of aggregation. In this thesis, the application of SM fluorescence will be used for the characterisation of the oligomeric populations present during fibril formation of HuL. In Chapter 2, the optimisation of the labelling of I59T HuL with A488 and A647, and the characterisation of the labelled-protein by different biophysical techniques will be presented. In Chapter 3, we will explore the use of a well-established SM microscopy approach [43] to study an enriched population of HuL oligomers before pursuing the characterisation of in situ formed HuL oligomers, produced using in vitro conditions that allow for the study of the effects of biomolecules (Chapter 4). Finally, the interactions between in situ HuL oligomers and the
extracellular chaperone, clusterin, will be explored using SM microscopy in Chapter 5. To the best of our knowledge, this thesis describes the first SM fluorescence study of the oligomeric population formed during fibril formation by a globular protein, and thereby allows for mechanistic details of amyloid formation and its regulation by a chaperone in a globular system to be compared to the aggregation pathways which have been characterised for IDPs related to misfolding diseases [42, 43, 90, 91].
Chapter 2: Fluorophore-labelling of human lysozyme

2.1. Introduction

In order to perform single-molecule fluorescence experiments with human lysozyme, a fluorophore needs to be introduced site-specifically to a single residue within the protein. In this chapter we explore the optimisation of this reaction as well as analyse the biophysical properties of the chemically-modified lysozyme and its feasibility for use in SM experiments.

2.1.1. Chemical modification of human lysozyme in a site-specific manner

Single fluorophores have been frequently incorporated into proteins by introducing single cysteine residues within a protein using site-directed mutagenesis [42, 118]. However, the eight cysteine residues within lysozyme are involved in the formation of four disulphide bridges, which are essential for the maintenance of the native-state structure and stability [141, 142], and hence they make it challenging to introduce a fluorophore at a specific cysteine residue. An alternative strategy involves targeting lysine residues, which has been shown to allow for selective labelling, using specific reaction conditions, due to the accessibility of individual lysine residues within a protein [143, 144]. Previous studies by Ahn and co-workers, 2012, showed that human lysozyme could be modified chemically in a site-specific manner at the lysine 33 residue (K33) [145]. This could be achieved due to the variability in the reactivities of the five lysine residues present in human lysozyme, with the K33 reactivity being kinetically favourable. This work also showed that the introduction of a biotin moiety into HuL at K33 was advantageous as the probe molecule was solvent exposed in the fibrils and hence labelling at K33 could potentially be used to characterise the aggregation of lysozyme by different techniques such as NMR or SM fluorescence. The reaction of human lysozyme with the selected moiety was, in this case, achieved under certain conditions using an N-hydroxysuccinimide (NHS) ester derivative of biotin. The same chemistry of conjugation applies for different NHS esters such as the fluorophores 5-carboxyfluorescein N-succinimidyl ester (F-NHS) and the N-succinimidyl ester derivatives of Alexa dyes, as well as nitroxide spin...
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labels used in NMR studies. The mechanism of the reaction consists of the side-chain amine group of lysine interacting with the carbonyl group of the NHS ester, resulting in the formation of an amide bond between the lysine side-chain and the fluorophore of interest (Figure 2.1). In this thesis, the method developed by Dr. Ahn and colleagues will be applied to the labelling of HuL with the two Alexa fluorophores already introduced in Chapter 1 (A488 and A647), which in turn will make it possible to apply fluorescence techniques to study the oligomeric species involved in HuL fibril formation and compare this process to previously reported oligomeric characterisation of disease associated IDPs [29, 42-44]. Given that the fluorophores themselves differ in both molecular mass, with A488 being 643.4 Da and A647, 842.02 Da, and overall charge i.e. A488 has two negative charges whereas A647 has three negative charges (Figure 2.1), it is likely that the reaction conditions for each fluorophore may need to be optimised in order to achieve the introduction of single-labels into the protein.

Figure 2.1. Labelling HuL with NHS-ester derivatives of Alexa fluorophores. The reaction between the lysine residue at position 33 (K33, highlighted in yellow) and the NHS-ester follows a SN2 mechanism where the bond between the NHS moiety and the Alexa fluorophore is broken simultaneously to the creation of an amide bond between the latter and the K33 side-chain. The structure of HuL was produced using PyMOL (PyMOL Molecular Graphics

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System, Version 1.7.4 Schrödinger, LLC), PDB entry 1JSF. The structures of A488 NHS-ester and A647 NHS-ester were drawn using ChemDraw Professional.

2.1.2. Chapter aims

This chapter will explore the labelling of I59T HuL with Alexa fluorophores in order to optimise the introduction of single-labels into the protein as it is required for SM experiments. Once this is confirmed, the biophysical properties of the labelled protein will be characterised to ensure that the introduced chemical moiety does not significantly alter the protein structure, native-state stability or the aggregation process. The labelled protein will then be used in the following chapters to further understand the mechanism of fibril formation in the lysozyme system using SM fluorescence.

2.2. Results

In order to use Alexa-labelled HuL for SM experiments, the labelling reaction needed to be optimised so that the formation of singly-labelled lysozyme was maximised. Singly-labelled implies that each protein molecule incorporates only one fluorophore. For the rest of this chapter, the non-natural variant I59T of human lysozyme will be used. As described in Chapter 1, this variant has similar properties to the naturally occurring amyloidogenic variant I56T, and its extensive characterisation makes it an excellent tool to study lysozyme aggregation [67].

2.2.1. Optimisation of the labelling of I59T HuL with Alexa fluorophores using different pH conditions and protein-to-dye ratios

The method developed by Ahn and co-workers, described above, involved the reaction of 7 μM WT HuL with an amine-reactive reagent containing a biotin moiety, N-(+)-biotinyl-6-aminocaproic acid N-succinimidyl ester (BioNSE), using 1:100 or 1:500 protein-to-dye ratios in 0.1 M MES buffer (pH 5.0), with stirring for 20 h at room temperature [145]. Although very successful for the single-labelling of HuL with biotin, these conditions require large amounts of reagent, which is not ideal for the use of F-NHS and Alexa fluorophores, due to their high cost. For this reason, the reaction conditions were optimised first using F-NHS in order to minimise the amount of dye required to singly-label HuL. An increase in the reactivity between the dye and HuL was achieved by increasing the pH. The reaction of WT HuL with F-NHS
Fluorophore-labelling of human lysozyme

was optimised to yield the single-labelled protein by using a protein-to-dye ratio of 1:15 in 0.1 M MES, pH 8.0 with stirring at 50 °C. In addition to similar spectroscopic properties to A488, F-NHS also contains negative charges at near-neutral pH. However, given that A488 has a slightly larger size, and A647 has a much larger size and is more negatively charged at near-neutral pH, the reactivity of the dyes was expected to be different due to steric hindrance as well as at different pH; therefore, it was necessary to optimise the conditions, exploring a range of pH from 5-8 (see Table 6 for dye sizes). The reaction for A647 is optimal at pH 5.0, 45 °C as this dye was found to be too reactive using higher pH i.e. I59T HuL would quickly incorporate two dye molecules. This result suggests that the steric hindrance of this larger dye moiety is not significant enough to reduce the reactivity. On the other hand, pH 8.0, 45 °C is optimal for A488 as only one molecule is incorporated into the protein. The protein-to-dye ratio was also investigated over a range from 1:1 to 1:100, and the optimised ratio was found to be 1:1 and 1:1.5 for A647 and A488, respectively. The initial concentrations for the reaction were set to 70 µM for I59T HuL and 70 µM and 105 µM for A647 and A488, respectively. The temperature of the reaction was initially set at 50 °C, but the optimised temperature was found to be 45 °C. The yield of the reaction was found to be 5 ± 2 % for A647 and 10 ± 3 % for A488. After purification by chromatography using a strong cation exchange resin (monoS column) to separate the desired product from other species, mass spectrometry was used to confirm the level of labelling presented within the protein. A summary of the reaction conditions investigated can be found in Table 2. For the optimised conditions, I59T HuL was confirmed to be singly-labelled (Figure 2.2, a and b). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the reaction products shows fluorescently labelled I59T HuL for both dyes when the gel was imaged after excitation with either a 488 nm laser or a 633 nm laser (Figure 2.2, insets).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Label</th>
<th>Labelling reagent</th>
<th>Protein-to-dye ratio</th>
<th>MW (Da) (labelling efficiency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-WT HuL</td>
<td>F-NHS</td>
<td>5-carboxyfluorescein N-succinimidy l ester</td>
<td>1:15</td>
<td>15078.93 (single)</td>
</tr>
<tr>
<td>F-I59T</td>
<td>F-NHS</td>
<td>5-carboxyfluorescein N-succinimidy l ester</td>
<td>1:15</td>
<td>15006.01 (single)</td>
</tr>
<tr>
<td>A488-I59T</td>
<td>A488</td>
<td>Alexa-488 N-succinimidy l ester</td>
<td>1:5</td>
<td>15714.5 (double)</td>
</tr>
<tr>
<td>A647-I59T</td>
<td>A647</td>
<td>Alexa-647 N-succinimidy l ester</td>
<td>1:15</td>
<td>17207.9 (triple) 18049.5 (quadruple) 18891.4 (quintuple)</td>
</tr>
<tr>
<td>A488-I59T*</td>
<td>A488</td>
<td>Alexa-488 N-succinimidy l ester</td>
<td>1:1.5</td>
<td>15196.84 (single)</td>
</tr>
<tr>
<td>A647-I59T**</td>
<td>A647</td>
<td>Alexa-647 N-succinimidy l ester</td>
<td>1:1</td>
<td>15521.30 (single)</td>
</tr>
</tbody>
</table>

*45 °C
**pH 5.0, 45 °C
Fluorophore-labelling of human lysozyme

**Figure 2.2. Labelling of I59T HuL with Alexa fluorophores.** a) Mass spectrometry (MS) analysis of A488-I59T HuL (7 µM) after reaction in 0.1 M MES buffer (pH 8.0) with a 1:1.5 lysozyme-to-A488 ratio. The peak at 15196.84 ± 0.49 Da corresponds to the single-labelled A488-I59T HuL. Inset: SDS-PAGE imaged by fluorescent emission of A488-I59T HuL. The band is located at around 14 kDa. b) MS analysis of A647-I59T HuL (7 µM) in 0.1 M MES, pH 5.0 with a 1:1 ratio, where the single-labelled peak appears at 15521.30 ± 0.46 Da. Inset: SDS-PAGE imaged by fluorescent emission of A647-I59T HuL. The band is located at around 14 kDa. The excitation of the dyes was achieved with a blue laser (488 nm) for A488-I59T HuL and a red laser (633 nm) for A647-I59T HuL. Both reactions were done at 45 °C with constant stirring in darkness. Both samples were purified by strong cation exchange chromatography prior to mass spectrometry analysis.
2.2.2. Characterisation of the stability and structure of Alexa-labelled human lysozyme

After successfully labelling I59T HuL with the Alexa fluorophores, the secondary structure and the thermostability of the labelled proteins were characterised by CD spectroscopy. CD spectroscopy measures the difference in absorbance between circularly polarised components and reports on the degree of chirality within a molecule, which can be used to observe the secondary and tertiary structures of proteins as well as to determine the midpoint temperature at which a protein unfolds [146]. Far-UV measures distinct spectra for the secondary structural elements such as α-helices, β-sheets and random coils. Near-UV CD monitors the tertiary structure, reporting on the conformation of aromatic residues found within the protein. Far-UV CD of the Alexa-labelled I59T HuL was monitored between 210-250 nm and compared to the I59T HuL monomer (Figure 2.3, a). Unfortunately, the presence of the Alexa dyes within the protein results in interference in the far-UV region, but it is possible to record the secondary structure of the labelled protein up to ~210 nm and from this analysis, it appears that the labelled protein is unaltered as compared to the unlabelled I59T HuL. In addition, thermal denaturation was monitored at 222 nm by CD spectroscopy. Calculating the temperature at the midpoint of the denaturation (where the protein is 50% unfolded) allows the comparison of native-state stability between the unlabelled and the labelled proteins. Thermal melts were fitted to a two-state model [74] and the denaturation midpoints (T_m) of A488-I59T HuL and A647-I59T HuL were calculated to be 71.3 ± 1.6 °C and 68.6 ± 1.6 °C, respectively. A comparison of these T_m values to those of the unlabelled I59T and WT HuL, as well as WT HuL labelled with F-NHS, can be found in Table 3. Although it appears, from the data, that the labelling with the Alexa dyes results in a destabilisation of the native-state thermostability of the I59T variant, it must be noted that the signal-to-noise ratio was quite low in the labelled protein samples, especially for A488-I59T and therefore it may have interfered with gaining accurate thermal denaturation curves (Figure 2.3, b-d). Due to the interference of the Alexa dyes in the CD measurements, a second technique was necessary to determine the unfolding transition. Differential scanning calorimetry (DSC) is a useful technique to identify transitions that induce a change in heat capacity and enthalpy and is useful for situations where samples are not well-resolved by spectroscopic techniques [74]. Unfortunately, the DSC was unavailable for further investigation of the Alexa-labelled I59T HuL native-state stability. It was possible, however, to measure the midpoint temperature for WT HuL and F-WT HuL, and both showed a single peak when the heat capacity was plotted against temperature (Figure 2.3, e). This result is characteristic of a two-state transition i.e. only one conformational transition
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was detected by DSC. According to previous studies, this conformational transition corresponds to the cooperative conversion of the native-state into the intermediate state, leading to the hypothesis of two first-order transitions, which questions the three-state model as explained in detail in reference [74]. The $T_m$ at the maximum heat capacity was found to be 77.2 ± 0.3 °C for WT HuL and 73.5 ± 0.7 °C for F-WT HuL. This data confirms that the F-label, unlike previous work with biotin labelling, has a slight destabilising effect on the WT HuL protein. This result also shows that the $T_m$ calculated from the denaturation followed by DSC is not significantly different from that obtained from the CD denaturation, even with the interference of the dye. Therefore, and due to the similarities between the fluorescein derivative and the Alexa dyes, it can be concluded that the temperatures at the midpoint of the denaturation calculated for the Alexa-labelled I59T HuL samples by CD denaturation are likely to be accurate enough for this characterisation. However, further analysis would be necessary for a more accurate characterisation, for example using tryptophan fluorescence.

Table 3. Comparison of the denaturation midpoints ($T_m$) of WT and I59T HuL as measured by far-UV CD thermal denaturation and DSC. The $T_m$ values correspond to an unfolded fraction of 0.5. The data are shown as the mean ± the standard deviation from three independent experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Technique</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>CD</td>
<td>79.9 ± 0.9</td>
</tr>
<tr>
<td>WT</td>
<td>DSC</td>
<td>77.3 ± 0.2</td>
</tr>
<tr>
<td>F-WT</td>
<td>CD</td>
<td>74.7 ± 0.9</td>
</tr>
<tr>
<td>F-WT</td>
<td>DSC</td>
<td>73.0 ± 0.1</td>
</tr>
<tr>
<td>I59T</td>
<td>CD</td>
<td>72.0 ± 0.4</td>
</tr>
<tr>
<td>A488-I59T</td>
<td>CD</td>
<td>71.3 ± 1.6</td>
</tr>
<tr>
<td>A647-I59T</td>
<td>CD</td>
<td>68.6 ± 1.6</td>
</tr>
</tbody>
</table>
Figure 2.3. Analysis of secondary structure and native-state stability of Alexa-labelled I59T HuL. a) Analysis of the secondary structure of I59T HuL (black), A488-I59T HuL (blue) and A647-I59T HuL (red) in terms of the Mean Residue Ellipticity (MRE) using far-UV CD spectroscopy. Both dyes interfere with the reading, making it impossible to record at wavelengths lower than 210 nm. The scans were recorded in triplicate, and the data shown is a representative data set. b) Thermal denaturation curve monitored in the far-UV region (222 nm) for A647-I59T HuL (red). The midpoint temperature was calculated by fitting the observed ellipticities to a two-state model [74] to be 68.6 ± 1.6 °C. c), d) Thermal denaturation curve monitored in the far-UV region (222 nm) for A488-I59T HuL (blue) in terms of ellipticity (c)
and fraction unfolded (d). The interference of A488 is noticeable, and hence the fitted data is presented in terms of ellipticity (c), but the data could be fitted and the midpoint temperature was calculated to be $71.3 \pm 1.6 \, ^\circ\text{C}$. e) Evolution of heat capacity over increasing temperature for F-WT HuL (dark cyan) in water. The curve corresponds to a two-state transition and the midpoint temperature was calculated as the temperature at which the heat capacity was at its maximum, and was equal to $73.0 \pm 0.1 \, ^\circ\text{C}$. This result is comparable to that calculated from CD thermal denaturation, $74.7 \pm 0.9 \, ^\circ\text{C}$, which is shown in (f). The $T_m$ from the CD thermal denaturation and DSC experiments (b-f) were calculated as the average from three independent experiments, and a representative data set of these experiments is shown.

### 2.2.3. Spectroscopic characterisation of Alexa-labelled human lysozyme

After probing their thermostability, the spectroscopic properties of the Alexa-labelled I59T HuL proteins were explored by different biophysical techniques (Figure 2.4 and 2.5). The absorbance spectra of 3.5 μM of A488-I59T and A647-I59T HuL were obtained between 200-800 nm and compared to that of unlabelled I59T HuL (Figure 2.4). As expected, a single absorbance peak located in the region of 280 nm is observed in the spectrum for I59T HuL. This peak is typically due to the tryptophan amino acids in the protein [147, 148]. In comparison, both A488-I59T and A647-I59T HuL show two absorbance peaks: one between 250-300 nm due to the presence of the protein and a second distinct peak with a maximum absorbance at approximately 490 nm and 651 nm which can be attributed respectively to the fluorophores A488 and A647 themselves (Figure 2.4). The excitation and emission spectra of labelled I59T HuL were also recorded (Figure 2.5). The excitation and emission maxima for A488-I59T HuL are 496 and 518 nm, respectively (Figure 2.5, a), and they are 657 and 672 nm, respectively, for A647-I59T (Figure 2.5, b). The excitation spectra were collected by setting a constant emission wavelength of 517 and 672 nm (± 5 nm slit width), respectively for A488-I59T and A647-I59T HuL, and recorded between 250-650 nm for A488-I59T and 250-700 nm for A647-I59T HuL. The emission spectra were collected by using an excitation wavelength of 494 and 651 nm (± 5 nm slit width), respectively, and emission was recorded between 500-650 nm for A488-I59T and 500-700 nm for A647-I59T HuL. The spectra for A488-I59T HuL were recorded at 600 V whilst those for A647-I59T HuL were collected at 500 V as the maximum intensity for the recording was reached at 600 V, which explains the difference in intensity (Figure 2.5, a and b). The excitation and emission maxima shown by both dyes in the labelled protein correlate perfectly with those observed for the free dye (Section 1.4.3) and therefore these data suggest that the dyes do not suffer any significant perturbation to their spectroscopic properties when bound to the protein.
Figure 2.4. Spectroscopic characterisation of labelled I59T HuL (I). Comparison of the absorbance of I59T HuL (black), Alexa488-I59T HuL (blue) and A647-I59T HuL (red) in water. The spectra for all the samples show a peak at ~280 nm, which is typically indicative of the presence of protein. The spectra for A488-I59T and A647-I59T HuL also show an extra peak at approximately 490 and 651 nm, respectively.
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Figure 2.5. Spectroscopic characterisation of labelled I59T HuL (II). a) Excitation (orange) and emission (blue) spectra of A488-I59T HuL in water with maxima at 496 and 518 nm, respectively. b) Excitation (green) and emission (purple) of A647-I59T HuL in water with maxima at 657 and 672 nm, respectively.

2.2.4. Fibril formation

In addition to characterising the native-state of the labelled proteins, their competence to form fibrils in the same manner as the unlabelled protein needed to be assessed. The conditions for fibril formation for destabilised variants of HuL were optimised by previous studies to be 0.1 M citrate pH 5.0, 60 °C with stirring and have been fully characterised for the unlabelled I59T
variant before [67, 69, 145]. The aggregation of a stoichiometric mixture of A488-I59T and A647-I59T HuL (3.5 μM of each dye) under these conditions was followed by light scattering at 500 nm (Figure 2.6, a) and the resulting aggregates were imaged by transmission electron microscopy (TEM) (Figure 2.6, b). Light scattering was used to follow the aggregation as the typically used probe to follow aggregation kinetics, ThT (introduced in Section 1.5.5), could not be used as its fluorescence spectrum overlaps with that of the Alexa dyes. The stoichiometric mixture was chosen as these are the conditions required for the SM experiments. The results show that, within error, the aggregation rate of the labelled I59T HuL mixture is the same as the unlabelled protein. The midpoint for I59T HuL aggregation was found to be 6.3 ± 1.1 h whereas that of labelled I59T HuL mixture was 5.7 ± 0.8 h. The midpoint was calculated as the time corresponding to a normalised light scattering signal of 0.5 i.e. the midpoint of the exponential increase. The fibrils of both unlabelled and Alexa-labelled I59T HuL show the same structural features when imaged by TEM (Figure 2.6, b and c).
Fluorophore-labelling of human lysozyme

Figure 2.6. Fibril formation of Alexa-labelled I59T HuL. a) In vitro aggregation monitored by light scattering at 500 nm for I59T HuL (black) and a stoichiometric mixture of A488-I59T and A647-I59T HuL (green) in 0.1 M citrate buffer, pH 5.0 with stirring at 60 °C. The data show the mean and the error bars represent the standard deviation in the midpoint of aggregation from three independent experiments. The normalised data was calculated using unity-based normalisation, where each point is calculated as \((x-x_{\text{min}})/(x_{\text{max}}-x_{\text{min}})\) where \(x\) is the measured raw data, and \(x_{\text{min}}\) and \(x_{\text{max}}\) are the minimum and maximum values of the raw data, respectively. b), c) TEM images of the endpoint of aggregation for I59T HuL (b) and Alexa-labelled I59T HuL (c), where the black scale bar represents 100 nm.
2.2.5. Incorporation of Alexa dyes into HuL fibrils

Previous research on the aggregation of α-synuclein showed a preference for A488-labelled monomer to be incorporated into the dually labelled fibrils [42]. Although this preference was only significant at the fibrillar level and did not affect the SM experiments used to follow the early stages of aggregation [42], the incorporation of the Alexa dyes into HuL fibrils was monitored in order to assess if a preference was also observed for this protein system. First, the concentration of labelled I59T HuL for each of the dyes needed to be measured accurately in order to ensure that a stoichiometric initial monomer concentration would be used. For this purpose, the concentration was checked by both amino acid analysis (AAA) at PNAC (Dept. of Biochemistry, University of Cambridge) and UV-vis spectroscopy. The results from AAA show no significant difference between this analysis and the data obtained by UV-vis spectroscopy and therefore the latter was used for concentration measurements of the labelled protein for the rest of this thesis due to ease and convenience of this methodology. Once the measurement of monomer concentration was proven to be reliable, the incorporation of the Alexa dyes was investigated using two different methods that assessed the concentration of monomeric A488-I59T and A647-I59T HuL at two different time points of the aggregation in which fibrils are already forming (9 and 24 h). The aggregation was set at a total initial monomer concentration of 7 μM (3.5 μM of each dye). The samples taken after 9 and 24 h were first centrifuged for 30 min at 11500 rcf to isolate the fibrils. The monomer concentration in the supernatant was then assessed by UV-vis absorbance and by fluorescence imaging of a SDS-PAGE analysis. This latter method involves the excitation of the sample with the appropriate laser and then imaging the fluorescence that is emitted by the fluorophore. After 9 h, the concentrations of A488-I59T and A647-I59T HuL were measured in triplicate by absorbance at 494 and 651 nm, respectively, and were found to be 3.47 ± 0.08 μM and 2.38 ± 0.06 μM, respectively. These data suggest that the labelled fibrils may have a preference for A647-I59T HuL monomers. The sample after 24 h showed a monomer concentration effectively equal to zero which suggests that all monomers had been incorporated in the fibrils by that time. The same supernatant samples were then analysed by SDS-PAGE and the resulting gel was imaged in the same manner as described in Section 2.2.1. The gel was first excited with a blue laser (488 nm), which illuminated A488-I59T HuL only, and then with a red laser (633 nm), exclusively illuminating A647-I59T HuL. The fluorescence emitted was, in both cases, lower for the mixture than for each of the dyes separately. No significant difference is observable between the fluorescence emitted by each of the fluorophores in the 9
h mixed time point when illuminated with the different lasers (Figure 2.7, a and b, lane 3). In order to investigate whether both dyes were incorporated into the fibrils and, additionally, whether they were able to produce FRET, the fibrils were imaged by Dr. Marie Bongiovanni using total internal reflection fluorescence microscopy (TIRFM). This method involves the observation of the fluorescence emitted in both the blue (A488) and red (A647) channels after the sample is excited with a particular laser, allowing the fibrils to be imaged [149]. The results show FRET and therefore it can be concluded that both dyes are indeed incorporated into the fibrils (Figure 2.7, c).

Figure 2.7. Incorporation of Alexa dyes into HuL fibrils. a), b) SDS-PAGE imaged by fluorescence using a 488 nm (a) and a 633 nm laser (b). The lanes are labelled for Ladder (L), 3.5 μM A488-I59T HuL (1), 3.5 μM A647-I59T HuL (2), 9 h (3) and 24 h time point (4). Both the 9 and 24 h time points were taken from an aggregation mixture of total initial monomer concentration equal to 7 μM (3.5 μM of each dye). In both cases, the mixture (lane 3) shows a
lower fluorescence than each of the dyes alone (lanes 1 and 2) which is consistent with monomer depletion by the formation of aggregates. No significant difference was observed for the fluorescence in lane 3 regardless of the laser used. c) TIRFM imaging of fibrils formed from equimolar concentrations of A488-I59T and A647-I59T HuL. When the sample was excited with a blue laser (488 nm), fluorescence was observed in both the A488 and the A647 channels meaning there was FRET and hence both dyes were present in the fibrils. Image taken by Dr. Marie Bongiovanni.

2.3. Conclusion and next steps

Single-labelling of the I59T lysozyme variant, a model system for lysozyme amyloidosis, was successfully achieved with both A488 and A647. A488 was found to label I59T in a single manner when reacted at pH 8.0, 45 °C with a protein-to-dye ratio of 1:1.5. A647 was found to be more reactive than A488 and therefore a lower pH and a lower protein-to-dye ratio were needed (pH 5.0, 1:1 ratio) to achieve single-labelling of I59T HuL. After purification by strong cation exchange chromatography, the labelled proteins were characterised by different biophysical techniques to determine the effect of the dyes on the attributes of lysozyme. This analysis shows that the introduction of Alexa dyes to I59T HuL results in a slight decrease in the native-state stability, although no changes to the secondary structure are observed. The in vitro fibril formation of the Alexa-labelled I59T HuL is comparable to that of unlabelled I59T HuL, with the midpoint of aggregation being $5.7 \pm 0.8\ h$ and $6.3 \pm 1.1\ h$, respectively, and the resulting fibrils show identical morphology. The investigation of the incorporation of the dyes into HuL fibrils, when present in an equimolar ratio, shows no significant preference for either of the dyes. This analysis also shows that FRET is observed in the fibrils, which confirms that both dyes are incorporated, and suggests that SM FRET should be a viable tool to follow the aggregation process. The facile nature of incorporating the Alexa-dyes into human lysozyme and its variants in a relatively controlled manner means that it is now possible to apply single-molecule fluorescence experiments to the lysozyme system in order to gain insight into the early events of fibril formation for a globular protein. The next chapters in this thesis will involve carrying out these investigations.
Chapter 3: Optimisation of a SM microscopy set-up to characterise HuL oligomers

3.1. Introduction

SM microscopy is a powerful technique to study the oligomeric species present in protein aggregation. Previous studies have shown how this technique can be used effectively to monitor the oligomeric populations present during the aggregation of a diverse range of systems, including the SH3 domain of PI3 kinase, α-synuclein, the amyloid-β peptide and tau [44, 118, 130, 150, 151]. To date, most studies have focused on the aggregation processes of IDPs and therefore, in order to investigate the aggregation process of the globular human lysozyme, the SM microscopy set-up required some optimisation. In this chapter, we will test the set-up using lysozyme samples enriched with oligomers, following aggregation protocols reported for HEWL in the literature.

3.1.1. Using oligomer-enriching conditions to optimise the SM microscopy set-up for HuL

As mentioned in Chapter 1, the early species present along the aggregation pathway of human lysozyme have not been fully characterised as their low concentration and transient nature have made them challenging to detect [63]. Also, unlike other systems previously studied by TCCD and SM-FRET, introducing a single fluorophore label into HuL posed a challenge. However, recent developments in site-specific labelling of human lysozyme [145] as well as the development of label-free methods for detecting lowly populated species [75, 152], have opened up the possibilities to gain insight into the early aggregation events of this system.

In order to optimise the SM microscopy set-up described in Section 1.5.4 to investigate HuL oligomers, we will use a positive control in which the oligomer formation is enriched. This
positive control relies on previous work on HEWL which showed that conditions containing high salt concentrations at acidic pH result in the formation of metastable oligomers [153, 154]. In those studies, the kinetic phase of HEWL was mapped as protein and salt concentrations varied (Figure 3.1). The results showed that as protein and/or salt concentrations increase, a critical concentration of oligomers is reached and this then nucleates the formation of, what seem like, curvilinear fibrils. This study involved monitoring aggregation with a range of HEWL concentrations from 6 μM to 1.4 mM, in the presence of 50 to 800 mM of NaCl [153].

![Figure 3.1. Metastable oligomer formation for HEWL. a) Kinetic phase of HEWL over different salt concentrations. Highlighted in purple is the region in which the formation of metastable oligomers and curvilinear fibrils is maximised. b) AFM imaging of the oligomers (top) and curvilinear fibrils (bottom). The scale bars represent 200 nm and 300 nm, respectively. Colour scale represents aggregate height in nm. Figure adapted with permission from references [153, 154].](image)

### 3.1.2. Chapter aims

As little is known about the population and stability of any oligomer species that are formed *in situ* under our standard HuL fibril forming conditions (pH 5.0, 60 °C), the SM microscopy set-up introduced in Section 1.5.4 will be optimised using samples enriched with oligomeric species of HuL (400 mM, pH 2.0 [153]). In this chapter, we will first optimise the oligomer-
enriching conditions on HuL (as the previous report used HEWL). Next, the detection of oligomeric species using SM fluorescence microscopy will be evaluated first using Alexa-labelled human lysozyme and then using label-free methods.

3.2. Results

The biophysical characterisation of WT HuL, and unlabelled and Alexa-labelled I59T HuL in the oligomer-enriching conditions was first investigated to ensure no significant alterations in their properties were observed before using these as a positive control for the SM techniques. Once the effect of these conditions on our system were established, their use for the optimisation of the SM microscopy set-up was pursued.

3.2.1. Spectroscopic characterisation of singly-labelled I59T HuL under oligomer-enriching conditions

Although the Alexa fluorophores are pH insensitive over a broad range of pH [155], their spectroscopic properties under oligomer-enriching conditions (pH 2.0, 400 mM NaCl) were investigated by measuring their absorbance. The absorption spectra of A488-I59T and A647-I59T HuL were obtained between 200-600 nm and compared in 400 mM NaCl at pH 2.0, 5.0 and 7.0 conditions. The concentration of NaCl was chosen to maximise oligomer formation whilst minimising the amount of protein needed, as well as avoiding the formation of amorphous aggregates (Section 3.1.1). As expected, the A488 and the A647 labelled protein spectra contain an absorbance peak in the region between 250-300 nm and a distinct maximum absorbance at around 490 nm and 650 nm, respectively, which can be attributed to the fluorophore (Figure 3.2). No absorbance shifts or significant changes in overall intensity are observed for the spectra recorded under these conditions (Figure 3.2). Even though chloride ions are known for being good quenchers and their effect in data collection by SM fluorescence techniques has been proved before [43], in this chapter the samples were dissolved in water before being injected into the SM set-up, which minimises the potential effect of said ions. It can therefore be concluded that these conditions (pH 2.0, 400mM NaCl) can be used without disturbing the signal of the Alexa dyes.
Figure 3.2. Spectroscopic comparison of Alexa-labelled I59T HuL under different pH conditions and with 400 mM NaCl. UV-vis spectra of A488-I59T HuL (a) and A647-I59T HuL (b) at pH 2.0 (red), 5.0 (green) and 7.0 (blue). Typically, a peak at ~280 nm is indicative of the presence of protein and the spectra show two extra peaks at approximately 490 nm (a) and 650 nm (b) due to the presence of the dye. No significant difference is observed for the different pH conditions.
3.2.2. Native-state stability and structure of unlabelled and Alexa-labelled HuL under oligomer-enriching conditions

Once the Alexa dyes were proven to be functional, the native-state stability of HuL was investigated in the 400 mM NaCl, pH 2.0 conditions. The secondary structure and thermostability of WT and I59T HuL were characterised by CD spectroscopy. Far-UV was monitored between 200-250 nm and compared to the data collected using the pH 5.0 conditions. The results for both WT and I59T HuL show a slight difference in the secondary structure, focused on the 210 nm region at pH 2.0 and pH 5.0 (Figure 3.3), which may be explained by the highly charged environment at pH 2.0, 400 mM NaCl conditions. Far-UV thermal denaturation was performed by monitoring the change at 222 nm with increasing temperature. As described in Section 2.2.2, the thermal melts were fitted to a two-state model [74] and the T_m of WT and I59T HuL in the pH 2.0 conditions were calculated to be 65.4 ± 1.8 °C and 58.7 ± 2.6 °C, respectively (Table 4). The T_m of WT and I59T HuL in the pH 5.0 conditions were calculated to be 79.9 ± 0.9 °C and 72.0 ± 0.4 °C, respectively [75]. Therefore, the thermal denaturation at pH 2.0 shows an average destabilisation of 13.3 °C for I59T HuL and of 14.5 °C for WT HuL (Figure 3.3 and Table 4). These results are in agreement with the difference in stability previously reported for the midpoint temperatures of HuL at pH 5.0 and pH 1.2 [74, 75, 156].
Figure 3.3. Analysis of native-state structure and thermostability of WT and I59T HuL monitored by CD spectroscopy in the far-UV region. a), b) Comparison of the secondary structure of WT HuL (a) and I59T HuL (b) in the pH 2.0, 400 mM NaCl conditions (red) and in the 0.1 M citrate, pH 5.0 conditions (green). c), d), e), f) Thermal denaturation of WT HuL (c, e) and I59T HuL (d, f) in the far-UV region (222 nm) using the pH 2.0 (c, d) and the pH 5.0 conditions (e, f). The unfolded fraction was calculated as described in reference [145]. In (a) and (b), the data show the mean and the error bars represent the standard deviation from three independent experiments.
Table 4. Comparison of the midpoint temperatures ($T_m$) of WT and I59T HuL as measured by far-UV CD thermal denaturation. The $T_m$ values correspond to an unfolded fraction of 0.5. The data are shown as the mean ± the standard deviation from three independent experiments.

<table>
<thead>
<tr>
<th>Conditions (pH)</th>
<th>$T_m$ (°C)</th>
</tr>
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<tbody>
<tr>
<td>WT 5.0</td>
<td>79.9 ± 0.9</td>
</tr>
<tr>
<td>WT 2.0</td>
<td>65.4 ± 1.8</td>
</tr>
<tr>
<td>I59T 5.0</td>
<td>72.0 ± 0.4</td>
</tr>
<tr>
<td>I59T 2.0</td>
<td>58.7 ± 2.6</td>
</tr>
</tbody>
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3.2.3. Optimising HuL concentration in order to maximise oligomer formation

After the native-state stability of both WT and I59T HuL was explored, the aggregation kinetics using different concentrations of WT HuL in 400 mM NaCl, pH 2.0 were followed by measuring ThT fluorescence over time in order to maximise the formation of oligomeric species. Since the purpose of these experiments was simply to be able to produce oligomer-enriched samples to inject into the SM set-up, the more stable WT protein was used, as it is more readily available and therefore allowed us to sample a variety of concentrations for this optimisation stage. Once the conditions were established for the WT protein, the Alexa-labelled I59T variant was used for further analysis by TCCD and SM FRET (Section 3.2.7).

To better observe the enrichment of oligomeric species, the kinetic traces were plotted as log ThT intensity as described by Hasecke and co-workers [34]. The results show a lag phase observable at 70 μM WT HuL, which then disappears into an exponential curve for higher concentrations of WT HuL (Figure 3.4). This early exponential region then reaches a short plateau before increasing again and reaching a second plateau, most clearly observable at 420 μM (Figure 3.4, d). The first exponential corresponds to the formation of metastable oligomers that lead to curvilinear fibrils, whereas the formation of rigid fibrils corresponds to the second exponential [153, 154]. In order to maximise the amount of oligomers and minimise the amount
of protein needed for further experiments, the rest of this chapter will focus on protein concentrations between 210 and 350 μM HuL, as stated in the corresponding sections.

![Figure 3.4](image)

**Figure 3.4. Kinetic characterisation of different concentrations of WT HuL in 400 mM NaCl, pH 2.0 conditions.** A flat lag phase is observed for 70 μM WT HuL (a). Progressive steepening is observable at 210 (b), 280 (c), and 420 μM (d). The logarithm of the ThT intensity is shown in order to highlight the first exponential as described in reference [153]. The data show the mean and the error bars represent the standard deviation from three independent experiments.

### 3.2.4. Characterisation of fibril formation using oligomer-enriching conditions

After choosing the HuL concentration necessary for maximising oligomer formation, the morphology and stability of the fibrils formed using 400 mM NaCl, pH 2.0 were investigated and compared to those of fibrils formed using pH 5.0 conditions. The aggregation of WT HuL (350 μM) was set at 52 °C in 400 mM NaCl, pH 2.0 and monitored by ThT fluorescence (Figure 3.5, a). The conformational stability of WT HuL fibrils formed under these conditions was established by depolymerisation using guanidine thiocyanate (GdnSCN) (Figure 3.5, b), giving
a midpoint of depolymerisation of 3.19 ± 0.07 M (data courtesy of Kimberley Callaghan). This result is in accordance with the midpoint of denaturation of 2-3 M GdnSCN reported in reference [156] for HuL fibrils formed under acidic conditions without salt. The resulting fibrils after 70 h were imaged by transmission electron microscopy (TEM), which shows linear fibrils that are distinctively longer than those formed by HuL using pH 5.0 conditions, and similar in size and structure to those formed without salt at pH 2.0 [156] (Figure 3.5, c). The fibrillar morphology and size are equivalent to those shown previously for the HEWL oligomer-enriching conditions [153, 154]. Finally, the ThT fluorescence of the aggregates at the endpoint of the aggregation reaction was measured in triplicate. The samples from the pH 2.0 conditions were taken after 17 h, which corresponds to the end of the first plateau (Figure 3.5, a), where previous studies have shown the presence of oligomers and curvilinear fibrils under these conditions [153, 154]. This time point was chosen because it corresponds to the end of the time period that will be studied by SM microscopy later in the chapter. For the pH 5.0 conditions, the time points were taken after 24 h, when all the monomer has been incorporated into fibrils (as measured by absorbance at 280 nm and SDS-PAGE analysis). The fibrils formed under the pH 5.0 conditions show a higher ThT fluorescence than the aggregates formed under the pH 2.0 conditions after 17 h (Figure 3.5 d). This result may be explained by the presence of a higher β-sheet content in the case of the fibrils formed at pH 5.0, as compared to that of those present after 17 h in the pH 2.0 conditions. This result may also be explained by the presence of oligomers and curvilinear fibrils at this time point in the pH 2.0 conditions as reported in references [153, 154].
Optimisation of a SM microscopy set-up to characterise HuL oligomers

Figure 3.5. Characterisation of fibrils formed using 400 mM NaCl, pH 2.0 conditions. a) Kinetic characterisation as followed by ThT fluorescence for 350 μM WT HuL. b) Stability of fibrils formed at pH 2.0 as measured by GdnSCN depolymerisation. Graph courtesy of Kimberley Callaghan. c) TEM image of fibrils formed after 70 h in 400 mM NaCl (pH 2.0). Inset: HuL fibrils formed using pH 5.0 conditions. The fibrils formed using pH 5.0 conditions are shorter than those formed using pH 2.0 conditions. The scale bars represent 100 nm. d) Comparison of the ThT fluorescence shown by the aggregates formed after 17 h in pH 2.0 conditions (red) and by the mature amyloid fibrils formed after 24 h in pH 5.0 conditions (blue). The fibrils formed at pH 5.0 show a slightly higher ThT fluorescence than those formed at pH 2.0. This result may be explained by the presence of oligomers and curvilinear fibrils at this time point in the pH 2.0 conditions [153, 154]. For (a) and (d), the data show the mean and the error bars represent the standard deviation from three independent experiments.

3.2.5. Characterisation of HuL oligomers formed in acidic conditions using conventional biophysical techniques

Once the native-state and the fibrillar state of HuL using oligomer-enriching conditions had been characterised by different biophysical techniques, the oligomeric species were characterised in bulk by atomic force microscopy (AFM) and dynamic light scattering (DLS).
DLS can be used to determine the size of molecules in solution by measuring their light scattering [157]. Although it is not very accurate for heterogeneous mixtures, as it is biased towards big molecules, it has been used here to observe whether any species, other than the monomer, was formed in the initial period of aggregation. Indeed, at 0 h only one peak was observed and it was centred at 1-2 nm, which is consistent with the size of a monomer (Figure 3.6, a). After 0.5 h, two peaks were observed, one was still consistent with the monomer (1-2 nm) whilst the second one was shifted to the right corresponding to a size of 3-4 nm (Figure 3.6, b). This second peak was even more distinct after 1.5 h, when the peak was clearly separated from the monomer with a size of 4 nm (Figure 3.6, c). Finally, another DLS measurement was taken after 7.5 h where only the peak centred at 4 nm was observed after centrifugation to remove the large aggregates (Figure 3.6, d). Before centrifugation, large aggregates could be observed in the region of 1-1.5 μm (Figure 3.6, c, inlet). These large aggregates were observed from 2.5 h, which is in accordance with previous results where curvilinear fibrils were formed after 2 h [153, 154]. The size of the oligomeric peak (approximately 4 nm in diameter) is also consistent with reference [154]. In addition, AFM images of the aggregates formed after 7.5 h show spherical oligomers of approximately 50-100 nm in diameter (Figure 3.6 d, inlet) which is consistent with what was previously published [153, 154].
Figure 3.6. Characterisation of HuL oligomers formed in acidic conditions using conventional techniques. DLS measurements of WT HuL after 0 (a), 0.5 (b), 1.5 (c) and 7.5 h (d). a) At 0 h, one peak is observed at 1-2 nm. b) After 0.5 h, two peaks can be observed, one still centred at 1-2 nm and a second peak at 3-4 nm. c) After 1.5 h, the two peaks centred at 1-2 nm and 4 nm are clearly distinct. Inset: DLS shows large aggregates of around 1 μm in diameter before centrifugation after 2.5 h. d) After 7.5 h, only the peak at 3-4 nm is observed after centrifugation. Inset: AFM image of oligomers formed after 7.5 h. The scale bar represents 500 nm and the colour scale represents heights with 0-2.5 nm in brown and 3-10 nm in white. AFM image provided by Ryan Limbocker.
3.2.6. Characterisation of HuL oligomers formed in acidic conditions using Alexa-labelled I59T HuL

After successfully detecting the presence of unlabelled oligomeric species using DLS and AFM, we next investigated whether we could monitor Alexa-labelled HuL fibril formation with the SM microscopy set-up, described in Section 1.5.4, to achieve three goals; 1) detect oligomeric species at picomolar concentrations, 2) obtain oligomeric distributions using TCCD and 3) use SM FRET to gain structural insight into the oligomeric species present in the early stages of aggregation. As discussed in Section 1.5.3, SM FRET requires the dyes to be in close enough proximity within the aggregates to transfer energy whereas TCCD only requires the two fluorophores to be present together within the same aggregate. Therefore, using TCCD initially seemed to be the best approach; however, TCCD proved to be very challenging due to issues with Alexa-labelled HuL sticking to the microfluidic device and the gel loading tips used to inject the sample into the microfluidic device, resulting in perturbation of the SM signal. In order to prevent the sticking, the gel tips were coated with 1 mg/ml of bovine serum albumin (BSA). This procedure greatly reduced any disturbance of the signal.

The Alexa-labelled I59T HuL was aggregated under the conditions described in Section 3.2.4 and different time points were taken and diluted to 25 pM before being injected into the microfluidic device at a flow rate of 0.61 cm s\(^{-1}\). The samples were maintained on ice (which prevents dissociation of the oligomers) until diluted. Analysis of the data from each time point was performed as described in Section 1.5.2. The results show that the number of oligomers increases with time for the first 2 h and then decrease after 7 h (Figure 3.7, a). This decrease may be related to the oligomeric species converting into fibrils. The oligomers detected can be further segmented into apparent size categories. Here, the size has been divided into three categories: very small oligomers (dimers to tetramers), medium oligomers (5-15 monomers) and large oligomers (16-50 monomers). Even though the total number of small oligomers does increase over time, the fraction of these small species decreases with time whilst that of medium and large oligomers increase over time (Figure 3.7, b). From these observations, it appears that the formation of small oligomers is happening at a slower rate than the formation of the larger oligomers. This suggests that the conversion from smaller to larger species may occur at a faster rate than the small species formation and hence the ratio of large-to-small species increases over time. In addition, the percentage of coincident bursts (here expressed as the percentage of oligomeric species) also increases with time, reaching 10% after 7 h (Figure 3.7,
c). Finally, the average size of the sample for each time point was calculated, resulting in approximately eight monomers per aggregate. Although the average size is only approximate, as explained in Section 1.5.3, this result is in accordance to the size estimated previously by AFM under these conditions [153, 154].

![Graphs](image)

Figure 3.7. TCCD characterisation of Alexa-labelled I59T HuL oligomers formed in acidic conditions. a) The numbers of oligomers detected by TCCD for each time point were classified by approximate size: 0 (black bar), 1 (orange), 2 (green) and 7 h (blue). The number of events detected for small species (apparent size of 2-4 monomers) increases between 0 and 2 h and decreases at 7 h, likely due to their conversion into curvilinear fibrils. b) Fraction of events categorised by approximate size for the different time points (0, 1, 2, 7 h in black, orange, green and blue, respectively). The proportion of small oligomers decreases over time whilst that of the medium oligomers (apparent size of 5-15 monomers) increases with time. The fraction of large oligomers (apparent size of 16-50 monomers) also increases in the first 2 h and then remains relatively constant after 7 h. c) Percentage of oligomeric species over time. The percentage of oligomers reaches 0.8% after 1 h, 5.0% after 2 h, and 9.7% after 7 h. d) Average apparent size over time in approximate number of monomers. The oligomeric species show an average size of about 8 monomers, which is in accordance to previous studies [153, 154].
With the encouraging results from the initial TCCD experiments, we next investigated whether we could apply SM FRET to our system. Previously, SM FRET has been used to highlight structural differences within oligomeric populations and this can give insights into transitions which may be present during the aggregation pathway [42, 43]. The same protocol as for TCCD was followed with the exception that the red laser was now turned off. The results show an increase in the size and the FRET efficiency after 1-2 h into the aggregation process (Figure 3.8). An increase in the FRET efficiency corresponds to a more efficient energy transfer between the Alexa fluorophores, which suggests a more structured and tightly packed oligomer structure. This result shows that SM FRET can be used to study the early stages of the aggregation pathway for the HuL system and gain insight into the structural and mechanistic details that govern it.
Figure 3.8. SM FRET characterisation of Alexa-labelled I59T HuL oligomers formed in acidic conditions. 2D plots of FRET efficiencies against the apparent oligomeric size in number of monomers for 0 (a), 1 (b) and 2 h (c). The FRET efficiency is proportional to the rigidity of the aggregate structure, with a higher FRET efficiency being indicative of a more structured species. The colour scale represents the amount of species detected in a logarithmic scale. After 1 and 2 h, both size and FRET efficiency increase.
3.2.7. SM fluorescence characterisation of HuL oligomers using extrinsic dyes

Although a fantastic tool to monitor the oligomer populations present during fibril formation of a protein, and to gain insight about the mechanism of fibril formation, using labelled samples has limitations as the presence of the fluorophores may alter the kinetics of the aggregation and the structural properties of the oligomeric species [158, 159]. For this reason, recent studies have developed methods to detect label-free aggregates \textit{in vitro} and \textit{in vivo}, opening up a range of new applications, including as diagnosis tools for the early detection of aggregates in human biological fluids [134, 152]. In this section, we will explore the use of label-free SM microscopy for the detection of WT HuL oligomers, formed under the conditions described in Section 3.2.4, using pFTAA and ThT as extrinsic dyes (for more information on these dyes, see Section 1.5.5). During the early stages of aggregation, different time points were taken and diluted to 10 μM, where the dilution was considered as a function of initial monomer concentration. The dye, either pFTAA or ThT, was then added to each time point for a final concentration of 30 nM and 5 μM, respectively. The samples were injected into the microfluidic device to monitor the appearance of pFTAA or ThT fluorescence, which corresponds to their binding to aggregates. The number of fluorescent bursts and their intensities were measured over time. The fluorescent signal from ThT and pFTAA can be used to reveal structural information about the aggregates as the fluorescence intensity is proportional to oligomer size and β-sheet content. In addition, the number of bursts or events can provide insights into the mechanistic details of the aggregation [134]. When ThT is used as the extrinsic dye, the number of events is effectively zero at 0 h, which is expected as monomers are not ThT-active (Figure 3.9, a). The number of events increases after 0.5 h and then remains relatively constant until the 6 h time point. The total intensity also increases within the first 0.5 h and continues to do so until 6 h (Figure 3.9, b), in a similar way to the increase in ThT signal observed using conventional ThT fluorescence measurements in a plate reader over the same time-course (Figure 3.5, a). The average intensity increases rapidly until 3 h, after which it remains relatively constant until 6 h (Figure 3.9, c). The average intensity for each time point was calculated as the total intensity divided by the number of events and it correlates to the average size and β-sheet content present in the sample at any particular time point. This result suggests that either the size and/or the β-sheet content present in the sample increase over time until 3 h, after which they remain constant. Further analysis using fluorophore-labelled protein and monitoring the ratio of pFTAA or ThT intensity to that of the fluorophore over time would be needed to assess whether both size and β-sheet content increase, as the intensity of the
fluorophore is proportional to size and not to β-sheet content. Finally, the data from this experiment also allows the classification of the aggregates observed at each time point according to their intensity, to determine how the proportion of low to high intensity oligomers changes with time. The number of intensity segments was established at 5 and the intensity ranges were distributed evenly so the same number of intensities was considered for each intensity range. The selection of the intensity ranges was made by dividing the difference between the maximum and the minimum intensities by 5, and then including the same number of intensities in each intensity range. The number 5 was chosen to minimise the number of intensity ranges whilst still allowing for the detection of any changes in the distribution of the intensity measured over time. The fraction of events corresponding to the lowest intensity range decreases whilst the proportion of higher intensity species increases over time (Figure 3.9, d). This result shows that over time there is a higher proportion of high intensity aggregates and hence the population distribution shifts towards larger sizes and/or higher β-sheet content over time. In fact, whereas around 50% of the events after 0.5 h correspond to higher intensity species, these account for 60% after 6 h (Figure 3.9, d).
Optimisation of a SM microscopy set-up to characterise HuL oligomers

Figure 3.9. SM characterisation of WT HuL oligomers formed under acidic conditions using ThT as the extrinsic dye. a) Number of events over time. For time 0 h, effectively no events are observed. After 0.5 h, the number of events increases and then plateaus until 6 h. b) Change in total intensity over time. The total intensity increases steeply from 0 to 3 h, when it starts increasing at a slower rate. This corresponds well to the ThT signal observed in bulk (Figure 3.5, a). c) Normalised average intensity over time. The average intensity was calculated as the total intensity divided by the number of events for each time point. The average intensity increases over time until approximately 3 h, after which it remains relatively constant. The normalised data was calculated using unity-based normalisation, where each point is calculated as $(x-x_{\text{min}})/(x_{\text{max}}-x_{\text{min}})$ where $x$ is the measured raw data, and $x_{\text{min}}$ and $x_{\text{max}}$ are the minimum and maximum values of the raw data, respectively. d) Change in the fraction of events corresponding to different intensity ranges over time. The fraction of species that fluoresce at the lower intensity range (140-200 photon counts, in blue) decreases over time whilst that of higher intensity species (200-260, in red; 260-320, in orange; 320-380, in yellow; and 380-440 photon counts, in green) increases over time. The graphs show the mean and the error bars represent the standard deviation from three independent experiments performed on different days.

The data obtained when pFTAA is used as the extrinsic dye shows that the number of events increases after 0.5 h and then remains constant until approximately 6 h, when it increases again until the 9 h time point (Figure 3.10, a). The total intensity for each time point follows a similar trend to the number of events (Figure 3.10, b) and is also similar to the ThT signal in bulk.
(Figure 3.5, a). The average intensity was then calculated as described above and the results show a steep increase after 0.5 h, after which it remains constant until 6 h when it decreases until 9 h (Figure 3.10, c). The fluctuations in the data may be explained by the heterogeneity of the mixture, especially after 4-6 h when the oligomers and curvilinear fibrils undergo the mechanistic changes to evolve into rigid fibrils [153, 154]. Finally, the aggregates observed by pFTAA fluorescence were segmented according to their intensity (Figure 3.10, d). As described before for ThT, the intensities were segmented evenly into 5 ranges and then the fraction of events corresponding to each range was calculated and plotted against time (Figure 3.10, d). The fraction of high intensity oligomers increases within 0.5 h and then the distribution of the population remains constant until 9 h. These data suggest that oligomers are formed within 0.5 h, after which the population distribution appears to be constant until the 9 h time point.

Figure 3.10. SM characterisation of WT HuL oligomers formed under acidic conditions using pFTAA as the extrinsic dye. a) Number of events over time. The number of events increases after 0 h, remains relatively constant from 0.5 to 6 h and then appears to increase between 6 and 9 h. b) Change in total intensity over time. The total intensity increases steeply between 0 and 0.5 h, when it starts increasing at a slower rate until 9 h. c) Normalised average intensity over time. The average intensity increases steeply 0-0.5 h and then remains constant.
until 6 h, after which it appears to decrease. The normalised data was calculated using unity-based normalisation, where each point is calculated as \((x-x_{\text{min}})/(x_{\text{max}}-x_{\text{min}})\) where \(x\) is the measured raw data, and \(x_{\text{min}}\) and \(x_{\text{max}}\) are the minimum and maximum values of the raw data, respectively.

d) Change in the fraction of events corresponding to different intensity ranges over time. The fraction of species that fluoresce in the lower intensity range (50-110 photon counts, in blue) decreases whilst that of the higher intensity species increases within 0.5 h (110-170, in red; 170-230, in orange; 230-290, in yellow; and 290-350 photon counts, in green). The graphs show the mean and the error bars represent the standard deviation from three independent experiments performed on different days.

The results shown using ThT and pFTAA as extrinsic dyes in this section can be compared to the fluorescent signal shown in bulk (Figure 3.11). This comparison shows that using SM microscopy with ThT allows for a slightly earlier detection of species than using the conventional method in a plate reader (Figure 3.11, a). For pFTAA, no significant changes in the fluorescence can be observed using the plate reader whereas SM microscopy shows an increase in total intensity after 0.5 h (Figure 3.11, b). The plate reader data shown in Figure 3.11 (b) represents the mean of a set of 5 samples ran simultaneously; however, more repetitions are needed to further determine whether pFTAA fluorescence is detectable using the pH 2.0 conditions in the plate reader.
Figure 3.11. Comparison of ThT and pFTAA fluorescent signals as monitored by SM microscopy and in bulk measurements. a) Normalised ThT intensity as detected by SM microscopy (orange) and by conventional ThT fluorescence measured in a plate reader (black). The high ThT intensity detected at 0 h is normal for measurements in the plate reader as the signal equilibrates, and is frequently shown in the literature [35]. The normalised data was calculated using unity-based normalisation, where each point is calculated as \((x-x_{\text{min}})/(x_{\text{max}}-x_{\text{min}})\) where \(x\) is the measured raw data, and \(x_{\text{min}}\) and \(x_{\text{max}}\) are the minimum and maximum values of the raw data, respectively. b) pFTAA total intensity as detected by SM microscopy (orange) and in the plate reader (black). In the plate reader, no significant change in the pFTAA fluorescence is observed whereas SM detects species after 0.5 h. The raw data is shown for clarity. The data shown from SM is the mean and the error bars represent the standard deviation from three independent repeats. The data from the plate reader measurements is the average and the error bars represent the standard deviation from five samples run simultaneously.
The results obtained using ThT and pFTAA are not directly comparable, as it appears that each dye binds different species along the aggregation pathway. This is consistent with previous studies where pFTAA was shown to be less specific for β-sheet structure and able to bind to earlier species in the aggregation pathway [138, 139]. Although this may or may not be the case here, it is clear that both dyes indicate that there are oligomeric species present early in the aggregation pathway under these conditions. Therefore, it is clear that we can detect label-free human lysozyme oligomers at μM concentrations using the SM microscopy set-up, and with these positive results, we then looked to analyse the in situ fibril formation of the I59T variant.

3.3. Conclusion and next steps

In this chapter, the use of the SM microscopy set-up described in Section 1.5.4 was explored to detect HuL oligomers formed under oligomer-enriching conditions. While the Alexa dyes show no disturbance in their properties under these conditions (400 mM NaCl, pH 2.0), the native-state stabilities of WT and I59T HuL show an average destabilisation of 13.3 °C for I59T HuL and of 14.5 °C for WT HuL as detected by CD spectroscopy, which is comparable to other studies using acidic conditions to induce lysozyme fibril formation [74, 75, 156, 160]. A slight change in the secondary structure in the 210 nm region is also detected. The morphology of the fibrils formed under these conditions was studied by TEM, which shows that these are longer than those formed under pH 5.0 conditions. Next, the detection of the oligomeric species was achieved by DLS and AFM. With the presence of oligomers confirmed, we moved on to perform TCCD analysis using Alexa-labelled I59T HuL samples and working in the picomolar concentration range. Although TCCD proved challenging, oligomers were successfully detected at 25 pM after 1-7 h of aggregation. The apparent size of these oligomers increases over time and the average size was calculated to be approximately 8 monomers, which is in agreement with previous work under these conditions [153, 154]. Finally, the feasibility of SM FRET was explored and the results show an increase in size and FRET efficiency after 1-2 h. Next, we explored the use of label-free SM microscopy methods using two extrinsic dyes, ThT and pFTAA, to determine whether unlabelled HuL oligomers could be detected using the SM microscope after dilution to μM concentrations, and also to confirm the presence of oligomers, as detected by TCCD and SM FRET. Both ThT and pFTAA show the formation of oligomeric species after 0.5 h and the size and/or the β-sheet content of these
species appear to increase over time. With the SM microscopy set-up optimised to follow HuL oligomeric species, we wished to focus our attention towards our favoured fibril forming conditions (pH 5.0, 60 °C), as the stable oligomers used in this chapter have a complex aggregation pathway and are not physiologically relevant (due to high protein concentrations, low pH and high salt concentration). As our group has used pH 5.0, 60 °C to extensively study biomolecules which inhibit lysozyme fibril formation, understanding the aggregation under these conditions would provide us with vital mechanistic information. The characterisation of HuL oligomers formed under our favoured conditions will be investigated in Chapters 4 and 5.
Chapter 4: Characterisation of in situ formed HuL oligomers using SM fluorescence microscopy

4.1. Introduction

In Chapter 3 we determined the feasibility of using the SM microscopy set-up to detect HuL oligomers at both micromolar and picomolar concentrations using an oligomer-enriched sample. In this chapter, we will use this set-up to investigate the structural nature of in situ oligomeric species formed under more physiologically relevant conditions that have been used extensively in our research group. Due to the native-state stability of the lysozyme variants, it is extremely challenging to make fibrils on a reproducible timescale, in vitro, using conditions which lend themselves to the study of how biomolecules modulate fibril formation. Over the years, a number of solution conditions have been successful for the formation of HuL and HEWL amyloid fibrils; however, for HuL, they have required harsh conditions including low pH with high temperatures and stirring [156, 160], moderate pH with high temperatures and the presence of denaturants [73] and physiological pH with high temperature and seeding [156]. The aggregation of HEWL, on the other hand, has been reported at pH 2 with high temperatures and stirring [161, 162], and at neutral pH with ethanol [163, 164], where the aggregation is more readily achieved after the reduction of the disulphide bonds [163]. Nevertheless, the recent investigations with I59T HuL [69] demonstrated that, for this variant, we could form in vitro fibrils in less than 24 h using relatively low concentrations of protein (7 μM, i.e. 0.1 mg/mL), pH 5.0 and 60 °C with stirring. These conditions, although at an elevated temperature, still allow for the inhibitory properties of biomolecules, such as extracellular chaperones e.g. haptoglobin, α₂M, clusterin [69, 79] and camelid antibodies e.g. cAbHuL-5 (De Genst and co-workers, unpublished), to be studied.
4.1.1. Chapter aims

As introduced in Section 1.2.2, HuL oligomers formed prior to amyloid fibrils during aggregation, under our extensively used pH 5.0, 60 °C conditions, have not been fully characterised. Understanding the mechanistic details regarding the population of oligomeric species in the early stages of this aggregation pathway is imperative if we wish to be able to regulate or inhibit aggregation. It is also of great interest to investigate how the oligomer distribution during fibril formation of a globular protein compares to well-studied IDP systems. In this chapter, we will use the SM microscopy set-up optimised in Chapter 3 to not only identify the oligomeric populations present along the aggregation pathway but also to gain insights into the structure and population distribution of these species.

4.2. Results

In the previous chapter, we showed that HuL oligomers formed under stabilising acidic conditions can be detected using SM fluorescence microscopy. The optimised SM microscope was used in this chapter to detect oligomers formed under more physiologically relevant conditions (7 μM, pH 5.0, 60 °C). In order to do this, the aggregation of 7 μM Alexa-labelled I59T HuL (3.5 μM of each dye) in 0.1 M citrate buffer, pH 5.0 was followed by ThT fluorescence in a conventional fluorimeter (Figure 4.1), and the resulting fibrils were imaged by TEM (Figure 4.1, inset). Different time points were taken during the aggregation, put on ice to prevent oligomer dissociation, and injected into the microfluidic device. As observed in Figure 4.1, the lag phase lasts for 4-5 h, at which point, the ThT fluorescence increases until reaching a plateau that is observable from around 10 h. For this reason, the results of this chapter will focus on the first 12 h of fibril formation as the goal was to observe oligomers formed in the early stages of aggregation.
Characterisation of HuL oligomers using SM fluorescence microscopy

Figure 4.1. Fibril formation of Alexa-labelled I59T HuL as monitored by conventional fluorescence spectroscopy. Normalised ThT fluorescence of stoichiometric amounts of A488-I59T and A647-I59T HuL in 0.1 M citrate buffer (pH 5.0), incubated with stirring at 60 °C. The lag phase ends at 4-5 h and a plateau is reached after approximately 10 h. The normalised data was calculated using unity-based normalisation, where each point is calculated as \((x-x_{\text{min}})/(x_{\text{max}}-x_{\text{min}})\) where \(x\) is the measured raw data, and \(x_{\text{min}}\) and \(x_{\text{max}}\) are the minimum and maximum values of the raw data, respectively. Inset: TEM image of the fibrils formed after 24 h. The scale bar represents 100 nm.

4.2.1. Characterisation of HuL oligomers by conventional methods

Before moving on to using the Alexa-labelled I59T HuL to investigate the early stages of aggregation with SM techniques, we first looked to see if we could identify the presence of oligomeric species using conventional techniques, keeping in mind that the oligomer population was likely to be heterogeneous and present in very low concentrations. In order to probe for the presence of oligomers, we looked at the evolution of the structural conformation of the ensemble of species present. The fluorophores, ThT, pFTAA (described in Section 1.5.5) and 8-Anilino-1-naphthalenesulfonic acid (ANS) were used to monitor the change in \(\beta\)-sheet content (ThT and pFTAA) and in hydrophobicity (ANS) by measuring their fluorescence.
emission at different time points during the aggregation process. ANS is a dye whose emission intensity has been shown to be proportional to the amount of exposed hydrophobic surfaces present [135, 165], and changes in its binding capabilities have been correlated to changes in cytotoxicity [89]. Therefore, ANS fluorescence provides a great tool for the direct evidence of the presence of exposed hydrophobic surfaces and its change over the course of the aggregation. The fluorescence emission of ANS for time points 0, 2, 5, 9 and 24 h was recorded in triplicate from 400 to 600 nm, after excitation at 350 nm, and the fluorescence maxima at 475 nm were plotted over time (Figure 4.2, e). The time points were taken from a 7 μM I59T HuL aggregation reaction under pH 5.0, 60 °C conditions. The ANS fluorescence at 475 nm increases with time, which suggests that the amount of exposed hydrophobic surfaces increased after 2 h (Figure 4.2, e). The ThT fluorescence emission for the same time points is shown in Figure 4.2 (a) and (b). Similarly to ANS, ThT fluorescence shows a slight increase in emission between 0 and 5 h, followed by a significant increase after 9 h, which corresponds with fibril formation (Figure 4.1). These data suggest that there is an increase in the hydrophobicity and β-sheet content of the sample at the early time points of the aggregation reaction. This result is different to previous research on a globular system in which no ThT or ANS binding was detected for initial aggregates [166]. Detecting ANS fluorescence is, however, in agreement with the study of Bolognesi and co-workers, which showed a positive ANS signal during the lag phase of the aggregation reaction of I59T HuL, which also correlated with an increased loss in cell viability [89]. Interestingly, we see an increased ANS fluorescence for the fibrillar species, which was not observed in the previous study. An increase in ANS fluorescence was also observed previously for the early time points of WT HuL fibril formation at pH 3, 60 °C [167]. In the case of pFTAA, the fluorescence emission increased over time after 3.5 h (Figure 4.2, c and d). The double-peak shape observed for the pFTAA fluorescence at later time points is characteristic of its binding to oligomers and fibrils [138]. In order to investigate whether a change in the secondary structure of I59T HuL could be detected as the aggregation proceeded, CD spectroscopy was used to scan the same time points. In order to minimise the concentration needed for the readings, a longer path length was used which, together with the citrate buffer, meant it was not possible to gain accurate spectra for the time points 2-9 h. Although the shape of the curve is not changing much, the intensity of the minima appears to change over time (Figure 4.3, a). The signals at 222 and 208 nm correspond to the presence of α-helical content whilst 215 nm generally corresponds to that of β-sheet [146]. The shape of the curve would undergo significant change if a significant conformational change towards β-sheet or random coil content could be observed [168]. After 24 h, the secondary structure of the sample shifted
from α-helical to β-sheet content (Figure 4.3, b). Although much less pronounced, this subtle change in secondary structure shown by CD spectroscopy is in agreement with the conformational change observed in previous studies of the globular systems of insulin [169], Sulfolobus solfataricus acylphosphatase (Sso AcP) [166] and an antibody domain [170], where their early time points of the aggregation process showed a significant conformational change that involved a disturbance in the 204-220 nm range, but yet the structure observed was native-like. In addition, the transient intermediate populated by HuL, which we believe interacts through unfolded β-sheet regions to then form amyloid fibrils, may retain native-like structure within the α-domain [75]. The lack of an obvious significant conformational change compared to other studies by CD spectroscopy may be caused by a significantly smaller population of the early species than those yielded from other systems, or these species being more structurally similar to the native-state. Given the lowly populated nature of the putative oligomers, it is clear that further repeats of these investigations would be beneficial and that these data are merely suggestive and cannot be overly interpreted.
Figure 4.2. Conventional biophysical characterisation of HuL oligomers in solution, formed at pH 5.0, 60 °C (I). a), b), c) and d) Fluorescence emission of ThT (a,b) and pFTAA (c,d) for time points 0 (black), 2 (red), 3.5 (orange), 5 (green), 9 (cyan) and 24 h (violet). The scale of the emission of ThT and pFTAA has been enhanced in (b) and (d), respectively, to observe clearly the time points 0 (black), 2 (red), 3.5 (orange), and 5 h (green). The fluorescence emission increases over time for both ThT and pFTAA. e) The normalised ANS fluorescence emission at 475 nm after 0 (black), 2 (red), 5 (green), 9 (cyan) and 24 h (violet) increases over time. f) Comparison of the normalised data for ANS (blue), ThT (orange) and pFTAA (green). The normalised data was calculated using unity-based normalisation, where each point is calculated as \((x-x_{\text{min}})/(x_{\text{max}}-x_{\text{min}})\) where \(x\) is the measured raw data, and \(x_{\text{min}}\) and \(x_{\text{max}}\) are the minimum and maximum values of the raw data, respectively. The data for 0 and 24 h were used as \(x_{\text{min}}\) and \(x_{\text{max}}\), respectively. The data show the mean and the error bars represent the standard deviation from one set (ThT, pFTAA) and from two sets of triplicate measurements (ANS).
Characterisation of HuL oligomers using SM fluorescence microscopy

Figure 4.3. Conventional biophysical characterisation of HuL oligomers in solution, formed at pH 5.0, 60 °C (II). a) CD signal at time 0 (black), 2 (red), 3.5 (orange), 5 (green), and 9 h (cyan). The spectra show interference, most likely due to the use of a longer path length and citrate buffer. b) CD measurements for I59T HuL after 0 (black) and 24 h (violet) without interference. The spectrum after 24 h shows a minimum at 215 nm, which typically corresponds to the presence of β-sheet content. The data for the 0, 2, 3.5, 5, and 9 h time points in (a) and 0 h in (b) show the mean and the error bars represent the standard deviation from one set of triplicate measurements. The data for the 24 h time points represents a single measurement.

4.2.2. Characterisation of HuL oligomers using TCCD

In order to characterise the species present in the early stages of fibril formation, TCCD was used to follow the aggregation of the Alexa-labelled I59T at picomolar concentrations. The time points were taken from the fibril formation reaction, diluted to 100 pM, injected into the microfluidic device and then infused at a flow rate of 0.61 cm s⁻¹. The resulting data shows an increase in the total number of coincident events (which correspond to oligomers) over time (Figure 4.4, a). The detected species were divided into three different categories according to their apparent size: small (2-4 monomers), medium (5-20 monomers) and large (21-100 monomers). The fraction of events corresponding to the medium and large oligomers increased over time, showing a significant fraction of large species (5-10%) from 9 h onwards (Figure 4.4, b). The percentage of oligomeric species in the sample was calculated as described by equation 1.1 in Section 1.5.2 and multiplied by 100 for each time point, and it shows an increase over time until reaching a maximum of 0.7-0.8% after 13 h (Figure 4.4, c). This result is significant because it means that for every 100 monomers, there is less than one oligomer molecule and hence the concentration of the oligomers will be, in principle, at least 100-fold lower than that of the initial monomer concentration. Finally, the average size, given as the
approximate apparent size in monomers, was calculated and showed an increase only after 9 h of aggregation (Figure 4.4, d). This average size is not accurate, due to the many different paths a molecule can take through the confocal volume and the stochasticity of the photoswitching of single fluorophores [171], but it gives further insight into how the average size of the species present in the sample changes with time as well as providing a comparison to the size histograms shown in Figure 4.4 (a).

**Figure 4.4. Characterisation of I59T HuL oligomers as followed by TCCD.** a) The number of coincident events increases with time. The species were divided into different apparent size categories where small (2-4 monomers), medium (5-20 monomers), and large oligomers (21-100 monomers) are shown in blue, orange and green, respectively. The number of medium oligomers increases with time whilst large oligomers are detected from 9 h onwards. b) The fraction of medium species increases over time, accounting for 40% of events after 4 h (c.f. 20% after 1 h). The fraction of large oligomers increases after 9 h, reaching a maximum of 10% after 11 h. c) The percentage of oligomers present in the sample increases with time. d) The average size increases after 9 h. This estimation is not accurate due to the many different paths a molecule can take through the confocal volume and the stochasticity of the photoswitching of single fluorophores.
From these results, it can be concluded that HuL oligomers formed in the pH 5.0, 60 °C conditions are detectable at 100 pM by SM microscopy, which is in itself a significant result as it means these oligomers are stable after dilution to picomolar concentrations. In addition, an increase in the number and size of the oligomers is reported. These results encouraged us to monitor the aggregation using SM FRET to gain more insights into the oligomer structures.

4.2.3. Characterisation of HuL oligomers using SM FRET

As described in Section 1.5.3, for FRET to occur, the donor and acceptor fluorophores must be within 10 nm distance. For this reason, unlike for TCCD, there is no chance coincidence and thus, SM FRET allows for higher concentrations to be used. For this reason, the different time points from the Alexa-labelled I59T HuL fibril formation were diluted to 750 pM for the rest of the SM experiments reported in the remaining chapters. As described in Section 1.5.3, SM FRET involves only the use of the blue laser whose excitation wavelength, 488 nm, corresponds to that of the donor (A488). The data obtained from this technique provides insight about the number and apparent oligomer size as well as the oligomer structure (related to the FRET efficiency). A more structured, compact oligomer will generally show a higher FRET efficiency. Different time points were taken from a 7 μM Alexa-labelled I59T HuL aggregation reaction, diluted to 750 pM and injected into the microfluidic device. The FRET efficiency given by the data is shown in a 2D plot with a colour scale to represent the number of oligomers, in a logarithmic scale, over time (Figure 4.5). These data show that the size and FRET efficiency as well as the total number of events detected increase with time. After 2 h, the 2D plot shows that the FRET efficiencies observed for the species of apparent sizes smaller and larger than 10 monomers are clearly distinct (Figure 4.5, b). The region of 2-10 monomers shows a broad range of FRET efficiencies that span from approximately 0.2 to 0.6, whereas the species larger than 10 monomers are detected at FRET efficiencies exclusively in the range of 0.6-0.7. These two distinct regions are observed for every time point until 9 h, when the lower FRET efficiency region virtually disappears (Figure 4.5, e), making the small oligomers almost indistinguishable from the large oligomers in terms of their FRET efficiency after 9-12 h (Figure 4.5, f). This result suggests that an oligomer may only be able to reach a certain size in a highly structured manner. Two main conclusions can then be drawn from these data: first, the oligomeric population can be divided in two distinct populations, small (apparent size of 2-10 monomers per oligomer) and large (10-150 monomers). Anything bigger than 150 monomers was ignored for this analysis as only pre-fibrillar species are of interest in this study.
This division also considers the observed FRET efficiency, as small oligomers show both low- and high-FRET and large oligomers only show high-FRET. Secondly, the population of small low-FRET oligomers appears to decrease with time until virtually disappearing after 12 h, when only high-FRET species are detected. The reason for this decrease may be explained either by these oligomers dissociating back to monomers, or by undergoing a conformational change to then convert into high-FRET oligomers. In order to find out more about these oligomer populations, the numbers of small and large oligomers were plotted against their FRET efficiencies (Figure 4.6). For small oligomers, there are two clearly distinct populations as per their FRET efficiencies, one population centred at a FRET efficiency of 0.2-0.3 and the second one centred at 0.6-0.8 (Figure 4.6, a). The number of low-FRET oligomers is highest after 2 h whereas the number of high-FRET oligomers is highest after 9 h (Figure 4.6, a). The time points between 2 and 9 h show a wider FRET efficiency distribution, which may be explained as the sum of two populations or as one population centred at approximately 0.5. Nevertheless, given the data for the earlier and later time points, the distribution observed for time points 3-8 h is more likely explained by the presence of two populations, where the low-FRET and high-FRET populations are those predominantly detected after 2 h and 9 h, respectively. The number of large oligomers increases with time and only one peak at a FRET efficiency of 0.6-0.8 is observed (Figure 4.6, b).
Figure 4.5. SM FRET 2D plots showing FRET efficiencies and oligomer size. The apparent size of the oligomers (in number of monomers) is plotted against their FRET efficiency for different time points of the aggregation of Alexa-labelled I59T HuL. Both the size and FRET efficiency increase over time. a) 0 h. b) 2 h. c) 5 h. d) 7 h. e) 9 h. f) 12 h.
Figure 4.6. Evolution of the distribution of I59T HuL oligomer populations by counts and FRET efficiency. The oligomers were separated into two different size populations: a) small (apparent size of 2-10 monomers), and b) large oligomers (apparent size of 10-150 monomers). The size classification for these two oligomer populations was chosen to have the minimum number of unique oligomer populations whose FRET histograms showed the minimum number of Gaussian distributions as detailed in reference [42]. For small oligomers, a shift towards higher FRET efficiencies is observed over time. The data for time points 2, 5, 6, 7, 9, and 12 h are shown in red, orange, yellow, green, cyan, and violet, respectively. The normalised data is shown to help visualise the shift in FRET efficiency. The data show the mean and the error bars represent the standard deviation from three independent experiments. The normalised data was calculated using unity-based normalisation, where each point is calculated as $(x-x_{\min})/(x_{\max}-x_{\min})$ where $x$ is the measured raw data, and $x_{\min}$ and $x_{\max}$ are the minimum and
maximum values of the raw data, respectively. b) Only one peak is observed for large oligomers (FRET efficiency equal to 0.6-0.8). The data for time-points 0, 2, 5, 6, 7, 9, and 12 h are shown in black, red, orange, yellow, green, cyan, and violet, respectively. The number of large oligomers increases with time.

Next, the population distributions for small oligomers were fitted to a two peak Gaussian in OriginPro 9.1, using the expression

$$y = y_0 + \frac{A e^{-\frac{(x-x_c)^2}{2w_1^2}}}{w_1 \times \text{sqrt} \left( \frac{\pi}{2} \right)} + \frac{(1-A) e^{-\frac{(x-x_c)^2}{2w_2^2}}}{w_2 \times \text{sqrt} \left( \frac{\pi}{2} \right)} \quad \text{(Eq. 4.1)}$$

where $y_0$ is the base of the curve, A is the amplitude, and $x_c$ and w are the centre and width for each of the distributions, respectively. Prior to this fitting to monitor the evolution of each of the oligomer populations over time, both the centre and width for each population were identified for all incubation times using the sum of two Gaussian distribution curves in OriginPro 9.1. The representative centres and widths were chosen from time point 4 h (Figure 4.7), as the population distributions at this time were representative enough for those detected at every time point, and the parameters were calculated to be $0.34 \pm 0.01$ and $0.60 \pm 0.01$ for the centres, and $0.217 \pm 0.012$ and $0.202 \pm 0.014$ for the widths for the low-FRET and high-FRET populations, respectively. The centres and widths were then kept fixed for the fitting of each time point in order to limit each population and accurately monitor their evolution over time. These parameters have physical meaning, as about 68% of oligomers of said populations will fall within the mean (centre) ± the standard deviation (width) of the distribution. The fittings for different time points have been shown in Figure 4.8. The colours used in this figure correspond to the colours in Figure 4.6 (a) for the same time points. It can be concluded from the data that low-FRET oligomers were formed first within 2 h of aggregation. As this population shifts towards higher FRET efficiencies over time, the low-FRET population decreases until disappearing completely after 12 h, when the fitting is unviable as the low-FRET population was not detected (Figure 4.8, f). This data further indicates that the low-FRET oligomers may be experiencing a conformational transformation to form the more structured high-FRET population during the aggregation.
Figure 4.7. Normalised number of oligomers as a function of FRET efficiency for time point 4 h. The data were fitted to a sum of two Gaussian curves (black line) from which the centres of each population were calculated to be $0.34 \pm 0.217$ (green line) and $0.60 \pm 0.202$ (red line). The data show the mean and the error bars represent the standard deviation from three independent experiments.
Figure 4.8. Evolution of small oligomers over time. a) 2 h (red). b) 5 h (orange). c) 6 h (yellow). d) 7 h (green). e) 9 h (cyan). f) 12 h (violet). The distribution of the population after 2 h is centred at a FRET efficiency of approximately 0.3, which then shifts towards higher FRET efficiencies until 9 h, when it is centred at approximately 0.6 (e). The data show the mean and the error bars represent the standard deviation from three independent experiments. The fittings represent the sum of two Gaussian curves (black) of which one corresponds to low-FRET oligomers (green line, centre fixed at 0.34 ± 0.217 for all time points) and the other one corresponds to high-FRET oligomers (red line, centre fixed at 0.60 ± 0.202 for all time points).
The process just described above allows for the monitoring of multiple populations, and the amplitude of the resulting Gaussian curves can be used to calculate the fraction of small low-FRET and high-FRET oligomers using the expression

\[ p_i = \frac{A_i}{A_L + A_H} \]  
(Eq. 4.2)

where \( p_i \) is the proportion or fraction of the population of interest (small low-FRET or high-FRET oligomers); \( A_i \) is the amplitude of the Gaussian distribution for the population of interest (small low-FRET or high-FRET oligomers); and \( A_L \) and \( A_H \) are the amplitudes of the low-FRET and high-FRET Gaussian distributions, respectively.

The fraction of small low-FRET oligomers was calculated using Eq. 4.2 and it decreases over time, and it was calculated to be approximately 90% after 2 h and 0% after 11 h, which in turn means that the high-FRET population increased from being lower than 10% after 2 h to 100% after 11 h (Figure 4.9, c). After 5 h, the fraction of high-FRET oligomers exceeds that of low-FRET for the first time. The fraction of the populations remains constant at approximately 50% after 5-7 h of aggregation (Figure 4.9, c). These proportions may be related to the rates of formation and depletion of both populations, as the low-FRET oligomers either dissociate into monomers or convert into high-FRET oligomers and the latter elongate in turn to form larger oligomers and eventually fibrils. The number of small low-FRET and high-FRET oligomers can then be calculated by simply multiplying each of the calculated fractions by the total number of small oligomers (Figure 4.9, a). The number of small low-FRET oligomers increases during the first 2 h of aggregation and then decreases until disappearing after 12 h, whereas the number of small high-FRET oligomers increases over the course of the aggregation (Figure 4.9, a). On the other hand, the population of large high-FRET oligomers increases over time, whilst the number of large low-FRET oligomers remains effectively zero throughout the aggregation reaction (Figure 4.9, b). In order to follow the shift of the oligomer population over time, the population distribution for each time point was fitted to one-peak and two-peak Gaussian curves, without putting any constraints on the centre or width of each curve. The one-peak fitting shows a shift towards higher FRET efficiencies over time, which correlates to the detection of more structured and compact species (Figure 4.9, d). The two-peak fitting shows two relatively constant populations that were clearly distinct, as per their FRET efficiencies, showing one population being centered at a FRET efficiency of 0.3-0.35, with another at

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approximately 0.6 (Figure 4.9, e). As the low-FRET oligomers completely disappear after 11-12 h, the two-peak fitting becomes unsuitable (as only one peak is observed) and hence the fitting shows a large error for time points 11-12 h (Figure 4.9, e). As mentioned only descriptively for Figure 4.6, after quantitative analysis the two-peak Gaussian curve fittings describes the data more accurately as it accounts for the change in the oligomer populations as it shifts from populating mostly the low-FRET species after 2 h to populating mostly the high-FRET species after 9 h, whereas a one-peak Gaussian curve cannot describe this shift (Figure 4.9). From this analysis, it can be concluded that as the small low-FRET oligomer population decreases, the small high-FRET oligomer population increases and therefore, we hypothesise that this result is most likely explained by the conversion of small low-FRET to high-FRET oligomers over time.
Figure 4.9. Evolution of low-FRET and high-FRET populations over time. a) Number of small low-FRET (green) and high-FRET (red) oligomers over time. The number of low-FRET oligomers increases for the first 2 h and then decreases over time, whereas the number of high-FRET oligomers increases over time. b) Number of large low-FRET (green) and high-FRET (red) oligomers over time. No large low-FRET oligomers were detected over the course of the aggregation, and the number of large high-FRET oligomers increases over time. c) Fraction of small low-FRET and high-FRET oligomer population over time. The fraction of low-FRET oligomers (green) decreases over time whereas the fraction of high-FRET oligomers (red) increases over time. After 5 h, the high-FRET population exceeds the low-FRET one. Both populations remain at approximately 50% from 5-7 h. d, e) Change in FRET efficiency over the course of aggregation. The oligomer distribution for each time point was fit to a one-peak
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(d) and two-peak (e) Gaussian curve and the value of the peak was then plotted over time. The FRET efficiency of the population increases over time for the one-peak fitting, whereas for the two-peak fitting the low-FRET and high-FRET populations remain relatively constant and centred at 0.3-0.35 and 0.6, respectively. The error at 11-12 h for the two-peak fitting can be explained by the existence of only one peak and hence making this fitting unsuitable for these two time points. The data show the mean and the error bars represent the standard deviation from three independent experiments.

4.2.4. Fitting SM FRET data to a kinetic model

In order to further understand the mechanistic details of HuL fibril formation, the data obtained using SM FRET in the section above were modelled and used to calculate different kinetic parameters. For this purpose, the numbers of oligomers of the three populations identified in the previous section (small low-FRET and high-FRET, and large high-FRET) were converted into concentrations. To do this, the fraction of oligomers was calculated as the number of oligomers divided by the average monomer count for the first 5 h of aggregation, when fibrils have not yet formed and hence monomer depletion can be ignored (Figure 4.1). The monomer count was calculated by multiplying the donor count by 2, as only the monomers labelled with A488 are visible in SM FRET experiments. The fraction of oligomers was then multiplied by the initial monomer concentration (7 μM) to obtain an estimate of the oligomer concentration. One of the limitations of this calculation is that the number of events, for both monomers and oligomers, is dependent on the intensity threshold applied on the analysis of the SM data. Nevertheless, this method to calculate oligomer concentration from SM microscopy data is widely applied [29, 40, 43], and the calculated concentrations are in agreement with the range of oligomer concentrations reported in the literature for other protein systems, when initial monomer concentrations in the low μM range were used [29]. The concentrations of the three different populations were then plotted against time (Figure 4.10), which shows that small and large high-FRET oligomers show the same kinetic behaviour, and therefore they will be considered as one oligomer type for the kinetic modelling in this section (Figure 4.11).
Figure 4.10. Oligomer concentration calculated from SM FRET data over time. The concentration of the small low-FRET oligomers is shown in green, and small and large high-FRET oligomers in red and orange, respectively. Both small and large high-FRET species show the same kinetic behaviour over time. The data show the mean and the error bars represent the standard deviation from three independent experiments.

The data were then fitted to a simple kinetic model, similar to that shown in references [29, 42-44] (Figure 4.11). From the ThT experiments performed in a fluorimeter under the same reaction conditions as the SM FRET experiments (Figure 4.1), we know that HuL monomer is not significantly depleted until after 5 h; therefore, we can utilise an early-time kinetic model, in which the monomer concentration is constant, up until this time point. This is advantageous since we do not know the mechanism by which monomer is depleted, and thus cannot devise a kinetic model that includes this phenomenon. Writing L(t) for the concentration of the low-FRET species, and H(t) for the high-FRET species, the rate equations under constant monomer conditions are

\[
\frac{dL}{dt} = \alpha - (k_c + k_d) L(t) \quad \text{(Eq. 4.3)}
\]
\[
\frac{dH}{dt} = k_c L(t) \quad (\text{Eq. 4.4})
\]

where \( \alpha \) is the rate of formation of low-FRET oligomers from monomer, and likely depends on monomer concentration as \( \alpha = k_{\text{oligo}} m(0)^2 \), \( k_c L(t) \) is the rate of formation of high-FRET oligomers from low-FRET oligomers, and \( k_d \) is the rate constant of the dissociation of low-FRET oligomers. Note that \( k_c \) is a pseudo-rate constant that may in fact have monomer dependence, but this cannot be determined under constant monomer conditions. Therefore, we cannot say for certain that the formation of high-FRET from low-FRET oligomers is a true unimolecular conversion reaction.

No depletion processes for high-FRET oligomers are included in this model because there is no evidence of high-FRET oligomer depletion: even after 5 h, when both monomer and low-FRET oligomer concentrations are decreasing, there is no decrease in high-FRET oligomer concentration. Moreover, the delayed appearance of high-FRET oligomers is evidence that they form from low-FRET oligomers and not directly from monomers. The model equations can be solved exactly analytically, yielding

\[
L(t) = \frac{\alpha}{k_c} \left( 1 - e^{-k_c t} \right) \quad (\text{Eq. 4.5})
\]

\[
H(t) = \frac{\alpha k_c}{k_c^2} \left( k_c t + e^{-k_c t} - 1 \right) \quad (\text{Eq. 4.6})
\]

where \( k_c = k_c + k_d \). This model fits the data reasonably well given the level of experimental variation, and yields the parameter values \( \alpha = 0.6415 \) nM h\(^{-1} \), \( k_c = 1.1209 \) h\(^{-1} \), and \( k_c = 0.1858 \) h\(^{-1} \). We see that although the model is only fitted to the data up to \( t = 5 \) h, these parameters are also consistent with the observed behaviour after \( t = 5 \) h. Namely, a relatively rapid equilibration of monomers and low-FRET oligomers, shown by the relatively high \( \alpha \) and \( k_d \), explain the relatively rapid drop-off in low-FRET oligomer concentration after \( t = 5 \) h, when monomer is depleted. The reduction in \( L(t) \) after \( t = 5 \) h is also consistent with a decrease in the rate of \( H(t) \) formation. A notable result of the modelling is that \( k_d \gg k_c \), i.e. low-FRET oligomers are approximately 5 times more likely to dissociate than to convert into high-FRET species. This is different to the results shown in references [29, 42-44], in which \( \alpha \)S data fit
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well to a model with no dissociation. The relatively rapid plateauing and decline of L(t) here cannot be explained by such a model, in which all L(t) loss is due to conversion to H(t). However, this result is, in fact, in line with all studies of oligomers formed by non-αS proteins [30, 31]. We thus have a distinction between unstable disordered low-FRET oligomers and later-forming, more structured and more stable high-FRET oligomers.

Figure 4.11. Kinetic traces for the different oligomer populations over time. Low-FRET species are shown in green and high-FRET species, in red. The data was fitted to an early-time kinetic model, over the first 5 h, when the monomer concentration is constant and there is no fibril formation (in accordance to ThT fluorescence in Figure 4.1). The data show the mean and the error bars represent the standard deviation from three independent experiments.

4.2.5. SM fluorescence characterisation of HuL oligomers using extrinsic dyes

Following the study of Alexa-labelled I59T oligomers, we wished to explore the use of label-free SM microscopy, for the reasons described in Section 3.2.7, using ThT and pFTAA as the extrinsic dyes to detect label-free I59T HuL oligomers at µM concentrations. As described in Section 1.5.5, whilst ThT is the dye most commonly used to monitor protein aggregation, pFTAA has been shown to bind aggregates earlier than ThT in the aggregation process of
amyloid-β [138]. Thus, we decided to use both dyes as the extrinsic dyes, for comparison. The samples taken at different time points during fibril formation of I59T HuL were diluted to 1 μM and then mixed with either pFTAA or ThT to a final dye concentration of 30 nM and 0.5 μM, respectively. The mixture was then injected into the microfluidic device, and infused at a flow rate of 0.61 cm s⁻¹. The data obtained from the experiments using pFTAA as the extrinsic dye shows an increase in the total number of pFTAA-active species over the first 8 h of aggregation (Figure 4.12, a). To further analyse the population distribution, the number of species was divided evenly into 5 different intensity ranges, and the results show that all intensity ranges increase in numbers with time (Figure 4.12, a). The selection of the intensity ranges was made by dividing the difference between the maximum and the minimum intensities by 5, and then including the same number of intensities per intensity range. The number 5 was chosen to minimise the number of intensity ranges whilst still allowing for the detection of any changes in the distribution of the intensity measured over time. The fraction of high intensity species increases with time, accounting for 40-50% of the detected species from 2 h onwards (Figure 4.12, b). The total intensity also shows an increase over time (Figure 4.12, c). The total intensity is the sum of the intensities of all the pFTAA fluorescent bursts detected over the course of the measurement. The average intensity is calculated as the total intensity divided by the number of species and hence, it is the increase in the number of events and the total intensity that leads to the increase in average intensity within the first 2 h (Figure 4.12, d). This data shows that pFTAA can be used to detect label-free oligomers, formed under physiologically relevant conditions, present at μM concentrations (assuming the dilution as a function of the initial monomer concentration). In addition, the increase in the intensity of individual events can result from either an increase in size or an increase in β-sheet content. The cause of this increase could be discerned by further analysis using fluorophore-labelled protein and monitoring the ratio of pFTAA intensity to that of the fluorophore over time, as the intensity of the fluorophore is proportional to size and not to β-sheet content.
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Figure 4.12. SM characterisation of I59T HuL oligomers using pFTAA as an extrinsic dye. a) The number of total pFTAA-active species increases with time. The number of species was classified into different intensity ranges with blue being 50-110 photon counts; red, 110-170; orange, 170-230; yellow, 230-290; and green, 290-350. The number of species in all intensity ranges increases over time. b) The fraction of higher intensity oligomers increases over time. c) The total intensity increases over time. d) The normalised average intensity appears to increase after the first 2 h, and it was calculated using unity-based normalisation, where each point is calculated as \((x-x_{\text{min}})/(x_{\text{max}}-x_{\text{min}})\) where \(x\) is the measured raw data, and \(x_{\text{min}}\) and \(x_{\text{max}}\) are the minimum and maximum values of the raw data, respectively.

When ThT was used as the extrinsic dye, the resulting data shows an increase in the number of ThT-active species with time (Figure 4.13, a), albeit the increase appears to be less smooth than the data obtained for pFTAA (Figure 4.12, a). Nevertheless, ThT shows a clear difference before and after 6 h, which correlates with the ThT fluorescence shown in the conventional fluorimeter assay (Figure 4.1). After 1 h, the number of ThT-active species deviates from that of the time points directly before and after and this is likely due to an error in the measurement and not related to the sample after 1 h being significantly different. However, these experiments would need to be repeated for further confirmation. To analyse the population distribution further, the ThT-active species were divided evenly into 5 intensity ranges, which were chosen
as described above (Figure 4.13, a and b). Although an increase in the number of high intensity species is observed (Figure 4.13, a), there appears to be no trend for the fraction of these species over time (Figure 4.13, b). This may be explained by a similar rate of formation of the ThT-active species regardless of their detected intensity. The total intensity remains relatively constant until approximately 6 h when it appears to increase (Figure 4.13, c). The average intensity did not show any distinct trend over the course of aggregation (Figure 4.13, d), which may be explained by the fluctuations in the total intensity data as well as in the total number of species detected, as the average intensity is calculated as the division of the two. Nevertheless, this data shows that ThT, like pFTAA, can be used to detect label-free HuL oligomers formed under physiologically relevant conditions using SM microscopy when diluted to a protein concentration of 1 μM.

Figure 4.13. SM characterisation of I59T HuL oligomers using ThT as an extrinsic dye.

a) The number of total ThT-active species appears to be constant until 6 h, after which it increases with time. The number of species was evenly divided into intensity ranges with blue being 80-130 photon counts; red, 130-180; orange, 180-230; yellow, 230-280; and green, 280-330. The number of species in all intensity ranges increases after 6 h. b) The fraction of higher intensity species does not show a clear trend over time, which may be explained by the rate of
formation of the ThT-active species being similar regardless of their intensity. c) The total intensity increases after 6 h. d) The normalised average intensity does not show a clear trend. The normalised average intensity was calculated using unity-based normalisation, where each point is calculated as 
\[(x-x_{\text{min}})/(x_{\text{max}}-x_{\text{min}})\] where \(x\) is the measured raw data, and \(x_{\text{min}}\) and \(x_{\text{max}}\) are the minimum and maximum values of the raw data, respectively.

The results shown in this section can be compared to the fluorescent signal shown by conventional fluorescence spectroscopy (Figure 4.14). The total intensity observed for ThT using SM microscopy follows the same trend as that measured in bulk with a fluorimeter. The fluorescence of pFTAA measured in bulk with a fluorimeter shows a steep increase after 1 h, whereas the increase in the total intensity observed using SM is gradual. This recording of pFTAA fluorescence in bulk has only been done once and therefore needs repeating. Nevertheless, the results shown in this section confirm that using extrinsic dyes to monitor fibril formation by SM microscopy, unlike the ensemble measurements performed by conventional methods, allows for more insight to be gained about the species formed during the aggregation reaction, as it reports on the number of dye-active species and their intensity distributions over time as shown previously in reference [134].
Figure 4.14. Comparison of ThT and pFTAA fluorescent signals as monitored by SM microscopy and fluorescence spectroscopy. a) Normalised ThT intensity as detected by SM microscopy (orange) and by conventional fluorescence spectroscopy (black). b) Normalised pFTAA intensity as detected by SM microscopy (orange) and by conventional fluorescence spectroscopy (black). The normalised data was calculated using unity-based normalisation, where each point is calculated as $(x - x_{\text{min}})/(x_{\text{max}} - x_{\text{min}})$, where $x$ is the measured raw data, and $x_{\text{min}}$ and $x_{\text{max}}$ are the minimum and maximum values of the raw data, respectively.
4.3. Conclusion and next steps

In this chapter we explored the characterisation of HuL oligomers formed in pH 5.0, 60 °C conditions, the more physiologically relevant conditions already widely used in our group and reported in the literature [67, 69, 145]. The characterisation of the early species present in the aggregation process was explored using conventional techniques (ANS, ThT, and pFTAA fluorescence, and CD spectroscopy). The results show positive ANS, ThT and pFTAA signals after 2 h (ANS and ThT) and 3.5 h (pFTAA). The data suggests that exposed hydrophobic surfaces are present as early as 2 h into the aggregation and that these species possess enough β-sheet structure to significantly bind with ThT. The CD data shows interference for time points 2, 5 and 9 h, which may be caused by the use of a longer path length and citrate buffer. Nevertheless, the secondary structures recorded for the different time points seem to remain very much native-like, similar to that observed for other globular systems [166, 169, 170].

Next, TCCD was used to investigate the oligomer population at picomolar concentrations. The data at 100 pM shows that both the number of oligomers and their size increase with time. The percentage of oligomers increases up to a maximum of 0.7-0.8% after 13 h. This low percentage of oligomeric species throughout the aggregation reaction may explain why the structural changes observed by CD spectroscopy were subtle and hard to discern. Following the results from TCCD, the structural properties of the oligomers were further explored using SM FRET. The results from this technique confirm that oligomers are formed within 1-2 h. The oligomer population was segmented by apparent size into small (2-10 monomers) and large (10-150 monomers). The data shows two types of small oligomers as per their FRET efficiencies (low-FRET and high-FRET population distributions). The low-FRET population distribution is centred at a FRET efficiency of 0.2-0.3, and its population increases within the first 2 h of aggregation and then decreases until disappearing completely after 11-12 h. The high-FRET population distribution is centred at a FRET efficiency of 0.5-0.7, and its population, unlike that of small low-FRET oligomers, remains present after 12 h. The population of large oligomers increases over time, and it shows a FRET efficiency distribution centred at 0.6-0.7 which suggests that these species are highly structured and compact. The earlier formation of small low-FRET oligomers may be explained by hydrophobic interactions forming more readily than β-sheet structure, in which hydrogen bond interactions take longer to form. In addition, the lack of large low-FRET oligomers suggests that a conformational change from
low-FRET to high-FRET is needed for the oligomers to be growth-competent. This type of prerequisite has been reported before for tau oligomers [33]. Finally, the data from SM FRET were fitted to an early-time kinetic model where the rate of formation of low-FRET oligomers (α), the pseudo-rate constant of formation of high-FRET oligomers from low-FRET oligomers (k_e), and the rate constant of dissociation of low-FRET oligomers (k_d) were calculated to be α = 0.6415 nM h⁻¹, k_e = 1.1209 h⁻¹, and k_d = 0.1858 h⁻¹. Interestingly, this indicates that low-FRET oligomers are approximately 5 times more likely to dissociate than to continue along the aggregation pathway to form high-FRET oligomers.

Once Alexa-labelled I59T HuL oligomers were confirmed to be detectable using the SM microscopy set-up, we used label-free SM microscopy methods involving the fluorophores ThT and pFTAA to determine whether the label-free oligomers could be detected upon dilution for SM analysis (in this case, to μM concentrations). Both ThT and pFTAA show the presence of oligomers at 1 μM (calculated as initial monomer concentration) which implies that the oligomers are stable enough after dilution. In addition, the data from the experiments with pFTAA shows that both the number and the size and/or the β-sheet content of the oligomers increase over time, whereas the data from the experiments with ThT shows that the number of species increases significantly only after 6 h. These results suggest that pFTAA may be reporting on the early-formed small oligomers whereas ThT may be reporting only on the large oligomers formed later in the aggregation reaction. Further repetitions would be needed as well as, as described before, further analysis using fluorophore-labelled protein and monitoring the ratio of pFTAA or ThT intensity to that of the fluorophore over time may make it possible to discern whether the increase in intensity observed is due to larger oligomer sizes or higher β-sheet content. This label-free method has recently been used to estimate the fragmentation rate of αS aggregates, a key process behind the multiplication of pathological aggregates [134], and therefore further analysis could involve the kinetic fitting of ThT and pFTAA data for I59T HuL.

In order to further explore the relevance and roles of these different oligomer populations in the HuL fibril formation, the next steps will include the study of their interaction with different biomolecules, and this will be explored in Chapter 5. In addition, different cell assays could be used to investigate the cytotoxicity of these oligomer populations and whether any differences can be observed on cellular toxicity.
Chapter 5: Characterisation of the interactions of HuL oligomers with the extracellular chaperone clusterin using SM fluorescence microscopy

5.1. Introduction

Understanding the role of different biomolecules on protein homeostasis is crucial if we want to be able to control and prevent the aggregation of proteins in the body. In order to do so, it is vital to further understand the mechanism of interactions that these biomolecules have with different species along the aggregation pathway.

5.1.1. Extracellular chaperones and their role in disease

Chaperones are proteins that help regulate protein homeostasis inside or outside the cell by assisting with the refolding of misfolded proteins to their native-state [172]. Chaperones can be found inside the cellular space e.g. chaperonins [173, 174], heat shock protein 90 (Hsp90) [175], and heat shock protein 70 (Hsp70) [176], and also in the extracellular space e.g. clusterin [69, 177], and α2M [95, 178]. Although both types of chaperones have a great variety of functions inside and outside of the cell, it is their capacity to inhibit or regulate protein aggregation that is most interesting in the context of this thesis. As described before in Section 1.3, it is currently thought that oligomers are the most toxic aggregate species towards cells [18], and that this is probably due to their exposed hydrophobicity [87, 89-91]. It is also currently believed that extracellular chaperones regulate aggregation by interacting with pre-fibrillar species early in the aggregation process [69, 79] and they do this, most likely, through hydrophobic interactions with the same exposed amino acid residues of the oligomers that are thought to be responsible for their toxicity [179]. Extracellular chaperones like clusterin, α2M and haptoglobin have been found to reduce the cytotoxicity of these species to cells [92, 94, 180]. A study using SM fluorescence also reported that clusterin forms soluble, stable
complexes with the aggregating protein implicated in Alzheimer’s disease, amyloid-β, and in doing so it prevents fibril formation [90]. Another study found that clusterin and α2M induce oligomers to form larger species, thereby reducing the overall toxicity of the oligomers [92]. Although these two chaperones have also been found to reduce the toxicity of amyloid-β oligomers [181, 182], other research reported an increase in toxicity when they are present during amyloid-β aggregation [93, 183]. Given this evidence, it is believed that the effect of extracellular chaperones in aggregation can be dependent on the condition details, such as the ratio of chaperone to protein of interest [179].

5.1.2. Clusterin and its role as an extracellular chaperone

As introduced in the previous section, clusterin is an extracellular chaperone present in blood plasma and cerebrospinal fluid in the human body [184]. Clusterin has a variety of functions including protease inhibition [185] and regulation of lipid transport [186], and it has also been shown to have a role in disease [179]. The expression of clusterin is upregulated for different conditions such as Alzheimer’s disease [187, 188] and diabetes [189] and it has been found to co-localise with the protein deposits found during disease [190-193]. In addition, clusterin has been shown to inhibit protein aggregation in vitro [69, 90, 91, 94, 194] and in vivo [195, 196]. Although the role of clusterin in disease is not yet fully understood [179], the literature shows that clusterin interacts with pre-fibrillar species, rather than with monomers or mature fibrils when having an impact on aggregation [69, 90, 94]. Kumita and co-workers investigated the influence of clusterin on HuL fibril formation [69] and showed that clusterin significantly inhibited the aggregation, and yet it did not interact with HuL in the monomeric and fibrillar states. In addition, HuL was still able to populate the transient partially-unfolded intermediate necessary for fibril formation [58, 197], and therefore the study concluded that clusterin must interact with oligomeric species present during the lag phase of HuL aggregation [69].

5.1.3. Chapter aims

In this chapter, we would like to revisit the ability of clusterin to inhibit HuL fibril formation using our newly acquired ability to identify oligomer populations of HuL using SM fluorescence. By examining the in vitro interactions between the extracellular chaperone clusterin and HuL oligomers, we will attempt to gain structural insight about what is truly occurring during the inhibition process.
5.2. Results

In Chapter 4, we showed the distinct oligomer populations present in the early stages of I59T HuL fibril formation. Due to the extensive research done previously on the role of clusterin in HuL aggregation and other systems [41, 69, 91, 94], we decided to further explore whether our newly established analysis of the lag phase of HuL aggregation could be used to further understand the mechanistic details of the interactions of these oligomer populations with relevant biomolecules, specifically, clusterin.

5.2.1. Characterisation of HuL fibril formation in the presence of clusterin using conventional methods

The aggregation of HuL in the presence of clusterin was reproduced from reference [69]. The aggregation was followed by light scattering at 500 nm and, whilst I59T HuL alone showed the classical sigmoidal growth, the sample with clusterin (1:10 clusterin-to-lysozyme molar ratio) did not (Figure 5.1, a). The resulting aggregates were imaged by TEM (Figure 5.1, b and c). Kumita and co-workers showed that clusterin forms a small amount of non-fibrillar aggregates when incubated alone in the pH 5, 60 °C conditions [69]. Therefore it is clear that clusterin is destabilised in these conditions, but it still retains inhibitory capabilities as shown by the kinetic assays and the TEM images. In fact, clusterin is still effective at inhibiting protein aggregation when present at 1:40 clusterin-to-lysozyme ratio under these conditions [69], where clusterin is about 50% unfolded as its melting temperature is ~45 °C (Kumita, unpublished results). Therefore, these results suggest that the inhibitory effect of clusterin is not related to its native structure as such. This conclusion is reinforced by the fact that its structure is not well known and some of its regions are considered intrinsically disordered, which is consistent with the current belief that it interacts with client proteins through hydrophobic interactions [179].
Figure 5.1. Characterisation of I59T HuL aggregation in the presence and absence of clusterin. Clusterin was present at a clusterin-to-lysozyme ratio of 1:10. a) Light scattering monitored at 500 nm. The curve for I59T HuL alone (black) shows the characteristic exponential growth after approximately 5 h and then reaches a plateau after 10 h. The curve for I59T HuL in the presence of clusterin (pink) does not show significant growth on this timescale. b), c) TEM images of the aggregates after 24 h for I59T HuL in the absence (b) and in the presence (c) of clusterin. The scale bar represents 100 nm. These data correlate well to those shown in reference [69].
5.2.2. Characterisation of the interactions of HuL oligomers with clusterin using SM FRET

Once the protocol for the aggregation in bulk was successfully reproduced, different time points along the aggregation pathway (0-12 h) were diluted to 750 pM, injected into the microfluidic device and flowed through the SM microscopy set-up at a flow rate of 0.61 cm s$^{-1}$. The apparent size of the oligomers was plotted against their FRET efficiency in a 2D plot for each time point. After 2 h, the FRET efficiencies shown by small oligomers (apparent size of 2-10 monomers) span from 0.3 to 0.7 (Figure 5.2, b), which is similar to the result obtained for the same time point in the absence of clusterin (Figure 4.5, b). However, the large oligomers detected after 2 h (apparent size of 10-150 monomers) appear at a much broader range of FRET efficiencies when compared to I59T HuL alone. The population distribution appears to be relatively unchanged until 7 h, after which the number of oligomers decreases for time points 8-12 h. Although small oligomers show a similar distribution as per their FRET efficiencies as compared to the results of I59T HuL in the absence of clusterin, this data suggests that, in the presence of clusterin, the formation of large oligomers does not necessarily result in a highly compact structure, as a broader range of FRET efficiencies appears to be accessible for large oligomers. In order to analyse the data further, the oligomers were divided into the same two apparent size categories described before, for direct comparison: small (2-10 monomers) and large oligomers (10-150 monomers); and the distribution of these two populations based on their FRET efficiencies was plotted over time (Figure 5.3). For small oligomers, only one Gaussian-like distribution is observed, and the number of oligomers increases within the first 2 h. This number then decreases over time until reaching its lowest point after 12 h (Figure 5.3, a). Very few large oligomers are detected at any time and their FRET efficiencies do not follow any trend in distribution (Figure 5.3, b). The histograms for the small population are shown for a clearer visualisation in Figure 5.4. To further investigate the differences in the evolution of the small oligomer populations in the absence and in the presence of clusterin over time, the oligomer distributions were fitted to a sum of two Gaussian distribution curves, whose centres and widths were fixed and equal to those used in Chapter 4 (with centres at 0.34, 0.60 and widths equal to 0.217, 0.202, for low-FRET and high-FRET oligomers, respectively, Eq. 4.1 and 4.2). The same centres were chosen as it is unlikely that the FRET efficiencies shown by the Alexa fluorophores would be affected by the presence of clusterin, due to the former being hydrophilic in nature whilst the latter is hydrophobic, and therefore the dielectric environment of the dye is not likely to be affected. In addition, when fitted to one-peak
Gaussian curves without any set boundaries, the average FRET efficiency for the centre of the population distribution for all time points is equal to 0.57 ± 0.01, which is comparable to that found for the small high-FRET population in Chapter 4. Therefore, we believe the direct comparison is feasible for the purpose of this analysis, in order to provide insights into the mechanisms by which clusterin is perturbing said distributions. The amplitude of each of the Gaussian curves was used to calculate the fraction of the low-FRET and high-FRET populations for each time point and then plotted over time (Figure 5.5). The fraction of the small low-FRET oligomers remains constant and equal to 0.15-0.20 over time (Figure 5.5, a). Thus, the fraction of the high-FRET population also remains constant over time (0.80-0.85) (Figure 5.5, b). In comparison, the fraction of the low-FRET population in the absence of clusterin shows a clear decrease throughout the aggregation (Figure 5.5, a), whereas the fraction of the high-FRET population increases over time (Figure 5.5, b). No significant amounts of large oligomers are detected in the presence of clusterin (Figure 5.5, c). Finally, the distributions of the oligomer population for each time point were fitted to one-peak Gaussian curves without any constraints on their centre or width, and the centres obtained after the fitting were plotted against time. The result shows that the population distribution remains constant and centred at a FRET efficiency of ~0.6 for all the analysed time points in the presence of clusterin, whereas the same fitting process shows an increase in the FRET efficiency during the aggregation process in the absence of clusterin (Figure 5.5, d).
Characterisation of the interaction of HuL oligomers with clusterin using SM microscopy

Figure 5.2. 2D plots of the size and FRET efficiencies observed for I59T HuL oligomers in the presence of clusterin over time. a) 0 h. b) 2 h. c) 5 h. d) 7 h. e) 9 h. f) 12 h. Size does not appear to increase over time. The FRET efficiency for both small and large oligomers spans from 0.2-0.8.
Figure 5.3. Evolution of oligomer populations over time. The oligomers are divided into two different apparent size categories: a) small (2-10 monomers) and b) large (10-150 monomers). a) For small oligomers, Gaussian-like distributions are observed and their centres are located at a FRET efficiency of ~0.6 for all time points after 0 h. The data for time points 0, 2, 5, 6, 7, 9, and 12 h are shown in black, red, orange, yellow, green, cyan, and violet, respectively. The number of oligomers increases within the first 2 h, after which it decreases until reaching its minimum after 12 h. b) For large oligomers, very few events are detected. The data for time-points 0, 2, 5, 6, 7, 9, and 12 h are shown in black, red, orange, yellow, green, cyan, and violet, respectively. The data show the mean and the error bars represent the standard deviation from three independent experiments.
Figure 5.4. Evolution of small oligomers over time. a) 2 h (red). b) 5 h (orange). c) 6 h (yellow). d) 7 h (green). e) 9 h (cyan). f) 12 h (violet). The centre of the population distribution is located at a FRET efficiency of ~0.6 for all time points. The number of oligomers decreases after 2 h. The data show the mean and the error bars represent the standard deviation from three independent experiments.
Figure 5.5. Comparison of the evolution of the low-FRET and the high-FRET oligomer populations of I59T HuL in the absence and in the presence of clusterin over time. a) Fraction of small low-FRET oligomers of I59T HuL alone (green) and in the presence of clusterin (blue) over time. In the presence of clusterin, the fraction of small low-FRET oligomers remains relatively constant at 0.15-0.20. b) Fraction of small high-FRET oligomers of I59T HuL alone (red) and in the presence of clusterin (orange) over time. In the presence of clusterin, the fraction of small high-FRET oligomers stays constant at 0.80-0.85. c) Number of large low-FRET and high-FRET oligomers over time where low-FRET oligomers are shown in green and blue in the absence and in the presence of clusterin, respectively, and the high-FRET oligomers are shown in red and in orange in the absence and in the presence of clusterin, respectively. In the presence of clusterin, the number of large low-FRET and high-FRET oligomers are virtually zero. d) Change in the FRET efficiency centre of the population distributions over the course of the aggregation time-course. The oligomer populations for each time point were fitted to a one-peak Gaussian curve and their centres were then plotted over time in the absence (black) and presence (pink) of clusterin. In the presence of clusterin, the FRET efficiency of the distribution centres remains constant at ~0.6 throughout the course of the aggregation. The data show the mean and the error bars represent the standard deviation from three independent experiments.
5.3. Conclusion and next steps

In this chapter we explored the interaction of clusterin with I59T HuL oligomers under physiologically relevant conditions using SM FRET. The results showed significant differences in the oligomer population distributions in the presence and absence of clusterin and hence this confirms that clusterin does interact with HuL oligomers during the course of aggregation. In the presence of clusterin, only one population of small oligomers (apparent size of 2-10 monomers) is detected, of which high-FRET oligomers account for 80-85%. For this reason, the two-peak fitting used for I59T alone is no longer viable and a one-peak Gaussian curve fitting describes the data more accurately. In addition, this oligomer population is present at a FRET efficiency centred at 0.6, which is corresponding to the small high-FRET oligomers present later in the aggregation process of I59T in the absence of clusterin. This data therefore suggests that clusterin may be promoting the conformational change of small oligomers from the low-FRET to the high-FRET species and then trapping them, consequently inhibiting the formation of large oligomers. At the same time, it may enhance the dissociation of small oligomers into monomers, as in its presence fewer small oligomers are detected. This type of behaviour in which clusterin traps oligomers, inhibiting further growth, has been reported before with respect to its interactions with amyloid-β oligomers [41, 90]. In addition, the interaction with low-FRET oligomers would be in agreement with the belief that extracellular chaperones like clusterin interact with oligomers through hydrophobic interactions, since low FRET efficiencies correspond to a less compact, more disorganised structure and hence, most likely, higher hydrophobicity [91, 179].

Further analysis on the effect of clusterin on the emission properties of the donor fluorophore is needed as a quenching effect may lead to a reduction in the donor emission, resulting in apparent lower sizes and high FRET values. However, fluorescence quenching was not a significant factor in previous studies using A488-αS and A647-clusterin [91]. Further analysis would also need to be done to confirm that the small high-FRET oligomers present with clusterin are indeed the same oligomers observed in the absence of this chaperone, for example using analytical ultracentrifugation. Labelling of clusterin with A647, as done in previous studies [41, 90], would allow us to determine specifically which species clusterin is interacting with by performing SM FRET using A488-I59T HuL to follow the intermolecular interactions. The ability of clusterin to regulate the oligomer population distributions could be further tested.
by adding it later in the aggregation, e.g. after 5 h, in order to see whether the dissociation of the oligomers into monomers or a conformational change would be promoted at that stage of the aggregation. This behaviour has been observed before for the chaperone Hsp70, which has been found to disaggregate amyloid-β oligomers [198]. However, this behaviour is unlikely as the addition of clusterin later in the lysozyme aggregation process has not demonstrated fibril dissociation [69]. In addition, the effect of clusterin on the ability of the oligomers to produce cell cytotoxicity would need to be explored in order to understand the role of their interaction in vivo.

Following the characterisation of the interaction of HuL oligomers with clusterin, investigating the interaction of the former with other relevant biomolecules would be paramount to explore more fully how the interactions with other biomolecules alter the oligomer structural properties, something that has been recently demonstrated for αS [91]. Other biomolecules of interest for the HuL system include the serum amyloid P component (SAP), which has been shown to inhibit the accumulation of HuL deposits in vivo [199]; and the camelid nanobody cAb-HuL5, which has been shown to encourage the formation of the partially-unfolded intermediate and yet inhibit amyloid formation [39].
Chapter 6: Conclusions and future work

In this thesis, the species present in the early stages of the aggregation of human lysozyme were investigated by SM confocal microscopy.

In Chapter 2, the Alexa-labelled I59T HuL was characterised by an array of biophysical techniques and two main conclusions could be reached from the data: first, Alexa-labelled I59T HuL undergoes aggregation in an equivalent manner to unlabelled I59T HuL; and second, both A488 and A647 are included in the fibrils and these show FRET. Therefore, these two conclusions confirm that Alexa-labelled I59T HuL is suitable for our SM experiments.

In Chapter 3, a well-established SM microscopy set-up was optimised for the HuL system by using conditions in which oligomer formation is maximised. The use of extrinsic dyes (ThT and pFTAA) and the techniques TCCD and SM FRET were utilised for this purpose. We were able to show that oligomers could be detected at micromolar and picomolar concentrations and the results obtained here compare well with previous characterisation of these metastable oligomers.

In Chapter 4, the focus switched to characterising HuL oligomers formed in situ using more physiologically relevant conditions (pH 5.0, 60 °C) with the set-up optimised in Chapter 3. The data shows that I59T HuL first forms disordered small oligomers (apparent size of 2-10 monomers, low-FRET). These oligomers can then undergo a conformational change to form more stable and structured oligomers (high-FRET). Such conformational changes have been shown using conventional techniques for other globular proteins such as insulin and Sso AcP [70, 166, 169]. This result makes sense as hydrophobic interactions can arise faster than the hydrogen bonds that sustain β-sheet structure can be formed. The data were then fitted to an early-time kinetic model, similar to those used in references [29, 42-44], and the rate of formation of low-FRET oligomers (α), the pseudo-rate constant of formation of high-FRET oligomers from low-FRET oligomers (kc), and the rate constant of dissociation of low-FRET oligomers (kd) were calculated. Interestingly, k_d>>k_c which indicates that low-FRET oligomers are approximately 5 times more likely to dissociate than to continue along the aggregation
pathway to form high-FRET oligomers. The formation of small disordered oligomers that can undergo a conformational change to form more structured oligomers during fibril formation has been previously observed for αS, tau and the yeast prion protein Ure2 [29-31, 42-44]. Although our data for I59T HuL shows different mechanistic dynamics to those reported for αS aggregation [29, 42-44], it is, in fact, in line with the studies of oligomers formed by non-αS proteins [30, 31]. In brief, the mechanism for αS aggregation was reported to fit well to an early-time model with no dissociation, that is, reverse reactions were excluded from the analysis. The resulting model described how monomers form low-FRET efficiency oligomers which then convert to ordered high-FRET efficiency oligomers prior to fibril formation [29]. On the other hand, the model for tau described the formation of an unstable low-FRET oligomer population in rapid exchange with the monomeric state, and another more structured high-FRET oligomer population, which is probably off-pathway. The disordered low-FRET oligomers can nucleate the formation of “fibrillar oligomers”, which can then form amyloid fibrils, and this nucleation can be suppressed early in the reaction [31]. In the case of the yeast prion protein, Ure2, the proposed model involved the formation of disordered low-FRET oligomers, via hydrophobic interactions between native Ure2 dimers, which can then dissociate back to the native-state or undergo a conformational change to form more structured high-FRET oligomers, which contain β-sheet structure, and can grow into fibrils [30]. Both tau and Ure2, like I59T HuL, showed the formation of these disordered low-FRET oligomers that predominantly dissociate but can also undergo a conformational change to form more structured high-FRET oligomers; and, unlike for tau, both populations have been identified as on-pathway to fibril formation for Ure2 and I59T HuL, as they are growth-competent and are most likely formed from the low-FRET oligomers later in the aggregation reaction. For I59T HuL, additionally, the role of high-FRET oligomers in the aggregation pathway is reinforced by our data in the presence of clusterin, since we showed that this population is sequestered by the extracellular chaperone, resulting in the inhibition of fibril formation (Chapter 5 of this thesis). In summary, the population of disordered low-FRET oligomers, formed from monomers, declines only as a consequence of the formation of a population of growth-competent, more structured oligomers (high-FRET) for αS whereas for tau, Ure2 and I59T HuL, the decline in the disordered low-FRET oligomer population cannot be explained by such a model, as these disordered oligomers are more likely to dissociate back into monomers than undergo a conformational change into the more structured high-FRET oligomers. Hence, while the majority of Ure2, tau and I59T HuL oligomers dissociate back to monomers and do not ultimately form fibrils, αS oligomers remain at a significant concentration, even after all the
monomers are depleted [42, 200] and this behaviour is also observed for amyloid-β (1-40) oligomers [90]. This discrepancy between the mechanistic dynamics shown by αS and amyloid-β, and those observed for other disordered proteins, such as tau and Ure2, and the globular I59T HuL may explain, in part, the differences of these systems in disease-related behaviour.

Finally, in Chapter 5 we investigated the interaction between HuL oligomers and clusterin, an extracellular chaperone which has been shown to inhibit HuL fibril formation by interacting with the species formed in the early stages of aggregation [69]. In the presence of clusterin, only small oligomers are significantly detected, of which high-FRET oligomers account for 80-85%. This data suggests that clusterin may be promoting the conformational change from small low-FRET to high-FRET oligomers and trapping them, consequently inhibiting the formation of larger oligomers and fibrils. This result is in agreement with the literature, where clusterin has been shown to inhibit fibril formation of other protein systems by sequestering small oligomers (<15 monomers in apparent size) and preventing their growth [41, 90, 91]. In addition, recent research by Whiten and co-workers showed that the interactions of clusterin with αS oligomers are dependent on the structure of the latter, and concluded that these interactions were mediated by the exposed hydrophobicity on the surface of the oligomers [91]. Beyond clusterin, other chaperones such as α2M and Hsp70 (first introduced in this thesis in Section 5.1.1) have been shown to trap small oligomers of αS and tau, respectively [91, 130].

The data presented in this thesis led us to revisit the aggregation pathway of human lysozyme and its inhibition by clusterin, that was first presented by Kumita and co-workers in 2007 [69], and our proposal is presented in Figure 6.1.

Regarding other globular proteins, it is worth comparing human lysozyme to β2-microglobulin and transthyretin, first introduced in Section 1.2.2. β2-microglobulin populates an intermediate state that can form intermolecular interactions leading to its oligomerisation and finally amyloid fibrils. β2-microglobulin forms small oligomers (dimer to tetramers) that undergo conformational changes into more stable, less dynamic structures which can then form fibrils [206, 207]. This is comparable to our findings, in which I59T HuL populates more disordered small oligomers before undergoing a conformational change towards a more structured oligomer population.
Conclusions and future work

On the other hand, the mechanism of aggregation of transthyretin consists of its dissociation from its native tetrameric state to an unstable monomeric state that can form intermolecular interactions, giving rise to small oligomers that are generally found to contain between 5-10 monomers (Figure 1.8) [213-215, 217-219]. These oligomers are the most cytotoxic species to human neuroblastoma cells, whereas the fibrils formed later on are not toxic [214, 215]. It would be interesting to check whether this is also the case for the cytotoxicity of human lysozyme oligomers and fibrils.

The aggregation of β2-microglobulin and transthyretin can be inhibited in the presence of different chaperones. The extracellular chaperone α2M interacts with the partially unfolded intermediate formed by β2-microglobulin, inhibiting oligomerisation and fibril formation [230]. The small heat shock protein αB-crystallin is also capable of inhibiting β2-microglobulin oligomerisation and fibril formation by competing with its self-assembly, and also enhancing the dissociation of β2-microglobulin oligomers back to monomers [231, 232]. In addition, clusterin has been shown to inhibit transthyretin aggregation by stabilising the tetrameric state [220], the monomeric state [209], and its oligomeric aggregation intermediates (Figure 1.8) [209, 221]. The latter is comparable to our findings, where clusterin interacts with lysozyme oligomers, increasing the population of the more stable and structured oligomeric species.
Figure 6.1. Proposed pathway for the aggregation of HuL and its regulation by clusterin. In the amyloidogenic mutational variants, the native-state stability is reduced, enhancing the population of a partially-unfolded intermediate. These intermediates can then interact to form unstable, disorganised oligomers which, in turn, can undergo a conformational change to form stable and structured oligomers. These oligomers will then grow to form amyloid fibrils. Our data shows that clusterin enhances the formation of small structured oligomers (high-FRET), and traps them over time, preventing them from growing into bigger aggregates. The way in which clusterin interacts with HuL oligomers is highlighted in pink.
The results shown for I59T HuL oligomers in Chapters 4 and 5 are significant as they give insight into the mechanistic details of fibril formation for a globular protein, as observed by SM microscopy techniques for the first time, thereby allowing for the comparison between these oligomer populations and those characterised for IDPs. In addition, these results raise questions that may be answered by further studies. Further insight about the degree of structural integrity and β-sheet content of the oligomer populations could be gained using proteinase K degradation coupled with SM microscopy. This method has previously shown that, for αS, the low-FRET oligomer population lacked a distinct structure whereas the high-FRET oligomers most likely contained a significant degree of β-sheet structure, albeit lower than that of the fibrils [42]. Interestingly, our data shows that the oligomer populations at 2 h and 9 h are quite defined. We therefore would like to explore whether we can see any differences in cellular toxicity using standard mammalian cell assays such as a MTT assay, in order to gain a better understanding of how these may have implications in vivo, as well as whether the oligomer population stabilised by clusterin alters cell viability. Additionally, A647-labelled clusterin could be used to further study the exact points of its interaction with I59T HuL oligomers using A488-labelled lysozyme.

Understanding the mechanistic details by which different biomolecules, such as SAP and the camelid nanobody cAb-HuL5, regulate HuL fibril formation, and how these differ from those seen for clusterin, will be needed to further understand how the different stages of the aggregation pathway can be modulated. In addition, in order to understand the role of these oligomer populations in vivo, it would be imperative to prove their toxicity in cells and investigate whether this toxicity is attenuated in the presence of clusterin or other biomolecules, as shown for other systems in the literature [91, 179].
Chapter 7: Materials and methods

7.1. Characterisation of the native-state of human lysozyme

This section will cover the material and methods used to characterise the monomeric state of unlabelled and fluorescently labelled HuL.

7.1.1. Expression and purification of human lysozyme in *Pichia pastoris*

Pre-cultures (10mL) of YPD medium (1% yeast extract, 2% peptone, 2% dextrose) were inoculated with *Pichia pastoris* cells containing the human lysozyme gene for each variant. After incubation for 48 h (230 rpm, 30 °C), these cultures were used to inoculate buffered minimal glycerol media (BMG; 400 mL, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 1% glycerol). After growing to a high density (48 h, 230 rpm, 30 °C), the cultures (200 mL) were centrifuged at 5000 x g (5 min, 4 °C) in a Beckman Avanti J centrifuge (Beckman Coulter Inc., Buckinghamshire, UK) in autoclaved 500 mL centrifuged pots. The supernatant was removed and the pellets were re-suspended in buffered minimal methanol media (BMM; 400 mL, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4.0 x 10^{-5}% biotin, 0.5% methanol) and incubated for 96 h (230 rpm, 23 °C) with the 0.5% methanol being replenished every 24 h. After incubation, the cultures were centrifuged (10 min, 5000 x g, 4 °C), and the pellets discarded. The supernatant was then centrifuged a second time (10 min, 5000 x g, 4 °C) and filtered with a 0.45 μm filter (Durapore membrane filters, Merck-Millipore, Hertfordshire, UK). Purification of lysozyme from the supernatant was performed on a HS20 cation-exchange POROS column (Applied Biosystems, Warrington, UK) on an ÄKTA Pure system (GE Healthcare, Little Chalfont, UK). Lysozyme was eluted at ~55 mS via a linear sodium chloride gradient. The protein peaks were analysed by SDS-PAGE on a 4-12% Bis-Tris NuPAGE gels (Life Technologies, Paisley, UK) using MES running buffer under reducing conditions, and the relevant fractions were dialysed using a 3.5 kDa MWCO membrane (Spectra/Por dialysis membrane, standard RC tubing, Spectrum Laboratories, Repligen Corporation, Massachusetts, USA) against water for 48 h and then lyophilised. The purity of the protein was confirmed by SDS-PAGE and molecular masses were determined by
MALDI mass spectrometry. The concentration of HuL was determined on a NanoDrop 2000c spectrophotometer (Labtech International Ltd., Uckfield, UK) using an extinction coefficient of 37,485 M⁻¹cm⁻¹ (Abs₂₈₀ = 2.55 for a 1 mg mL⁻¹ protein solution).

7.1.2. Characteristics of the fluorophores

The spectral properties of the fluorescent dyes used in this thesis are shown in Table 5.

Table 5. Spectral properties of the fluorophores

<table>
<thead>
<tr>
<th>Property / Dye</th>
<th>Fluorescein derivative</th>
<th>A488</th>
<th>A647</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mw (gmol⁻¹)</strong></td>
<td>473.39</td>
<td>643.4</td>
<td>842.02</td>
</tr>
<tr>
<td>Extinction coefficient, ε (cm⁻¹M⁻¹)</td>
<td>~70,000</td>
<td>73,000</td>
<td>270,000</td>
</tr>
<tr>
<td>Excitation wavelength, λ_max (nm)</td>
<td>492</td>
<td>494</td>
<td>651</td>
</tr>
<tr>
<td>Emission wavelength, λ_max (nm)</td>
<td>520</td>
<td>517</td>
<td>672</td>
</tr>
<tr>
<td>*Lifetime (ns)</td>
<td>4.1</td>
<td>4.1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Quantum yield (ϕ)</strong></td>
<td>~0.95</td>
<td>0.92</td>
<td>0.33</td>
</tr>
</tbody>
</table>

*Lifetime measurements were made in water at 22°C, data provided by ISS Inc. (Champaign, IL). **Quantum yield measurements for the Alexa dyes were made in PBS (50 mM potassium phosphate, 150 mM NaCl, pH 7.2) at 22°C relative to fluorescein in 0.01 M NaOH, data provided by ThermoFisher (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

7.1.3. Fluorescent labelling of HuL

Human lysozyme (HuL, 6.8 µM) was dissolved in MES buffer (100 mM) in pH 5.0 for the A647 succinimidyld ester derivative and pH 8.0 for the fluorescein and A488 succinimidyld ester derivatives with stirring in a glass vial (3 mL). The fluorescein derivative and the Alexa fluorophores succinimidyld ester derivatives were dissolved in DMSO (1 mg in 300 µL) and the dye concentrations were determined using UV-vis absorbance at the corresponding excitation
wavelength for each fluorophore on a NanoDrop 2000c spectrophotometer (Labtech International Ltd.). Samples were incubated for 18 h with stirring (1000 rpm) at 50 °C for the fluorescein derivative and at 45 °C for the Alexa dyes derivatives. The buffer was exchanged to de-ionised water after 24 h using centrifuge cut-off filters (3 kDa) (GE Healthcare) before purification (Section 7.1.4).

7.1.4. Purification of labelled HuL

The end points of the reaction were dissolved in 50 mM Tris buffer, pH 8 and purified using a monoS ion exchange column (GE Healthcare) on an ÄKTA Pure purification system (GE Healthcare). The protein was eluted using a sodium chloride (NaCl) gradient (0-1.5 M). The buffer of the fractions containing the desired product was exchanged to de-ionised water using centrifuge cut-off filters (3 kDa) (GE Healthcare). The protein integrity and purity were confirmed by mass spectrometry by Dr. Janet Kumita using Electrospray mass spectrometry on a Xevo G2 mass spectrometer (Waters UK, Elstree, Hertfordshire, UK) within the mass spectrometry facilities at the Dept. Chemistry, University of Cambridge. The concentration of the sample was determined on a NanoDrop 2000c spectrophotometer (Labtech International Ltd.) using the extinction coefficients of the corresponding fluorophores (Table 5), and then flash-frozen in aliquots with liquid nitrogen and stored at -20 °C until further use. The molecular masses of labelled HuL are shown in Table 6.

Table 6. Molecular weight of labelled HuL

<table>
<thead>
<tr>
<th>Sample</th>
<th>Label</th>
<th>MW (Da) of the fluorophore</th>
<th>MW (Da) of singly-labelled HuL</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-WT HuL</td>
<td>F-NHS</td>
<td>473.39</td>
<td>15166</td>
</tr>
<tr>
<td>F-I59T HuL</td>
<td>F-NHS</td>
<td>473.39</td>
<td>15154</td>
</tr>
<tr>
<td>A488-I59T HuL</td>
<td>A488</td>
<td>516.47</td>
<td>15198</td>
</tr>
<tr>
<td>A647-I59T HuL</td>
<td>A647</td>
<td>842.02</td>
<td>15523</td>
</tr>
</tbody>
</table>
Materials and methods

7.1.5. SDS-PAGE analysis of Alexa-labelled HuL

Polyacrylamide gel electrophoresis (SDS-PAGE) analysis of labelled HuL was done on a 4-12% Bis-Tris NuPAGE gels (Life Technologies) using MES running buffer under reducing conditions. The gels were then imaged on a Typhoon Trio Variable Mode Imager System (GE Healthcare) measuring the fluorophore emissions after excitation at 488 and 633 nm for A488 and A647, respectively.

7.1.6. Spectroscopic characterisation of Alexa-labelled HuL

The absorbance spectra of WT human lysozyme and F-WT HuL (in water) were recorded between 200-800 nm in a 1 cm cuvette on a Cary 400 scan spectrophotometer (Agilent Ltd., Oxford, UK) at room temperature.

The excitation and emission spectra of Alexa-labelled I59T HuL were recorded in a Cary Eclipse spectrofluorimeter (Agilent Ltd.) at room temperature. The excitation spectra were recorded by setting a constant emission wavelength of 517 and 672 nm (± 5 nm slit width), respectively for A488-I59T and A647-I59T HuL, and recording between 250-650 nm for A488-I59T and 250-700 nm for A647-I59T HuL. The emission spectra were recorded using an excitation wavelength of 494 and 651 nm (± 5 nm slit width), respectively, and recording the emission between 500-650 nm for A488-I59T and 500-700 nm for A647-I59T HuL.

7.1.7. Circular dichroism (CD) spectroscopy

CD spectroscopy was performed on a Jasco J-810 spectropolarimeter (JASO Ltd., Great Dunmow, UK). Far-UV data were measured in a 0.1 cm path-length cuvette with a protein concentration of 13.6 µM in 0.1 M sodium citrate, pH 5, and the scans were recorded between 215 and 250 nm. Scans were recorded at 50 nm min⁻¹ with a 1 nm band width and a 4 s response time. The mean residue ellipticity (MRE) was calculated as described in reference [146]. In brief, the MRE at a certain wavelength (λ) is calculated as

\[
\text{MRE} = \text{MRW} \times \frac{\theta_\lambda}{10} \times d \times c \quad \text{(Eq. 7.1)}
\]
where MRW is the mean residue weight, $\theta_\lambda$ is the observed ellipticity (degrees) at wavelength $\lambda$, $d$ is the path length (cm), and $c$ is the concentration (g/mL). The MRW is calculated as

$$\text{MRW} = \frac{M}{N - 1} \quad (\text{Eq. 7.2})$$

where $M$ is the molecular mass of the polypeptide chain (in Da), and $N$ is the number of amino acids in the chain.

Far-UV thermal denaturation was monitored at 222 nm. During thermal denaturation, the temperature was increased from 20 to 95 °C at a rate of 0.5 °C min$^{-1}$. Ellipticity values were normalised to the fraction of unfolded protein ($F_u$) using

$$F_u = \frac{\theta - \theta_N}{\theta_U - \theta_N} \quad (\text{Eq. 7.3})$$

where $\Theta$ is the observed ellipticity, $\Theta_N$ is the native ellipticity and $\Theta_U$, the unfolded ellipticity. $\Theta_N$ and $\Theta_U$ were extrapolated from pre- and post-transition baselines at the relative temperature.

Experimental data were fitted with a sigmoidal expression, using OriginPro 9.1 (OriginLab Corporation) where $T_m$ is defined as the temperature where the fraction of unfolded protein is 0.5. This method was described in detail in reference [67].

7.1.8. Differential Scanning Calorimetry (DSC)

The stabilities of F-WT HuL and WT HuL were investigated by measuring the heat capacity ($C_p$) as a function of temperature over the range of 15-95 °C/h using a VP-DSC MicroCalorimeter (MicroCal. LLC, Milton Keynes, UK), with protein concentrations of 7 μM. The baseline obtained by heating a buffer solution was subtracted from the protein sample. The data were fitted to a two-state model as described in reference [74],

$$N \leftrightarrow U$$

where $N$ is the native-state and $U$ is the unfolded state. The average excess heat capacity ($<\Delta C_p>$) was derived from the average excess enthalpy $<\Delta H>$ using the equations
### 7.2. Characterisation of the fibrillar state of human lysozyme

This section will cover the material and methods used to characterise the fibrillar state of unlabelled and fluorescently labelled HuL formed under different conditions and in the presence and in the absence of clusterin.

#### 7.2.1. Preparation of ThT stock solution

Stock ThT solutions were prepared by dissolving ThT in phosphate-buffered saline (PBS), pH 7.4 and then filtering through a 0.22 μm filter to give a concentration of 2.5 mM. The solutions were aliquoted, covered in aluminium foil, and stored at -20 °C until used. Once thawed, any ThT leftovers were discarded.

#### 7.2.2. Fibril formation of HuL under acidic conditions

HuL was dissolved in 400 mM NaCl, 25 mM KH$_2$PO$_4$ buffer, pH 2.0. A 200 μL aliquot of the sample was placed in a 96-well half-area black plate, and ThT stock solution was added for a final concentration of 50 μM. The samples were incubated at 52 °C with shaking at 100 rpm for 5 s every 10 min in a BMG POLARstar Omega plate reader (BMG Labtech Ltd., Aylesbury,
UK). ThT was excited at 440 nm and its fluorescence emission was recorded at 480 nm. The protocol was first described in reference [153].

7.2.3. Fibril formation of HuL under pH 5 conditions

Alexa-labelled I59T HuL, 7 μM (3.5 μM of each dye) was incubated in a 1 cm path length quartz cuvette in 0.1 M sodium citrate, pH 5.0, with stirring at 60 °C in a Cary Eclipse fluorescence spectrophotometer (Agilent Ltd) and light scattering was monitored at 500 nm (slit width of 5 nm). The aggregation of unlabelled HuL was also monitored by recording ThT fluorescence in the presence of 62.5 μM ThT and using an excitation wavelength of 450 nm (slit width 5 nm) and an emission wavelength of 480 nm (slit width 5 nm).

7.2.4. Fibril formation in the presence of clusterin

The protocol followed here was described before by Kumita and co-workers in 2007 [69]. The aggregation was set-up as described in Section 7.2.3 in the presence of 1:10 clusterin-to-lysozyme molar ratio. The clusterin was a gift from Prof. Mark Wilson at the University of Wollongong (Wollongong, Australia).

7.2.5. Transmission electron microscopy (TEM)

Fibril samples (5 μL) were collected at the endpoints of the aggregation reactions and applied to carbon-coated copper grids. The samples were stained with 2% (w/v) uranyl acetate, and imaged using a FEI Tecnai G2 transmission electron microscope (CAIC, Department of Physiology, Development and Neuroscience, University of Cambridge, UK). Images were analysed using the SIS Megaview II Image Capture system (Olympus).

7.2.6. Measurement of fibril stability by guanidine thiocyanate depolymerisation

Samples of HuL fibril formed under acidic conditions (100 μL) were centrifuged in a benchtop centrifuge (13000 rpm, 30 min, 4 °C) and the supernatant was removed. The fibrils were washed with dH2O and centrifugation was repeated (13000 rpm, 30 min, 4 °C), discarding the supernatant. The fibril pellets were re-suspended in a guanidine thiocyanate (Gdn.SCN)
titration of concentrations ranging 0-6 M in 0.25 M increments and incubated overnight at 25 °C. The samples were then centrifuged (13000 rpm, 10 min) at room temperature and the supernatant was saved. The concentration of monomer in the supernatant was determined by recording the absorbance at 280 nm for each sample and the monomer concentration was plotted as a function of GdnSCN concentration. These experiments were performed by Kimberley Callaghan.

7.2.7. ThT analysis of the endpoint of aggregation

Samples of fibrils formed under acidic conditions (100 μL, 350 μM) were diluted in PBS, pH 7.4 to a total volume of 1 mL; whereas samples of fibrils formed under pH 5.0 conditions (100 μL, 7 μM) were diluted in 0.1 M citrate buffer, pH 5.0 to a total volume of 1 mL, then 2.5% v/v of 2.5 mM ThT (62.5 μM) was added in both cases. ThT binding was monitored by excitation at 440 nm (slit width 5 nm) and recording the emission between 450 nm and 600 nm in a 1 mL quartz cuvette using a Cary Eclipse fluorescence spectrophotometer (Agilent Ltd.). The measurement of a sample with only the corresponding buffer was subtracted from all samples. The samples were stirred for 5 min before collecting the data.

7.3. Characterisation of oligomers using conventional methods

7.3.1. Dynamic light scattering (DLS)

DLS measurements were performed at 25 °C in a Zetasizer Nano S (Malvern Instruments, Worchestershire, U.K.) with a Peltier temperature controller using fixed parameters (attenuator 7, cell position 4.20 mm) using a low volume (45 μL) quartz cell with 3 × 3 mm path length.

7.3.2. Atomic force microscopy (AFM)

AFM was done by Ryan Limbocker, under the supervision of Dr. Simone Ruggeri, following the same methodology as described in [83].
7.3.3. ANS measurements of time points from I59T HuL aggregation

Aliquots from different time points of the 7 μM I59T HuL aggregation reaction in pH 5.0, 60 °C conditions were diluted in pH 5.0 buffer to 2 μM (monomer equivalents) for a total volume of 70 μL, where ANS was added to a final concentration of 23 μM. The emission spectra, after excitation at 350 nm, were recorded from 440-600 nm at room temperature in a 96-well half-area black plate using a BMG Clariostar plate reader (BMG Labtech Ltd.). A control with only ANS and buffer was measured and subtracted from all samples.

7.3.4. ThT measurements of time points from I59T HuL aggregation

Aliquots from different time points of I59T HuL aggregation in pH 5.0, 60 °C conditions (100 μL, 7 μM) were analysed following the protocol described in 7.2.6.

7.4. Characterisation of oligomers using SM fluorescence microscopy

7.4.1. Label-free SM fluorescence microscopy using ThT and pFTAA as extrinsic dyes

The preparation of ThT stocks solutions was described in Section 7.2.1. The pFTAA stock solutions were prepared in 25 mM Tris buffer, 100 mM NaCl, pH 7.4, as described in reference [134]. The concentrations of the extrinsic dyes for the SM microscopy experiments were optimised [134] and, in this thesis, 0.5 μM and 5 μM of ThT were used for the pH 5.0 and pH 2.0 conditions, respectively, whereas 30 nM of pFTAA was used for both conditions. The methodology for these experiments is described in detail in reference [134]. Briefly, aliquots of different time points were taken during I59T HuL aggregation and diluted to micromolar concentrations in the corresponding buffer (10 and 1 μM for the aggregation under acidic conditions and the pH 5.0 conditions, respectively) and one of the extrinsic dyes for a final concentration of ThT of 5 μM (acidic conditions) and 0.5 μM (pH 5.0 conditions) and 30 nM of pFTAA. The samples were then injected into the microfluidic device described in Section 1.5.4, and infused at a flow rate of 0.61 cm s⁻¹ as controlled by a syringe pump (Harvard Apparatus PhD Ultra, Cambridge, UK) attached to the outlet port via Fine BorePolyethylene Tubing (0.38 mm inner-diameter, 1.09 mm outer-diameter; Smiths Medical International, Hythe, Kent, UK). A 488 nm laser (1.5 mW, OFL68, OdicForce) was used for the excitation
of the sample by directing it onto the back aperture of an inverted microscope (Nikon Eclipse TE2000-U, Nikon Ltd., Kingston upon Thames, UK). The beam was focused through an objective (Apochromat 60X, NA 1.40, Nikon) by reflection on a dichroic mirror (Di01-R488/543/594, Laser 2000), to a concentric spot with limited diffraction, 10-15 μm into the sample being flown through a channel of the microfluidic device. The fluorescence from the sample was collected with the same objective, reflected into the same dichroic mirror, and passed through a 50 μm pinhole (Melles Griot) to remove any out-of-focus light. The emission was filtered (FF01–510/84) and directed to an avalanche photodiode (APD, SPCM-14, PerkinElmer Optoelectronics). A field-programmable gate array (FPGA, Colexica), was used to detect the signals from the detectors and transform these into time bins, which were selected according to the expected residence time of molecules passing through the confocal volume.

At each time point, data were collected for 10 min (100,000 time bins, bin width 0.2 ms). The experimental output data were collected by use of an FPGA card and analysed in Igor Pro 7 (version 7.0.2.2 64-bit) by use of customised code written by Dr. Mathew Horrocks. The code only accounts for bursts that show higher photon counts than an applied threshold, which was selected as described in Section 1.5.2, and then calculates the total number of events, the total intensity, and the average intensity for each time point.

7.4.2. SM microscopy using Alexa-labelled HuL

The Alexa-labelled I59T HuL and the SM microscopy set-up described in Section 1.5.4 were used following the methods described before in detail [43, 121]. The methodology described in Section 7.4.1 applies for the labelled experiments with the difference of the use of two overlapping beams, at 488 nm (OFL68, OdicForce) and at 633 nm (Melles Griot, 25LHP151, Cambridge, UK). In these experiments, after passing through the 50 μm pinhole, the emission from the samples is separated into two different channels using a second dichroic mirror (FF573-Di01, Laser 2000), which separates the fluorescence from the two different fluorophores. The longer wavelength is then focused by a lens (Plano apo convex, focal length = 50 mm, Thorlabs Inc., Ely, UK) through a set of filters (695AF55, Omega Optical Filters, Omega Optical Inc., Brattleboro, VT, USA) onto the ADP detector. The shorter wavelength is focused through a second set of filters (535AF55 Horiba, 540LP Omega) onto the second ADP. The signals from the two ADPs are connected to a customised field-programmable gate array, FPGA (Colexica), which combines them into time bins as described in the section above (7.4.1). For SM FRET experiments, only the 488 nm laser is switched on. The data were
analysed in Igor Pro 7 using the methodology described in Sections 1.5.2 for TCCD and in 1.5.3 for SM FRET. The data was modelled as described in Section 4.2.5.

**7.4.3. Threshold selection for SM microscopy**

As described in Section 1.5.2, the threshold used in the data analysis of these experiments was determined manually. To facilitate the choice, the number of events was plotted against the different thresholds. The resulting graph is an exponential decay in which the sufficient threshold is located right before the plateau (Figure 7.1).

![Figure 7.1. Visualising the choice of threshold in SM experiments. The number of events for time point 0 h is plotted against the threshold used, resulting in an exponential decay in which the sufficient threshold to use is located right before the plateau (here highlighted with a green arrow).](image-url)
Chapter 8: Bibliography

Bibliography


