Single-particle Kinetic Measurements and Structure Characterization of PrP Fibril Elongation Using Super-Resolution Microscopy

Yuanzi Sun

For the degree of Doctor of Philosophy

MRC Prion Unit at UCL, Institute of Prion Diseases
University College London (UCL)
Declaration

I, Yuanzi Sun, confirm that the work presented in my thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

06/09/2022
Abstract

In prion diseases, benign cellular prion protein (PrP\textsuperscript{C}) is converted to PrP\textsuperscript{Sc}, fibrillar assemblies of misfolded PrP, which self-propagate by recruiting PrP\textsuperscript{C} into the growing fibril. Using total internal reflection (TIRF) microscopy, this study analyses elongation kinetics of synthetic and authentic PrP fibrils on a single-particle level to reveal polymorphic fibril populations, featuring structural and dynamic heterogeneity similar to prion strains, which were previously hidden in ensemble measurements.

MoPrP 91-231 fibrils elongated along a preferred direction with an intermittent ‘stop-and-go’ pattern. Fibrils fell into three main populations, types I, II and III, which displayed distinct structural and dynamic properties, and elongated by different mechanisms. They maintained their properties even under elongation conditions favouring a different fibril type. Type I and II fibrils incorporated folded or partially folded PrP molecules; type III fibrils recruited unfolded monomers with a pronounced inhibition at high PrP concentration. The elongation of authentic fibrils of two strains, RML and ME7, in the presence of MoPrP 91-231 monomers, were slower than synthetic fibrils under the same condition. RML fibril elongation dynamics displayed heterogeneity as well.

The discovery of polymorphic fibril populations of amyloid and prions growing in competition suggests that prions may present as quasi-species of structural isomorphs and that the replication environment may tilt the balance between amyloid species and prion isomorphs.

The structures of the heterogeneous fibrils after elongation were measured at an enhanced resolution using transient amyloid binding (TAB) and polarised-TAB super-resolution microscopy, revealing unique structural features associated with each fibril type.
Impact Statement

Prion diseases are a group of fatal neurodegenerative diseases and have drawn substantial public health concerns. The diseases are linked with the infectious amyloid species, $\text{PrP}^\text{Sc}$, formed by the misfolding and aggregation of cellular prion protein ($\text{PrP}^\text{C}$). $\text{PrP}^\text{Sc}$ can self-propagate by recruiting $\text{PrP}^\text{C}$ and transforming them into the misfolded form. Studies on other more common neurodegenerative diseases, such as Alzheimer’s Disease and Parkinson’s Disease, show that, similar to prion diseases, amyloid fibrils are formed, which propagate by a prion-like mechanism. Studying the propagating mechanism is important to understand disease development and to identify possible treatments.

This study focused on the growth of individual synthetic or authentic prion protein fibrils, revealing that structurally and dynamically heterogeneous PrP fibrils co-exist and compete under homogenous conditions, which were hidden in bulk analyses of amyloid kinetics. Polymorphic fibrils propagate their structures with fidelity; the environment shifts the equilibrium between competing isomorphs. This observation supports the theory that prions exist as quasi-species rather than acquire a homogeneous structure. It has important significance in understanding the dynamic equilibrium between different PrP species and developing possible therapeutic interventions. Designing drugs targeting a specific fibril type is likely not able to stop disease propagation since other fibril isoforms can outcompete the targeted fibril morphology.

Detailed kinetic analysis was conducted for synthetic PrP seeds, allowing the identification of multiple types of fibrils by their characteristic growth dynamics. Fibrils of different types also acquired specific morphologies. The two prion strains, RML and ME7, acquired different structures from the synthetic fibrils and elongated with slower rates. This is, to our knowledge, the first time that a direct link was established between the elongation kinetics of PrP fibrils observed in situ and their specific morphologies.

The experimental setup and analysis scheme can be further applied to study the elongation of amyloid seeds under other genetic backgrounds and in other
amyloidogenic protein systems. Further application to observe amyloid propagation in cellular environments can provide valuable information on the propagation rates and mechanisms in vivo, as well as the role of cellular machinery during the process.

Transient amyloid binding (TAB) super-resolution imaging technique was applied here to image structurally distinct, elongated PrP fibrils, revealing their unique properties, including brightness and periodic change in brightness. The polarised-TAB (p-TAB) technique developed here provides a simple approach to estimating the orientation of single dye molecules, revealing the non-uniform dye binding orientation and possible twisting substructure of certain fibrils. Both approaches allow the estimation of fibril type. These techniques can be further applied to identify misfolded aggregates of different structures and study their dynamic conversion.
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Statement of collaboration

Part of the work in this thesis was carried out in collaboration with Dr. Matthew Lew’s lab at Washington University in St. Louis. Specifically, TAB and p-TAB imaging techniques were developed through the collaborative work of both labs. Dr. Matthew Lew’s lab provided the analysis script for p-TAB data, including two-channel registration, pairing localisations in two channels and calculating the linear dichroism (LD) value of each blinking event and pixel. In the thesis work, I applied TAB and p-TAB techniques to image different PrP fibrils. I further developed the scripts to estimate the orientation of dye molecules and track the brightness or LD profile along the fibril axis to reveal structural features. The mentioned work is in Chapter VII.
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<td>α-Synuclein</td>
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<td>Aβ</td>
<td>Amyloid beta</td>
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<td>AD</td>
<td>Alzheimer’s Disease</td>
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<td>AF</td>
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<td>AFM</td>
<td>Atomic force microscopy</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<td>β2M</td>
<td>β2-microglobulin</td>
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<td>BALM</td>
<td>Binding-activated localisation microscopy</td>
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<td>Fluorescence lifetime imaging</td>
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<td>FT</td>
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<td>IDP</td>
<td>Intrinsically disordered proteins</td>
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<td>IPTG</td>
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<td>MoPrP</td>
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PA-FP  Photoactivatable fluorescent protein
PALM  Photoactivated localisation microscopy
PBS  Polarisation beam splitter
PBS buffer  Phosphate-buffered saline
PD  Parkinson's Disease
PDD  Parkinson's Disease Dementia
PFOTS  Trichloro-(1H,1H,2H,2H-perfluorooctyl)silane
PHF  Paired helical filaments
PIRIBS  Parallel in-register intermolecular \( \beta \)-sheet
PK  Proteinase K
PMCA  Protein Misfolding Cyclic Amplification
PrP  Prion protein
PrP\(^C\)  Cellular prion protein
PrP\(^{Sc}\)  Scrapie form of the prion protein
PSF  Point spread function
p-TAB  Polarised-TAB
RML  Rocky Mountain Laboratory prion strain
ROI  Region of interest
RT_QuIC  Real-Time Quaking-Induced Conversion
SEC  Size exclusion chromatography
SMLM  Single-molecule localisation microscopy
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<td>Spectrally-resolved Points Accumulation for Imaging in Nanoscale Topography</td>
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Chapter I. Introduction

1.1 Prion Diseases

1.1.1 Introduction to prion diseases

Prion diseases are fatal and transmissible neurodegenerative diseases affecting humans and other mammals.

The first recognised prion disease is scrapie in sheep and goats discovered over 200 years ago [1]. Prion diseases existing in other animals were later discovered, including bovine spongiform encephalopathy (BSE, also known as mad cow disease), chronic wasting disease (CWD) in deer and elk, and diseases in other mammals such as mink. As the name suggests, ‘spongiform encephalopathy’ in the brain is a typical feature of prion diseases. Another striking feature is the transmissibility of the diseases [1] within the same species or across species. Transmission occurs through natural processes or inoculation of the diseased animals’ brain homogenates into healthy animals.

The first identified type of prion disease in humans, Creutzfeldt-Jakob disease (CJD), was reported by two neurologists in the 1920s and named after them. Infectious and genetic types were identified later, including variant Creutzfeldt-Jakob disease (vCJD), kuru, Gerstmann-Straussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). Human prion diseases are rare, with an incidence of 1-2 : 1,000,000. They are typically represented by a clinically silent incubation period, and fast progression after disease onset, eventually leading to death. The most typical neuropathological feature is the spongiform change in the brain; other features include neuronal loss, gliosis and plaque lesions [2, 3].

The cause of prion diseases had been under debate for decades. Early studies on scrapie considered a transmissible ‘scrapie agent’ as the cause [4]. An unconventional ‘slow virus’ was initially considered as the agent [5] due to the transmissibility resembling the property of the virus. This theory was challenged, however, as the pathogen was resistant to chemicals, heat and radiation, which were able to disrupt nucleic acids and kill viruses [6]. In the 1960s, researchers proposed that the scrapie agent was an abnormal self-
propagating protein instead of a virus [7]. This theory, called the 'protein-only hypothesis', was supported by the isolation of infectious, disease-causing, protein-rich scrapie agents [4] and has been widely accepted. The disease-causing agent was termed 'prion' [6], standing for 'proteinaceous infectious particle'.

Prion formation in diseases could be spontaneous, for example, sporadic CJD in humans was caused by *de novo* formation of prions for unknown reasons; prion diseases could also be a result of autosomal dominant inheritance of related mutations in the PRNP gene encoding PrP\(^C\) (GSS, FFI); infection with prions was another cause for the disease, such as consumption of BSE-contaminated food (leading to vCJD) or infection with surgical instruments [1].

The precursor of prions is a benign cellular protein called cellular prion protein (PrP\(^C\)). It is encoded by the PRNP gene and is widely expressed in the central nervous system and other tissues [8]. This gene is highly conserved among mammals [9], with a ~90% homology among humans, mice, hamsters and rats [10]. After expression, the 253 amino-acid long protein is cleaved to form mature PrP\(^C\) containing amino acids 23-231 (human sequence). PrP\(^C\) is present in a di-, mono- or unglycosylated form through glycosylation at two possible sites, N180 and N196. PrP\(^C\) attaches to the outer cell surface through a glycophaspatidylinositol (GPI) anchor at its C terminus. Although the physiological function of PrP\(^C\) is not well characterised, researchers have found evidence that PrP\(^C\) protects cells against oxidative stress, binds copper ions and assists in transmembrane signaling [8].

The structure of PrP\(^C\) has been resolved by Nuclear magnetic resonance (NMR) spectroscopy [11, 12] (Figure 1-1) and X-ray crystallography [13]. PrP\(^C\) contains an unstructured N-terminal domain (involving amino acids 23 to 121 [12]) and a globular C-terminal domain consisting of three α helices and two-stranded antiparallel β sheets. In the C-terminal domain, there are two glycosylation sites for N-glycans (N180 and N196) and a disulfide bond linking C179 and C214 (human sequence).
The disease-causing agents, prions, consist of multi-molecular assemblies of conformationally misfolded forms of PrP. They can propagate by seeded replication, meaning they can serve as templates and convert PrP\textsuperscript{C} into the misfolded form. This property underlies the infectious nature of prions. As will be discussed in Section 1.1.2, prions refer to all species that are infectious and can lead to the diseases [14], including PrP scrapie (PrP\textsuperscript{Sc}) and other PrP species that meet this definition. The transition from PrP\textsuperscript{C} to prions involves a significant change in secondary structure. However, the detailed mechanism has not been explicitly characterised.

It has been established that more than one type of misfolded PrP plays a role in disease development. For example, the infectious agents and neurotoxic species were shown to be distinct [15]. Understanding what those species are, how they are formed, and how they could be eliminated are important aspects to tackle prion diseases.

**1.1.2 Different misfolded PrP species and their role in prion transmission**

In the 1980s, one misfolded species, PrP scrapie (PrP\textsuperscript{Sc}), was isolated from diseased animal brains but was not present in control samples [16, 17]. PrP\textsuperscript{Sc} was found to be infectious, characterised by partial proteinase K (PK) resistance and detergent insolubility. Inoculation of PrP\textsuperscript{Sc} into healthy lab
animals led to the development of prion diseases [16]. Thus, PrP<sup>Sc</sup> meets the definition of ‘prions’, and early studies considered PrP<sup>Sc</sup> as the major component of prions [18]. It was found later that some disease-causing, infectious PrP species were PK sensitive [19]. For an extreme case, nearly all disease-related PrP species in the DY strain could be digested by PK [19]. The PK-sensitive, disease-related PrP also meets the definition of prions.

The PK-resistant core of PrP<sup>Sc</sup> contained N-terminal truncated PrP and was termed PrP 27-30 as their apparent molecular weight was 27-30 kDa measured by gel electrophoresis.

PrP 27-30 was first purified by Prusiner and co-workers [4, 17] in 1982 and was shown to be rod-like amyloid. Its amyloid nature was confirmed by Congo red staining [20] and the unique X-ray fibre diffraction pattern [21]. Prusiner’s work provided early evidence in support of the ‘protein-only hypothesis’ in prion diseases. The biophysical and biochemical properties of PrP 27-30 were thoroughly investigated afterwards.

Prions were shown to exist as different strains. Each strain can be faithfully propagated in lab animals and exhibit distinct incubation times and neuropathological features [22]. Differences between strains were proposed to be encoded in PrP<sup>Sc</sup> conformations [19].

Studies on purified PrP<sup>Sc</sup> from multiple strains have confirmed the conformational differences: (1) the level of PK resistance and the size of the PK resistant core measured by gel electrophoresis were different for different strains, suggesting exposure of distinct PK cleavage sites [22, 23]; (2) strains differed in secondary structures probed by infrared spectroscopy [24] or conformation-dependent immunoassay [19]; (3) strains had unique thermal or chemical denaturation curves [25]. Since 2021, cryo-electron microscopy (cryo-EM) structures have been solved for PrP 27-30 from three prion strains: RML [26], ME7 [27] and 263K [28], and the folding of PrP subunits was indeed different in each strain. RML and ME7 propagate in mice while 263K propagates in hamsters, and the amino acid sequence is slightly different for the two species, which partially explains the differences in PrP<sup>Sc</sup> conformation. However, PrP sequences for RML and ME7 are the same, indicating that PrP
of the same amino-acid sequence can adopt multiple misfolded conformations.

Prions can easily infect individuals of the same species with the same PrP sequence due to their autocatalytic nature. However, transmission barriers usually exist when infecting other species or the same species with slightly different PrP sequences [29]. Infection is unable to occur or occurs with less efficiency, as seen by a longer incubation time or a lower attack rate; the efficiency increases in subsequent passages.

This can be explained by the 'conformational selection model', stating that there are a range of possible PrPSc conformations, but only a subset of them are compatible with each PrP primary structure [29, 30]. Transmission can easily occur between species or individuals if there is a large overlap of permissible PrPSc conformations, while a substantial transmission barrier exists when there is no such overlap [29, 30].

Strain mutation, that a distinct strain is propagated in the host [30], is often observed when transmission occurs between species or between individuals with different PrP sequences. This can be explained by the conformational selection model as well utilising the theory of prion quasi-species. The model of quasi-species [31] was initially built to describe the evolution of genetic species, such as replicating DNA or RNA. A species consists of many individuals with slightly different sequences caused by imperfect replication, which co-exist under neutral conditions. Upon changing the conditions, a shift of the population's mean (consensus) sequence takes place until a new equilibrium has been reached. Analogous to that, a prion strain corresponds to a 'species', which contains structurally different substrains (quasi-species). Here, they refer to structurally different misfolded PrP assemblies. A strain can breed true in the new host if the host can propagate the same dominating PrP assemblies as in the original strain; however, an apparent strain 'mutation' is seen when the new host preferentially propagates some minor assemblies of the original strain [29, 30]. This behaviour resembles the population shift of genetic quasi-species when the environment changes.
Recent studies revealed the dissociation of prion infectivity and neurotoxicity and proposed that different types of misfolded species were involved. One study suggests that prion propagation in infected mouse brains occurs in two phases (Figure 1-2) [32]. During the initial prion replication phase, the prion titer (infectivity, dotted lines) increases exponentially until reaching a maximum. The second phase then starts and lasts until the clinical onset. During the second phase, prion titre remains at maximum, while the PK-sensitive disease-related PrP isoforms (toxicity, solid lines) increase linearly, with a rate nearly proportional to the PrP<sup>C</sup> expression level. The duration of this phase is inversely proportional to the PrP<sup>C</sup> expression level. They proposed a mechanism change from the generation of infectivity (phase 1) to the production of toxicity when the infectious species acts as a catalytic surface (phase 2) [32]. A recent study [15] showed PK-resistant fibrils were highly infectious yet not directly neurotoxic to cultured neurons. The neurotoxic PrP species, not infectivity, could be destroyed by treatment with sarkosyl. The search for the toxic species (termed PrP<sup>L</sup>, L stands for lethal) is still ongoing.

**Figure 1-2.** Diagram showing two phases of prion propagation. The three mouse lines have different PrP<sup>C</sup> expression levels: Prnp<sup>+/−</sup> is with 50% wild-type PrP<sup>C</sup> expression level, Prnp<sup>+/+</sup> with wild-type expression and Tg20 with ~eightfold overexpression. The figure is taken from [32].
1.1.3 Experimental models to study the mechanism and kinetics of prion formation and propagation

Several experimental models have been developed and utilised to tackle different aspects of prion diseases.

Animal models [33] are the most relevant models to study disease progression, disease phenotype and transmission barriers *in vivo*. The most commonly used animals are rodents, including hamsters, mice and bank voles. Originally, wild-type mice were used to study species barriers by injecting sheep scrapie. The approach also generates different mouse-adapted scrapie strains such as ME7 and RML discussed above. Mouse models have greatly advanced since the development of the transgenic technique. The generation of the Prnp knock-out (prnp0/0) mice [34] in 1992 was a milestone in prion research. One direct outcome was to prove that PrP$^C$ is essential for prion propagation. It also allowed the generation of mouse lines expressing PrP sequences of other species or PrP with mutations by various transgenic and gene targeting technologies. Mouse lines expressing PrP sequences of other species removed the transmission barriers efficiently; various mouse lines were developed to study the effect of genotypes, mutations and PrP expression levels and test drugs [33]. Overall, animal models were essential to study prion transmission, reveal disease-causing mechanisms and develop therapeutic approaches. However, this approach incurs high costs, usually takes a long duration and is not well suited to analysis on the molecular level.

Cell-based models [35] provide a cost-efficient way to study prion infection and propagation. They were used to elucidate the synthetic and degradation pathways of PrP$^C$, cellular uptake mechanisms and PrP$^{Sc}$ replication sites. Multiple cell lines [35, 36] were developed to adapt different prions strains and study transmission barriers, strain mutations and therapeutic approaches. One disadvantage is that cell lines have different susceptibilities to different strains, making a direct comparison difficult. Besides, the number of cell lines that can propagate human prions is limited [37]. Cell-based assays couldn't capture the complicated mechanisms *in vivo*. In the context of drug development, drugs
found efficient in cell models are usually ineffective in vivo; drug treatment can also promote the emergence of drug-resistant prion strains [37].

Soto and coworkers developed the cell-free Protein Misfolding Cyclic Amplification (PMCA) technique [38, 39] that amplifies PrPSc seeds to generate new PK-resistant PrP and new infectivity. This technique utilises PrPSc from infected brain samples as seeds and uninfected brain homogenates as substrates to provide PrPC. The assay amplifies PrPSc using repeating sonication and incubation cycles [38]. The amplified PrPSc was shown to retain the strain-specific properties after inoculation into wild-type mice [40], namely the incubation times and neuropathological features. PMCA technique is a powerful diagnostic tool; it is also applied to study transmission barriers, reveal the effect of cofactors and identify possible drugs. However, in the current form, this technique lacks a real-time quantitative read-out needed for kinetic and thermodynamic analysis.

Another cell-free in vitro assay widely used is the Real-Time Quaking-Induced Conversion (RT-QuIC) assay [41, 42]. It utilises recombinant PrP expressed by Escherichia coli as the substrate and amplifies misfolded PrP from animal tissues [43] or body fluids [41, 42] by automatic shaking cycles. The amplification of amyloid materials is monitored by the binding of the amyloidophilic dye Thioflavin T (ThT). RT-QuIC has wide application in disease diagnosis and screening of possible inhibitors. It is a high throughput method; however, the end product usually lacks infectivity.

A wide range of recombinant PrP species has been generated, including full-length (FL) PrP of different species, their truncated forms and PrP with point mutations. They were able to form misfolded products such as amyloid by aggregation de novo, usually under quite harsh conditions involving low pH, agitation, elevated temperature or denaturants [44-47]. They were widely used in vitro to study the mechanism of de novo or seeded aggregation, the conformational conversion mechanism, and the kinetic and thermodynamic effects of PrP mutations [44]. Amyloid fibrils formed by truncated mouse PrP (MoPrP) can induce prion-like disease in transgenic mice expressing truncated MoPrP, albeit with a long incubation time [48]. Fibrils formed by full-
length MoPrP using PMCA protocol in the presence of cofactors induced prion diseases after intracerebral injection into wild-type mice [49]. Both studies suggest an important role of recombinant protein in prion research. However, the environment of a test tube cannot capture the complexity of the cellular environment; thus, it is somewhat challenging to translate results from \textit{in vitro} assays into information directly relevant to human diseases.

1.2 Introduction to protein amyloid

1.2.1 What is amyloid?

Amyloid is a protein fibril whose repeating substructure consists of β strands perpendicular to the fibril axis, forming a cross-β sheet architecture [50]. The cross-β sheet is held by backbone hydrogen bonds between adjacent β strands. Their structure features lead to a specific X-ray fibre diffraction pattern: a strong meridional reflection at ~4.7 Å representing the distance between β strands and an equatorial reflection at ~6–11 Å correlating to the distance between β sheets (Figure 1-3) [50, 51]. Amyloid fibrils can be stained with Congo red and exhibit a green birefringence under cross-polarised light [52, 53]. The characteristic diffraction pattern and Congo red binding pattern have been used as gold standards to identify amyloid.

![Cross-β structure](image)

\textbf{Figure 1-3.} The characteristic cross-β diffraction pattern of amyloid fibrils shown in X-ray fibre diffraction. The figure is from [51].
Many peptides and proteins can form amyloids in vivo. Certain amyloids have functional roles. For example, some peptide hormones are stored in the amyloid form in secretory cells, with the advantage of long storage time and quick release upon receiving a triggering signal [50]. Other amyloids are caused by protein misfolding and aggregation and are directly linked to diseases. Alzheimer’s Disease (AD) is the most prevalent neurodegenerative disorder affecting elderly people. The pathological hallmarks are extracellular amyloid deposits formed by Amyloid β (Aβ) peptide and intracellular neurofibrillary tangles formed by tau protein [54]. Other neurodegenerative diseases involving amyloid formation include, but are not limited to, Parkinson’s Disease (PD), Dementia with Lewy Bodies (DLB) and Huntington’s disease.

In the case of prion diseases, PrP 27-30 fibrils purified from diseased animal brains were shown to be amyloid based on their tinctorial property upon Congo red binding [20] and cross-β X-ray fibre diffraction pattern [55]. Atomic models were recently built for RML [26], ME7 [27] and 263K [28] prions by cryo-EM measurements and indeed exhibited parallel in-register intermolecular β-sheet (PIRIBS) amyloid structures. Thus, understanding the mechanism of amyloid formation could inform the mechanism for PrP misfolding and help understand the causes of prion diseases.

1.2.2 Amyloid generation and its pathologic role in vivo

Proteins have to fold correctly in order to fulfil their function. However, they sometimes acquire a misfolded state [56], especially in a crowded cellular environment. Proteins are more prone to misfolding when there is a relevant change in protein’s primary sequence caused by sequence expansions or mutations or an increase in concentration due to gene duplication [56]. A series of cellular machinery is employed to guide protein into the correct fold and to eliminate misfolded species. Several examples of the cellular machinery include molecular chaperones [57], proteasome, autophagy and endoplasmic reticulum associated degradation [58, 59]. However, when these
systems fail to control the generation of misfolded species, misfolded species will accumulate and possibly cause damages [59].

Protein aggregation is a complicated process involving multiple misfolded species, such as oligomers (on-pathway or off-pathway to amyloid formation), amyloid fibrils and amyloid plaques [60]. The nature of the toxic species and the mechanisms of toxicity have been widely discussed but are still poorly understood and controversial.

Disease-associated amyloid fibrils cause damage [61, 62] by interacting with cellular membranes [63], blocking nucleocytoplasmic transportation and damaging lysosomes to release toxic oligomeric species [60]. Amyloid fibrils formed by one single protein type could adopt different morphologies, which can have different cellular toxicities in vivo [60].

Although numerous studies have proved the toxicity of amyloid fibrils, some amyloid fibrils are not directly toxic or even have a protective role [64]. As an example, PrPSc of the RML strain was not directly toxic to cultured neurons even though they exhibited a high level of infectivity [15]. Amyloid fibrils could act as a sink of more toxic oligomeric species, thus reducing the overall toxicity. Disease severity was not always linked to amyloid load [65], which suggests toxic roles of species other than amyloids.

It was hypothesised that some oligomeric intermediates are the main toxic species [66]. Toxic oligomers could damage cellular membranes, react with membrane receptors [67] or produce reactive oxygen species (ROS), along with other negative effects [60, 68]. However, not all oligomers are toxic, and some types could be benign [68].

The toxic amyloid fibrils and oligomers [68, 69] could be transported between cells, thus ‘infecting’ other cells and causing broader damage.

Whether disease-associated protein amyloids are the direct cause or the outcome of diseases is controversial. Prions are considered as the cause of prion diseases, supported by disease development after inoculation in numerous transmission studies. However, emerging evidence suggests that this causality might not be true for many other neurodegenerative diseases.
For example, it was proposed that amyloid fibrils were likely the outcome of disease development in AD instead of the direct cause [70].

Prions can self-replicate by converting PrP$^C$ to the misfolded form, spread between cells and propagate among different tissues. They also show transmissibility within a species or even across species under certain circumstances. Amyloid fibrils formed by other proteins can acquire some ‘prion-like’ properties, such as the autocatalytic conversion [71] and the ability of cell-to-cell spreading [72]. However, unlike prion diseases, there is no epidemiological evidence showing the transmissibility of other amyloid-associated neurodegenerative disorders [14, 73]. The structural and mechanistic reason for the unique transmissibility of prions is still being investigated. Prions might replicate faster than other amyloids by efficient fragmentation and elongation, which outcompetes the removal by clearance and dilutional effect by cell division [29]; their relatively stable structures might provide higher stability against degradation.

1.2.3 Models of amyloid formation

The exact amyloid formation mechanism is still under debate. Iadanza and co-workers [60] reviewed the currently well-recognised mechanism, as shown in Figure 1-4. A slow primary nucleation process leads to the formation of oligomers from monomers. Those oligomers are transient and heterogeneous and could be either on-pathway to produce amyloid fibrils or off-pathway species. Oligomers of a particular size are recognised as the critical nucleus, the most unstable species. Once the critical nucleus is formed, it can rapidly recruit monomers and elongate, forming β-sheet-rich amyloid fibrils. Fragmentation of the existing fibrils and surface-catalysed secondary nucleation substantially increase the number of aggregates and the rate of amyloid formation. Fast elongation leads to a rapid increase of fibril mass. Depolymerisation also takes place at a much slower rate than elongation. Amyloid formation eventually plateaus, and fibrils can finally deposit as large, inert plaques [60].
Figure 1-4. Schematic figure showing key steps of amyloid formation. (A) The overall aggregation mechanism. The figure is revised from [60]. (B) Sketches of individual steps. Each orange ellipse represents a protein monomer; the stacked green rectangles represent amyloid fibrils, with each green rectangle representing a building block converted from a protein monomer. The figure is modified from [74].

Protein aggregation may involve all or a subset of the processes discussed above. The simplest model, nucleation-dependent polymerisation (NDP), including primary nucleation and elongation (growth), was initially described
by Oosawa and Asakura [75] to account for the formation of protein polymers. Ferrone and co-workers [76] later proposed the involvement of secondary nucleation in addition to the NDP mechanism for the polymerisation of sickle haemoglobin. This model can describe the aggregation of Aβ42 as well [77]. The Knowles Group [78] later built models considering nucleation, fibril growth and fragmentation, which can describe the aggregation kinetics of insulin, β2-microglobulin (β2M) in vitro and prion propagation in vivo.

For PrP misfolding and aggregation, Prusiner [79] proposed one of the earliest models to describe the formation of disease-related PrP. In the model, PrP<sup>C</sup> and PrP<sup>Sc</sup> differ in their conformation. A partially unfolded monomer, denoted as PrP<sup>*</sup>, is generated by the fluctuation in PrP<sup>C</sup> conformation, and it serves as an intermediate to form PrP<sup>Sc</sup>. In sporadic prion diseases, PrP<sup>*</sup> might accumulate to form PrP<sup>Sc</sup> under a very rare circumstance; in inherited prion diseases, PrP<sup>C</sup> is destabilised and can form PrP<sup>*</sup> more easily, leading to the formation of PrP<sup>Sc</sup> with a higher probability. In acquired prion diseases, the pre-existing PrP<sup>Sc</sup> serves as the seed to structurally convert PrP<sup>*</sup> into the misfolded form. At that time, it was unclear whether PrP<sup>Sc</sup> was a misfolded monomer or aggregated assembly.

The Lansbury model [80] considers PrP<sup>Sc</sup> as an aggregate whose formation follows the nucleation-dependent mechanism of amyloid formation. Unlike short peptides or intrinsically disordered proteins (IDPs), native PrP has a compact fold; thus, a (partial) unfolding step is required prior to the misfolding process. The precursor, a partially unfolded PrP<sup>U</sup>, nucleates to form PrP<sup>Sc</sup>.

The current understanding of prion replication is largely based on the combination of both models, as shown in Figure 1-4 A. Masel and co-workers [81] built a model that included primary nucleation, elongation of nuclei and fibrils, fragmentation of fibrils and degradation of monomers and fibrils. They estimated parameters by fitting the model to published experimental data. The data included infectivity or PrP<sup>Sc</sup> accumulation over time or incubation time in animal brains (1) infected with different strains, (2) infected with different doses of PrP<sup>Sc</sup> and (3) using different mice models expressing different levels of PrP<sup>C</sup>. Their model was found to agree with the experimental data and served
as a good candidate to explain the mechanism of prion formation and propagation in vivo [82].

1.3 Amyloid fibril structures at atomic resolution

1.3.1 Structures of prion fibrils

A. RML

Figures 1-5. Schematic representations of the core structures of subunits in RML (A), ME7 (B) and 263K (C) strains. Each amino acid side chain is depicted as a circle. Each β-strand is plotted with a line with an arrowhead. The figures are taken from [26-28].

The atomic structure of amyloid has been a critical question to solve. Structural information can help understand why they are toxic, provide indirect but vital information about how misfolding occurs and can be used to design inhibitors. However, amyloid fibrils are insoluble, heterogeneous and unable to form...
crystals, making it impossible to determine their structures by conventional methods such as X-ray crystallography and NMR. In recent years, the cryo-EM technique has rapidly developed and become a valuable tool for studying amyloid structures.

The atomic structures of several prion fibrils from multiple sources have been experimentally determined. The biologically most meaningful structures are authentic prion fibrils purified from diseased animal brains, and researchers have derived structure models for RML [26] and ME7 [27] prion strains propagating in mice and 263K strain [28] propagating in hamsters. Structures of two amyloid fibrils formed in vitro from recombinant full-length or truncated human PrP (HuPrP) were also elucidated [83, 84].

Purification of RML from brain homogenate [26] involved PK digestion, and the purified fibrils were essentially PK-resistant cores (PrP 27-30), including residues 89 to the C terminus. Banding patterns on Western blot revealed the relative abundance of different glycosylated species was mono- > di- > non-glycosylated PrP. 90% of RML fibrils were single-strand. They adopted left-handed twisted, helical structures, with a width of ~10 nm and a half-pitch length of ~135 nm. The other 10% were paired, two-strand structures with a width of ~20 nm and a half-pitch length of 150 to 180 nm.

The atomic model was obtained at a 2.7-Å-resolution for residues T94 to Y225 for single-strand fibrils (Figure 1-5 A). Fibrils adopted parallel in-register intermolecular β-sheet (PIRIBS) structures. Each ‘rung’ of fibril was composed of one PrP molecule with 15 identified β-strands. The N terminal region (from T94 to roughly S169) adopted a double-hairpin conformation; the C terminal region adopted a single-hairpin structure connected by a disulfide bond, with N-linked glycans and GPI anchor pointing outwards of the fibril into the solvent. The compact fold of the PrP molecule was further stabilised by the intramolecular hydrophobic effect and polar interactions. The PrP subunit was not coplanar; the N-terminal region interacted with the C-terminal lobe of the consecutive rung at the interface.

Structures of ME7 fibrils (Figure 1-5 B) [27] shared many similarities with RML with certain differences. ME7 predominantly existed as single-strand fibrils
with a half-pitch length of ~158 nm, and a small fraction was double-strand. ME7 contained a larger fraction of PrP in the di-glycosylated state compared to RML. The PK-resistant core involved the same residues as in the RML strain. A PIRIBS architecture was identified with 15 β-strands. In each PrP subunit, the N and C terminal lobes acquired a double-hairpin and single-hairpin structure, respectively, similar to the corresponding structures in RML. However, differences existed, especially in the N terminal lobe involving residues A112-G130, which might explain the narrower angle between the two lobes in the ME7 strain. A ~half-rung distance was seen for the two lobes at their interface.

Both RML and ME7 strains propagated in the same species, and the two studies revealed that PrP of the same sequence could fold into different structures, which encodes the properties of different strains.

For the 263K strain [28], the atomic model was obtained at a 3.1-Å-resolution for the core region (residues 95-227, hamster sequence) of PK-treated fibrils (Figure 1-5 C). 263K fibrils were left-handed and single-strand with a width of ~13 nm. They adopted PIRIBS architectures, and one molecule with 11 β-strands made up one ‘rung’. The conformation of the N terminal lobe was similar to that of the RML strain, with an extra hydrophobic ‘Greek key’ motif

between residues 112 to 134 (hamster sequence). The C-terminal conformation of the 263K strain was remarkably different from that of the RML strain: a double-hairpin conformation was observed in 263K instead of a single-hairpin in RML, and densities from K194 to E196 were poorly resolved, suggesting these residues might be flexible. The groove between the N terminal lobe and the C terminal lobe was wider in 263K fibrils, which might provide larger space for glycans at N181 and account for mainly di-glycosylated PrP in 263K. N-linked glycans and GPI anchors pointed outwards into the solvent, similar to RML fibrils.

Studies using animal models indicated that the transmission of the 263K strain into mice exhibited a transmission barrier [85]. In the first passage of mice inoculated with the 263K strain, no clinical sign was observed for 2 years. However, subclinical infection was detected (PrPSc was first detected in a
mouse on day 310). Stain adaption occurred in later passages: mice injected with a certain inoculum developed clinical disease. The transmission barrier could come from difficulties of templating mouse PrP\textsuperscript{C} into 263K PrP\textsuperscript{Sc} structure – even though only 8 residues are different between mouse and hamster PrP sequences within the fibril core region of the 263K strain.

In contrast to the prion quasi-species model [30] stating that a cloud of structurally heterogeneous PrP assemblies co-exist in a strain, here, cryo-EM only identifies one or two fibril types in each strain. The possible reason is that some sub-species are less populated or relatively unstable and are lost during the purification process. In addition, the less populated structures in the purified samples, if any, might not be identified by cryo-EM.

Structures of PrP amyloid generated \textit{in vitro} were determined and were markedly different from authentic prion fibrils.

Glynn and co-workers [83] presented a cryo-EM structure of amyloid fibrils formed by unglycosylated HuPrP 94-178. Fibrils generated by agitating recombinant PrP under partially denaturing conditions (4 M urea, pH 4.0) were subjected to PK treatment. Unbranched and twisted fibrils were the most abundant species, whose atomic structure was obtained at a 3.5-Å-resolution for residues K106 to Y145. Fibrils were left-handed, made of two symmetric protofilaments and acquired PIRIBS architectures. Each rung in a protofilament consisted of one PrP molecule. The molecule folded into a β arch with four β strands, generating a tight and hydrophobic intramolecular interface. The intermolecular interface between protofilaments involved residues 107 to 125 of opposing PrP subunits in the two protofilaments, where hydrophobic side chains formed a tight and dry interface.
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<th><strong>Table 1.</strong> Comparison of structure models for different PrP fibrils.</th>
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Wang and co-workers [84] generated PrP fibrils from full-length HuPrP under denaturing conditions (2M GdnHCl, pH 7.4). An atomic model was built for core regions involving residues 170-229 at a 2.7-Å-resolution. Fibril width was ~25 nm, and the width of the fibril core was ~14 nm. Similar PIRIBS structures were observed. Full-length fibrils were left-handed. They contained two protofilaments with one PrP subunit making up a rung in each protofilament. Each PrP monomer folded into a hydrophilic outer surface and a hydrophobic core. The intermolecular interface involved only two residues of each subunit (K194 and E196) forming salt bridges, different from the large interface seen in HuPrP 94-178 fibrils [83].

The structural properties obtained from cryo-EM models for the five types of fibrils are summarised in Table 1. All fibrils acquire PIRIBS structures typical for amyloids and exhibit left-handed twisting morphologies. Structures for the five types of fibrils are different, with some shared properties, while polymorphism in a sample was often observed. Authentic fibrils have a larger PK-resistant core. Most of them are made of only one strand. They are glycosylated to a different level and with a GPI anchor for membrane attachment. As a comparison, the two fibrils formed in vitro have a much smaller PK-resistant core, which might help explain the less or no infectivity of in vitro generated fibrils since they might be eliminated by the degradation machinery more easily. These synthetic fibrils are composed of two intertwining protofilaments with no post-translational modifications.

1.3.2 Structural heterogeneity of amyloid fibrils formed by other amyloidogenic proteins

High-resolution atomic structures have been solved for multiple amyloid fibrils, including synthetic fibrils aggregated from recombinant proteins [86-89] and authentic fibrils purified from patients [90]. These structures are reviewed in [91, 92]. Similar to the case of PrP, one protein could fold into fibrils with several distinct structures, suggesting structural heterogeneity might be a common feature for amyloid fibrils.
One system that has drawn considerable attention is amyloid fibrils formed by Aβ peptides. Aβ peptides in humans come from amyloid precursor protein (APP) after cleavage by beta- and gamma-secretase and can be 39-43 amino-acid long [93, 94]. The two most common Aβ peptides contain 40 or 42 amino acids [95], termed as Aβ40 or Aβ42, respectively. Amyloid fibrils formed by them are a major component of extracellular plaque found in the brain of patients with AD. Aβ deposits are present in other clinical diseases, such as Parkinson’s Disease Dementia (PDD) and Dementia with Lewy bodies (DLB). Aβ deposits are also found in clinically normal elderly individuals [96].

Atomic structures of Aβ42 amyloid fibrils [97] from patients with different pathological conditions were identified by cryo-EM, revealing two major fibril types. Type I fibrils were left-handed, consisting of two identical protofilaments adopting pseudo-2:1 symmetry. Residues 9-42 formed the ordered core with six β-strands, where residues 19-42 adopted an S shape, and residues 9-18 formed an extended arm. A large and mainly hydrophobic interface was formed between two protofilaments, involving part of the outer surface of the S-shaped domain and the extended arm. Another fibril type, type Ib, was made by two type I fibrils packing side by side.

Type II fibrils were left-handed and were made of two protofilaments packed with C2 symmetry. The ordered core involved residues 12-42. Residues 20-42 adopted a similar, but not identical, S shape as in type I fibrils. The interfaces between two protofilaments were much smaller than type I fibrils [97].

The two types of fibrils had different prevalence in patients with distinct disorders [97]. In patients with sporadic AD, type I fibrils were the sole or major type identified. Type Ib or type II fibrils were present in some patients at a small percentage. In familial AD, type II fibrils were the only or main type. In patients with other disorders such as DLB or PDD, only type II fibrils were observed [97]. The fact that one type of fibril is dominant in a type (or a subtype) of disorders suggests a possible link between fibril structures and disease phenotypes.
Aβ42 fibrils formed *in vitro* exhibited different structural features compared to authentic fibrils. One type of fibrils was generated by incubating recombinant monomer solution containing organic cosolvent at low pH [86]. All 42 residues were properly located in the model built by cryo-EM. Structurally, fibrils showed an approximate 2₁ screw symmetry consisting of two twisted protofilaments. Each molecule folded into an LS shape. The first open circle of the S-shaped region at the C terminus resembled that of authentic type I and II fibrils [97] with minor differences. However, larger differences were seen in other parts, especially in the N terminus, where the first 8 or 11 residues were disordered in authentic type I or type II fibrils, respectively.

An earlier cryo-EM study [98] on Aβ42 fibrils generated under neutral pH showed a different internal packing and intermolecular interface. Fibrils were made of two protofilaments and acquired a two-fold symmetry with left-handed twists. The C terminal region of each molecule was located at the fibril core, forming β-sheets. This region also formed the intermolecular interface between protofilaments. The N terminal domain protruded outwards from the core, forming hairpin-like morphologies. However, a detailed structural model was not obtained due to the lower resolution (~7 Å).

Solid-state NMR studies [99, 100] revealed more structures that Aβ42 fibrils could adopt. Overall, structural studies on Aβ42 fibrils from different sources have confirmed that one peptide could misfold and aggregate into amyloid fibrils with distinct structural arrangements.

Structural polymorphism was observed for other peptides and proteins such as Aβ40 [90, 101], tau [102] and α-Synuclein (α-Syn) [103, 104]. The polymorphism arises from different folding within a single polypeptide chain, distinct interfaces and staggering patterns between protofilaments, and varied numbers of protofilaments.

1.4 Project rationale

Prion diseases are associated with different misfolded assemblies of PrP<sup>C</sup>, among which PrP<sup>Sc</sup> is the main infectious species. The established models of
prion propagation in vivo [78, 81] emphasises the critical role of elongation and fragmentation of existing PrPSc. PrPSc elongates by recruiting and transforming PrP\textsuperscript{C} into the misfolded form. However, the mechanism and kinetics of the elongation process have not been well understood.

Here, using recombinant MoPrP 91-231 as a model system, I studied the elongation kinetics of synthetic amyloid seeds on a single-particle level using TIRFM. The aim was to understand the elongation mechanism of amyloid fibrils and to test whether fibrils were structurally and mechanistically homogeneous or whether multiple fibril structures and assembly mechanisms could co-exist. The established method was then applied to study authentic prion fibril elongation to test the propagation properties of disease-associated PrP fibrils.

Structural polymorphism was observed for amyloid fibrils of PrP, either recombinant fibrils or authentic PrP\textsuperscript{Sc}. In this work, I imaged fibrils using novel super-resolution microscopy, including transient amyloid binding (TAB) microscopy and polarised TAB microscopy, aiming to extract unique microscopic properties of the structurally distinct amyloid fibrils from heterogeneous fibril populations.
Chapter II. *In vitro* dynamic analysis to study kinetics and thermodynamics of amyloid formation and propagation

2.1 Spontaneous aggregation assays conducted in bulk solution

Kinetic and dynamic studies of amyloid formation were initially carried out in bulk solution *in vitro*. The formation of aggregates under controlled conditions can be monitored by multiple approaches [105], including light scattering, tryptophan fluorescence and fluorescent dyes such as ThT. Each approach has limitations. Light scattering measurements couldn’t distinguish amyloid or non-amyloid aggregates such as amorphous aggregates; the signal of large aggregates can mask the small ones. ThT fluorescence is not sensitive to the formation of small oligomers and is affected by exogenous compounds [106]; the presence of ThT in solution might interfere with aggregation kinetics [107]. Several amyloidogenic polypeptides, such as Aβ and α-Syn do not contain tryptophan residues.

Assays carried out in bulk solution provide valuable information on the overall aggregation kinetics, while it is challenging to determine the exact mechanism, i.e. the molecular processes involved. Molecular processes in aggregation [108] include events that increase the number of aggregates (primary nucleation, secondary nucleation and fragmentation) and events that change aggregate mass (mainly elongation and dissociation). Secondary nucleation and elongation sometimes involve multiple elementary steps, i.e. attachment of reactants and structural rearrangement, and these steps might need to be considered separately. Both nucleation and elongation are essential for *de novo* aggregation; the main question to solve is whether secondary nucleation or fragmentation is dominant and whether elongation or secondary nucleation needs to be considered as a multistep process.

Perturbation analysis [109] is traditionally used to determine whether secondary processes are dominant. This method analyses the kinetic in the initial aggregation phase. For a primary nucleation-dominated mechanism, the kinetics in the initial phase can be described by a cos(t) function; if one secondary process dominates, the formation of new aggregates by secondary
pathways leads to a sudden increase in aggregate mass after a lag phase, and the kinetics in this phase is consistent with a \( \cosh(t) \) function.

One useful parameter, the scaling exponent \( \gamma \), can infer the aggregation mechanism. \( \gamma \) is obtained from the relationship between aggregation half time \( (t_{50}) \) and initial monomer concentration \( (m_0) \) \cite{110}:

\[
t_{50} \propto m_0^{\gamma}
\]

\( \gamma \) describes the monomer dependence of the dominating process \cite{110}. Since each dominating process is related to a unique or a range of \( \gamma \) values, determination \( \gamma \) can narrow down the choice of models. In certain models, \( \gamma \) changes with monomer concentration, reflecting a changing monomer dependence of the dominating process \cite{108, 110}.

The aggregation mechanism could be inferred by fitting rate equations defined by different models to the experimental data. Recently, a global fitting approach, the web-based software AmyloFit \cite{108}, was developed for this purpose. The best-fit model may be obtained from fitting, as well as kinetic parameters such as rate constants and reaction orders. However, as with all bulk assays, this approach can only yield average kinetic values and masks heterogeneities in the fibril population.

### 2.2 Seeding assays

As discussed, amyloid formation involves primary nucleation, growth (elongation), dissociation and possible secondary processes (fragmentation and secondary nucleation). The slow primary nucleation step can be bypassed by adding preformed seeds into the monomer solution. By carefully tuning conditions for certain protein systems, secondary processes can become negligible \cite{111}. As a result, elongation dominates seeded aggregation assays under specific conditions and aggregation can be greatly simplified, allowing elongation related kinetic parameters to be directly obtained. A number of studies on amyloid seeding assays have been carried out and summarised in an extensive review by Buell \cite{105}.  

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Seeding assays can be performed in bulk solution by adding a small number of preformed aggregates into monomer solution and monitoring the kinetics. Surface-based techniques are another way to perform ensemble measurements, which utilise biosensors such as surface plasmon resonance (SPR) [112] or quartz crystal microbalance (QCM) [113]. SPR sensitively measures the change in the refractive index of the medium on the sensor surface upon molecule binding [114]. QCM detects the change in mass by measuring the resonance frequency of the quartz crystal oscillator [115]. The ability to attach amyloid fibrils onto the sensor surface and study the size/mass change makes these techniques excellent choices for studying amyloid fibril elongation and dissociation.

However, the ensemble approaches cannot provide information on individual amyloid fibrils. Another disadvantage is related to the assumption that elongation is the only process taking place; other processes can alter the kinetics while information about them can't be reliably obtained in ensemble measurements. On the other hand, single-particle experiments can track every single particle and eliminate the uncertainty caused by multiple processes, since any process other than elongation could be directly identified (limited by the resolution limit of the method used).

*In situ* atomic force microscopy (AFM) or total internal reflection fluorescence microscopy (TIRFM) have been utilised in single-particle experiments. Their ability to monitor the change of a single particle in real-time at a high spatial resolution not only enables the determination of kinetic parameters but also gives information on the growth pattern (e.g. growth directionality, steady growth or ‘stop-and-go’ growth, monomer or oligomer addition), and structural or kinetic heterogeneity [116-120]. All of the information is valuable to understanding the molecular mechanism underlying elongation but cannot be obtained from ensemble measurements. If structural information is also available, a link could be built between fibril structure and kinetics.

There are three major ways to perform a single-particle study: (a) monitor seed elongation on mica surface *in situ* using AFM, or more recently, high-speed AFM; (b) observe seed elongation on coverslip surface *in situ* by TIRF.
microscopy; (c) perform seeding in solution, take out aliquots at multiple time points and monitor the progress of elongation by TIRF, AFM or TEM.

The first method has been utilised over decades [116, 118, 121]. The high resolution of AFM makes it possible to distinguish different structures and determine fibril length with high precision. However, measurements are technically challenging and slow, the charged mica surface might interfere with fibril assembly, and the contact between the probe tip and the specimen during scanning could cause fibril breakage.

Method (b) is easy to set up and has less disturbance to the system compared to AFM. The glass coverslip surface can be easily modified to study how different surface properties influence amyloid growth. However, the glass surface possibly influences protein aggregation as well. Since the resolution of TIRF imaging is limited by light diffraction, it is difficult to differentiate distinct structures and determine fibril length with high precision. This resolution limit is greatly overcome with newly developed super-resolution techniques [122, 123].

The solution-based assays (method (c)) eliminate the influence of substrate surface on fibril growth. They can be conducted in two ways. The first approach measures the fibril length distribution for seeded samples after different incubation times [124]. However, seeds cannot be identified, making it impossible to obtain the elongated length for each fibril. Alternatively, seed particles can be labelled so that seeds and elongated segments are distinguishable [123, 125, 126]. The labelling can be done using fluorophores (for TIRF imaging [123]), antibodies (for AFM [125]) or nano gold particles (for TEM [126]). This approach allows the determination of fibrils’ growth lengths. The average rate, growth directionality and kinetic heterogeneity among fibrils can then be derived. However, sample withdrawal might fragment fibrils by shear forces. Without in situ observation, the information provided by this kind of study is limited to ensemble data as it cannot analyse the kinetics of individual fibrils.

The study of single-aggregate dynamics, including oligomer formation and fibril elongation, has been performed for many amyloidogenic peptides or
proteins, such as Aβ40 [116-118], Aβ42 [119, 120, 127, 128], amylin [129], α-Syn [123, 130-132], yeast prion Sup35 [125, 126, 133-136], and PrP [137-139]. Those studies shed light on the molecular mechanism of amyloid formation. The discrepancies observed for different proteins and also for the same protein in different studies reveal the complexity of amyloid formation.

2.3 Kinetic models used for seeding assays

2.3.1 Determining elongation/dissociation rate constants using simple reaction models

Elongation and dissociation are not one-step reactions; instead, they are more complex, including several elementary steps. However, a few simple reaction models agree with many experimental findings, suggesting they capture the real reactions under the experimental conditions. The analysis using simple models is easy to carry out and provides a useful way to compare kinetic parameters obtained from different experiments. Here, I present kinetic models that have been proposed in the literature. The following analysis is based on the hypothesis that elongation (with or without dissociation) is the only reaction taking place, meaning there is no primary nucleation, secondary nucleation and fragmentation.

(1) Bimolecular reaction model with a nearly constant monomer concentration

The two reactants in elongation are monomer in solution (M) and fibril end (E).

The simplest model considers elongation as an irreversible bimolecular reaction, \( M + E \rightarrow P \), where \( P \) represents the product which is equivalent to fibril end \( E \). The elongation reaction rate can be expressed as:

\[
 r = k C_M C_E 
\]

where \( r \) is the rate, \( k \) is the rate constant (assumed to be the same for all fibrils), and \( C_M \) and \( C_E \) are the concentrations of monomers and fibril ends, respectively. Under the assumption that the number of seed fibrils doesn’t change, \( C_E \) is a constant.
If the concentration of soluble proteins can also be considered a constant, the calculation of the rate constant $k$ is straightforward:

$$k = \frac{r}{C_M C_E}$$

The assumption of a constant monomer concentration is valid if the seed concentration is very small compared to the monomer concentration, and only the initial rate is studied when a small portion of monomer is consumed. Reaction rates can be measured indirectly by ThT fluorescence or light scattering representing the increase of fibril mass, by the fibril mass change on a surface using QCM or by the length increase of individual surface-bound seed fibrils using AFM or TIRFM. The measured parameters need to be converted to the absolute reaction rate $r$. Thus, $k$ can be calculated at specific monomer and seed concentrations.

The model predicts elongation rate $r$ to increase linearly with monomer concentration $C_M$ when seed concentration is kept unchanged between experiments, which can be easily tested experimentally.

(2) Bimolecular reaction model with a depleting monomer concentration

If the change of monomer concentration is substantial, the reaction rate can be described as:

$$r = -\frac{dC_M}{dt} = kC_M C_E$$

Solving this differential equation yields the monomer concentration ($C_M$) as a function of time ($t$):

$$C_M = C_{M,0} \exp(-kC_E t),$$

where $C_{M,0}$ is the initial monomer concentration. Thus, when $C_E$ is constant, the reaction can be treated as a pseudo-first-order reaction, and $C_M$ follows an exponential decay. $k$ is then calculated from the slope of $\ln C_M$ against $t$:

$$\ln C_M = -kC_E t + \ln C_{M,0}$$

One difficulty here is to measure the change in monomer concentration sensitively. One way is to centrifuge reaction mixtures taken at different time points and measure the concentration in the supernatant. Alternatively, in ThT
assays, the free monomer concentration can be calculated from ThT fluorescence. However, this approach needs to be treated with caution since not all monomers may be converted into aggregates at the plateau phase of aggregation, and ThT fluorescence may not be proportional to monomers incorporated into fibrils.

(3) Reversible reaction model

As is clear from the studies on amyloid formation [60], fibril elongation is a reversible process, including a dissociation reaction. Hereafter, the rate constant for elongation will be written as \( k_+ \) (which is \( k \) in the previous sections), and the dissociation rate constant is \( k_- \).

If considering dissociation as a simple unimolecular reaction with fibril ends as the reactant, its rate can be expressed as:

\[
 r_- = k_- C_E
\]

Fibril dissociation can be monitored experimentally [140] by diluting a solution of mature fibrils in buffer and measuring the monomer concentration \( C_M \) at multiple time points. At the beginning phase of this experiment, monomer concentration is relatively low, so that the elongation reaction can be neglected. The concentration of fibril ends is considered a constant. Thus:

\[
 r_- = \frac{dC_M}{dt} = k_- C_E
\]

\[
 C_M = C_{M,0} + k_- C_E t
\]

where \( C_{M,0} \) is the initial monomer concentration. \( k_- \) can then be determined.

When the reverse reaction is added to the elongation reaction scheme \( (M + E \rightleftharpoons P) \), \( E \) acts as a reactant for the forward reaction and \( P \) as a reactant for the reverse reaction, while both represent equal numbers of fibril ends. Thus, \( C_P \) and \( C_E \) are equivalent. Assuming the number of fibrils doesn’t change with time, the reaction rate can be written as:

\[
 r = -\frac{dC_M}{dt} = k_+ C_M C_E - k_- C_P = k_+ C_M C_E - k_- C_E
\]

Solving this differential equation, we get:
\[ \ln \left( \frac{C_{M,0} - C_{M,eq}}{C_M - C_{M,eq}} \right) = k_+ C_E t \]

where \( C_{M,eq} \) is the monomer concentration at equilibrium, \( C_{M,eq} = \frac{k^-}{k^+} \). \( k_+ \) can be determined once monomer concentrations at multiple time points, including at equilibrium, are experimentally determined.

(4) Elongation as a two-step reaction

The models discussed above assume that elongation can be simplified to a one-step process. However, this assumption is not always valid. The currently wide-accepted two-step ‘dock-lock’ model for amyloid fibril elongation [141] proposed an initial reversible ‘dock’ of a monomer onto the fibril end and a subsequent irreversible ‘lock’ when the attached monomer is incorporated into the fibril by structural rearrangement. The locking step is usually slow, involving a high-energy transition state.

This reaction scheme is analogous to the Michaelis-Menten (MM) mechanism in enzyme reactions, which also includes two individual steps: the binding of the substrate to the enzyme and a structural conversion from the enzyme-bound substrate to the product with the aid of the enzyme.

The one-step reaction model is only reasonable at low monomer concentrations when the absolute rate of the docking step (monomer attachment) is much slower than the locking step (structural rearrangement). At high monomer concentrations, the structural rearrangement, whose rate is not dependent on monomer concentration, can become rate-limiting. This leads to a ‘saturation’ of the overall rate as a function of monomer concentration.

The two-step reaction scheme can be expressed as follows:

\[ F_{n-1}E + M \xrightarrow{k_1} F_{n-1}EM^* \xrightarrow{k_2} F_nE \]

where \( F_{n-1}E \) represents a fibril made of \( n \) monomers, one of which is the active fibril end prone to elongation, \( M \) represents the monomer, and \( F_{n-1}EM^* \) is the docked intermediate, i.e. the monomer-bound-fibril complex. Since only the
fibril end E of a fibril enters the reaction, the reaction scheme can be simplified to:

\[ E + M \xrightarrow{k_1/k_{-1}} EM^* \xrightarrow{k_2} E \]

Similar to rate equations for the MM kinetics, the overall reaction rate as a function of substrate (monomer) concentration can be described by the following equation:

\[ r = \frac{R_mC_M}{K_m + C_M} \]

where \( r \) represents the pause-free rate at a monomer concentration \( C_M \), \( R_m = k_2 \cdot C_{E,\text{total}} \) represents the maximum rate when all fibril ends are occupied by monomers in the form of \( EM^* \), and \( C_{E,\text{total}} \) is the concentration of fibril ends in the system. \( K_m \) stands for a composite rate constant \( K_m = (k_{-1} + k_2) / k_1 \). By fitting the above elongation to rates at various monomer concentrations, \( k_2 \) and \( K_m \) can be derived.

### 2.3.2 Determining thermodynamic parameters

(1) Equilibrium constant and Gibbs free energy change

Since amyloid elongation/dissociation is a reversible reaction (refer to the model in 2.3.1 (3); \( M + E \rightleftharpoons P \)), the standard free energy change \( \Delta G^0 \) can be calculated from the equilibrium constant \( K \):

\[ \Delta G^0 = -RT \cdot \ln K \]

where \( R \) is the molar gas constant.

The equilibrium constant \( K \) is obtained by the following equation:

\[ K = \frac{k_+}{k_-} = \left( \frac{C_{P,eq}}{C_{M,eq}C_{E,eq}} \right) = \frac{1}{C_{M,eq}C_{E,eq}} \]

\( K \) can be determined once \( k_+ \) and \( k_- \) are obtained experimentally. \( K \) can also be calculated as the reciprocal of the monomer concentration at equilibrium, \( C_{M,eq} \). This concentration is also known as the critical concentration \( C_r \).
Theoretically, \( C_r \) could be derived from either the aggregation reaction (spontaneous or seeded aggregation) or the fibril dissociation reaction. It is not affected by the seed concentration or total protein concentration in the system, provided the total protein concentration is higher than \( C_r \). One way to determine \( C_r \) is to accurately measure the monomer concentration of an aggregation or dissociation assay at different time points and find the asymptotic value when the system approaches equilibrium. However, \( C_r \) is usually low and difficult to measure accurately.

An alternative strategy to overcome this difficulty is introducing denaturants into the reaction system. In analogy to protein folding, the simplest model describing the effect of denaturants is that free energy change of elongation reaction increases linearly with denaturants concentration,

\[
\Delta G_D = \Delta G^0 + m \cdot C_D
\]

where \( \Delta G_D \) represents the free energy change of the reaction at a specific denaturant concentration \( C_D \). \( m \) is a constant.

A higher \( \Delta G_D \) in the presence of denaturants means a lower equilibrium constant and a larger critical concentration so that it can be measured more accurately. \( \Delta G_D \) at a specific denaturant concentration \( C_D \) can be obtained by the measured critical concentration \( C_{r,D} \):

\[
\Delta G_D = -RT \cdot \ln \frac{1}{C_{r,D}}
\]

From a linear fit of \( \Delta G_D \) and \( C_D \), \( \Delta G^0 \) and \( m \) are determined.

It’s worth noting that the different amyloid fibril isomorphs formed by the same peptide could acquire unique \( K \) values.

(2) Activation energy obtained from the temperature dependence of elongation rate

The relationship between reaction rate constant \( k \) and temperature \( T \) is usually described by an empirical equation, Arrhenius law:

\[
k = A \exp \left( -\frac{E_a}{RT} \right)
\]
where $A$ is a pre-exponential factor; $E_a$ is activation energy which is the minimum energy needed for a reaction to take place; both of them are usually considered constant in the temperature range studied for amyloid growth.

This equation implies that the rate constant $k$ increases with temperature. Generally, higher activation energy represents a smaller rate constant and means the rate constant is more sensitive to temperature change.

The above equation can be converted to:

$$\ln k = \ln A - \frac{E_a}{R} \cdot \frac{1}{T}$$

When elongation rate constants $k$ under multiple temperatures are experimentally obtained, $A$ and $E_a$ can be directly derived through a linear fit of $\ln k$ and $\frac{1}{T}$. Note that $T$ needs to have a unit of K; $E_a$ is not influenced by the chosen unit of $k$.

(3) Activation enthalpy $\Delta H^\ddagger$, activation free energy $\Delta G^\ddagger$, and activation entropy $\Delta S^\ddagger$

Transition state theory can be used to describe the reaction process of an elementary reaction. It states that reactant molecules approach each other and form ‘activated complexes’. They are in quasi-equilibrium with the reactants and can convert into products: $A + B \rightleftharpoons AB^\ddagger \rightarrow P$. Those complexes $AB^\ddagger$ are in the ‘transition state’. They have the highest potential energy on the reaction path.

One can define activation enthalpy $\Delta H^\ddagger$, activation free energy $\Delta G^\ddagger$, and activation entropy $\Delta S^\ddagger$ as the differences in enthalpy, free energy, and entropy, respectively, between the activated complexes and the reactants.
Figure 2-1. Illustration of activation free energy $\Delta G^\dagger$ and Gibbs free energy change $\Delta G$.

For liquid-phase reactions, $\Delta H^\dagger$ can be derived as:

$$\Delta H^\dagger = E_a - RT$$

where $E_a$ is the activation energy of the overall reaction. RT is usually much smaller than $E_a$ in the temperature range we are interested in, so in practice, we use:

$$\Delta H^\dagger \approx E_a$$

The relationship between reaction rate constant $k$ and $\Delta G^\dagger$ is derived as:

$$k = B \exp\left(\frac{-\Delta G^\dagger}{RT}\right)$$

For the seeding experiment, the relationship was further derived in literature [142]:

$$\frac{dN}{dt} = \Gamma \exp\left(\frac{-\Delta G^\dagger}{RT}\right)$$

where $N$ is the number of monomers aggregated in the fibrils; $\Gamma$ is a diffusive pre-factor, which is the number of monomers entering the reaction volume per unit time. $\Gamma$ can be calculated under certain conditions from monomer concentration, diffusion coefficient and the effective reaction volume at fibril ends [143]. Here, the elongation reaction is seen as a result of reactants
diffusing and crossing a single free energy barrier \cite{113}. Thus, $\Delta G^\ddagger$ can be calculated from:

$$\Delta G^\ddagger = -RT \cdot \ln \left( \frac{dN}{dt} \right)$$

$\Delta S^\ddagger$ can then be easily calculated:

$$\Delta S^\ddagger = \frac{1}{T} (\Delta H^\ddagger - \Delta G^\ddagger)$$

2.3.3 Studies on fibril elongation in literature

Here, I present \textit{in situ} single-molecule studies on amyloid elongation for multiple proteins in literature, emphasising the information that can only be derived from this type of studies, such as fibril growth patterns and any structural or kinetic heterogeneities.

(1) Aβ40

An early study \cite{116} utilised AFM to study \textit{de novo} aggregation of the Aβ40 peptide and the elongation of mature fibrils. In a fresh Aβ solution, oligomers were observed immediately, followed by worm-like protofibrils forming after $\sim$2 h. Neither oligomers nor protofibrils could form mature fibrils directly. Protofibrils grew either unidirectionally or bidirectionally with stall phases. No mature fibrils were observed for up to 12 h.

In the presence of surface-attached mature fibrils and a monomer solution, they observed the formation of oligomers as well as intermittent elongation of seeds with a maximum growth rate of $20 \pm 12$ nm/min, much faster than the protofibrils. Mature fibrils had an axial periodicity of 80 to 130 nm. Interestingly, TEM images of fibrils formed in bulk solution revealed one more fibril type with a shorter periodicity (25–28 nm).

In another study, the elongation of surface-bound seeds was monitored by TIRFM \cite{117}. A solution containing Aβ40 monomer, preformed seed and 5 μM ThT was sealed in a chamber. Small seeds on the coverslip surface rapidly grew into long fibrils or fibril clusters along the surface. Fibril growth was
unidirectional and acquired a ‘stop-and-go’ behaviour with an almost constant rate of ~300 nm/min in the growth phase. This rate was about 15 times faster than observed in [116], which makes sense considering the ~25-fold difference in monomer concentrations.

A more extensive study on Aβ40 seed elongation was conducted using AFM [118]. Preformed Aβ seeds were deposited on mica, and the monomer solution was replenished to maintain a constant monomer concentration. Seeds grew bidirectionally with similar rates at both ends. Contrary to other studies, fibrils grew steadily with no significant stall phases. However, there are possible stall phases when carefully examining the fibril end displacement versus time traces. The fibril height distribution was bimodal with two maxima at 3 nm and 9 nm, possibly suggesting the existence of two fibril structures consisting of one or two protofilaments, respectively. Growth rates among the thicker fibrils were relatively consistent, while rates among thin fibrils had a broader distribution.

Elongation rate was studied in a monomer concentration range from 0 to 10 μM. Steady fibril dissociation was observed when no monomer was present. Elongation rates increased linearly with monomer concentration, suggesting elongation could be considered a bimolecular reaction between fibril end and monomer in this system. The critical concentration, 0.44 ± 0.07 μM, was used to calculate ΔG⁰, -36.5 kJ/mol. A rate constant of 1.8 x 10⁴ M⁻¹ s⁻¹, which was much lower than the monomer diffusion limit, suggested monomer diffusion was not rate rate-limiting.

(2) Aβ42

The elongation of Aβ42 seeds was studied using high-speed AFM (HS AFM) [119]. Two fibril morphologies were observed: coiled fibrils with ~100 nm periodicity and 5-10 nm in height and straight fibrils with ~5 nm in height. A third type was hybrid fibrils containing both morphologies. AFM measurements showed that elongation followed a ‘stop-and-go’ pattern; elongation of all types of fibrils was bidirectional and polarised, i.e., with one fast end and one slow end. They studied the durations of each growth event and pause event. The distribution of both durations can be described by exponential equations,
which was strong evidence that the fibril end could adopt two states: a growth competent state and a ‘blocked’ state when the fibril was incapable of elongation.

For most fibrils, elongation templated seed structures with no change in fibril morphology during elongation, as predicted by the ‘dock-lock’ model (Section 2.3.1 (4)). Interestingly, a ‘switch’ in fibril morphology was occasionally observed during elongation, leading to the formation of hybrid fibrils. The frequency of the switch was affected by buffer salt. Most fibrils were coiled in NaCl; however, when using KCl, the frequency of coiled fibrils decreased, while straight fibrils and hybrid fibrils increased. This morphological switch can’t be explained by a simple ‘dock-lock’ model, as the model implies that the structure of newly elongated fibrils is entirely determined by the seed structure. They argued there may be limited structural fluctuations at the fibril ends which can be templated when a new peptide is incorporated.

Another study utilised TIRFM to observe in situ elongation of Aβ42 seeds [120], using surface-attached HiLyte Fluor (HF) 647 labelled seeds and HF 488 labelled monomer solution. Seed fibrils grew either unidirectionally or bidirectionally with strong polarity; multiple stall phases were observed. The fast-end pause-free rate was studied further. Pause-free rate followed a normal distribution at 76.2 ± 17.9 nm/min (mean ± S.D.) at 10 µM monomer concentration. At different monomer concentrations, the average rate increased linearly with Aβ42 concentration in a low concentration range (2.5 µM and 5 µM) and then saturated, resembling the MM kinetics. At temperatures of 24–37°C, the reaction rate constant followed Arrhenius law with an activation energy of 47 ± 17 kJ /mol.

A recent study utilised super-resolution microscopy to study the aggregation of Aβ42 and, interestingly, how PrP^C inhibited Aβ42 aggregation [127]. Aβ42 seed, monomer and PrP^C were labelled with fluorescent dyes with unique spectral characteristics so that the fluorescence of each species could be individually imaged. Incubation was conducted in solution, and samples were withdrawn for imaging at sequential time points. Consistent with the observation in [120], Aβ42 seeds either elongated unidirectionally or
bidirectionally with polarity. The study found that PrP<sup>C</sup> inhibited the elongation step of Aβ42 aggregation by binding to and blocking the fast end of the fibril, while it didn’t interact with the slower end. This was explained by the unsymmetric structures of the opposing fibril ends.

_in situ_ TIRF imaging [144] revealed the essential role of secondary nucleation in amyloid amplification, which wasn’t observed in the above studies. A mixed monomer and seed (0.8%–2.5%) solution in a multiwell plate was monitored by TIRFM at 23 °C. Some fibrils elongated with pause phases while some fibrils didn’t grow at all; elongation rate distribution showed a single peak with significant variance. The concentration dependence of elongation rate was consistent with the MM kinetics. In addition to seed elongation, they also observed the appearance of new fibrils at places close to existing fibrils. This could indicate that new nuclei were formed by secondary nucleation and attached to the nearby coverslip surface before subsequent elongation. The existence of secondary nucleation was confirmed by the fact that the number of new fibrils scaled with the increase of total fibril mass. The rate of secondary nucleation saturated at high monomer concentrations, consistent with a multi-step mechanism.

Recently, elongation and secondary nucleation were both observed for _de novo_ aggregation of Aβ42 by single-molecule fluorescence lifetime imaging (FLIM) [128] on coverslip surfaces. The authors incubated Alexa Fluor (AF) 594 labelled Aβ42 monomers and measured the fluorescence intensity and lifetime during incubation. Deep learning was applied to separate fibril clusters into images of single fibrils. For each separated fibril, its photon number and fluorescence lifetime were collected, allowing the estimation of the number of protofilaments. Fibrils were clustered into three groups made of 2, 3 or 4 protofilaments, respectively. Each group of fibrils can be divided further into short or long fibrils.

The study observed substantial heterogeneity in rate (length), fluorescence lifetime and growth pattern (polarity) of fibrils within each group, among different groups, and between different batches of experiments. For elongation, a stop-and-go behaviour was observed, and most fibrils grew
unidirectionally. They also observed possible secondary nucleation on the surface of oligomers, but the emerging new fibrils weren’t necessarily the same type as the original oligomers.

(3) α-Synuclein

α-Synuclein (α-Syn) is an intrinsically disordered protein with an α-helix-containing amphipathic N terminus, a hydrophobic central region (NAC) and a highly acidic C-terminal region.

The formation of different aggregated α-Syn species was observed by AFM on the mica surface [130]. At neutral pH (7.5), α-Syn rapidly formed sheet-like structures with a height of 1.1 ± 0.2 nm, which were proposed to be single β-sheet assemblies formed by the N-terminal and central regions on the negatively charged mica. The sheets could elongate on the surface in two preferred directions.

When preformed mature fibrils were present with monomers at pH 7.0, the mature fibrils themselves didn’t elongate; instead, a few fibrillar structures, hypothesised to be protofibrils, grew out of the mature fibrils with a ‘stop-and-go’ elongation pattern.

At low pH (pH 5.0) and 45 °C, fibrils formed quickly, followed by deposition of amorphous aggregates, which became bigger and higher (up to 150 nm). It might be explained by a reduced repulsion between molecules since the charged C-terminus was partially neutralised at low pH.

α-Syn seed elongation was observed in situ by TIRFM [131] at ~25 °C in a mixed solution of monomers (pH 6.0), 0.65% seeds and ThT. They observed fibrils growing out of seed clusters, for which reason the growth directionality couldn’t be determined. A ‘stop-and-go’ pattern was observed. The distributions of stall time and growth time of individual events follow exponential distributions, supporting the two-state model at fibril ends as observed in [119]. The growth rate varied both within and between fibrils.

Another study investigated α-Syn seed elongation in bulk solution [123]. Seed fibrils labelled with AF 568 were incubated with monomers partially labelled with AF 647 in solution at 37 °C without shaking. Samples taken out at multiple
time points were subjected to two-colour super-resolution imaging to distinguish seeds and newly grown fibrils with high precision. The study didn’t observe fragmentation or *de novo* formation of fibrils. Although images taken at earlier time points showed initial unidirectional growth, all elongated fibrils at later time points grew bidirectionally. A fraction of fibrils didn’t elongate at all. The average fibril growth rate showed a wide distribution, with a mean value of 1.4 ± 1 nm/min, and the fastest could be > 5 nm/min. They brought up two explanations for the variance in rates and incapability for some fibrils to elongate: seeds were heterogeneous, and some of them were not efficient in seeding; fibril ends might be blocked or damaged for the seeds that didn’t elongate.

HS AFM was used to study the self- and cross-seeding of two α-Syn variants, wild-type (WT) and E46K, at two pH values, 5.8 and 7.4 [132]. WT fibrils formed at either pH (termed as WT-pH5.8 and WT-pH7.4) and E46K fibrils formed at pH 7.4 (E46K-pH7.4) were straight with different thicknesses/widths caused by the lateral association of different numbers of thin fibrils; E46K fibrils at pH 5.8 (E46K-pH5.8) acquired a helical structure.

Elongation of straight fibrils (WT-pH5.8, WT-pH7.4, E46K-pH7.4) was observed when the fibrils were incubated with the same monomers as in seeds. Elongated fibrils usually maintained the seed structure (as thick, straight fibrils), and solution pH mainly affected growth rates. However, a thin filament was observed to elongate from a thick seed under certain conditions. When monomers in solution were different from those in seeds, no or little growth was observed, except when WT-pH5.8 seeds and E46K monomers were used, thin fibrils different from seeds occasionally grew.

Helical seeds (E46K-pH5.8) could recruit WT or E46K monomers at the same pH to elongate into the same structures as the seeds, while self-seeding took place at a higher rate. Interestingly, fibril elongation was observed in the presence of E46K monomers at pH 7.4, and striated segments occasionally elongated from helical seeds, making hybrid fibrils. The authors proposed that elongation involved monomer addition, structural rearrangements, as well as conformational fluctuation of the added monomer or fibril ends which led to
changes in fibril morphology, as discussed in [119] for the elongation of Aβ42 seeds.

(4) Yeast prion protein

Several yeast proteins can acquire either a normal state or multiple transmissible amyloid states, each of which is related to a distinct phenotype and can be propagated faithfully. Ex vivo studies on the elongation properties of yeast prions have been carried out [125, 126, 134-136] and will be briefly reviewed here.

The elongation of amyloid fibrils formed by Sup35 NM domain [126] in solution was visualised by TEM. The seeds were formed by an NM derivative and labelled with nano gold. Seed elongation was carried out in solution in the presence of WT NM monomers. TEM images showed that most elongated fibrils had a short, gold-labelled seed region, with long, newly elongated parts at both ends. Unidirectional elongated fibrils were occasionally detected, which was explained by the blocking of a fibril end by gold particles or improperly folded protein molecules.

An AFM-based method was developed to study the elongation in solution [125], leading to a different observation. Two Sup35 variants were generated, one allowing antibody binding while the other blocking it. In the bulk seeding experiments, seeds and monomers contained different Sup35 variants, so seeds and elongated fibrils could be distinguished by antibody binding. AFM measurements for the elongated fibrils taken at different time points showed that seed elongation was heterogeneous in terms of growth polarity and rate. Elongated fibrils were classified into four major categories: (1) short fibrils, (2) asymmetric fibrils with one short end and one intermediate-length end, (3) highly asymmetric fibrils with one short end and one long end, (4) symmetric fibrils with intermediate to long length. Fibril polarity and growth rate were maintained in the second round of seeding. They proposed that multiple fibril types with unique structures coexisted. However, no obvious morphological differences, such as height and periodicity, were observed by AFM for fibrils in categories (2), (3) and (4).
An *in situ* study on Sup35 NM seed elongation using fluorescence microscopy [134] also showed the growth to be asymmetric. ~70% of fibrils grew from one end, ~15% had bidirectional growth with strong polarity, and ~15% had similar growth from both ends. The study argued that unidirectional growth was not caused by an inactive end since similar fractions of sonicated seeds with fresh ends showed unidirectional growth.

Whether NM fibrils grew by monomer or oligomer addition was studied by both bulk assays and a single-fibril elongation assay using TIRFM [135]. Experimental findings supporting the monomer addition hypothesis included: (1) the lack of detectable oligomers during fibril formation; (2) the linear increase of growth rates with monomer concentration before saturation, which didn’t support a rare oligomer to be the direct precursor of elongation; (3) all observed addition events measured by TIRFM had similar intensity profiles when monomer solutions were at different labelling ratios; while the oligomer addition assumption would lead to a different distribution of this intensity.

Taking advantage of the high temporal and spatial resolution of HS AFM, Kongo and co-workers [136] observed the dynamics of Sup35 NM monomers, the formation of oligomers, and the fragmentation and elongation of fibrils.

AFM images showed that monomeric Sup35 NM contained a globular core and two highly flexible, unstructured tails belonging to intrinsically disordered regions. HS AFM detected the formation of oligomers long before fibrils were formed, suggesting oligomers weren’t the direct precursors for fibril formation.

After depositing seeds onto mica and adding monomer solution, elongation was observed within 3 min. Fibril elongation was asymmetric, and the growth rate showed heterogeneity within one fibril at different time points and between fibrils. They proposed that fibril elongation occurred by monomer addition instead of oligomers since elongation was smooth instead of step-wise and took place before oligomers could form.

They observed gaps separating individual oligomers (~6 nm) and between fibrils and surrounding oligomers (~10 nm), likely caused by the extension of unstructured regions surrounding oligomers and at the side of fibrils. Those structures might explain why no fibril branching was observed.
<table>
<thead>
<tr>
<th>Protein/peptide</th>
<th>Research</th>
<th>Directionality</th>
<th>Pattern</th>
<th>Concentration dependence</th>
<th>Elongation rate constant $M^{-1} s^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ40</td>
<td>Goldsberry, 2005 [116] AFM</td>
<td>Protofibril: unidirectional/ Bidirectional</td>
<td>Stop-and-go</td>
<td>3.6 x 10^4 *</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibril: --</td>
<td>Stop-and-go</td>
<td>8.3 x 10^5 *</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ban, 2004 [117] TIRFM</td>
<td>Unidirectional</td>
<td>Stop-and-go</td>
<td>6.4 x 10^5 * (625 by ThT assay *)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xu, 2019 [118] AFM</td>
<td>Bidirectional, not polarised</td>
<td>Steady</td>
<td>Linear (0-10 µM)</td>
<td>1.8 x 10^4</td>
</tr>
<tr>
<td>Aβ42</td>
<td>Watanabe-Nakayama, 2016 [119] HS AFM</td>
<td>Bidirectional, polarised</td>
<td>Stop-and-go</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young, 2017 [120] TIRFM</td>
<td>Unidirectional/ Bidirectional, polarised</td>
<td>Stop-and-go</td>
<td>MM</td>
<td>9.3 x 10^5 (at 37 °C)</td>
</tr>
<tr>
<td></td>
<td>Amin, 2021 [127] Solution</td>
<td>Unidirectional/ Bidirectional, polarised</td>
<td>Stop-and-go</td>
<td>MM</td>
<td>~10^6</td>
</tr>
<tr>
<td></td>
<td>Zimmermann, 2021 [144] TIRFM</td>
<td></td>
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<tr>
<td></td>
<td>Meng, 2022 [128] FLIM</td>
<td>Unidirectional (majority);</td>
<td>Stop-and-go</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Syn</td>
<td>Wordehoff, 2015 [131] TIRFM</td>
<td></td>
<td>Stop-and-go</td>
<td></td>
<td>8.6 x 10^3</td>
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<tr>
<td></td>
<td>Pinotsi, 2014 [123] Solution</td>
<td>Unidirectional/ Bidirectional</td>
<td></td>
<td></td>
<td>1 x 10^3</td>
</tr>
<tr>
<td>Sup35 NM</td>
<td>Scheibel, 2001 [126] Solution</td>
<td>Bidirectional (majority)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DePace, 2002 [125] Solution</td>
<td>Bidirectional, heterogeneous</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Inoue, 2001 [134] Solution / Fluorescence Microscopy</td>
<td>Unidirectional (majority) / Bidirectional</td>
<td></td>
<td></td>
<td>8.7 x 10^5 * assuming the fibril is single-strand.</td>
</tr>
<tr>
<td></td>
<td>Konno, 2020 [136] HS AFM</td>
<td>Unidirectional (majority)</td>
<td>Monomer addition</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Collins, 2004 [135] Solution, TIRFM</td>
<td></td>
<td>Monomer addition</td>
<td>MM **</td>
<td></td>
</tr>
</tbody>
</table>
*: rate constants were calculated using data in the reference, assuming a bimolecular reaction model: \[ k = \frac{r}{C_E} \cdot \frac{1}{C_M} = \frac{\text{rate (in nm/s) \cdot (number of strands)}}{0.48 \text{ (in nm)}} \cdot \frac{1}{C_M}. \]

**: an MM type of kinetics was estimated based on the data shown in the paper.

(5) Discussion

Traditional single-molecule approaches such as TIRFM and AFM, together with the development of new techniques such as super-resolution microscopy and HS AFM enable the observation of amyloid elongation \textit{in situ} with enhanced spatial and temporal resolution. Single-aggregate studies have provided unique insights into protein aggregation mechanisms and allowed the direct calculation of kinetic and thermodynamic parameters.

\textbf{Growth patterns}

Amyloid fibril elongation was commonly observed for multiple proteins discussed above and for other proteins such as Aβ25-35 [145], insulin [146] and human lysozyme [8]. Large discrepancies exist in growth patterns such as directionality even for the same protein. One example is the elongation of Sup35 NM fibrils, which showed unidirectional growth [134, 136], bidirectional and polarised growth [125], or bidirectional and unpolarised growth [125] in different studies. For Aβ42, both bidirectional, polarised growth and unidirectional growth were seen [119, 120, 127, 128]. The uneven growth at fibrils' opposing ends indicates different structures and free energy barriers for the elongation reaction.

In most studies, a ‘stop-and-go’ growth was observed. The presence of stall phases is compatible with a model that fibril ends can adopt two states [119, 120, 131]: a growth competent state and a blocked state. The blocked state is caused by an incorrectly incorporated molecule, which needs to detach or undergo a structural rearrangement before growth can proceed. Another study [123] argued that this model couldn’t explain some very long stall phases lasting over hours. Physical obstacles, inefficient seeds and damaged fibril ends are alternative explanations.
Studies about Sup35 NM [135, 136] seed elongation indicated that fibrils grew by monomer addition instead of oligomers. A direct assembly of protofilaments/protofibrils into mature fibrils was not observed. However, another study [147] proposed that protofibrils can directly form mature fibrils by hierarchically associating with each other.

**Kinetic analysis**

The concentration dependence of elongation rate usually exhibits an MM-type kinetic [120, 135, 144] or a linear relationship [118]. The MM-type mechanism can be explained by the ‘dock-lock’ model [105, 120, 135, 141, 144], stating that elongation involves reversible binding of monomers to fibril ends and subsequent irreversible structural conversion, as discussed in Section 2.3.1 (4). The structural conversion might involve more than one single step. Cannon and co-workers [148] studied Aβ40 seed elongation using SPR and derived a 3-step model including a rapid and reversible monomer attachment to fibril ends followed by two consecutive conformation changes. Interestingly, the elongation of Aβ42 [119] and α-Syn E46K [132] fibrils showed occasional morphology switches, indicating a supplement to the ‘dock-lock’ model that the fibril end structure can fluctuate between a limited number of conformations.

Elongation rates of fibrils usually involve a large degree of heterogeneity within a study, as seen in [120, 123, 128, 144]. The elongation rate constants obtained for one protein from different studies also varied to a large extent. The heterogeneity in kinetics could result from unique fibril structures, different sources of proteins, varied buffer compositions and different types of surfaces.

**Thermodynamic parameters**

In addition to the calculation of free energy of elongation for Aβ40 [118] and activation energy/enthalpy for Aβ42 [120] discussed above, numerous other studies have focused on the thermodynamic parameters for amyloid elongation.

Using QCM, activation enthalpy ΔH‡, activation free energy ΔG‡, and activation entropy ΔS‡ were obtained for the elongation of insulin [146], Aβ42, α-Syn, β2M, lysozyme and other proteins [149]. ΔH‡, ΔG‡ and TΔS‡ were all
positive, suggesting that the formation of activated complexes was entropically favourable but enthalpically unfavourable. It was proposed in [149] that the entropy gain largely came from the net breakage of hydrogen bonds with water molecules during rearrangement; desolvation of hydrophobic regions played an important role in $\Delta S^\ddagger$. $\Delta H^\ddagger$ is primarily contributed by weak interactions and unfolding of the peptide.

**Conformation of elongated fibrils**

Usually, amyloid elongation can be considered a templating event, i.e. the newly incorporated monomers are structurally templated by the fibril. This is supported by several AFM studies [118, 136], showing that the elongated fibrils have the same morphology as the seeds. The most convincing evidence of structural templating comes from cryo-EM measurements for both *ex vivo* SAA1.1 seeds and fibrils after seed elongation. 98% of the elongated fibrils adopted a structure highly similar to one type of *ex vivo* fibrils; the rest was likely from *de novo* aggregation, confirming that elongation templated the atomic structure of seeds [150]. The energy barrier for elongation is much smaller than *de novo* aggregation, even though the structure of seeds might not be the most stable under the specific condition.

Propagation of a different fibril morphology from the beginning of a seeding assay or switch of fibril structure during elongation was occasionally observed. This could involve a change in fibril morphology from straight to twisted fibrils or vice versa [119, 132] or a reduction in the number of strands [132]. In [119], the morphology switch occasionally occurred when the seeding assay was conducted in the same buffer as seed production. The frequency increased when seeding was performed in a different buffer. As discussed, a change in fibril morphology might indicate that the structures of fibril ends or newly added monomers fluctuate within a few possible conformations. When the buffer changes, the fibril end could favour a thermodynamically more stable state, leading to an increased frequency of structural switch. Thus, both seed types and solution conditions could affect the structure of elongated fibrils.

The elongation of PrP seed has been studied both in bulk and at the single-molecule level and provided valuable information on PrP seeding
mechanisms. However, the single-molecule analysis mainly focused on oligomeric species and didn’t show the formation of long amyloid fibrils. I will next review these studies on PrP seeding assays.

2.3.4 Seeding assays on Prion protein

The aggregation of full-length HuPrP was studied in situ by AFM [137] under two pH values: 4.5 and 7.2. At pH 4.5, monomers, dimers and trimers on the mica surface can be distinguished by their height profiles. The three assemblies were basic units that can associate with each other forming oligomers by hydrogen bonding. These oligomers could further combine, exhibiting elongation of ring-like oligomers. At pH 7.4, PrP monomers could combine with each other by intermolecular hydrogen bonding and deposit onto the surface as ball-like aggregates. However, these species were usually small and didn’t exceed 100 nm in length.

Sang and co-workers studied the seeded replication [138] and de novo aggregation [139] of recombinant full-length MoPrP in vitro at the single-aggregate level. The seeds were produced under partially denaturing conditions and centrifuged to separate soluble seeds (supernatant) and insoluble aggregates (pellet). Seeding assays using soluble or insoluble seeds were monitored in situ on the coverslip surface in a buffer with no denaturant. Less than 10% of the seeds elongated and less than 1% of the seeds fragmented. The elongation of soluble seeds led to conversion from mostly PK-sensitive aggregates into an increasing fraction of PK-resistant aggregates, suggesting structural conversion took place during elongation. Fragmentation rate was proportional to fibril length. Fragmentation caused a loss in PK resistance for initially PK-resistant fibrils. However, the ‘fibrils’ described here only had sizes of a few pixels, precluding a real quantitative kinetic analysis.

De novo aggregation [139] was studied in the presence of 2 M GdnHCl with agitation. Aliquots were taken out from the assay solution and imaged by TIRFM in the presence of ThT or by spectrally-resolved points accumulation for imaging in nanoscale topography (sPAINT) using fluorescent dye Nile red
which probed the hydrophobicity of the aggregate surface. Early time point samples contained both ThT-active oligomers and ThT-inactive oligomers (observed by NR fluorescence) which were smaller in number and shorter in length. The length of those species increased with time. ThT-inactive species acquired increasing hydrophobicity with time, which was higher than ThT-active oligomers that maintained a roughly constant level. Mature fibrils appeared at later time points, whose hydrophobicity was similar to ThT-active aggregates.

PK resistance of oligomers was probed by the remaining ThT fluorescence after PK treatment. ThT-active oligomers acquired PK resistance with time. The authors proposed an aggregation mechanism that ThT-inactive oligomers and PK-sensitive (ThT-positive) oligomers were in equilibrium with monomers; PK-sensitive oligomers could convert to PK-resistant species and eventually fibrils.

Milto and co-workers [151] studied the elongation of fibrils formed by truncated MoPrP 89-230 by seeding assays in bulk at different GdnHCl concentrations (0.5–2.5 M) and temperatures (40–65°C). The elongation rate constant k was derived by fitting an exponential function to the normalised ThT fluorescence curve (for reaction \( M + E \rightarrow P \) with depleting monomer concentration, Section 2.3.1 (2)).

At 0.5 M GdnHCl, \( \ln k \) decreased linearly with 1/T on an Arrhenius plot in the temperature range from 40°C to 60°C, when most monomers were folded. The activation energy \( E_a \) for elongation was calculated to be 166 ± 10 kJ/mol. At 2.5 M GdnHCl, almost all protein was unfolded at all temperatures, a linear relationship was seen, and \( E_a \) for elongation was much smaller, 51 ± 3 kJ/mol. At each GdnHCl concentration between the extreme values, data points fell into two lines with \( E_a \) roughly corresponding to the values when unfolded or folded monomers were abundant, respectively. Those findings suggest that PrP monomer unfolding is essential for fibril elongation.

At a given temperature, the elongation rate versus GdnHCl concentration (0.5–2.5 M) plot showed a ‘bell-shaped’ relationship, similar to the trend observed by Knowles [146] for insulin elongation. Knowles proposed that the
initial increase in rate with GdnHCl concentration indicated that a (partially) unfolded monomer instead of native monomer was the direct precursor for elongation. The rate decreased at high denaturant concentrations because of decreasing intramolecular/intermolecular interactions and decreasing possibilities to form critical contacts.

Honda and co-workers [152] studied full-length HuPrP seeding kinetics by systematically varying the concentrations of seed fibrils, monomers, and GdnHCl at 37 °C. The initial increase rate of ThT fluorescence was used for analysis. Similar to the findings in [151], the elongation rate had a bell-shaped relationship with GdnHCl concentration, with the highest rate occurring at 2.3 M GdnHCl.

They argued that the unfolded PrP ('U') was in equilibrium with the natively folded PrP ('N'); unfolded PrP was the direct precursor for elongation. In the high GdnHCl concentration range (2.3–3.5 M), almost all PrP was in the U state. Elongation rates at increasing monomer concentrations followed the MM-type mechanism and can be explained by the two-step ‘dock-lock’ model for unfolded monomers to add to fibril ends. At higher GdnHCl concentrations, rates increased linearly with monomer concentrations within the PrP concentration range they studied.

Interestingly, at a lower GdnHCl concentration (1.3–2.0 M), the fraction of PrP in the N state was substantial; the elongation rate increased rapidly with monomer concentration and then decreased. They proposed that native PrP interacted with either a U-and-fibril complex or the fibril itself (with or without a bound U PrP) and inhibited elongation. A complete model for elongation was then established.

Overall, this chapter summarises the established methods to study amyloid elongation kinetics and reviews seeding studies on several amyloidogenic protein systems. The kinetic study on PrP fibril elongation on the single-aggregate level is currently lacking. My study will apply these approaches to investigate the elongation kinetics of PrP and compare the kinetics to findings in the literature.
Chapter III. Novel microscopy methods used for dynamic and structural analysis

3.1 TIRFM

Total internal reflection fluorescence microscopy (TIRFM) [153] has long been used in optical imaging. This technique significantly reduces background fluorescence compared to conventional epifluorescence microscopy and offers enhanced resolution in the z-axis of imaging.

When light encounters an interface between two media with different refractive indices, it will be partially reflected and partially refracted. All incident light will be reflected if it meets the requirements [153]: (a) the light travels from a high-refractive index medium (with an index $n_1$) to a low-refractive index medium (with an index $n_2$), and (b) the incidence angle $\theta_1$ is larger than a critical value $\theta_c = \sin^{-1}(n_2/n_1)$. In this case, no refractive light beam exists in the low-refractive index medium; however, a thin electromagnetic field is generated in this medium by the reflected light. Its intensity decays exponentially with the distance from the interface. The depth of this electromagnetic field, i.e. the penetration depth, is affected by light wavelength, incidence angle and refractive indices of the two media. Considering a system in which the illuminating laser beam travels from a glass coverslip to a biological sample, the penetration depth is usually within 100–200 nm. Only fluorophores within this region are excited; thus, background fluorescence caused by exciting fluorophores in bulk solution is largely suppressed.

3.2 Image-reconstruction Super-resolution Microscopy

Optical microscopy is a useful tool to study biological systems with minimal damage to the specimen. However, the resolution is limited by light diffraction. Because of the wave nature of light, light emitted from a point source is no longer a point on the image plane after going through lenses but acquires a shape called an Airy disk: a bright circular region in the centre surrounded by less bright concentric rings [154]. If two point sources are close enough, their
images in the image plane partially overlap, so the two ‘points’ can no longer be resolved. A widely used approach to determine resolution is Rayleigh criterion, stating that the resolution limit is the distance of two point objects if one object’s first trough in its Airy disk falls exactly on another object’s peak in the image plane [155]. The mathematical expression is:

$$\text{Rayleigh resolution limit} = \frac{1.22\lambda}{2N_A}$$

where $\lambda$ is the wavelength of illumination light and $N_A$ is the numerical aperture of the objective. The resolution limit is ~250 nm for visible light using a high-power oil immersion microscope objective. Researchers often use full width at half maximum (FWHM) of a point object as a measure of resolution [155].

In order to overcome this resolution limit in fluorescence microscopy, three main strategies have been developed: (1) image reconstruction microscopy such as stochastic optical reconstruction microscopy (STORM) and photoactivated localisation microscopy (PALM), (2) super-resolved confocal microscopy by stimulated emission depletion, and (3) structured illumination microscopy. Image reconstruction microscopy is the basis of super-resolution imaging in this work and will be briefly reviewed here.

In image reconstruction microscopy, measures are taken to switch on a subset of isolated fluorophores sequentially. Even though the image of a single fluorophore is ~250 nm in size due to light diffraction, its centre position can be obtained with high precision if enough photons are detected from the fluorophore. The centre positions of all single fluorophores in the image frame are mapped onto a reconstructed image. By repeating this procedure of exciting isolated fluorophores, determining centre positions and mapping the centres to the same reconstructed image, a reconstruction of the original structure can be generated at a high resolution.

The localisation precision of each localisation event is mainly determined by the emitted photons [156], while determining the resolution is challenging. Researchers often use FWHM [157, 158] to describe the resolution in image reconstruction microscopy, but it should be noted that the resolution used here is affected by both localisation precision and localisation density [155].
Figure 3-1. Principles of STORM imaging [159, 160]. (A) The labelled structure to be imaged. (B) The diffraction-limited image of the structure. (C)-(E) Frames of STORM imaging. The red dots show isolated fluorophores excited in each frame; the black cursors represent the localised central positions. (F) The reconstructed image.

The widely used stochastic optical reconstruction microscopy (STORM) was developed in the Zhuang lab [159, 160]. They utilised photoswitchable fluorophores conjugated to the structures of interest. In the initial work, Cy3-Cy5 dye pairs were used as photoswitchable fluorophores, which could reversibly adopt fluorescent or dark states. Red laser illumination stimulates fluorescence emission of Cy5 and also switches it to a stable dark state. Upon green laser illumination, Cy3 is stimulated and is able to convert Cy5 back to the fluorescent state if Cy5 is in close contact with Cy3. This process makes a ‘cycle’ which allows only a small fraction of Cy5 molecules to be excited in each cycle. Ideally, excited fluorophores are well-separated. Their centre
positions are determined by fitting a Gaussian intensity profile to the point spread function (PSF) of excited fluorophores. After thousands of cycles, a reconstructed image is obtained from all calculated centre positions (Figure 3-1). The authors reported a greatly enhanced imaging resolution down to approximately 20 nm. They illustrated that fluorophores labelled on double-strand DNA with ~40 nm spacing could be resolved. This technique was further applied to three-dimensional imaging [161] and multicolour imaging [162] using other photo-switchable probes.

(2) PALM

Betzig and co-workers [163] developed photoactivated localisation microscopy (PALM) to reveal intracellular structures at a high resolution. Photoactivatable fluorescent protein (PA-FP) is used as fluorophores. They are tagged onto a target protein and expressed intracellularly. During imaging, a large fraction of fluorophores is initially in an inactivated state. A subset of resolvable fluorophores is activated using a corresponding excitation laser and then imaged until they are photobleached. A brief laser pulse at 405 nm activates new fluorophores when the activated fluorophores are too sparse. This cycle is repeated until all fluorophores have been activated and photobleached. By fitting a Gaussian PSF to the acquired image of each fluorophore, its central location and position uncertainty can be obtained. These two parameters are used to construct a new Gaussian profile with a much smaller standard deviation than the original PSF. A high-resolution image is then generated by summarising the new Gaussians for all imaged fluorophores.

(3) PAINT

The points accumulation for imaging in nanoscale topography (PAINT) technique [164] was originally developed to achieve super-resolution imaging of lipid bilayers and vesicles, reaching a spatial resolution of ~25 nm. The dye molecules, Nile red, are added to the buffer solution. The diffusion of the dye molecules causes collisions between them and the targeted structures, generating high fluorescence until the dye molecules detach from the structures or are photobleached. Isolated blinking events are achieved by adjusting the dye concentration and laser power. A reconstruction of the centre
positions yields a high-resolution image of the target structure. A large reservoir of dye molecules in the solution ensures enough localisation events to be collected over a long period.

DNA-PAINT [165] was developed based on PAINT and has wide applications. This technique utilises two types of single-stranded DNA oligomers with complementary sequences. The docking strands are conjugated to the structures of interest, and the imager strands are labelled to the dye molecules, which can move freely in the buffer. During imaging, the imager strands bind transiently to the docking strands, allowing the attached dye molecules to be imaged. The blinking is controlled by the binding and dissociation of the complementary DNA strands, not by the dye molecules’ photophysics. This technique reaches a resolution of < 30 nm [165]. It has been applied to image DNA origami and protein structures, with the advantage of controllable binding kinetics, high resolution and the ability to image different types of structures on a sample [165, 166].

(4) BALM

Schoen and co-workers [157] developed binding-activated localisation microscopy (BALM) to super-resolve DNA utilising DNA-binding dyes added to the buffer solution. Those dyes are dark in buffer and are hundreds of times brighter once binding to DNA. Isolated binding events with high photon emission are achieved by tuning the solution conditions. The repeated dye binding, localisation and bleaching cycles generate a high-resolution image of the target structure. The authors imaged DNA molecules at an enhanced resolution of ~14 nm (FWHM) using YOYO-1 dye in an optimised buffer with β-mercaptoethanol (BME) or a reducing-oxidising system. They successfully validated this method by imaging the chromosomal organisation in fixed cells using a different dye, PicoGreen.

Ries and co-workers [167] extended the BALM technique to image amyloid fibrils. They used a luminescent conjugated oligothiophene (LCO) dye, p-FTAA, to image α-Syn fibrils. A resolution of 14 nm was reported, but the observation time was limited to a few minutes due to the irreversible binding of dye molecules to the amyloid structures and subsequent photobleaching.
Transient amyloid binding (TAB) microscopy [158] developed in our lab in collaboration with Dr. Matthew Lew is also based on BALM to image amyloid fibrils. The traditional amyloid dye ThT was initially used as the fluorophore. Since ThT binds to amyloid reversibly, photobleached dye molecules are constantly recycled. Thus, amyloid structures can be observed over a long period, making the technique a good candidate for dynamic imaging. TAB imaging has been utilised to image Aβ42 fibrils at a resolution of ~60 nm, observe Aβ42 fibril remodelling by the drug epi-gallocatechin gallate (EGCG) [158] and Aβ42 fibril elongation over a long period [122]. The latter study used Nile red as the amyloid binding dye instead of ThT.

3.3 Polarised super-resolution structural imaging

Amyloid dyes bind to the amyloid fibril surface at a preferred orientation. In the systems studied so far, ThT binding orientation is preferentially parallel to the long axis of the amyloid β-sheet [168, 169]. Experimental evidence supporting this includes molecular dynamic simulation of ThT binding to Aβ16-22 protofibrils [168] and confocal imaging of amyloid spherulites under polarised excitation light [169]. Thus, determining the orientation of the dye molecule in each blinking event can provide information on the fibril axis orientation.

Several approaches have been developed to obtain the orientation of dye molecules using fluorescence microscopy with some modifications to the imaging system. The dye molecules are usually modelled as dipoles. Each dye molecule absorbs light during excitation through the absorption dipole moment and emits fluorescence through the emission dipole moment [170]. As a result, implementing the excitation polarisation in the illumination path, adding a polarisation beam splitter (PBS) in the detection path, or combining the above two measures [170] can provide information on the polarisation of fluorescence emission and the orientation of the dye molecule. This theory has been applied to measure the orientation and wobble of DNA binding dyes SYTOX Orange [171].
Shaban and co-workers [172] conducted polarised d-STORM (direct STORM) imaging for ThT-coated insulin amyloids by splitting the emission fluorescence into two channels polarised perpendicular to each other. First, they confirmed that ThT could be used as a super-resolution dye with adequate photochemistry in d-STORM in the presence of a reducing agent and oxygen scavenging system, which revealed the twisting helical substructure of insulin fibrils. Next, images acquired by polarised d-STORM were analysed to determine the orientation and localisation of each single ThT molecule. They observed substantial orientational flexibility (wobble) of the fibril-bound ThT molecules, even within the 30 ms exposure time. After correcting for the wobbling angle, ~60° from their analysis, the orientation of the identified ThT molecules again underlines a twisted substructure of the fibril.

More recently, our collaborators [173] performed polarised TAB (p-TAB) imaging utilising a similar detection method, i.e., detecting the polarised emission fluorescence in two polarised channels, to image Aβ42 fibrils in the presence of Nile red (NR) dye. Using a sophisticated analysis procedure, they were able to determine the localisation, orientation in three-dimensional space and wobbling of each NR molecule with relatively high precision and calculation speed. They showed that most NR molecules lay on the x-y plane along the fibril’s long axis, especially for the thin fibrils. For the thick fibrils, dye molecules’ orientation with respect to the fibril axis was more disordered. Considering that NR bound to the grooves formed by amino acid side chains along the β sheet, the results implied that the thick fibrils were composed of multiple intertwining protofilaments providing orientationally heterogeneous binding sites, while the thin fibrils were likely single-strand.

Another parameter, the wobbling area, described how tightly the dye molecules bound to the amyloid surface. They found that NR bound to thin fibrils more tightly than thick fibrils, which could be explained by higher binding-site densities on the thick fibrils. Their study revealed unique structural heterogeneities in amyloid fibrils that couldn’t be obtained by traditional super-resolution imaging.
3.4 Amyloid-binding dyes used in TAB imaging in this work

ThT is a widely used amyloid-binding dye. Traditionally, it was used to identify amyloid structures and to monitor amyloid-forming kinetics in vitro. Structurally, the ThT molecule contains two connected benzylamine and benzathiole rings [174] (Figure 3-2 A). The two rings can rotate with respect to one another around the shared C-C bond.

A. ThT

B. Nile red

C. Nile blue

D. A cartoon showing the principle of TAB imaging. The figure is adapted from [158]. The twisted green fibre represents an amyloid fibril; the arrowhead lines represent dye molecules, which are bright upon binding onto the amyloid surface (orange) and dark in solution (grey).

Free ThT in solution displays a low fluorescence quantum yield, with an excitation maximum at 385 nm and an emission maximum at 445 nm. In the presence of amyloid fibrils, ThT fluorescence is greatly enhanced, with
excitation and emission maxima red-shifted to 450 nm and 482 nm, respectively. Recent studies have revealed the mechanism of ThT binding onto amyloid fibrils and the high fluorescence emission upon binding.

Quantum-chemical calculations [175] indicated that in the electronic ground state, the potential-energy minimum is reached when the dihedral angle of the C-C bond is 37°, while the maximum is at 90°, i.e. when the two rings are perpendicular to each other. When excited, the state at 37° dihedral angle, termed as the radiative locally excited (LE) state, can generate fluorescence and return to the ground state. The potential-energy minimum of the excited ThT molecule is at a 90° dihedral angle, and this state is called the nonradiative twisted internal charge-transfer (TICT) state. Excited molecules in the LE state can cross to the TICT state if there are no other steric constraints. Molecules in this dark TICT state relax through a nonradiative process back to the ground state. This rotational property around the C-C bond makes ThT a 'molecular rotor'.

When there is no steric constraint on ThT molecules, for example, for free ThT in solution without amyloid fibrils, the two rings rotate freely with respect to their connecting C-C bond. Thus, the excited state which emits high fluorescence is easily quenched [175]. When ThT binds to the amyloid fibril surface, it is trapped sterically with reduced rotational freedom. Once excited, ThT molecules are in the radiative state and can't rotate into the dark TICT state, thus causing a great enhancement in fluorescence emission.

ThT was shown to bind to the amyloid surface at a preferred orientation, with its axis aligning along the axis of amyloid fibrils. Specific side chains across neighbouring β-strands on amyloid surfaces form channel-like motifs [174], which serve as binding sites to accommodate ThT molecules. ThT preferentially binds to hydrophobic side chain channels primarily formed by aromatic amino acids, while it binds poorly to charged amino acid residues. The binding is reduced at low pH, possibly due to electronic repulsion. ThT binding to amyloid requires a flat β-sheet surface made of at least four or five neighbouring strands to accommodate the whole length of the ThT molecule [174, 176]. This requirement might explain why ThT does not bind to some β-
sheet-rich globular proteins. However, ThT binds to not only amyloids but also hydrophobic pockets of globular proteins [177], lipid membranes and other fibrils such as collagen [178].

Under a fluorescence microscope, amyloid fibrils are visible in the presence of ThT with a ~440 nm excitation laser (or LED) and appropriate filters. More recently, ThT was utilised in super-resolution microscopy to image amyloid structures at an enhanced resolution [158] and hint at the sub-structure of amyloid fibrils [172].

Another two dyes, Nile red (NR) and Nile blue (NB), belong to a family of Benzophenoxazine dyes (Figure 3-2 B and C). NR was initially used to dye clothing and later to stain lipid membranes by biologists. The excitation and emission wavelength maxima of NR depend greatly on the polarity of its local environment. Spectroscopic studies [179] of NR in organic solvents revealed that excitation and emission maxima are red-shifted with increasing solvent polarity. In pure methanol, the excitation maximum of NR is ~560 nm, and the emission maximum is 625 nm. In an aqueous (water) solution, the fluorescence emission maximum of free NR shifts to 650 nm [180].

In the presence of amyloid fibrils, NR binds to the fibril surface and generates strong fluorescence emission, making it a good candidate for structural imaging. Since NR binding sites on the amyloid surface have lower local polarity (higher hydrophobicity) than the buffer, the emission maximum of NR is blue-shifted [180]. The emission maximum varies among amyloid fibrils formed by different proteins or fibrils formed by the same protein under different conditions, indicating that NR binding sites on different amyloid fibrils have distinct hydrophobicity. It was shown that NR binds to amyloid fibrils formed by HuPrP 90-231 under native conditions and also to monomeric HuPrP, albeit to a much less extent [180].

Utilising this unique spectrum shift property on a single-molecule level, a hydrophobicity map of the aggregate surface can be obtained by analysing the NR emission spectrum of individual blinking events [181, 182]. This technique was termed sPAINT (spectrally-resolved PAINT). For each fluorescence emission generated by a single NR molecule binding transiently onto the
Amyloid surface, the spatial position of the molecule was obtained, and information about the emission spectrum was gathered simultaneously by an inserted transmission diffraction grating before the camera. Thus, the local hydrophobicity of the surface could be derived. Bongiovanni and co-workers [181] and Lee and co-workers [181, 182] utilised this method to observe the surface hydrophobicity of individual oligomers and fibrils formed by Aβ42 or α-Syn and monitored its change during aggregation.

Compared to ThT, NR could also be used to image protein aggregates by either TIRFM or super-resolution microscopy, with certain advantages. ThT fluorescence upon amyloid binding strongly depends on solution pH [180], with decreasing fluorescence emission at acidic pH. NR, however, is less sensitive to pH and could be used over a broad pH range. NR can also detect ThT inactive, early-stage oligomers [139] formed in the lag phase of the amyloid formation process. However, since NR binds strongly to species other than amyloid fibrils such as lipids, it is not suitable to detect amyloid in cells or tissue samples.

NB (Figure 3-2 C) is a positively charged dye molecule with higher water solubility than NR [183]. Similar to NR, the excitation and emission maxima of NB are red-shifted in a polar solvent. In water, its excitation maximum is 635 nm, and the emission maximum is 674 nm [183].

ThT, NR and NB have been used to image amyloid fibrils in the literature. In this work, I tested their performance when imaging amyloid fibrils formed by MoPrP, and utilised them to observe in situ seed elongation and perform super-resolution imaging.
Chapter IV. Materials and Methods

4.1 Microscope setup

Figure 4-1. Schematic representation of the microscope system. L1 – L4: lasers; M1 – M6: mirrors; D1 – D4: dichroic mirrors; CL: collimating lens; EF: emission filter; PBS: polarisation beam splitter.

Figure 4-1 shows the setup of the microscope system used for TIRF, TAB and p-TAB imaging in this work. The bespoke microscope system was assembled by Cairn Research Ltd. based on a Nikon Eclipse Ti2-E inverted microscope with a 100X objective (Nikon, CFI SR HP Apochromat TIRF 100XC/1.49 NA Oil) and iLas spinning TIRF illumination (Gattaca System).

Lasers of four wavelengths are employed: 405 nm (OBIS LX laser with 100 mW of output power), 473 nm (Cobolt MLD laser, 300 mW laser power), 561 nm (OBIS LS laser, 150 mW laser power) and 638 nm (Omicron LuxX. Diode laser, 200 mW laser power). In this work, the 473 nm laser was employed to image ThT fluorescence in the presence of amyloid structures; the 561 nm laser was used to visualise NR fluorescence, and the 638 nm excitation laser was for imaging NB fluorescence and AF 647 fluorescence.

A dual collimator (CL in Figure 4-1, Gattaca Systems) allows a quick switch between high-intensity and large-field imaging modes. TAB and p-TAB
imaging utilised the high-intensity mode, and time-lapse TIRF imaging of elongation utilised the large-field imaging mode.

Our microscope utilises ‘azimuthal TIRF’ provided by Gataca Systems to reduce interference illumination patterns and illumination nonuniformities compared to conventional TIRF. The Galvo mirrors in the iLAS unit scan the laser circularly on the back aperture of the objective. The focused laser beam at each spot of the circular path at the back focal plane then generates collimated laser beam from the objective with a specific azimuthal angle. The rapid spinning of the incident laser beam thus averages out illumination artifacts even within a short exposure time.

Excitation filters, dichroic mirrors and emission filters (Chroma) used for the four imaging channels are listed in Table 3. They allow the desired laser light to reach the sample and ensure only the desired emission fluorescence to reach the camera (Photometric 95b CMOS camera).

Table 3. Excitation filters, dichroic mirrors and emission filters used for the imaging channels.

<table>
<thead>
<tr>
<th>Laser line (nm)</th>
<th>Excitation filter</th>
<th>Dichroic mirror</th>
<th>Emission filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>405</td>
<td>ZET405/10x</td>
<td>ZT405rdc</td>
<td>ET460/50m</td>
</tr>
<tr>
<td>473</td>
<td>ZET473/10x</td>
<td>ZT488rdc</td>
<td>ET500lp</td>
</tr>
<tr>
<td>561</td>
<td>ZET561/10x</td>
<td>ZT561rdc</td>
<td>ET575lp</td>
</tr>
<tr>
<td>638</td>
<td>ZET638/10x</td>
<td>ZT640rdc</td>
<td>ET655lp</td>
</tr>
</tbody>
</table>

The MetaMorph software installed on the PC is used to control image acquisition. The perfect focus system of the microscope as well as the MetaMorph software control are employed to maintain the sample in focus during a time-lapse image acquisition.

The optional Optosplit II unit (Cairn Research) in the emission pathway (shown in the purple rectangle in Figure 4-1) contains a polarisation beam splitter (PBS), which can split the emission fluorescence into two orthogonally-
polarised channels and project the two channels to different portions of the camera.

An enclosure box (OKO-Labs) built around the main body of the microscope maintains a constant temperature of the sample.

4.2 Protein expression and purification

4.2.1 Expression and purification of MoPrP 91-231

The mouse PrP gene was amplified using polymerase chain reaction and was inserted into the expression vector pTrcHisB (Invitrogen) using the restriction sites BamHI and HindIII [184]. The construct was transformed into Escherichia coli BL21 (DE3) strain. Recombinant truncated MoPrP 91-231 was expressed and purified using a protocol modified from [184]. Bacteria culture was grown at 37 °C with 100 μg/mL ampicillin, and the expression of poly-Histidine tagged (His-tagged) MoPrP 91-231 was induced by adding isopropylthio-β-galactoside (IPTG) to 1 mM when the culture reached an OD$_{600}$ nm of ~0.6. After growing overnight at 37 °C, cells were harvested by centrifugation. The successful expression of MoPrP 91-231 was confirmed by gel electrophoresis shown in lanes 1-5 in Figure 4-2. Cell pellets were resuspended in the extraction buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.1% Tween-20, 50 U/ml benzonase, 10 μg/ml lysozyme) and disrupted by sonication. The suspension was centrifuged at 9,900 g for 30 min. The resulting pellets were washed again in the extraction buffer by repeating the resuspension, sonication and centrifugation steps. Solubilisation of protein was performed by resuspending the pellets in the solubilisation buffer (6 M GdnHCl, 50 mM Tris-HCl pH 8.0, 0.8% β-mercaptoethanol (BME)), followed by disruption by sonication and centrifugation at 21,000 g for 45 min. The supernatant was saved, and the pellet was solubilised again. Supernatants from both solubilisation steps were pooled and filtered through a 0.45 μm filter. The solution was loaded onto a 30 ml nickel-nitrilotriacetic acid (Ni-NTA) column pre-equilibrated with buffer A (10 mM Tris, 100 mM Na$_2$HPO$_4$, 6 M GdnHCl, 10mM Glutathione (GSH), pH 8.0). Oxidative refolding was carried out on the
column by running a 30-column volume (CV) 0–100% buffer A to buffer B (10 mM Tris, 100 mM Na₂HPO₄, pH 8.0). After refolding, the protein was eluted with a linear gradient from buffer B to buffer C (10 mM Tris, 100 mM Na₂HPO₄, 1 M imidazole, pH 5.8), and the protein fraction was collected. Imidazole was removed from the collected protein fraction by dialysis into 25 mM Tris buffer, pH 8.4. The His-tag was removed by Thrombin (12 µL, Novagen, Cat.No. 69671-3) cleavage at room temperature overnight, in the presence of 2.5 mM CaCl₂. The samples pre- and post-cleavage were examined by SDS-PAGE gel (lanes 6 and 7 in Figure 4-2, respectively). The cleaved protein was loaded onto a HiPrep SP FF cation exchange column (Cytiva, Cat.No. 28-9365-44) pre-equilibrated with 10 mM HEPES, pH 8.2. Protein was eluted with a linear gradient of 0–1 M NaCl. The eluted fraction was dialysed against 10 mM HEPES buffer, pH 7.5, aliquoted, and frozen for storage. The yield was ~30 mg PrP from 5 L of culture.

**Figure 4-2.** Expression and purification of MoPrP 91-231 analysed by gel electrophoresis. M represents the protein ladder; lane 5 represents the cell lysate before induction; lanes 1–4 are loaded with the lysate of cells that had been inducted and incubated overnight. The existence of a band at ~25 kDa in the post-induction samples (pointed by the black arrow) suggests a successful expression of His-tagged MoPrP 91-231. Lanes 6 and 7 represent the protein samples before and after thrombin cleavage, respectively. Lane 8 and lane 9 represent the product after and before spinning through a 100 kDa filter, respectively.
Monomeric PrP was prepared by spinning the protein through a 100 kDa membrane filter (Amicon®, Cat.No. UFC5100). The flow-through was aliquoted and frozen.

The final product was examined by SDS-PAGE gel shown as lanes 8 and 9 in Figure 4-2. The presence of only one protein band at a correct molecular weight confirmed the successful production of pure MoPrP 91-231.

4.2.2 Expression and purification of MoPrP 23-231

The expression and purification of full-length prion protein (MoPrP 23-231) followed a similar procedure as MoPrP 91-231, with the following modifications. (1) After the protein was eluted from the first column, the collected protein fraction was dialysed against 20 mM BisTris, pH 6.5. After dialysis, the protein was cleaved as described. (2) The cleaved protein was loaded onto a second Ni-NTA column pre-equilibrated with 20 mM BisTris plus 25 mM imidazole, pH 6.5, and eluted with a gradient of 25 mM–600 mM imidazole (0–60% of a buffer containing 20 mM BisTris, pH 6.5 and 1 M imidazole). (3) The protein peak was then collected and dialysed against 10 mM BisTris buffer, pH 6.5. The dialysed protein was aliquoted and frozen for storage. (4) The monomeric PrP for aggregation assays was prepared by spinning the thawed protein solution through a 30 kDa (Amicon®, Cat.No. UFC8030 or UFC 5030) membrane filter unless otherwise indicated. If the protein concentration in the flow-through was low, then the solution was concentrated by spinning in a 3 kDa filter (Amicon®, Cat.No. UFC5003) and the retentate was used.

4.2.3 Expression and purification of MoPrP 144C

MoPrP 144C was expressed in *Escherichia coli*, extracted and solubilised similarly to wild-type MoPrP 23-231. The expression and purification were confirmed by gel electrophoresis (Figure 4-3). Briefly, PrP from the cell pellet was solubilised and loaded onto a Ni-NTA column. After refolding on the column, the protein was eluted and dialysed against 20 mM BisTris buffer, pH
6.5 for 1 h. The protein solution was then filtered through a 0.2 \(\mu\)M syringe filter, and the concentration was measured to be 36.5 \(\mu\)M. The protein was not cleaved in this step.

The protein eluted from the Ni-NTA column was loaded on lanes 2 and 3 of the gel. 2x Laemmli loading buffer was used in lane 2, and 4x LDS loading buffer was used in lane 3. Laemmli buffer contains BME, a reducing agent able to break the disulfide bond, while LDS loading buffer doesn’t contain BME. As a result, PrP in lane 2 was monomeric, while PrP in lane 3 without BME contained both monomeric fraction and dimeric fraction formed by a disulfide bond linking residues 144C of two PrP molecules.

![SDS-PAGE gel](image)

**Figure 4-3.** SDS-PAGE gel showing the purified MoPrP 144C. Lane1 represents the protein ladder. Lanes 2 and 3 are the eluted protein using 2x Laemmli loading buffer or 4x LDS loading buffer, respectively.

4.3 Labelling of MoPrP 23-231

4.3.1 Labelling of MoPrP 23-231 with Alexa Fluor 647 NHS Ester

The labelling experiment was first conducted at pH 8.0–9.0 using 100 mM NaHCO\(_3\) (final concentration) in the buffer to adjust the pH. This pH range is optimal for the coupling of NHS ester dyes. The AF647 NHS ester (Invitrogen™, Cat.No. A20006) dye dissolved in DMSO was added to 30 \(\mu\)M into the pH adjusted, 20 \(\mu\)M PrP solution. The mixture was stirred overnight at
4°C for the labelling reaction to occur, and blue precipitates were seen afterwards. The solution was filtered through a 30 kDa filter to remove precipitates and aggregates (if any). The flow-through containing labelled PrP and excess free dyes was concentrated by spinning inside a 3 kDa filtration unit. Free dye was removed by repeatedly adding 10 mM BisTris to the retentate where PrP was at and spinning the solution through the same 3 kDa filter.

The final product – labelled PrP – was in the retentate, and UV-Vis measurement gave the dye concentration to be 4.7 µM and PrP concentration to be 11 µM. The labelling ratio was ~40%.

To make labelled fibrils, labelled PrP monomer was mixed with unlabelled PrP at 5% (use 4.7 µM as the labelled protein concentration in the calculation) and aggregated for 87 h using the standard condition for MoPrP 23-231 aggregation: 10 µM total protein in 50 mM MES, 2 M GdnHCl, 20 µM ThT, with agitation at 37°C (see Section 4.4.1 for details on the aggregation conditions). The kinetic traces were consistent with that of unlabelled PrP and confirmed the formation of ThT-positive aggregates.

PrP wasn’t stably soluble in the slightly alkalic environment optimal for NHS ester coupling. In the second labelling attempt, the pH of the protein solution was adjusted to 7.4 using Na-phosphate (NaP) buffer after dialysing the protein solution to remove BisTris. PrP was more stable at this pH, and the conjugation reaction was still able to occur. A mixed solution containing 7.7 µM PrP and 15.4 µM dye was stirred at room temperature for 1 h, allowing the labelling reaction to occur. Precipitates were seen on the stir bar afterwards. Precipitates were dissolved by adding the same volume of 8 M GdnHCl solution into the solution. Free dye in this solution was removed by dialysis against 10 mM Bistris buffer, pH 6.5. However, blue precipitates were seen again on the dialysis membrane. After taking out the solution from the dialysis cassette, solid GdnHCl was added to the solution, reaching a concentration of 6 M to dissolve precipitates. This solution was concentrated by spinning inside a 3 kDa Amicon filter. In order to remove any aggregates, the concentrated solution was span through a 30 kDa filter. It was worth mentioning that the
retentate was more coloured than the flow-through, suggesting a large proportion of protein didn't go through the 30 kDa filter. In contrast, for unlabelled protein in the native buffer, ~70% of protein could pass the 30 kDa filter. This discrepancy was possibly due to a larger apparent size that PrP adopted in the unfolded state in 6 M GdnHCl. Another explanation was that PrP had aggregated; however, the high GdnHCl concentration in the solution made this theory less likely. Monomeric flow-through was then concentrated again, and the retentate was the labelling product, denoted as PrP a. The final PrP concentration was 4.3 µM, and the dye concentration was 11.2 µM.

In a repeat labelling experiment, the solution after labelling was mixed with precipitates that were solubilised in 6 M GdnHCl. The mixture was dialysed against 6 M GdnHCl overnight to remove the free dye. The dialysed solution was filtered through a 100 kDa filter. The flow-through was concentrated by spinning in a 3 kD filter, reaching a dye concentration of 25.6 µM and a PrP concentration of 23.9 µM. This labelled PrP was denoted as PrP b.

![Figure 4-4](image)

**Figure 4-4.** Unstained (left) and Coomassie-stained (right) gel pictures of unlabelled and labelled MoPrP 23-231. Lane 2: 9.9 mg unlabelled PrP; lane 3: 2.5 mg unlabelled PrP; lane 7: 2.3 mg labelled PrP subjected to Trichloroacetic acid (TCA) precipitation to remove GdnHCl prior to mixing with gel loading buffer.
Gel electrophoresis was performed for PrP b to confirm that labelling was successful. The unstained and Coomassie-stained gel is shown in Figure 4-4. Three samples were loaded onto the gel: unlabelled PrP (lanes 2 and 3) as controls and labelled PrP (lane 7).

Before staining, in lane 7 with labelled PrP, a weak, light blue band was observed migrating at ~28 kDa (the black arrow in Figure 4-4 left), indicating the presence of AF647 labelled protein. After Coomassie staining (Figure 4-4 right), the PrP bands in lanes 2 and 3 appeared, and the band in lane 7 was stained as well. The bands in the three lanes were at almost the same molecular weight. The molecular weight of a dye molecule is small (~1.3 kDa), and the incorporation of a dye molecule led to a slightly higher band position on the gel.

This labelled PrP band in lane 7 was fainter than the unlabelled PrP band in lane 3. Possible causes were (1) the TCA precipitation step for labelled PrP might lead to some protein loss, and (2) the concentration measurement of labelled PrP by UV-vis was not reliable due to the low concentration of protein and the influence of AF 647 spectrum at 280 nm where protein concentration was calculated.

Labelled aggregates were then produced by aggregating 10 µM unlabelled MoPrP 23-231 monomer with 5% PrP a or 5% PrP b. The aggregation time was 88h to make aggregates a and 139 h to make aggregates b.

Aggregates a were subjected to ultracentrifugation (Section 5.4.1). 50 µL sample was ultracentrifuged at 100,000 g for 30 min at 4°C, the supernatant was collected, and the pellet was resuspended in 90 µL aggregation buffer.

4.3.2 Labelling of MoPrP 144C with AF 647 C2 maleimide

MoPrP 144C expressed and purified as described in 4.2.3 was used for labelling. Tris (2-carboxyethyl) phosphine (TCEP) was added to the protein solution to a concentration of 150 µM. After 15 min incubation, AF647 dye (Invitrogen, Cat.No. A20347) dissolved in DMSO was added to the protein solution at a 1.5:1 ratio, and the mixture was put on a rocker overnight. After
labelling, large amounts of blue precipitates were seen. The solution was removed, and a high concentration of urea solution was added to dissolve the precipitates. I proceeded with the solubilised precipitate fraction.

I tried to cleave the His-tag off from the labelled protein. Since PrP in the native buffer tended to precipitate, I maintained PrP in a buffer with 2 M urea and tested if thrombin cleavage worked. The manufacturer's instruction suggested thrombin was active under this condition.

After thrombin digestion, precipitates were observed and were pelleted by centrifugation. Gel electrophoresis (data not shown) showed that the supernatant and pellet resuspension contained only a trace amount of cleaved protein; most of the protein was uncleaved. Thus, thrombin didn’t work as expected under this condition.

Both the supernatant (with 2 M urea) and the pellet resuspension dissolved in 6 M GdnHCl were dialysed against 20 mM BisTris to remove the denaturant, causing the protein to precipitate again. The supernatant of the two solutions was mixed; CaCl₂ and thrombin were added to cleave the protein. The two pellets were dissolved in 7.2 M urea, mixed and diluted with BisTris buffer to make a urea concentration of 0.51 M. Cleavage was performed for this solution as well.

After overnight cleavage, precipitates were observed for both samples. Solid urea was added to dissolve precipitates. All cleaved protein was mixed, filtered through a 0.45 µm syringe filter and purified by a Ni-NTA column. All running buffers contained 2 M GdnHCl to prevent protein precipitation. Three fractions near the protein peak were collected.

Samples of the three peaks were loaded onto lanes 7, 8 and 9 on the gel (Figure 4-5) after GdnHCl was removed by TCA precipitation. LDS loading buffer was used; all samples with loading buffer were boiled for 10 min before loading onto gel. Before staining (left), faint, light blue bands showed on the gel, suggesting successful labelling. After Coomassie staining, a major cleaved protein band was seen; however, a small fraction of the protein wasn’t cleaved. A faint dimer band was observed as well. The labelling ratio was calculated to be 80% by the concentration measurement (Figure 4-6);
unlabelled PrP possibly dimerised through the formation of a disulfide bond linking the 144C residues of two unlabelled PrP molecules, leading to this dimer band.

**Figure 4-5.** Purification and labelling of MoPrP 144C. Lanes 1-3 are the same as in Figure 4-3. Lane 1 represents the protein ladder. Lanes 2 and 3 are the uncleaved protein eluted from the Ni-NTA column, boiled with 2x Laemmli loading buffer or 4x LDS loading buffer, respectively. Lanes 7-9 are the cleaved and labelled protein eluted from the second Ni-NTA column.

Fraction 2 of the eluted protein (lane 8) was concentrated by spinning in a 3 kDa filter; imidazole was removed by repeatedly adding 2 M GdnHCl solution and centrifuging in the same filter for a total of four times. The final retentate was filtered through a 100 kDa filter and was the labelling product.

The labelled PrP was named cPrP\(^\text{C}\), standing for cleaved, labelled PrP\(^\text{C}\). The concentration measured by Nanodrop (Figure 4-6) was 8.6 µM for PrP and 6.9 µM for dye. They were used to make aggregates 5C shown in Figures 5-26 and 5-27 A.
I repeated the labelling experiment using a procedure similar to the previous attempt but omitted the cleavage step since it caused a precipitation problem. The labelled protein solution was dialysed against 20 mM BisTris buffer overnight. After dialysis, precipitates were seen. Concentration measurement showed that the solution fraction only contained a small amount of protein, and the labelling ratio was low, so I proceeded with the precipitates. After washing the precipitates with buffer to remove TCEP, 6 M GdnHCl was added to dissolve them. I measured its concentration using Nanodrop and found that the dye concentration was about two-fold of protein, possibly suggesting free dye also precipitated during dialysis. This solution was filtered in a 10 kD spin filter, and the flow-through was shown to contain only dye and no protein by concentration measurement. Free dye was further removed by repeatedly adding 6 M GdnHCl and spinning inside the 10 kD filter.

The final solution (uPrP<sup>C</sup>, standing for uncleaved, labelled PrP<sup>C</sup>) above the membrane was filtered through a 100 kDa filter. The concentration of the flow-through was measured to be 23 µM for both PrP and dye. This batch of labelled PrP was used to make aggregates 5U, as shown in Figure 6-27 B.

4.4 *In vitro* aggregation assays

4.4.1 Aggregation assays of MoPrP 23-231

Monomeric PrP was diluted to the desired concentration into the aggregation buffer (50 mM MES, pH 6.0, 2 M GdnHCl) with 20 µM ThT. The protein solution was pipetted into a 96-well plate (Corning, Cat.No. 3651) with 3 Zirconium (Zr)
spheres (with a 0.5 mm diameter) inside each well. The plate was sealed and inserted into a plate reader (BMG Clariostar). Spontaneous aggregation was initiated by shaking the plate at 700 rpm with 100 s on / 20 s off cycles at 37°C unless otherwise indicated. ThT fluorescence was recorded every 10 min to monitor aggregation kinetics until aggregation curves for most wells plateaued. Generally, at least triplicates were performed for each experimental condition.

For seeding assays, 10 µM monomeric PrP was used, and a specific fraction of seeds ranging from 5% (w/w) to 0.00005% was added to the protein solution in the aggregation buffer. The aggregation condition was the same as the spontaneous aggregation assays.

4.4.2 Aggregation assays of MoPrP 91-231

The procedures to conduct spontaneous and seeded aggregation assays were similar to those for MoPrP 23-231, except that the aggregation buffer was 50 mM NaP, pH 7.4, 2 M GdnHCl, 300 mM NaCl, and the temperature was 42°C unless otherwise indicated.

4.4.3 Perturbation analysis

Perturbation analysis [109] was conducted for aggregated samples which had reached the plateau phase when the experiment stopped. Fitting the sigmoidal function (Equation 5.1) to each kinetic trace yields the initial fluorescence f₀ and amplitude A. The first 15% of fluorescence increase was selected for fitting. It was defined as the kinetic data from 0 h to the time point t₁₅ when fluorescence first reached (f₀+A*15%). The selected data were divided by A for normalisation. Two equations, $\Delta = A(\cos |B| t - 1) + C$ and $\Delta = A(\cosh B t - 1) + C$, were fitted to the normalised data using MATLAB, to identify the better fit and obtain parameters A and B.

Flat lines seen in Figure 5-22 A for two traces were caused by a rapid increase in fluorescence near t₁₅. The fluorescence remained low until a time point when it increased to be much larger than 15% of the amplitude. The latter time point was not included.
4.4.4 Generation of MoPrP 91-231 seeds for elongation assays

Frozen aliquots of monomeric PrP were thawed and diluted into the buffer to a final composition of 10 µM rPrP in the aggregation buffer (50 mM NaP, pH 7.4, 2 M GdnHCl, 300 mM NaCl) with 20 µM ThT. The protein solution was pipetted into the 96-well plate with 3 Zr spheres in each well. The plate was incubated in a BMG Clariostar plate reader with shaking at 700 rpm with 100 s on / 20 s off cycles at 42 °C, and ThT fluorescence was recorded. The assay was stopped at 68 h when the kinetics of selected wells had reached a plateau. Selected samples were collected. The aggregates were used as seeds for the pilot elongation experiment shown in Section 5.5.2(3) after a 1:6.7 dilution.

Subsequently, a seeding assay was conducted, with the end product of the previous assay as seeds. The seed solution was prediluted 1:10 and sonicated for 10 min in a water bath sonicator. Then seeds were added at 0.1% to the protein solution, whose composition was the same as the previous assay. Seeded aggregation was conducted under the same condition as spontaneous assays. Aggregates were collected after two days, aliquoted and used as seeds for elongation assays shown in Chapter 6.

4.4.5 Sequential seeding assays in a 96-well plate

A sequential seeding assay of MoPrP 91-231 was performed in buffers containing either 1 M (condition a) or 2 M GdnHCl (condition b), plus 50 mM NaP, pH 7.4 and 300 mM NaCl. The monomer concentration was 1 µM, and the seed concentration was 0.1% (w/w) with respect to the monomer.

In the first seeding assay, a mixed solution with seeds and monomers in a specific buffer a or b was pipetted into plate wells with 3 Zr beads in each well and incubated in a BMG Clariostar plate reader at 37 °C with agitation. The seeded products were labelled based on their buffer conditions, a or b, and were used as seeds for the second assay.

In the second assay, using two seed types (seeds a and b) and two solution conditions (buffers a and b) generated four experiment combinations and four
end products: aa, ab, ba and bb. Here the first letter represented the seed type (i.e. the buffer type of the first seeding assay), and the second letter represented the buffer condition of the second assay.

4.5 Purification of authentic prion fibrils

Ex Vivo RML and ME7 prions were purified from scrapie-infected mouse brains by a lab member as previously described [185]. Briefly, 10% (w/v) brain homogenate was pre-treated with Pronase, EDTA, sarcosine and Benzonase. Sodium phosphotungstic acid (NaPTA) was added to 0.3%. After incubation, NaPTA precipitation was performed in the presence of 35% iodixanol. The pellets (containing contaminant proteins) and the surface layer (comprised mainly of lipid and contaminant proteins) were discarded. The supernatant was filtered through a 0.45-µm microcentrifuge filter and diluted two-fold with 0.3% NaPTA + 2% sarkosyl. After centrifugation, PrPSc was enriched in the pellet. The pellet was washed twice by resuspending in 17.5% iodixanol + 0.3% NaPTA and centrifugation. The pellet after the last centrifugation was resuspended in 0.1% sarcosine, aliquoted and frozen for storage. No PK digestion was performed.

4.6 Characterisation of PrP monomer and aggregates

4.6.1 SDS-PAGE gel

Protein samples were mixed with 4x LDS loading buffer (Invitrogen, Cat.No. NP0007) at a 4:1 ratio or 2x Laemmli loading buffer (BIO-RAD, Cat.No. 1610737) at a 1:1 ratio. The mixture was boiled at 100°C for ~10 min. Samples were loaded onto a 10-well NuPAGE 4%-12% Bis-Tris gel (Invitrogen, Cat.No. NP0335BOX) and run in MES running buffer (Invitrogen, Cat.No. NP0002) at 190 V for ~45 min. The gel was then stained in Coomassie solution for ~1 h before destaining.

For samples containing GdnHCl, TCA precipitation was performed to remove GdnHCl from the sample.
For cultured cells, 1 ml culture was spun down at 16.1 kG for 5 min. The pellet was resuspended in 200 µL 1x loading buffer and boiled for ~20 min before being loaded into the gel.

**4.6.2 Protein concentration measurement**

Protein concentration was measured by the UV absorbance at 280 nm using a Nanodrop spectrophotometer or a BMG Clariostar plate reader. The extinction coefficients used are: 62,268 M⁻¹ cm⁻¹ for MoPrP 23-231, 25,577 M⁻¹ cm⁻¹ for MoPrP 91-231, and 5,960 M⁻¹ cm⁻¹ for α-Syn.

**4.6.3 Circular dichroism spectroscopy**

Circular dichroism (CD) spectra were obtained using a Jasco J-715 spectropolarimeter (Jasco) in the wavelength range of 190-250 nm using a 2 mm Quartzglass Suprasil cuvette (Hellma, Cat.No. 110-2-40) with PTFE stopper lids. The scan rate was set at 100 nm/min; data pitch was 0.5 nm with 2 nm bandwidth.

The temperature was controlled by the PTC-423L Peltier controller (Jasco Corporation, Japan). Before each measurement, the sample was left to equilibrate for 60 s. 10 scans were taken and averaged to get the complete spectra that were background corrected.

Chemical denaturation of MoPrP 91-231 monomer was conducted by titration experiments using a 6.4 M GdnHCl solution containing 50 mM NaP, pH 7.4 and 300 mM NaCl at 37°C. The ellipticity was monitored at 222 nm. The first measurement was acquired using the 250 µL sample containing 10 µM PrP<sup>C</sup> in buffer with NaP and NaCl (no GdnHCl); a total of 23 measurements were obtained following the sequential addition of 25 µl of GdnHCl solution until a final concentration of 4.4 M was reached. Once the denaturant was added, the solution was gently mixed four times, and the sample was allowed to adjust for 60 seconds before every measurement. A total of three titration experiments were performed to obtain reliable data.
The sample used for thermal denaturation was 5 µM PrP\(^C\) in the aggregation buffer. Thermal denaturation was studied between 10°C and 60°C by monitoring the ellipticity at 222 nm. The temperature interval was set at 0.1°C, and the heating rate was 1°C/min.

4.6.4 CD sample preparation after sequential seeding assay in plate

Samples aa and bb described in Section 4.4.5 were used (1 ml each) to prepare CD samples. Each was spun at 16,100 g for 2 h at room temperature. 700 µL supernatant was taken out and saved for CD measurements. The pellet was resuspended and sonicated for 15 min in a water-bath sonicator and a further 10 min before CD measurements.

5 µM PrP monomer solutions in either buffer, a or b, were prepared as control samples. CD spectra were recorded for the buffer, monomer, pellet resuspension of aggregates and supernatant of aggregates. Spectra were then blank corrected by subtracting the corresponding buffer spectrum. Spectra for the two pellet resuspension samples were further baseline corrected. Finally, all spectra were smoothed using Adaptive-Smoothing filtering with a 5 nm window in Jasco software.

4.6.5 Negative stain electron microscopy

2 µL aggregated protein samples were loaded onto carbon-coated 300 mesh copper grids (Electron Microscopy Sciences, Cat.No. CF300-CU) that were glow discharged for 40 seconds using a PELCO easiGLOW™ glow discharge unit (Ted Pella Inc, USA). Samples were left to bind for 2 minutes, blotted, washed briefly in four water drops with blotting in between, and then stained with NanoW stain (Nanoprobes, Cat.No. 2018-5ML) for 2 × 1 min, then blotted and air-dried. Grids were imaged on an FEI Talos electron microscope.
4.6.6 TIRFM imaging of PrP aggregates

5–10 µL samples were deposited into one well of a plasma-glowed chamber and incubated for ~10 min. The unbound aggregates were washed away by the buffer. 200 µL imaging buffer was added to the well. The chamber was then sealed and ready for imaging.

4.7 Elongation assays on surface

4.7.1 Microscope chamber cleaning

8-well cell culture chambers with #1.5 high-performance glass coverslip bottom (Cellvis, Cat.No. C8-1.5H-N, 170 ± 5 µm thickness) were used for imaging. To remove fluorescent contaminants and to facilitate seed adhesion, chambers were immersed in 2% Hellmanex II (Hellma, discontinued) solution for 2–3 h at room temperature with occasional stirring, rinsed thoroughly with H₂O, followed by immersion in methanol for 5–10 min and rinsed with H₂O. The cleaned chambers were left on bench to dry, then sealed with parafilm and stored in a closed container. The washed chambers were usually used within a week. Before each imaging or elongation experiment, the chamber was glow-discharged for 2 min using a PELCO easiGLOW™ glow discharge unit.

For chamber silanisation, the 8-well chamber was cleaned with Hellmanex II detergent and plasma-glowed for 5 min right before use. Silanisation was conducted by incubating the chamber inside a desiccator with trichloro-(1H,1H,2H,2H-perfluorooctyl)silane (PFOTS) under vacuum for 40 min. The silanised chamber was rinsed with methanol and dried on a hot plate.

4.7.2 Preparation of NR and NB solutions

NB powder (Sigma-Aldrich, Cat.No. 370088) was dissolved in Ethanol. The dye concentration was measured by a Nanodrop spectrophotometer, using an extinction coefficient of 76,800 M⁻¹ cm⁻¹ at 627.5 nm [186]. The measured
concentration was 40 µM. The container was wrapped with aluminium foil during storage to protect the solution from light.

NR powder (Sigma-Aldrich, Cat.No. 72485) was dissolved in Methanol. For concentration measurement, a small volume of the dye solution was mixed with an equal volume of H2O. The concentration of the mixture was measured by a Nanodrop spectrophotometer, using an extinction coefficient of 38,000 M⁻¹ cm⁻¹ [187] at the absorption maximum, which was 582 nm in this measurement. The concentration of the original NR solution was 50 µM.

4.7.3 Chamber assembly for elongation experiments

To prepare the monomer solution, monomeric PrP was diluted in a specific buffer to reach a desired final composition. NB was added to 400 nM for the visualisation of PrP fibrils.

Aliquoted recombinant seeds were thawed and diluted into the aggregation buffer at a 1:80 or 1:120 dilution ratio. 10 µL diluted solution was deposited onto the coverslip surface inside one well of an 8-well microscope chamber and incubated for 30–45 s. For authentic prion fibrils, the sample was incubated for 5–10 min. After incubation, the seed solution was taken out, and the well was washed with 200 µL aggregation buffer twice. 200 µL protein monomer solution was pipetted into the well. The chamber was sealed by double-wrapping in parafilm (one piece was wrapped on the top of the chamber, and another stripe was wrapped around the side) or filling the chamber lid with Twinsil silicone (Picodent). The sealed chamber with the sample inside was then ready to be imaged under the microscope.

4.7.4 Time-lapse imaging of fibril elongation

The assembled chamber with surface-attached seeds and the monomer solution was placed on the microscope for ~20 min for temperature equilibration. Positions of multiple fields of views (FOVs) with identified bright seeds were chosen using the multi-dimensional acquisition module in
MetaMorph imaging software. The software recorded images of those FOVs every 15 min for over 2 days, usually at 5% laser power at large-field mode with 1 s exposure time.

After the acquisition, images taken at each location were imported into ImageJ as a time-lapse image stack. The image stack was drift corrected by ImageJ plugins StackReg [188] and Image Stabilizer [189]. The middle 1,000x1,000 pixel-sized region was used for analysis.

The fibril selection criteria were detailed in Section 6.4.2.

A kymograph was generated for each fibril to extract the kinetic information by drawing a segmented line along its axis and running the reslice command in ImageJ. The fibril edge in the kymograph was drawn as a segmented line, and its coordinates were saved. The saved fibril edge positions were analysed using custom scripts written in MATLAB to identify the growth/stall phases and calculate the overall rate, pause free rate, stall phase percentage and other parameters.

Fibril brightness was extracted from each kymograph. Since brightness fluctuated during fibril growth, the time point to take intensity measurement for a fibril was set to be the end time of its last growth event. Brightness was measured in the last frame if a fibril was in the growth phase when the experiment stopped. The brightness value was calculated as the average pixel intensity of the middle half of the fibril’s length.

Since laser illumination was not flat but adopted a Gaussian profile in each FOV, the measured fibril brightness needed to be corrected for the difference in illumination intensity, depending on the fibril’s location in the FOV. > 200 points were selected from one FOV at background positions, i.e. at positions where no fibrils existed. A 2D Gaussian function was fitted to (x,y) coordinates of selected points and their intensity values. A brightness correction factor was calculated for any point (x,y) to be:

\[
\text{Correction factor} = \frac{G_{\text{max}}}{G_{(x,y)}}
\]
where $G_{\text{max}}$ was the maximum value of the fitted Gaussian function, and $G_{(x,y)}$ was the value at coordinates $(x,y)$. Thus, a corrected fibril brightness was calculated by multiplying the brightness value by the correction factor at the fibril’s $(x,y)$ coordinates. Here we assumed that the fibril intensity was linearly related to the background intensity.

For one experiment where the illumination profile was not a regular Gaussian function (caused by a not-perfect TIRF calibration), the FOV was split into 10x10 grids. The correction factor of each grid was calculated by:

$$\text{Correction factor} = \frac{G'_{\text{max}}}{G_{\text{grid}}}$$

where $G_{\text{grid}}$ was the mean intensity value of background points in the grid, and $G'_{\text{max}}$ was the maximal mean value of all grids.

### 4.7.5 Sequential surface seeding assay

The first elongation assay in the microscope chamber was carried out with surface-attached seeds and a 200 µL solution containing 1 µM PrP monomer, 1 M GdnHCl, 50 mM NaP pH 7.4, 300 mM NaCl and 400 nM NB. After incubating at 37 °C for 71 h, the chamber was opened, the solution was taken out, and the surface was washed with buffer. A new monomer solution with 2 M GdnHCl (the other components were the same as in the previous assay) was added to the same well. The chamber was sealed again and put on the objective for time-lapse imaging.

Intensity difference within a FOV caused by Gaussian illumination needed to be adjusted. A pseudo background image was generated in ImageJ by running the Gaussian blur command with a radius of 50 pixels for the original image. The corrected image $i$ was obtained using the Calculator plus command by the ‘divide’ operation: $i = (i_1/i_2)*k_1+k_2$, where $i_1$ was the original image, $i_2$ was the background image, $k_1$ was the mean intensity of $i_2$, $k_2 = 0$. Fibril intensity was directly determined from the corrected image $i$. 
4.7.6 Seeding on the surface of TEM grids

TEM grids used for seeding assays were carbon-coated Au grids (Electron Microscopy Sciences, Cat.No. CF300-Au) as they were inert in the aggregation buffer while the copper grids were not. Grids were glow discharged for 40 s right before use. Recombinant seeds were diluted at 1:3.2 in the buffer. 2 µL sample was pipetted onto a grid and incubated for 5–10 min allowing seed adsorption, in a closed container to prevent evaporation. The remaining sample was blotted. The grid was washed briefly in two buffer drops with blotting in between and put into a well of a 96-well plate. 100 µL PrP monomer solution was added to the well. The plate was sealed and incubated in a 37 °C incubator for a desired period.

After incubation, the solution was pipetted out from the well, and the grid was taken out carefully, washed with three water droplets and stained with NanoW stain solution for 2 × 1 min, then blotted and air-dried. Grids were imaged on an FEI Talos electron microscope. Care was taken when handling grids as they were easily bent, and the carbon film was easily peeled off during a multiple-step experiment.

Typically, fibrils longer than 500 nm were analysed to obtain a width distribution. Fibrils imaged by TEM were straightened in ImageJ and oriented horizontally. A 10-pixel wide, vertical line was drawn across each fibril, and the intensity profile along the thick line was generated by ImageJ. Fibril edges were assumed to be at the two dip positions in the intensity profile, and the fibril width was calculated accordingly.

4.8 Super-resolution imaging and data analysis

4.8.1 TAB imaging

TAB imaging was performed for fibrils elongated on coverslip surfaces. The chamber assembly procedure was the same as in 4.7.3, except that the solution contained a reduced concentration of dyes to ensure better blinking. 50 nM NR and the 561 nm excitation laser was used for the imaging of recombinant fibrils. The excitation lasers were at full laser power in the high-
intensity mode. Stacks of 5,000 or 10,000 frames with 20 ms exposure time were acquired for each region of interest.

The captured image stacks were first analysed using the ThunderSTORM plugin [190] in ImageJ to generate localisation information on all blinking events. The reconstruction was a direct output of ThunderSTORM or was generated by a MATLAB script using the localisation information, with the brightness of each pixel representing the number of localisations that fell inside that pixel.

The following steps were conducted to trace the change in fibril brightness along the fibril axis.

(1) The list of all localisations generated by ThunderSTORM was imported to MATLAB; the coordinates for each localisation were divided by the pixel size (20 nm) for an easier calculation for the following steps; the calculated values were used hereafter.

(2) The fibril or fibril segment of interest was selected by drawing a rectangle covering it. The largest isolated fibril or fibril segment in this rectangle was selected.

(3) All localisations belonging to the selected fibril or fibril segment were scatter plotted; a polynomial or spline fit for those points was conducted to find a curve describing the fibril axis. The polynomial fit was conducted up to order 8; to better track the fibril axis, a spline fit was performed with \( k = 4 \) and \( l \) between 10 to 40, depending on the fibril’s length and shape.

(4) Two points on the fibril axis near the two ends were selected as the new start and end points of the fibril. Localisations within the small regions outside the end positions were discarded since fitting at the end was often unreliable.

(5) Fibril straightening. For each localisation, an orthogonal projection onto the fibril axis was made, and coordinates of the projection point were calculated. The next step was to calculate which pixel this localisation fell into in the straightened image, which was determined by the distance along the fibril axis from the left end to the projection point, and the perpendicular distance from the localisation to the fibril axis, as discussed in Section 7.1.3.
(5) The intensity profile was then generated from the straightened image of the fibril: the brightness at each axial position was the sum of all pixels in the column direction (i.e., pixels in the fibril cross-section), and it was plotted against the axial position. This profile was put through fast Fourier transform (FFT) using MATLAB to identify any periodicity peaks.

4.8.2 p-TAB imaging and image analysis

p-TAB imaging was performed for elongated fibrils on coverslip surfaces. Before imaging, the Cairn integrated polarisation beam splitter (PBS) cube was inserted into the Optosplit II image splitter (Figure 4-1) and adjusted so that each orthogonally polarised channel occupied half of the camera sensor. An ROI containing structures of interest in both channels were selected. A stack was then acquired using the same parameters as in TAB image acquisition.

Reconstructions were generated for each channel using the ThunderSTORM plugin. ThunderSTORM also outputted information about identified blinking events, including estimated (x,y) positions, widths of the PSF (σ) and photons.

Further analysis was conducted using a MATLAB script adapted from Dr. Matthew Lew’s lab in WUSTL [158] with minor changes, and the workflow is shown in Figure 4-7. The main difference was in the initial registration of single emitters from the 2 channels. The goal of this step was to ensure that the pairs of photon bursts used to calculate linear dichroism (LD) originated from the same single molecule. A rough registration was done by eyes before a refined registration using MATLAB, thus eliminating the calibration step using fluorescent beads in [158]. The refined registration step was conducted as described in [158] to obtain a transformation function. Single molecules from the two channels were paired if the distance between a molecule’s position in one channel and the transformed position of a molecule from the other channel is minimal and also within a limit of 3 times the localisation precision. Photons detected for each localisation event were calculated by summing all photons in a 5x5 pixel-sized square centred at the (x,y) position of the localisation, followed by background correction using the average photons per pixel in the
one-pixel wide surrounding region of the square. The precision of each localisation was calculated [191] using the photons calculated above, and localisations with localisation uncertainty over 20 nm were deleted.

Figure 4-7. Flowchart of the MATLAB script to generate an LD map.

For paired single-molecules, photons emitted by each of them were a sum of the photons from both channels; the LD of each emitter was calculated by the equation:

\[ LD = \frac{I_x - I_y}{I_x + I_y} \]

where \( I_x \) and \( I_y \) were photons detected in x and y channels, respectively. For unpaired single-molecules that were present in either x or y channel, the
transformed position in another channel was calculated. Photons at the transformed position were obtained, allowing LD to be calculated.

An LD map with a 20 nm pixel size was obtained: each pixel’s LD value was the median value of all single emitters (including paired and unpaired localisations) that fell in that pixel. LD was set as 0 if no single molecules were detected in one pixel. The LD map was then plotted with the colour bar indicating both the number of localisations (brightness) and LD value (colour) of each pixel.

Next, the change of LD value along the fibril axis was analysed. Similar to the analysis described for TAB images, a segment of fibril was selected and processed in MATLAB so that each localisation could be located in the straightened, pixelated image. In the straightened image, the LD value of a pixel was the median LD of all localisation events located in that pixel.

To track how LD changed along the fibril axis, an average value at each axial position was calculated by averaging the LDs of all localisations in the cross-sectional pixels. The average LD versus axial position plot was smoothed using the smooth function in MATLAB and put through FFT to identify any peaks.

To correct the change in LD value caused by the change in fibril backbone direction, a revised LD (LD’) was computed for each molecule to represent the angle between the dye molecule axis and the local orientation of the fibril axis. For each localisation event, the dye molecule’s azimuth angle was \( \beta = \pm \sin^{-1} \sqrt{\frac{1-\text{LD}}{2}} \). The azimuth angle of the fibril axis is the slope of the fibril axis at the projection point (P): \( \alpha = \tan^{-1} f'(x_P) \in (-\frac{\pi}{2}, \frac{\pi}{2}) \), where \( f \) represents the fibril axis. Thus, the angle between the two orientations, \( \theta \), was \( ||\alpha| - |\beta|| \), where \( \beta \) was chosen to yield the smaller of the two possible \( \theta \) angles.

A revised LD of each localisation was \( \text{LD}' = 1 - 2 \sin^2 \theta \). The change in LD’ along the fibril axis was then analysed.
Chapter V. The aggregation of full-length and truncated MoPrP in bulk solution: spontaneous aggregation assays and seeding assays

The first step of my work was to generate recombinant seeds. Two types of recombinant PrP, full-length MoPrP 23-231 or truncated MoPrP 91-231, were used to generate aggregates, and their aggregation kinetics were analysed.

The seeding ability of the generated different types of seeds was tested by seeding assays in bulk solution and single-particle elongation assays. The goal was to find a good seed candidate and experiment setup to study the elongation mechanism and kinetics.

5.1 Spontaneous aggregation of MoPrP 23-231

5.1.1 Aggregation assay kinetics

In literature, fibrillar aggregates of PrP were produced in vitro under several protocols: (1) incubating PrP under partially denaturation conditions [44, 45] with GdnHCl or urea, or a mixture of GdnHCl and urea at an elevated temperature with agitation; (2) incubating PrP in the presence of detergents, for example, 0.03% SDS [192] for a relatively long time (35 days in this reference); (3) incubating PrP under an acidic condition in the presence of a low concentration of denaturant, for example, HuPrP 90-231 formed fibrillar aggregates in 1 M GdnHCl at pH 4.0 [47].

I utilised the first method to generate fibrils and studied the aggregation kinetics [193]. Monomeric MoPrP 23-231 was filtered through a 30 kDa Amicon membrane filter to remove any pre-formed aggregates. Even though the molecular weight of PrP (23.4 kDa) is only slightly smaller than the cut-off size of the membrane, most of the protein (~70%) was able to pass through the membrane, confirmed by the concentration measurement of PrP before and after filtration. It indicated that the protein in the stock buffer (10 mM HEPES, pH 7.5) was mostly monomeric.
Figure 5-1. Aggregation kinetics of MoPrP 23-231. (A) The measured ThT fluorescence traces of 9 wells (left panel) and the corresponding sigmoidal fits (right panel). Traces with the same colour in the two panels represent the same well. (B) The fluorescence trace and sigmoidal fit overlaid for each well, plotted separately among different wells.

The flow-through was then concentrated in a 3 kDa Amicon filter and was diluted in a partially denaturing buffer, reaching a final composition of 10 µM PrP in the buffer containing 50 mM MES pH 6.0 and 2 M GdnHCl (referred to
as the standard aggregation buffer in Section 5.1). The solution was incubated in a 96-well plate with three single-use Zirconium beads in each well with intense agitation at 37 °C. The aggregation kinetics were recorded by reading the ThT fluorescence (Figure 5-1).

A sigmoidal function (Equation 5_1) was fitted to kinetic curves for wells that had fully aggregated using MATLAB. Half time ($t_{50}$, defined as the time needed to reach 50% of the amplitude), lag time ($t_{lag}$, the duration of the lag phase), elongation rate constant ($k$) and fluorescence amplitude ($A$) were extracted. Due to the fluorescence rapidly dropping after maximum, information about the maximum fluorescence could not be fitted accurately.

$$F = f_0 + \frac{A}{1 + e^{-k(t-t_{50})}}$$

$$t_{lag} = t_{50} - \frac{2}{k} \quad \text{Equation 5_1}$$

Kinetic traces in Figure 5-1 show considerable heterogeneity in lag time, from less than 20 h to over the measurement duration (113 h). The heterogeneity was previously observed for the aggregation of Aβ40 [194] and Hamster PrP 90-231 [195]. The phenomena were attributed to either a stochastic event occurring during nucleation [194] or the formation of different types of nuclei kinetically and structurally, leading to the formation of distinct fibrils [195].

Three approaches were tested to reduce the heterogeneity:

(1) Changing bead type

The assay mentioned above used three Zirconium (Zr) beads with a 0.5 mm diameter to enhance agitation and promote aggregation. No ThT-positive aggregates were detected in wells without beads, suggesting the presence of beads is essential for PrP aggregation. The acceleration of aggregation by gyrating beads could have multiple causes, including an increased air-water interface area promoting nuclei formation [196, 197], and the presence of bead-water interface promoting fragmentation once the critical nuclei are formed [197, 198]. The degree of acceleration is affected by the bead shape, size, mass, material, surface hydrophobicity and the number of beads used.
However, Zr beads are not always spherical and identical in shape, which adds uncertainty to the experimental settings.

Glass beads are another candidate for spheres. They are larger in size with a 2 mm diameter, and their shape is better controlled, leading to less heterogeneity among glass beads than Zr beads. We previously used glass beads (one in each well) to facilitate the aggregation of α-Syn and observed overlayed kinetic traces within replicates. Here, I tested whether using glass beads led to more consistent replicates for PrP aggregation.

A comparison of kinetics using three Zr beads or one glass bead in each well is shown in Figure 5-2. One glass bead could facilitate PrP aggregation; however, the lag time of duplicates wasn’t consistent, indicating that changing the sphere type didn’t efficiently reduce the heterogeneity of PrP aggregation.

![Figure 5-2. MoPrP 23-231 aggregation kinetics when using three Zr beads or one glass bead as the stirring spheres.](image)

(2) Changing the monomer pre-treatment method

In the literature, researchers often pre-treated the monomer with 6 M GdnHCl [44] to completely unfold the protein before diluting it into the aggregation buffer and starting aggregation assays. This pre-treatment likely provided a uniform starting point for all PrP molecules.

To test whether the pre-treatment reduced the variance in kinetics, monomers with or without denaturation treatment were used in the aggregation assay. Under condition (a), monomers in the native storage buffer were diluted
directly into a buffer with GdnHCl, so that protein was eventually in the standard aggregation buffer. Under condition (b), monomers were first diluted in a buffer containing 8 M GdnHCl to reach a GdnHCl concentration of 6 M to unfold the protein monomer. This solution was further diluted by a native buffer to reach the standard aggregation buffer condition. Aggregation kinetics using monomer preparation (a) are plotted in Figure 5-1; kinetics using pre-treated monomers (b) are shown in Figure 5-3. The blue and orange curves are from two separate experiments under identical conditions.

Unfortunately, this pre-treatment didn’t yield tighter replicates. Aggregation kinetics using monomers with or without the pre-treatment didn’t show obvious discrepancies: similar sigmoidal curves with heterogeneous lag times were observed. The fluorescence amplitude was affected by different fluorescence reading settings; thus, values couldn’t be directly compared between experiments.

![Figure 5-3. MoPrP 23-231 aggregation kinetics using pre-treated monomers.](image)

(3) Changing buffer type

The third attempt was to slightly adjust the composition of the aggregation buffer by adding NaCl. Aggregation was conducted under three conditions: (a) 10 µM PrP aggregating in the standard buffer (pH 6.0), (b) 10 µM PrP in the standard buffer with 300 mM NaCl, and (c) 20 µM PrP aggregating in the standard buffer with 300 mM NaCl. The kinetic traces and the fitted sigmoidal curves are plotted in Figure 5-4 A-C, respectively.
Figure 5-4. MoPrP 23-231 aggregation kinetics under multiple conditions and the sigmoidal fits. Monomer in this assay was the flow-through of a 50 kDa membrane filter instead of a 30 kDa filter as used in the previous assays. (A) Kinetics of 10 µM PrP aggregating in the standard aggregation buffer (condition (a), the left panel) and the sigmoidal fits (the right panel). The one curve (dark blue) fitted with a much slower slope in the elongation phase compared to other curves was likely caused by a fitting error due to the very noisy original data. (B) Kinetics of 10 µM PrP aggregating under condition (b). (C) Kinetics of 20 µM PrP aggregating under condition (c).
Condition (a) was essentially the same as the previous assay in Figure 5-1, except that the monomer was filtered through a 50 kDa filter. The final fluorescence was relatively consistent between wells except for the yellow curve. Variance in lag time was also observed in this dataset, albeit smaller than in the assay shown in Figure 5-1.

The consistency among replicates wasn’t improved in the presence of NaCl (condition (b)). Interestingly, the final fluorescence was shown to be different among wells. 6 out of 9 curves shared a similar level of final fluorescence, which was also similar to that under condition a. However, two curves (the yellow and green curves) had an over two-fold increase in final fluorescence.

At a higher PrP concentration (20 µM) in the presence of NaCl (condition (c)), the discrepancies in lag time existed but were smaller than in the other conditions. However, only three replicates were tested here; I couldn’t rule out the possibility that a larger degree of heterogeneity would be observed if more replicates were tested.

Overall, simply adding NaCl to the aggregation buffer couldn’t effectively reduce the heterogeneity in lag time.

(4) Changing the buffer type and temperature simultaneously

![Graph](image)

**Figure 5-5.** MoPrP 23-231 aggregation kinetics in a different buffer system and at an elevated temperature.

In the last attempt, I tested a buffer system adapted from RT-QuIC assays [41, 199] in the presence of denaturant: 50 mM NaP pH 7.4, 150 mM NaCl and 2M GdnHCl. The solution at 9.7 µM PrP concentration was incubated at 50°C with
agitation. The kinetic traces (Figure 5-5) show that the lag time was shorter and tighter within triplicates, while the final fluorescence varied. Overall, this condition yielded the most reproducible kinetics and was used in kinetic assays studying the effect of PrP concentration (Section 5.1.3).

5.1.2 Kinetic analysis revealed the involvement of secondary processes

Nucleation and elongation are necessary for the aggregation to take place; the following analyses test whether secondary processes, including fragmentation, secondary nucleation and lateral association, are also involved in PrP fibril formation under my assay conditions.

A perturbation method [109] is traditionally used to analyse kinetic data of protein-polymer formation and answer this question. In this method, only the initial aggregation phase (for example, the first 15% of the final fluorescence) is analysed. Simplifications could be made for this phase since the concentration change of certain species is typically small.

Several assumptions are made in this model: (1) the critical nuclei are in equilibrium with monomers, and their concentration is negligible compared to free monomers or the protein incorporated into fibrils; (2) elongated fibrils cannot dissociate back to the nucleus state.

Two differential equations [109] describe the change in fibril concentration and polymerised monomer concentration in the model. The change in fibril concentration is considered as a result of both primary nucleation and secondary processes; the change in polymerised monomer concentration is assumed to result from only fibril elongation and dissociation. The two kinetic equations cannot be solved analytically; however, approximations can be obtained using the perturbation method. All terms in the equations are expanded in power series, and only first-order terms are retained, allowing first-order solutions to be derived. The first-order approximations are generally applicable for the initial phase of aggregation, where the concentration of each species only varies slightly [109]. Two parameters, A and B, are a combination
of rate constants for nucleation, elongation and secondary processes, initial and critical concentrations, and nucleus size.

If the contribution of secondary processes is small, the incorporated monomer concentration is given by:

\[
\Delta = A \cos(B|t - 1|) - \frac{1}{2} B^2 At^2 \quad \text{Equation 5.2}
\]

If the contribution of secondary processes is nonnegligible, the equation becomes:

\[
\Delta = A \cosh Bt - 1 \quad \text{Equation 5.3}
\]

Equation 5.3 implies that at the very beginning of the aggregation process when \(Bt \ll 1\), \(\Delta \rightarrow \frac{1}{2} B^2 At^2\), indicating primary nucleation dominates in this phase; when \(Bt \gg 1\), \(\Delta \rightarrow \frac{1}{2} AB^2t\) meaning secondary processes dominate.

**Figure 5-6.** Fitting of the aggregation kinetics in the initial phase. Equation 5.2 (left) or 5.3 (right) was fitted to normalised ThT fluorescence traces; a constant \(C\) was added to the equations to correct for a nonzero starting value. Fitting \(\Delta = A \cosh Bt - 1 + C\) to data was very sensitive to the initial guesses of the three parameters.

I used this method to analyse the aggregation kinetics shown in Figure 5-1. For the six wells where aggregation had plateaued, the above two equations were fitted to the initial 15% fluorescence increase to find a model that better described the data.
The fitting results shown in Figure 5-6 indicate the kinetics of the initial phase can’t be followed by a t^2 function (Equation 5.2) while can be satisfactorily captured by a cosh(t) function (Equation 5.3). Thus, secondary processes played an important role in the spontaneous aggregation of MoPrP 23-231.

5.1.3 Concentration dependence of de novo PrP aggregation

As has been established in 5.1.1, triplicates were the most consistent in a buffer system with 50 mM NaP pH 7.4, 150 mM NaCl and 2 M GdnHCl at 50°C. Under this condition, I studied the dependence of aggregation on PrP concentration ranging from 1.6 µM to 9.7 µM. The kinetic curves and analysis are plotted in Figure 5-7.

The dependence of amplitude A, elongation rate constant k and half time on PrP concentration are plotted in Figure 5-7 C.

Amplitude exhibited a linear dependence on PrP concentration except for the data point at the highest concentration (9.7 µM), indicating the amount of ThT positive aggregates at the plateau phase was nearly proportional to the initial monomer concentration.

Elongation rate constant k didn’t show a strong dependence on PrP concentration, which was characteristic of a nucleation-polymerisation kinetic model. A weak positive slope was observed between 1.6 µM and 5.7 µM, followed by a weak negative slope at higher concentrations which could indicate an inhibition effect playing an increasing role. However, it was difficult to draw any conclusion due to the variance of fitted k within triplicates.

Half-time at the lowest PrP concentration (1.6 µM) was significantly larger than the other data points; interestingly, there wasn’t a further decreasing trend for half-time at concentrations higher than 2.4 µM.
Figure 5-7. Kinetic traces and analysis of MoPrP 23-231 aggregation at different PrP concentrations. (A) The kinetic traces (left) and the corresponding sigmoidal fits (right). (B) Normalised sigmoidal curves. Normalisation was conducted by dividing each fitted curve by \((f_0 + A_{\text{sigmoid}})\) so that the maximum of each curve was 1. \(A_{\text{sigmoid}}\) represents the amplitude \(A\) from the sigmoidal fit. (C) Concentration dependence of fitted parameters.
The slope in the log(half time) versus log(PrP concentration) plot gives the scaling factor $\gamma$. $\gamma$ is a useful parameter to determine the aggregation mechanism [110]. Mechanisms involving primary nucleation, elongation, with or without secondary nucleation and/or fragmentation have their unique, constant $\gamma$ value. In this case, $\gamma$ took a positive curvature. $\gamma$ defined by the first 2 data points was -1.2, which could result from multiple mechanisms, such as primary nucleation with a nucleus size of $\sim$2-3, or a combination of primary nucleation with secondary nucleation with a nucleus size of 1-2 [77, 110]. $\gamma$ changed to $\sim$0 for the last 3 data points at high PrP concentrations, implying a switch of the dominating mechanism. Several hypotheses could account for the low concentration dependence, such as the inhibition by some PrP species [152], the formation of off-pathway oligomers, the presence of concentration-independent rate-limiting steps, or the PrP concentration reaching a ‘supercritical concentration’ [200].

When analysing the dataset using a web-based kinetic fitting software AmyloFit [108], none of the listed models can adequately follow the traces at all concentrations. This suggested that the listed mechanisms (primary nucleation and elongation with optional fragmentation and/or secondary nucleation; elongation and secondary nucleation could be either a single-step or a two-step process) couldn’t describe the aggregation mechanisms of PrP. The noisiness of the curves and the remaining discrepancies within replicates also added complexity to the fitting.

The perturbation method [109] was then applied to study the initial phase of amyloid formation, i.e. the first 15% increase in ThT fluorescence. Either a $t^2$ function (Equation 5.2) or a cosh($t$) function (Equation 5.3) was fitted, and the results are shown in Figure 5-8. Even though the $t^2$ fit (Figure 5-8 A) might seem adequate for a few data sets, it failed to capture the rapid increase of fluorescence around $t_{15}$ in a few panels. However, the cosh($t$) fit (Figure 5-8 B) captured the trend in all panels reasonably well. $R^2$ was calculated for each fit. The mean $R^2$ value for all $t^2$ fits was 0.69, and that for cosh($t$) fits was 0.80. A better fit to the cosh($t$) function suggested that secondary processes were nonnegligible, consistent with the findings in Section 5.1.2 where aggregation took place in a different buffer system.
Figure 5-8. Fitting the $t^2$ (A) or cosh(t) (B) function to the initial phase of aggregation. Data in the first panel fluctuated significantly in the lag phase, preventing the determination of $t_{15}$, so this panel was removed from the fitting.
One parameter generated in the perturbation method, $B^2A$, depends only on the primary nucleation process by the following equation [109].

$$\log \frac{B^2A}{c_0(c_0 - c_s)} = \log(k^+_i K_i) + i \log c_0 \quad \text{Equation 5.4}$$

where $c_0$ represents initial monomer concentration, $c_s$ is the critical concentration, $k_+$ is the elongation rate constant, $K_i$ is the equilibrium constant for nucleus formation, and $i$ is the nucleus size.

Ideally, the nucleus size could be determined from a log-log plot of $\frac{B^2A}{c_0(c_0 - c_s)}$ versus $c_0$ as the slope, as shown in Figure 5-9. Here, A and B were obtained from cosh(t) fits, and $c_s$ value was set to be 0. $\frac{B^2A}{c_0(c_0 - c_s)}$ was found to vary within replicates to a maximum of over 3 log units. With such a large variance, the slope couldn’t be reliably determined.

![Figure 5-9](image)

**Figure 5-9.** Analysis of the nucleus size during MoPrP 23-231 aggregation.

### 5.1.4 Aggregate structures measured by TEM and TIRFM

Figure 5-10 shows TEM images of PrP aggregates at the end of a fibril formation assay in the standard aggregation buffer. Typical fibrillar structures of amyloid are seen, including isolated fibrils (usually short in length), fibril bundles and tangled fibrils. Large, heavily stained clusters were seen as well. Different fibril morphologies were observed. Most fibrils exhibited a twisting morphology with varied pitch lengths (Figure 5-10 by the blue arrows). A few small segments appeared to be straight, for example, the fibril pointed by the yellow arrow in Figure 5-10 A.
Figure 5-10. Typical TEM image of MoPrP 23-231 aggregates. The scale bar represents 200 nm. (A) A TEM image of PrP aggregated in the standard buffer for 90 h. (B) TEM images of PrP aggregates aggregated from 6M GdnHCl pre-treated monomer for 63 h.

Figure 5-10 B shows a TEM image of aggregates produced using 6 M GdnHCl pre-treated monomer. The structure of these aggregates was comparable to the aggregates generated under the standard condition, indicating the pre-treatment didn’t have a profound effect on the aggregate structure.
Figure 5-11. TIRF image of MoPrP 23-231 aggregates. The scale bar represents 5 µm.

A TIRF image of the aggregates formed in the standard aggregation buffer is shown in Figure 5-11. 50 nM NB was used here as the imaging dye. Short fibrils with ~1 µm in length and fibril clusters were abundant. However, due to the resolution limit, no further structural information could be obtained from the TIRF image. A detailed, fluorescence microscopy-based imaging and structural analysis of PrP fibrils will be presented in Chapter 7.

5.2 Seeding assays of MoPrP 23-231 in bulk solution

5.2.1 Seeding assays at different seed concentrations

Adding pre-formed amyloid aggregates into the monomer solution can bypass the slow primary nucleation step and thus can reduce, even completely remove, the lag phase. Here, I studied the aggregation kinetics when adding pre-formed seeds at concentrations ranging from 5% (w/w) to 0.00005%. Seeding assays were conducted under the same condition used for spontaneous aggregation assays: 10 µM PrP with seeds in the standard buffer. However, gain settings and focal height for fluorescence reading were different from the previous experiments, so the absolute fluorescence was not comparable.
Figure 5-12. MoPrP 23-231 seeding assay kinetics measured by ThT fluorescence. (A) Kinetics for seeding assays at 5% seed concentration. (B) Kinetics at 0.005% seed. (C) A zoomed-in view of panel B.

Figure 5-12 shows kinetics at 5% and 0.005% seed concentrations, confirming that the lag time was significantly shortened and the heterogeneity was greatly reduced in the presence of seeds. With 5% seeds, the lag phase was nearly abolished, making the sigmoidal fit unreliable. Indeed, for the two blue traces in panel A, negative values for lag time and half time were obtained from the fit and were removed from later analysis. In contrast, for three control wells
without seed, one sample aggregated with a half time of ~80 h, while 2 out of 3 wells didn’t aggregate until the end of the measurement (118 h).

A careful examination of the initial increase of fluorescence (Figure 5-12 C) revealed that the sigmoidal fit couldn’t perfectly track the data in this range. The initial fluorescence of the fit was higher than the actual value, and the fit failed to capture the slow increase in fluorescence at ~3–6 h. This fitting error was likely caused by the noisy nature of the dataset and implied that the lag time obtained from the sigmoidal fit was larger than the actual value. In the following analysis, half time (t50) was used instead of lag time to avoid problems related to this fitting error.

The kinetics of seeding assays provided valuable insights into PrP aggregation mechanisms. At the initial phase of the seeding assay (within hours after the experiment started), primary nucleation was not expected to occur. If elongation were the only process, the number of aggregates would remain constant while the mass of aggregates increased. ThT fluorescence should increase linearly with time at the beginning of the experiment when the monomer was abundant and approached saturation when the monomer was gradually depleted (Section 2.3.1, models (1) and (2)). The slope of the initial increase should be proportional to the concentration of added seeds. However, the measured kinetics at seed concentrations lower than 5% showed a sigmoidal trend instead of an initial linear increase, indicating that secondary processes also took place, which increased the number of aggregates. Indeed, the perturbation method [109] stated that in the presence of secondary processes, seeding kinetics should display a similar cosh(t) trend, while the change in seed concentration only affected the apparent lag time. Another model [201] assumed that the generation of new fibril ends depended only on the existing fibrils (via branching or fragmentation, for example), and monomer concentration was constant. An analytical solution was derived: both the fibrils’ number and mass increased exponentially with time, with seed concentration affecting the lag time.

This observation again confirmed the findings obtained from spontaneous aggregation assays in 5.1.2.
I repeated the seeding assay with a larger variety of seed fractions. After fitting the sigmoidal equation to the kinetic traces, the parameters from the fits are plotted in Figure 5-13.

![Figure 5-13](image)

**Figure 5-13.** Kinetic analysis for MoPrP 23-231 seeding assays. Data points with the same colour were from one experiment. The blue data points represented the assay discussed above in Figure 5-12. In the assay represented by the orange dots, triplicates were performed for each seed fraction, except at 5% seed, only duplicates were performed.

Both amplitude A and elongation rate constant k didn’t change much with seed concentration. Half time and lag time were significantly shortened in the presence of seed, even at the lowest seed concentration tested (0.00005%). Interestingly, half time was only weakly dependent on seed concentration. This observation was in contrast to the findings in the literature, either by modelling [109, 201] or experiments [202], that lag time or half time decreased linearly with the log of seed concentration, except at extremely low seed concentrations, kinetics became indistinguishable to the unseeded assay. In the study of Aβ42 [202], the linear relationship was observed when seed
percentage was more than ~0.008%, while half time became similar to spontaneous aggregation at lower seed concentrations.

To further probe the dependence of half time on seed concentration, 14 different seed concentrations were tested, ranging from 6.3% (labelled as S0, seed fraction was 6.3x10^{-2}) to 6.3x10^{-13}% (labelled as S13, seed fraction was 6.3x10^{-15}). Duplicates were performed under each seed concentration. PrP concentration was 8 µM in a buffer with 40 mM MES, 1.6 M GdnHCl and 16 µM ThT. Half times of each kinetic trace were obtained from a sigmoidal fit and plotted in Figure 5-14. At the highest seed concentration (S0), the fitted lag time of one curve was negative, and this condition was discarded. For the last three columns in Figure 5-14 (yellow columns) with seed S12, seed S13 or without seed, aggregation took place in only one of the duplicates within 70 h, and half time for that well is shown.

Figure 5-14 shows a significantly reduced half time when using seed S1–S8 compared to unseeded reactions. Under these conditions, the lag phase was nearly abolished, and half time didn’t change significantly with seed concentration, suggesting a high seeding efficiency of a trace amount of seed. A sharp transition of half time was observed at S9 when half time was comparable to the unseeded condition. This might suggest that no seed particle existed in the ‘seed’ due to dilution (30,000 PrP monomer equivalent of seed in each well at S9 as a result of dilution only) and aggregate adsorption to the surface of tubes and pipette tips. This hypothesis might be reasonable if the aggregates tended to stick and form large particles in the seed solution. At lower seed concentrations (S10-S12), the half time was larger than that using seeds S1-S8, but heterogeneity was observed among these samples. This likely suggested that (1) different numbers of seed particles were randomly distributed into these wells due to the low seed concentration or wall adsorption, or (2) there were no seed particles in those samples and a spontaneous aggregation was observed.
5.2.2 The smallest seeding-competent species

Infectious prion rods are estimated to contain at least 400-1,000 PrP molecules [203]. Some low molecular weight species purified from prion-infected brain homogenate containing > 5 PrP molecules were infectious, and oligomers with 14-28 PrP molecules were the most infectious particles per mass [204]. Infectious trimers [205] and possibly monomers [206] have been reported for the tau protein. Here, I tested whether low-molecular-weight species – monomers and small oligomers – were able to seed PrP aggregation. They were prepared by spinning the aggregated PrP solution through Amicon centrifugal filters with different cut-off sizes of 100 kDa, 50 kDa or 30 kDa and taking the corresponding flow-through (marked as FT). It is worth mentioning that the Amicon filters are designed to concentrate biological samples and don’t guarantee a precise separation of species with different molecular weights.

I also tested whether denaturing the flow-through by 6 M GdnHCl for 2.5 h affected the seeding ability (samples marked FT DN). For comparison, the retentate was used as seeds as well (marked as Re).

One difficulty is determining the concentration of aggregates in the flow-through or the retentate after the filtration step. Here, when using the untreated
seed solution, flow-through or retentate at a specific seed fraction, I calculated the volumes corresponding to equivalent fractions of the original seed.

The half time under each condition was extracted from the sigmoidal fit and plotted in Figure 5-15.

![Figure 5-15](image)

**Figure 5-15.** Half time of seeding assays with different seed treatment methods. ‘Re’: retentate; FT: flow-through; FT DN: denatured flow-through. Triplicates were conducted for each condition. The green bar using ‘total seed’ showed the fitted value of only one well since the sigmoidal fit of the other two wells yielded negative values of half time. For the ‘30kD FT’ and ‘No seed’ samples, only one out of three wells aggregated under each condition, and that value was plotted.

Figure 5-15 shows that all retentates were seeding competent, which was plausible as large, seeding competent aggregates couldn’t pass through the membrane and stayed in the retentate.

Interestingly, the flow-through of 100 kDa and 50 kDa filters were seeding competent as well, while the flow-through of a 30 kDa filter was not. The molecular weight of MoPrP 23-231 was 23.4 kDa. 30 kDa was slightly larger than the size of a PrP monomer; 50 kDa and 100 kDa corresponded to the
weight of small oligomers, suggesting that some low-molecular-weight oligomers, likely dimers, trimers or tetramers, might be able to seed PrP aggregation. However, since half time is insensitive to seed concentration, quantification of the amount of seeding competent aggregates in the flow-through couldn’t be performed. Alternatively to the hypothesis that the oligomers in the flow-through initiated the seeding reaction, it is also possible that a trace amount of aggregates larger than the cut-off size went into the flow-through and was responsible for the seeding ability of 100 kDa and 50 kDa filter flow-through samples.

Previous experiments showed that a 30 kDa cut-off membrane allowed the native-state monomer to pass through with ~70% recovery. Here, the 30 kDa flow-through was not seeding competent, suggesting the monomer itself couldn’t form a nucleus on the pathway to amyloid formation. However, one possibility couldn’t be ruled out that a low amount of misfolded monomers didn’t pass through the membrane during filtration.

Interestingly, treating the flow-through of 100 kDa and 50 kDa filters with 6 M GdnHCl couldn’t destroy the seeding ability. This suggested that the seeding-competent species in the flow-through, such as the misfolded oligomers or a trace amount of seeds larger than the membrane’s cut-off size, were tightly packed and at least partially resistant to GdnHCl denaturation.

I then conducted a seeding assay using the 100 kDa flow through at six different seed concentrations ranging from 5% to 0.00005%. As a comparison, the seeding assay with different concentrations of untreated seed was repeated. Triplicates were performed except at 5% untreated seed, where only duplicates were performed.

Figure 5-16 plots the dependence of half time on seed concentration (on a log scale) for the two types of seeds. Surprisingly, when using the 100 kDa flow-through as seed, half time decreased with seed concentration. The dashed line in Figure 5-16 shows a linear fit which captured the trend well. This decreasing trend was consistent with the behaviour of other amyloidogenic proteins in the literature. In contrast, unfiltered seeds displayed shorter half
times than filtered seeds and no such dependence on seed concentration, similar to the results shown in Figure 5-14.

![Graph showing dependence of half-time on seed concentration.](image)

**Figure 5-16.** Dependence of half-time on seed concentration.

The results using unfiltered seeds can be explained by large seed clusters being the dominating seeding-competent species. Small numbers of seed clusters can efficiently reduce the half time, which is only plausible when secondary pathways such as surface-catalysed secondary nucleation dominate in the seeding assay. This is supported by the perturbation analysis shown in Figures 5-6 and 5-8.

In the 100 kDa flow-through, no large particles exist, and seed particles are most likely isolated oligomers that can seed aggregation rapidly in a concentration-dependent manner.

Overall, small PrP oligomers and tiny amounts of mature aggregates are both highly efficient in seeding PrP aggregation.

### 5.3 Aggregation of truncated MoPrP 91-231

#### 5.3.1 Aggregation assay kinetics of recombinant MoPrP 91-231

The expression and purification of monomeric MoPrP 91-231 were described in Section 4.2.1. The protein solution was filtered through a 100 kDa filter to remove pre-formed large aggregates.
In literature, truncated PrP aggregation was usually induced in the presence of denaturants, similar to the methods used for full-length PrP. Truncated PrP could also aggregate in a native-like, no denaturant buffer [207] (50 mM NaP pH 7.3, 100 mM NaCl, 50 mM KCl) with agitation, forming ThT insensitive amorphous aggregates prior to ThT positive amyloid fibrils. ThT fluorescence didn’t show a typical sigmoidal trend in this case.

Here, I tested three partially denaturing buffers for PrP aggregation (data not shown): (a) 50 mM sodium acetate buffer at pH 5, 3 M urea, 200 mM NaCl, adapted from [48]; (b) 50 mM MES buffer pH 6, 2 M GdnHCl, 300 mM NaCl, similar to the buffer used in MoPrP 23-231 aggregation assays with additional NaCl; and (c) 50 mM NaP pH 7.4, 2M GdnHCl, 300 mM NaCl [193]. 18 μM PrP in the corresponding buffer was incubated with strong agitation at 37°C. No aggregation was detected using buffer (a) by the end of the measurement (142 h). In buffer (b), one out of three wells displayed increasing fluorescence at 20 h, while the other two wells only started to aggregate near the end of the measurement. All three samples using buffer (c) aggregated, with a lag time between 60 h to 90 h. This pilot experiment showed that buffer (c) was the best option for PrP to aggregate with relatively reproducible kinetics. Buffer (c) was then used as the standard aggregation buffer for MoPrP 91-231 in Section 5.3. Note that the standard buffers used for the aggregation of MoPrP 23-231 or MoPrP 91-231 are different.

I next tested whether PrP in the native-like buffer (50 mM NaP pH 7.4 and 150 mM NaCl) was able to aggregate. A partially denaturing condition with buffer (c) was used as a control. The protein solution under each condition contained 10 μM PrP and was incubated with agitation at 42°C.

Aggregation was nearly completed within 20 h for PrP under the partially denaturing condition (Figure 5-17 A). TEM measurements (Figure 5-17 B) confirmed the formation of fibrillar aggregates, which tended to form clusters and huge clumps of aggregated material (not shown); fibrils with different widths and numbers of strands were observed. In contrast, none of the wells in the native buffer aggregated by the end of the experiment (~70 h).
Figure 5-17. Aggregation of MoPrP 91-231 under the partially denaturing condition. (A) ThT kinetics of aggregation. (B) TEM images of the aggregates. The scale bars represent 100 nm. The thin arrows point to thin, single-strand fibril segments, whereas the fat arrows point to wide, multiple-strand fibril segments.

Kinetic traces of PrP aggregation (Figure 5-17 A) show that both the half time and final ThT fluorescence varied among replicates. To further study this heterogeneity, five repeat experiments were conducted with 6~8 replicates in each experiment. ThT fluorescence was measured on the same plate reader with the same settings, allowing the amplitude to be directly compared.
Figure 5-18. Scatter plot of lag time versus fluorescence amplitude in MoPrP 91-231 aggregation assays.

In three experiments, one well didn’t aggregate by the end of the measurement (68 h, 49 h and 26 h, respectively). Aggregation were observed for the rest 32 wells in total, and their amplitudes and half times are scatter plotted in Figure 5-18, with unique markers representing different experiments. In three experiments represented by yellow circles, red triangles and purple diamonds, the variation within replicates was small, and they appeared in a small cluster in the plot. However, in the other two experiments represented by the blue and green markers, large variations were seen for both amplitude and lag time.

Figure 5-19. Box plots for half time (left) and amplitude (right) extracted from sigmoidal fits.
Figure 5-19 shows the analysis for the fitted half time and the amplitude of all 32 wells. For both parameters, most of the data points fell into a small range, while there were few outliers. These results show that, similar to MoPrP 23-231, the aggregation of MoPrP 91-231 exhibited high level of heterogeneity in kinetics, which could lead to the formation of structurally distinct mature fibrils, as seen by the different fluorescence amplitude.

5.3.2 Concentration dependence of MoPrP 91-231 aggregation

Aggregation of MoPrP 91-231 was studied in a concentration range from 0.03 µM to 20 µM in the standard buffer. The aggregation kinetics and the fitted sigmoidal curves are shown in Figure 5-20.

No aggregation was observed at or below 0.3 µM PrP concentration by the end of the measurement (95 h); only one well aggregated at 0.5 µM and two wells aggregated at 1 µM. This suggested that the critical concentration for MoPrP 91-231 aggregation was lower than 0.5 µM under this condition. However, the exact value couldn’t be determined as aggregation might be able to take place at concentrations lower than 0.5 µM had the solution been incubated for longer.

At higher PrP concentrations, all samples aggregated and the kinetic traces were similar to those for MoPrP 23-231: fluorescence increased sharply after the lag phase, rapidly dropped after reaching a maximum and then remained plateau. Compared to the traces shown in Figure 5-17, the current dataset was noisier, and the drop was more pronounced. The difference might be explained by different settings of fluorescence reading, such as the focal height. This quick drop of fluorescence at the beginning of the plateau phase and the noisy nature of this dataset made the fitting of the growth rate k highly inaccurate. This parameter could vary over 200 fold within triplicates, whereas half time and fluorescence amplitude were relatively consistent within replicates.
A. Raw kinetic data for aggregation

![Raw kinetic data for aggregation](image)

B. Sigmoidal fits

![Sigmoidal fits](image)

**Figure 5-20.** MoPrP 91-231 aggregation at different monomer concentrations. Triplicates were performed for each condition. (A) Raw kinetic traces. (B) Fitted sigmoidal curves.
Two parameters, amplitude $A$ and half time were extracted from the fits and plotted as a function of PrP concentration (Figure 5-21). Wells that didn't aggregate were excluded from the plot.

Figure 5-21 A reveals that amplitude increased almost linearly with PrP concentration except at the highest concentration. In literature, the x-intercept of this fitted line is the critical concentration. However, the x-intercept value obtained here was negative, suggesting that the critical concentration was very small.

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Figure 5-21.** Dependence of amplitude and half time on PrP concentration in MoPrP 91-231 aggregation assays.

Half time was shown to decrease with PrP concentration. The scaling factor $\gamma$ was the slope of the fitted straight line in the log-log plot (Figure 5-21 C) and was -0.68. The last three data points at higher concentrations (5, 10 and 20
µM) adopted a smaller absolute slope than the fitted line. A similar positive curvature was observed for MoPrP 23-231 aggregation, as discussed in Section 5.1.3: γ was ~1.2 at low PrP concentrations and approached 0 in the high concentration range from 5.7 to 9.7 µM. However, the data quality here couldn’t support any concrete conclusion.

A. \[ \Delta = A(\cosh Bt - 1) + C \]

B.

Figure 5-22. Analysis of the initial 15% of fluorescence increase. (A) A fit of the cosh(t) function to the data (Equation 5_3). (B) A log-log plot of \( \frac{B^2A}{c_0(c_0-c_s)} \) versus initial monomer concentration \( c_0 \). The two flat lines shown in (A) were not included in this analysis.
In the perturbation analysis (Figure 5-22 A), a $\cosh(t)$ function can be fitted to the initial phase of aggregation well, while a $t^2$ fit cannot (not shown), indicating that secondary processes played an important role in aggregation. The slope of $\frac{B^2A}{c_0(c_0-c_s)}$ versus $c_0$ in a log-log plot (Figure 5-22 B) represented the nucleus size of primary nucleation. The slope of the fitted line was 3.8; however, a large variation of $\frac{B^2A}{c_0(c_0-c_s)}$ existed under each $c_0$, which added uncertainty to the determined slope.

Overall, the aggregation assay of MoPrP 91-231 in bulk solution revealed heterogeneity in terms of kinetics and fibril morphologies. Perturbation analysis indicated that both primary nucleation with a nucleus size of ~4 and secondary processes were important to generate new aggregates.

5.3.3 Seeding assay of MoPrP 91-231 in bulk

Seeding assays were performed in bulk at two seed concentrations: 1% and 0.1% (w/w). At 1% seed, fluorescence fluctuated to a large extent after a short lag phase (usually within 1 h; date not shown) before remaining at a constant level at later time points. The fluctuating data could be explained by the fast accumulation of large aggregates in the presence of abundant seeds, leading to quick precipitation of aggregates which interfered with fluorescence measurements and caused a drop of ThT fluorescence in solution.

At 0.1% seed concentration, ThT fluorescence fluctuated in a few wells; kinetics in other wells displayed a sigmoidal shape with a lag phase shorter than 1 h, even though the data was noisy. Here, wells that displayed sigmoidal kinetics are plotted (Figure 5-23 A). Different levels of final fluorescence were observed for initially identical samples, and a box plot of the final fluorescence is shown in Figure 5-23 B. The variation in final fluorescence might be explained by different aggregated species being amplified in separate wells, or the fluorescence reading of a well was affected by its position in a 96-well plate, causing a different reading for identical samples.
A. 

Figure 5-23. MoPrP 91-231 seeding assays in the presence of 0.1% seed. (A) The kinetic traces of 13 wells. The orange and yellow traces are from two different experiments. (B) Box plot of the final ThT fluorescence shown in (A).

5.4 Development of the solution-based seeding assays observed using TIRFM

5.4.1 Producing labelled full-length MoPrP seeds

To resolve possible heterogeneities in fibril formation, I next studied the seeding of MoPrP 23-231 aggregates on a single-particle level. The first strategy was to perform seeding in bulk under quiescent condition and to take out samples at different time points to image by TIRFM. I produced
fluorescently labelled seeds so that the seeds and newly formed or elongated fibrils can be differentiated during imaging. Seed segments can be imaged using the labelled fluorophore, while the whole amyloid fibrils can be imaged using amyloid-binding dyes freely moving in the imaging buffer. The labelled fluorophores and amyloid binding dyes should be chosen carefully so that their excitation and emission spectra don't interfere with each other.

(1) Producing labelled MoPrP 23-231 seeds using NHS-ester labelled PrP monomer

N-hydroxysuccinimide (NHS) ester reacts with primary amines of protein located at the N terminus and lysine side chains. The 11 lysine residues in MoPrP sequence provide multiple reaction sites for NHS ester labelling. This labelling strategy is the first choice since wild-type PrP can be used directly, avoiding the additional steps to make PrP mutants required by other reactive groups. However, the labelling ratio and the exact labelling site are difficult to control.

NHS ester dyes were previously used in our lab to label α-Syn monomers. We were able to make labelled aggregates by incubating a mixture of labelled monomers with an abundance of unlabelled monomers, suggesting that labelling didn't change the aggregating property of α-Syn [208].

The labelling procedure was described in Section 4.3.1, using Alexa Fluor (AF) 647 NHS Ester dyes. The labelling ratio for the product was kept at ~40% to avoid labelling one protein with multiple dye molecules.

To make labelled fibrils, 10 µM PrP containing 5% labelled monomer was aggregated in the standard MoPrP 23-231 aggregation buffer with agitation at 37°C for 87 h. The ThT kinetic traces were consistent with that of unlabelled PrP.

To test whether labelled PrP was incorporated into fibrils, the aggregated sample was adsorbed onto a coverslip and imaged in the presence of 5 µM ThT on a Zeiss Elyra microscope (Figure 5-24). In the 488 nm (green) channel showing ThT fluorescence, fibrils and large clusters were observed. In the 642 nm (magenta) channel showing AF 647 fluorescence, many fluorescent dots
were present. However, a large portion of them didn’t overlay with amyloid fibrils in the 488 channel.

![Image](image_url)

**Figure 5-24.** Composite image of MoPrP 23-231 aggregates made with 5% labelled monomer. ThT signal is shown in green and the AF 647 signal is shown in magenta. The scale bar represents 5 µm.

The presence of random dots in the 642 nm channel suggested labelled PrP molecules weren’t efficiently incorporated into amyloid fibrils; instead, they tended to remain as monomers or form small oligomers. In the second PrP monomer labelling attempt, I tried to reduce the possibility of PrP aggregating into oligomers during labelling by dissolving precipitates formed in this step with GdnHCl. Two batches of labelled PrP were made; each was in a solution containing 6 M GdnHCl and was filtered through either a 30 kDa filter (the flow-through was referred to as PrP a) or a 100 kDa filter (referred to as PrP b) to remove any pre-formed aggregates. Aggregates were prepared using 5% PrP a or 5% PrP b with 95% unlabelled PrP monomer, and they were referred to as aggregates a and aggregates b, respectively.

To study whether labelled PrP was incorporated into amyloid fibrils, ultracentrifugation was performed for aggregates a to separate large fibrils from monomeric and oligomeric species. UV-Vis measurement showed that ~25% protein and ~93% dye were in the supernatant. The resuspended pellet
was loaded onto the coverslip and imaged using a Zeiss LSM 700 confocal microscope in the presence of 5 µM ThT. I observed large aggregates when imaging ThT fluorescence in the 458 nm channel; however, these structures didn’t appear in the red channel showing the fluorescence of AF 647. These observations suggested that amyloid fibrils in the pellet fraction didn’t contain labelled monomers.

To confirm this conclusion, the morphologies of aggregates were examined by TEM. TEM images (Figure 5-25) show the presence of fibrils, fibril clusters and large amounts of small dots. Fibrils and fibril clusters were similar to the morphologies of aggregates formed by purely unlabelled PrP. The dot-like structures, however, were not commonly observed in unlabelled fibril samples and were attributed to the monomers or oligomers of labelled PrP.

Taken together, it was concluded that monomeric PrP labelled by NHS ester could not be efficiently incorporated into amyloid fibrils.

Figure 5-25. TEM images of MoPrP 23-231 aggregates with 5% labelled PrP. Scale bars represent 500 nm.

(2) Producing labelled full-length MoPrP seeds using Maleimide labelled PrP monomer

Since PrP monomers labelled by NHS ester were not able to form fibrils, another labelling method was required. Maleimide is a thiol-reactive probe which reacts with the thiol group of cysteine residues. PrP has two cysteine residues, forming a disulfide bond in its native fold. In order to label PrP by
maleimide dye, one more cysteine residue needs to be introduced into the PrP sequence. I used MoPrP 144C to perform labelling by AF 647 C2 maleimide in this work.

MoPrP 144C was expressed in *Escherichia coli* and purified by a Ni-NTA column similarly to wild-type MoPrP 23-231. The protein eluted from the column was used for labelling without proteolytically cleaving off the His-tag. To prepare for labelling, a reducing agent, TCEP, was added to the protein solution. TCEP ensures that the 144C residue is reduced instead of forming a disulfide bond with another PrP molecule [209-211] while doesn’t interfere with the native disulfide bond. The labelling procedure is described in Section 4.3.2. MoPrP 144C was not stable and tended to precipitate during labelling.

Two batches of labelled PrP monomer were made. In the first batch, the labelled PrP was subjected to thrombin cleavage to remove the His-tag, and the labelled and cleaved product was denoted as cPrP<sup>C</sup> (meaning cleaved PrP<sup>C</sup>). In another batch, the labelled PrP was not cleaved to reduce the number of steps involved; the product was denoted as uPrP<sup>C</sup> (uncleaved PrP<sup>C</sup>). cPrP<sup>C</sup> was eluted in 2 M GdnHCl and filtered through a 100 kDa filter to remove aggregates; the final concentration was 8.6 µM for PrP with an 80% labelling ratio. uPrP<sup>C</sup> was maintained in 6 M GdnHCl and was filtered through a 100 kDa filter as well; the final concentration was 23 µM for PrP with a ~100% labelling ratio.

Labelled fibrils were made by aggregating a mixture of 10 µM unlabelled PrP and 5% labelled PrP (either cPrP<sup>C</sup> or uPrP<sup>C</sup>) under the standard conditions with agitation. The formation of amyloids was confirmed by ThT fluorescence measured at the end time point. The aggregates formed using cPrP<sup>C</sup> or uPrP<sup>C</sup> were referred to as aggregates 5C and 5U, respectively.
Figure 5-26. 5C aggregates imaged with 638 nm excitation. The scale bar is 5 µm.

The aggregated samples were imaged using TIRFM to verify the successful production of labelled fibrils. 20 µM ThT was added to the imaging buffer to visualise amyloid structures, but the ThT signal with 473 nm excitation was very weak. When imaging in the 638 nm channel, numerous fibrils and huge, bright blobs were observed for both 5C and 5U samples. Fibril density of 5U was very high, and the ‘film’ of fibrils masked the observation of any other possible species on the surface. The 5C fibrils on the coverslip surface were not as dense (Figure 5-26). Unlike the aggregates made by NHS ester labelled PrP (Figure 5-24), no small labelled species were commonly found. Thus, labelling MoPrP 144C with maleimide dye was successful, and the labelled PrP could be incorporated into fibrils.

To determine whether incorporating labelled PrP changed fibril morphologies, fibril structures were measured by TEM. TEM images (Figure 5-27) show that fibrils in aggregates 5C and 5U were indistinguishable from those formed by unlabelled PrP (Figure 5-10).

Another batch of labelled, uncleaved monomer was prepared and employed to make labelled fibrils with 5% labelled PrP, which were used in the bulk seeding assay in Section 5.4.2.
5.4.2 Imaging samples from a seeding assay with labelled fibrils as seeds

I performed a seeding assay using labelled seeds and unlabelled monomers and imaged aggregates taken at different time points under TIRFM. The assay took place under a quiescent condition.

If elongation is the only process, each fibril should contain a red seed due to the fluorescence of AF 647 and elongated regions with no red signal whose length increases with time. If secondary processes such as fragmentation and secondary nucleation also occur, new aggregates that don’t contain a red signal will form. Previous kinetic assays have established that oligomers as small as dimers could seed aggregation. This observation adds complexity to the theory, as the small seeding competent oligomers might not contain any labelled monomer, thus leading to the elongated fibrils that are invisible in the red channel.
The seed solution was subjected to ultracentrifugation to remove free labelled monomers. The pellet resuspension was sonicated for 5 min, then added to 5% into the monomer solution. This solution in the standard aggregation buffer was incubated in a 96-well plate at 37°C. Samples from different wells were taken at sequential time points and observed using TIRFM in the presence of ThT (Figure 5-28).

At 2.5 h, most of the red signal from AF 647 in seeds and the green signal from ThT binding to amyloid colocalised. Signals shown in the red channel only were caused by poor ThT signal of those aggregates. Some dot-like structures were only visible in the ThT channel. It was unable to determine whether they were newly formed aggregates or short elongated fibrils from a small seed invisible in the red channel.

![Image](image-url)

**Figure 5-28.** Two-channel images of seeded MoPrP fibrils taken at different time points. The 638 nm (red) channel image is coloured magenta, and the 473 nm channel image is shown green. The scale bar is 5 μm.

After incubation for 48 h, short fibrils were observed; some of them had a red end, such as the fibril shown in the zoomed-in region in Figure 5-28; however, some fibrils were only visible in the ThT channel.
At 90 h, large fibril clusters were seen whose size ranged from a few micrometres to tens of micrometres (not shown). Those clusters were visible in both red and ThT channels. In regions without those clusters, fibril density was less than in samples incubated for a shorter time. Strikingly, most of those isolated fibrils contained no red signal.

Taken together, the presence of fibrils with a red end suggested elongation took place; at a longer incubation time, the emergence of fibrils without any red signal possibly indicated the involvement of secondary processes. However, since small oligomers were likely seeding competent, seeding could also be initiated by a small seed which didn’t contain any labelled PrP, leading to an elongated fibril without a red signal. Fibrils tended to form large clusters after prolonged incubation.

For these reasons, I could not obtain valuable kinetic data from this experiment. For the next step, I conducted in situ experiments to image the change of isolated seed particles on the coverslip surface by TIRFM, so that individual fibrils could be tracked and the fibril clustering problem could be overcome.

5.5 In situ seeding assays

5.5.1 Set up for single-molecule seeding assay

(1) Coverslip cleaning

It is essential to ensure that the coverslip is clean before use to avoid impurities that interfere with fluorescence measurements and protein assembly. Glass can be cleaned by (a) chemicals, such as acids (HCl or HNO₃), bases (KOH or NaOH), detergents, or harsh oxidising solutions (Piranha solution, chromic acid); (b) a plasma or ozone cleaner; (c) a combination of different methods.

I imaged the coverslip of freshly opened microscopy chambers in air to check whether impurities existed. TIRF images were acquired using 405, 473, 561 and 638 nm lasers with the corresponding filters in the large FOV mode. Fluorescent ‘dots’ were observed in all channels tested, indicating the
coverslip was not clean enough for single-molecule studies. The density of the dots was the lowest in the 638 nm channel.

The first cleaning method tested was plasma cleaning. During plasma cleaning, high-energy plasma is generated in the machine and reacts with organic contaminants on the glass surface. The small products are simultaneously removed by vacuum pumping. Plasma cleans the surface efficiently, leaving it hydroxylated and hydrophilic. Here, I used a PELCO easiGlow glow discharge cleaning system optimised for TEM grid cleaning.

After 5 min plasma glowing at 0.24 mbar, the chamber was imaged in buffer (50 mM NaP and 150 mM NaCl). TIRF images show very few dots in each channel. However, when fluorescent dyes (300 nM NR or 50 nM NB) were present, numerous fluorescent particles were observed (Figure 5-29). It suggested that plasma cleaning alone couldn’t remove all the organic impurities, some of which were not self-fluorescent but could stimulate fluorescence emission of NR or NB.

![Figure 5-29](image)

**Figure 5-29.** TIRF images of coverslip surfaces after 5 min plasma cleaning in the presence of dyes. (A) Surface imaged in buffer with 300 nM NR in the 561 nm channel. The scale bar represents 10 μm. (B) Surface imaged in buffer with 50 nM NB in the 638 nm channel.
I then explored cleaning methods using chemicals. Chemicals used for cleaning need to be compatible with the chambers, which are made of a glass bottom and a polystyrene frame. This prevented the use of an efficient cleaning chemical, piranha solution. In a pilot experiment, piranha solution was shown to react with the frame, turn the frame yellow and bend the chamber.

I tested other candidates: 5 M NaOH solution, 1 M HCl and 2% Hellmanex II detergent solution. Hellmanex II is a concentrated alkaline detergent designed to clean glass cuvettes and optical parts. Chambers (or wells) were filled with different solutions for 1-2 h before thoroughly rinsing with H₂O. The dried chambers were plasma glow-discharged for 40 s. When imaging in the buffer using TIRFM, the surface cleaned with Hellmanex II detergent contained the smallest number of fluorescence dots in the presence of NB, showing a significant improvement compared to the chambers cleaned by plasma only.

The cleaning method hereafter was to combine a 2 h Hellmanex detergent cleaning and a 2 min plasma glowing. As shown in Figure 5-30, the cleaned coverslip surface displays only few fluorescent impurities in the presence of NB or NR.

**Figure 5-30.** Images of the cleaned coverslip surfaces. The scale bar represents 10 µm. (A) Images taken in the 638 nm channel with 200 nM NB; (B) Images taken in the 561 nm channel with 300 nM NR.
5.5.2 Surface treatment and functionalisation

In literature, bare coverslip surfaces were frequently used to conduct *in situ* elongation experiments [117, 131, 212]. However, fibril growth might be hindered by the surface since the fibrils are close to the surface. Surface properties such as hydrophobicity and charge might interfere with aggregation kinetics [213, 214]. Coverslip surfaces are often passivated to reduce the surface effect and non-specific protein adsorption. Several detergents, proteins and polymers can be used for passivation, including BSA [135], PEG [215, 216] and Pluronic F-127 [120]. Pluronic F-127 is a triblock PEG-PPG-PEG copolymer. The middle hydrophobic PPG block can attach to the hydrophobic, silanised glass surfaces, and two PEG blocks form flexible arms extending in solution and preventing non-specific protein adsorption [217]. Seeds are attached to the passivated surfaces by biotin-streptavidin linkages or antibodies. The biotin-streptavidin linkages require that both the passivation agents and the seeds are covalently labelled with biotin.

Here, I tested three surfaces to identify one that enabled seed elongation: (1) use the untreated bare surface, and a mixed solution of seeds and monomers [117, 131, 212]; (2) use surface passivated with F-127, surface-attached seeds by antibodies [120] and monomer solution with no seeds; (3) deposit seed solution onto the cleaned bare coverslip surface, then add monomer solution.

(1) Elongation assay on the untreated bare surface

Two types of incubating chambers were used (Figure 5-31):

A. A flat chamber composed of a flat glass slide and a coverslip, sealed either by silicone glue (twinsil) or double-sided tape.

B. A chamber composed of a glass coverslip and a depression slide (a glass slide with a concavity on one surface), so a larger sample volume could be filled in. The system was sealed by twinsil.
A.

![Figure 5-31](image)

**Figure 5-31.** Sketches of the assembled chambers shown in the top and side views. (A) The chamber assembled with a flat glass slide and a coverslip. On the left panel, a small droplet of seed and monomer mixture was deposited onto the centre of a glass slide; then, a clean coverslip was gently put on to cover the droplet; a silicone glue, twinsil, was applied to the edge of the coverslip to seal the system. On the right panel, a piece of double-sided tape with a circular hole in the centre was stuck to the glass slide. The hole was filled with the assay solution. The coverslip was then pressed on the tape and solution. (B) The chamber made of a depression slide and a coverslip.

α-Syn was used as the model protein in this test. α-Syn aggregation was performed by incubating 250 µM monomer in 20 mM NaP buffer, pH 7.4, with agitation at 37°C for ~2 days. The formation of fibrillar aggregates was confirmed by AFM (Figure 5-32). The aggregates were diluted and probe sonicated for 30 s to make seeds. The solution for the elongation assay contained 150 µM monomer and 0.005% (w/w) mixed seed; 7 µM ThT was added for TIRF imaging. The seed fraction used here was much smaller than that in the literature (0.63%) [131] since large fibril clusters were seen on the
surface as well as in solution after incubation at the larger seed concentration in a pilot test.

![AFM image of α-Syn aggregates](image)

**Figure 5-32.** AFM image of α-Syn aggregates.

Multiple flat chambers were assembled (Figure 5-31 A), each containing 8 µL of the mixed solution and were incubated at 37°C. Figure 5-33 shows images of the chambers after different incubation times, revealing the presence of fibrils and fibril clusters on the surface, which grew longer with time. However, ThT signal faded rapidly due to photobleaching and the limited supply of ThT in a small sample volume, which prevented long-term, *in situ* observation.

![TIRF images of elongated α-Syn fibrils in different flat chambers at multiple time points. The scale bar represents 5 µm.](image)

**Figure 5-33.** TIRF images of elongated α-Syn fibrils in different flat chambers at multiple time points. The scale bar represents 5 µm.
I then employed a chamber assembled by a depression slide and coverslip (Figure 5-31 B). It held 25 µL sample, providing a larger reservoir of ThT. In this test, the buffer used for seed production and seeding assay both contained 150 mM NaCl and 20 mM NaP. The assembled chambers were incubated at room temperature for 3 days.

Images of the chamber were taken at the end time point (Figure 5-34 A), revealing an abundance of large aggregates which protruded into the solution, causing a fuzzy background around the bright regions and preventing quantitative analysis. As a comparison, samples incubated in a flat chamber (Figure 5-34 B) didn't show the presence of large aggregates. The large clustered aggregates in the depression slide were likely caused by a larger number of seed particles growing and sticking together, which might take place near the coverslip surface, or in solution followed by the clusters depositing onto the surface. In the flat chamber, the limited z dimension and the smaller number of seed particles disfavoured the formation of these large clusters.

![Figure 5-34](image)

**Figure 5-34.** Images of seeded α-Syn aggregates in a depression slide (A) and a flat chamber (B) taken at the end time point. The scale bar represents 5 µm.
(2) Elongation assay on the passivated glass surface

I tested a second strategy, attaching the seed particles onto an F-127 coated coverslip surface by antibodies [120], in order to track the change of a single attached seed in situ. The seeds were attached to the surface instead of in the solution, thus avoiding the clogging problem or deposition of new fibrils.

In this experiment, MoPrP 23-231 seeds and monomers were used.

The cleaned 8-well chamber was silanised by trichloro-(1H,1H,2H,2H-perfluorooctyl)silane (PFOTS), then passivated with 5% F-127 solution for 13 min. First, I tested the protein blocking efficiency of the coated F-127 layer. After incubating 1:400 diluted MoPrP aggregates on the treated surface for 10 min, only few fluorescent particles were observed (Figure 5-35), suggesting a good protein repellent property of the F-127 coated surface.

Figure 5-35. Images of the passivated chamber surfaces after seed incubation. 500 nM NR was present in the imaging buffer. The scale bar represents 5 μm.

Seed adsorption onto the passivated surface required the assistance of antibodies. PrP antibody 5B2 was diluted at 1:200 and incubated on the silanised surface for 8 min. After washing to remove unattached antibodies, the surface was passivated by 5% F-127. Seed solution (with 1:200 dilution)
was incubated for 10 min allowing seeds to attach to antibodies. 200 µL monomer solution with 10 µM MoPrP 23-231 was then added.

TIRF images were acquired after 19 h and 75 h incubation at 37 °C (Figure 5-36). At 19 h, multiple short fibrils were observed. However, there were dot-like background patterns which interfered with the fluorescence of aggregates. Images acquired at 75 h for the same position revealed that a few fibrils elongated between the two time points, while most of them didn’t grow substantially.

The background fluorescence was typically high after silanisation by PFOTS, possibly due to the hydrophobic surface promoting dye binding to the surface. The background fluorescence was partially reduced by treatment with F-127 but was still higher than the untreated bare surface.

![Figure 5-36](image)

**Figure 5-36.** Images of surface-attached MoPrP 23-231 seeds after 19 h (left) and 75 h (right) incubation in the monomer solution. Arrows point to two fibrils that had elongated between the two time points. The images were corrected for uneven illumination. The solution contained 10 µM PrP monomer, 50 mM MES, 2 M GdnHCl and 250 nM NB. The scale bar is 5 µm.

I applied the same method to study α-Syn seed elongation and successfully observed the elongation of most attached seed particles. Overall, treatment with antibody and F-127 worked in terms of seed attachment and elongation.
This method could be a candidate for elongation assay; however, issues related to high background and background patterns remain to be solved.

(3) Elongation of surface-attached seeds on a bare glass surface

MoPrP 91-231 was used in this test; the 8-well chamber was cleaned with detergent and was plasma glow-discharged without any further treatment. 10 µL 1:6.7 diluted PrP seeds were incubated on the cleaned chamber surface for 20 min. After washing, 200 µL monomer solution containing 10 µM PrP with 106 nM NB was added. The solution was in the standard buffer for MoPrP 91-231 aggregation. The chamber was sealed and imaged in situ at 42°C. Figure 5-37 shows TIRF images of a small region at four different incubation times. Dot-like or short fibrillar aggregates were observed at earlier time points, which gradually grew into long fibrils. It was also interesting to note that some fibrils ceased growing while others were growing.

![Figure 5-37. TIRF images showing the elongation of MoPrP 91-231 seeds. The four images were taken after incubation of 2 h, 4 h, 6 h and 8 h, respectively. The scale bar is 5 µm.](image)

Since this simple setup using the untreated coverslip surface and deposited seeds worked, I preferred to use this method in future experiments to study elongation kinetics. MoPrP 91-231 elongated faster than MoPrP 23-231 and thus was chosen to investigate further. A few parameters still required improvement: seed density was high in the pilot test; thus, seed concentration and/or seed incubation time need to be adjusted; signal-to-noise ratio in Figure
5-37 was low, suggesting a need to adjust dye concentration or illumination settings.

5.6 Discussion

(1) De novo aggregation of MoPrP 23-231 and MoPrP 91-231 display kinetic heterogeneity

The aggregation kinetics of MoPrP 23-231 and MoPrP 91-231 showed significant heterogeneity in the half time (equivalent to lag time in this discussion) and the fluorescence amplitude. For the spontaneous aggregation of MoPrP 23-231, the lag time within replicates varied from less than 20 h to over 110 h (Figure 5-1), while the fluorescence amplitude could have a two-fold difference (Figure 5-4 B). Similarly, for MoPrP 91-231, the lag time ranged from 4 h to over 68 h, while the amplitude could have an over three-fold difference (Figure 5-18). The presence of heterogeneity makes AmyloFit not applicable.

A similar phenomenon was observed in literature for the aggregation of Aβ40 [194] and Hamster PrP 90-231 [195]. The studies proposed that the nucleation process might involve one or more stochastic steps, leading to heterogeneity in kinetics. However, the vast amount of monomers [218] and the rapid formation of nuclei [219] argue against this theory.

The second possible factor affecting aggregation heterogeneity is the purity and state of PrP monomers used in the assays. For the aggregation of Aβ 42 [220], pure, monomeric peptide is required to ensure satisfactory reproducibility. The authors proposed to use multiple rounds of size exclusion chromatography (SEC) to remove impurities, and one more round right before conducting the assays to remove pre-formed aggregates [220]. In my study, the protein was not purified by SEC. Even though the protein was filtered by a 30 kDa or a 100 kDa membrane filter, trace amount of pre-formed oligomers might still exist due to the imperfect separation ability of the filters.

The heterogeneity could also arise from other discrepancies existing among different wells, such as the shape and surface area of agitating beads, the
number and surface area of air bubbles in solution, or the presence of contaminants.

The differences in fluorescence amplitude among replicates in my study likely reflect the formation of heterogeneous amyloid structures. The nuclei formed could acquire distinct structures, which eventually lead to the formation of structurally different mature fibrils.

Several approaches were tested to reduce the heterogeneity in de novo aggregation of MoPrP 23-231. Changing the type of agitating spheres or changing the monomer pre-treatment protocol wasn’t effective. The heterogeneity in half time was reduced by increasing PrP concentration or by simultaneously changing the buffer condition and increasing the temperature. However, it wasn’t completely removed in this study.

The concentration-related decrease in half time, along with the decrease in heterogeneity, can be seen in Figures 5-4 B, C, and 5-7 for the aggregation of MoPrP 23-231. The half time decreased with PrP concentration in a low concentration range (at and below 3.2 µM), while further increasing PrP concentration didn’t significantly alter the lag time. Heterogeneity in lag time decreased at PrP concentrations at or above 8.1 µM. In [194], the heterogeneity and lag time decreased simultaneously with increasing Aβ40 concentration.

When using the aggregation condition adapted from an RT-QuIC assay, i.e. using a buffer at pH 7.4 and an elevated temperature, the aggregation lag time was significantly reduced to below 5 h. The heterogeneity was reduced simultaneously (Figure 5-5).

These results suggest that certain reaction conditions can reduce heterogeneity in aggregation kinetics. However, other strategies are required to reduce it further, such as a better control of the initial state of the protein.

(2) Secondary processes are involved in the aggregation mechanism.

Analysis using the perturbation method revealed that secondary processes such as secondary nucleation and fragmentation played a vital role in the spontaneous aggregation of full-length and truncated MoPrP (Figures 5-8 and
5-22). The secondary processes are responsible for the fast increase in fluorescence right after the lag phase, which couldn’t be traced by a mechanism involving only primary nucleation and elongation. The seeding assay kinetics of MoPrP 23-231 (Figure 5-12) confirmed the involvement of secondary processes by its sigmoidal shape since a mechanism without secondary processes would have led to an initial linear increase in fluorescence followed by a slow saturation.

The nucleus size of primary nucleation for MoPrP 23-231 couldn’t be determined, while that for MoPrP 91-231 was estimated to be 4 (Figure 5-22 B).

The aggregation of full-length MoPrP was studied by the Udgaonkar group [44]. They concluded that no secondary processes were involved.

In their work, the purification of the bacterial-expressed PrP involved a final SEC step [44, 221, 222]. PrP was denatured using 6 M GdnHCl before being diluted into the native buffer. Both steps could remove pre-formed aggregates.

Their final aggregation buffer contained 50 mM Tris (pH 7.4) and 2 M GdnHCl. It was similar to one buffer used in this work: 50 mM NaP (pH 7.4), 2 M GdnHCl with 150 mM NaCl (Figure 5-5). Aggregation assays were then conducted with up to 100 µM PrP at 37°C with continuous shaking.

The first 5% fluorescence increase was analysed by perturbation analysis [44]. The fluorescence could be captured by a cos(t) or t² function, suggesting spontaneous aggregation was the main pathway to generate new aggregates in the concentration range they studied. From the log-log plot of \( \frac{B^2A}{c_0(c_0-c_s)} \) versus \( c_0 \), a nucleus size was determined to be 1. Thus, they proposed that full-length wild-type PrP aggregation followed the nucleation-dependent polymerisation (NDP) mechanism with PrP monomer misfolding to a critical nucleus by conformational rearrangement.

Perturbation analysis for the aggregation of MoPrP G126V revealed that a secondary process, determined to be heterogeneous nucleation, played a role. A remarkable difference in aggregation mechanisms was thus obtained for wild-type or G126V protein, even though only one amino acid was different.
The reason for the contrasting aggregation mechanisms for wild-type MoPrP obtained by them and in my work is challenging to determine. Several possible explanations are as follows. (1) The amino-acid sequences involved in these two studies were slightly different due to the slightly different constructs used [44, 221, 222]; (2) their assays were conducted under agitating conditions but without the presence of beads, while three Zr beads were used in my assays. This might lead to a reduced degree of shaking in their assays, and consequently, less involvement of secondary pathways, especially fragmentation; (3) the 96-well plates used in the two studies could be different; and (4) the buffer systems were different.

Other discrepancies were observed as well between the two studies. They determined a critical concentration for full-length MoPrP to be ~4 µM. However, although I could not precisely determine the critical concentration in my work, it was lower than 1.6 µM as PrP aggregation took place at 1.6 µM. This discrepancy could be explained by the slightly different experimental conditions or the formation of thermodynamically different aggregates.

In their study, the kinetic traces within replicates were highly consistent with nearly no heterogeneity in half time. This likely resulted from the pure and monomeric PrP as the starting state for aggregation, achieved by SEC and GdnHCl denaturation [44]. My study lacked the SEC purification step. Even though PrP stocked was denatured before using in the assay in a test (Figure 5-3), aggregation kinetics still showed discrepancies. This step alone was unable to remove contaminants and might be insufficient to remove all pre-formed aggregates.

They observed a nearly unchanged lag time (~3.3 h) and half time (~5 h) over the concentration range studied (10-100 µM), which could be explained by the critical nucleus size being 1. The dependence was weakly positive, possibly caused by the formation of off-pathway oligomers competing with the formation of fibrils. They indeed observed transient oligomeric species which were detected only before the formation of fibrils. The formation of off-pathway oligomers precluded the analysis of aggregation kinetics using AmyloFit.
Interestingly, in my study, half time decreased with PrP concentration below 5.7 µM; it remained at a similar value (~5 h) in a concentration range from 5.7 µM to 9.7 µM. Even though I didn’t study the aggregation at higher concentrations, I expected that the nearly constant lag time would still be true. In this case, this trend on half time would be consistent with theirs in the high concentration range.

(3) Small oligomers of MoPrP 23-231 might accelerate aggregation

After centrifuging the MoPrP 23-231 aggregates through the membrane filters, the flow-through of a 50 kDa or a 100 kDa filter was seeding competent, while the flow-through of a 30 kDa filter was not (Section 5.2.2). The monomer of MoPrP 23-231, with a molecular weight of 23.4 kDa, can pass through the 30 kDa membrane filter in a test experiment. Small oligomers, including dimers, trimers and multimers, are not likely to pass through the 30 kDa filter due to their larger sizes. On the other hand, the flow-through of a 50 kDa filter might contain monomers and dimers. However, the cut-off size does not guarantee a perfect separation of PrP species in the flow-through and the retentate. Some aggregates with molecular weights slightly larger than the membrane cut-off sizes possibly bleed through the membrane, leading to a trace amount of larger aggregates in the flow-through.

As a result, I speculate that the flow-through of a 30 kDa filter contains only or dominantly monomers, which are not seeding competent. The flow-through of a 50 kDa or a 100 kDa filter contains monomers and oligomers which are smaller than the cut-off size, while it could also contain trace amounts of slightly larger aggregates. The fact that it is seeding-competent reveals the seeding ability of oligomeric or possible tiny fibrils that can pass through the filters. The nature of the seeding-competent species in the flow-through need to be further investigated to identify the smallest seeding-competent PrP species.

In [44], the analysis of the aggregation kinetics revealed that the critical nucleus for full-length MoPrP aggregation had a size of 1. This implied that a monomer could misfold by itself and transform other normal monomers into the misfolded form. However, I wasn’t able to obtain the size of a critical
nucleus for full-length MoPrP in this study. The nucleus for MoPrP 91-231 was estimated to contain ~four monomers. This evidence supports the hypothesis that a small misfolded oligomer can initiate seeding.

Interestingly, the Diamond group showed that misfolded tau trimers and monomers were seeding competent [205, 206]. Tau trimers can be internalised by cells and seed intracellular tau aggregation [205]; a misfolded tau monomer was later shown to be seeding competent in both cell models and in vitro assays [206] and was the smallest seed species. This misfolded monomer, called Ms, acquired a different internal packing compared to the inert monomers (Mi). The purity of the Ms fraction was confirmed by several approaches, leading to a conclusion that the seeding ability of the monomer fraction was intrinsic and unlikely caused by the contamination of oligomers or other large-molecular-weight species.

Figure 5-16 shows that seeding assays with a serial seed dilution of MoPrP 23-231 aggregates display a near-constant half-time insensitive to the seed concentration. However, when the aggregates were filtered through a 100 kDa filter and then used as seeds, the half time increased with the seed dilution factor. The constant half time when using different concentrations of unfiltered aggregates as seeds doesn’t agree with the common amyloid formation mechanism. It indicates a very high efficiency of generating new aggregates in the presence of even a tiny amount of seed (as little as one seed cluster).

(4) The unique features of recombinant PrP aggregation compared to the authentic prion fibrils.

One interesting hypothesis brought up in this study was that the oligomers of recombinant MoPrP were likely seeding-competent.

The smallest-seeding competent species has been discussed for authentic prion fibrils. In the early hetero-dimer hypothesis, Prusiner [223] hypothesised that there were seeding competent monomeric prions. However, later studies argued against this model. PrP species purified from authentic infectious prions with 5 or less than 5 PrP molecules were shown to be not infectious, while larger oligomers were infectious [204]. The purified, infectious prion rods were estimated to contain at least 400-1000 PrP molecules [203]. Thus, the
propagation of authentic prions clearly needs a seed which consists of multiple PrP molecules.

Traditional prion propagation models suggest that both fibril elongation and fragmentation (fission) are essential for prion propagation in vivo [29]: prion fibril elongation increases the fibril load while fragmentation increases the number of seeding-competent aggregates; here, in vitro seeding assays confirm that secondary processes are vital for the formation of recombinant fibrils as well, while the exact process, secondary nucleation or fragmentation, couldn't be determined.

(5) Seeding assays conducted on bare coverslip surfaces can reveal elongation kinetics.

With a simple experiment setup, i.e. depositing seeds on the coverslip surface and monitoring their change in the presence of monomers using TIRFM, I observed the elongation of MoPrP 91-231 seeds. This method allows an in situ observation of individual seed particles, as previously shown for other protein systems [117, 120]. The elongation kinetics will be studied further in the following chapter using this setup.

A surface modification method was tested. Here, F-127 was coated on the coverslip surface [120], which in theory can reduce the interference of the coverslip surface on the elongation kinetics and reduce non-specific protein adsorption [217]. However, a dot-like fluorescence pattern and an increased background fluorescence were observed for the modified surface, which restricted its application in this study.

MoPrP 23-231 seeding assay was also conducted in bulk solution under quiescent conditions, using fluorescently labelled seeds and unlabelled monomers. Images for the samples withdrawn after different incubation times showed elongated fibrils, huge fibril clusters, and fibrils that didn’t contain identifiable seed regions. Multiple processes could take place, including the elongation of red seed fibrils, the elongation of small seeds which didn’t contain labelled PrP, the dissociation of unlabelled or labelled PrP monomer from fibrils, fibril fragmentation leading to labelled or unlabelled shorter fibrils, primary or secondary nucleation yielding unlabelled fibrils, and fibril clustering
forming large labelled aggregates. However, the exact processes taking place couldn’t be identified. This experiment only gives information on an ensemble of fibrils, while quantitative analysis couldn’t be obtained due to two major reasons: (1) small oligomers could act as seeds, which makes identifying the seed regions difficult; (2) PrP fibrils tend to stick together and form large clusters, preventing the observation of each composing fibril.

In this chapter, the aggregation kinetics of MoPrP 23-231 and MoPrP 92-231 were studied in bulk, revealing unique kinetic features. MoPrP 91-231 seeds were successfully made, and a simple experimental setup to observe seed elongation was established, enabling the study of elongation kinetics in situ on a single-aggregate level, which will be described in the following chapter.
Chapter VI. Single-particle elongation kinetics of MoPrP 91-231 observed by TIRFM

In the previous chapter, I established the method to monitor the elongation of single seed particles \textit{in situ} and revealed heterogeneity among fibrils during elongation. In this chapter, I applied the method to study the elongation of MoPrP 91-231 seeds under varying experimental conditions (PrP monomer concentrations, GdnHCl concentrations and temperatures), which provides valuable information on the elongation mechanism. I also developed analysis methods to analyse elongation kinetics quantitatively.

6.1 Seed production for elongation assays

I produced MoPrP 91-231 aggregates as described in Chapter 5 by spontaneous or seeded aggregation [193], which can be used as seeds to study elongation kinetics.

Ideally, seeds should be identical in all experiments, i.e. they should come from equal aliquots of aggregates, which are from either one well/tube of the solution, or pooled aggregation assays performed under the same condition. However, because of the heterogeneity observed in lag time and final fluorescence, it is questionable whether the aggregates from different wells are identical. Besides, as this chapter will demonstrate, even seeds from a single well are not identical.

Seeding assays are expected to produce reproducible kinetics for most amyloids. However, for the seeding assays of MoPrP, significant heterogeneity was seen in the final fluorescence (Figure 5-23). Several hypotheses could explain the heterogeneity: (1) fluorescence readings were not accurate, (2) the seeds were a heterogeneous mixture leading to a slightly different population added in each well, or (3) slight differences were present in the aggregation condition of each well, for example, the difference in the shape of agitating spheres might lead to a varied degree of shaking.
Figure 6-1. Production of MoPrP 91-231 seeds for the surface-based elongation assays. (A) Kinetics of the spontaneous aggregation assay (grey trace) and two seeding assays (blue and orange traces). The products of the spontaneous assay were used as seeds for the two seeding assays. The products of the seeding assays were used as seeds for the surface-based elongation assays. The two seeding assays were performed in different experiments but under the same conditions. (B) Typical TEM images of seeds used for elongation assays. Seeds were diluted before adsorption onto EM grids. The scale bars represent 200 nm. The figure is adapted from [193].

In the end, I chose to use the products from the seeding assay as seeds for the surface-based elongation assays. 20 μM ThT was present in each assay solution to monitor the kinetics. The products used were from two wells with similar kinetic profiles (Figure 6-1 A, orange and blue traces), and they were aliquoted individually rather than pooled together. The detailed procedure is in Section 4.4.4. Seed aliquots represented by the blue trace were used for the elongation assays studying the influence of PrP<sup>C</sup> concentration; seeds
represented by the orange trace were used for experiments studying the effect of GdnHCl concentration and temperature. TEM images (Figure 6-1 B) show that diluted seeds were fibrillar aggregates which tended to be in clusters.

6.2 Chamber assembly for elongation assay

Chamber preparation includes seed adsorption, monomer solution preparation and addition, and chamber sealing [193]. A schematic representation is shown in Figure 6-2 A.

(1) 10 µL diluted seed solution was incubated on the coverslip surface for adsorption. The dilution ratio and incubation time were adjusted to obtain a good seed density on the surface. A dilution ratio of 1:80 or 1:120 and incubation time of 30–45 s usually gave a satisfactory result. After incubation, excess solution was removed, and the surface was washed with buffer twice.

(2) Monomer solution with a specific concentration of PrP and buffer composition was prepared, with 400 nM NB added for TIRF imaging. 200 µL solution was pipetted into the chamber well.

(3) Chamber sealing was initially conducted by parafilm. One piece of parafilm covered the top of the whole chamber, with its edges pressed onto the side of the chamber; another piece was a long stripe wrapping around the side surfaces of the chamber and covering the edges of the first parafilm. In most cases, this sealing method worked well to prevent evaporation; however, leakage occasionally occurred at the edges of the second long stripe. The main reason was that plasma glowing rendered the chamber highly hydrophilic, so monomer solution tended to climb the wall at the four corners. If the solution reached the parafilm and permeated between the parafilm and the chamber’s top surface, it could spread underneath the parafilm and sometimes leak from the edge.

To avoid this problem, I later sealed the chamber with twinsil, a two-component silicone glue. The two parts were mixed at a 1:1 ratio and poured into the chamber’s lid to cover its surface. The lid was left untouched for ~5 min until
the mixture started to set. The chamber was then covered with the lid with a slight press and kept immobile until the glue was completely dry.

6.3 Performing in situ elongation assays

I started with the assay condition using 1 μM MoPrP 91-231 monomers in the standard buffer for MoPrP 91-231 aggregation (containing 50 mM NaP, pH 7.4, 300 mM NaCl and 2 M GdnHCl) at 37 °C.

A.

![Procedure for seed elongation experiments](image)

B.

![Seed elongation in one region of interest (ROI) measured by TIRFM](image)

Figure 6-2. Procedure for seed elongation experiments. (A) A schematic representation of the experimental procedure. (B) Seed elongation in one region of interest (ROI) measured by TIRFM. The figure is adapted from [193].

After the chamber was assembled, the selected fields of views (FOVs) were monitored using TIRFM over at least 2 days, and images were taken every 15 min. Figure 6-2 B shows seed elongation in one region, where dot-like seeds at 0 h gradually grew into long fibrils or fibril clusters. Signal to noise ratio was
reasonably good. Several interesting findings were observed from this imaging stack [193]:

(1) Elongation was the only, or at least the major, process observed. Almost all growing fibrils grew from an identifiable seed at 0 h, suggesting that elongation played a dominating role while primary nucleation rarely took place. This is reasonable, as our bulk assays indicated that spontaneous aggregation of PrP required harsh agitation. Rare growth from positions where no ‘dot’ was present was likely due to a tiny seed which didn’t show up in TIRFM imaging. Occasionally, fibrils appeared suddenly on the surface, possibly caused by detached seeds elongating in solution before depositing onto the surface.

However, some fluorescent dots showing up at the initial time point didn’t elongate. They could be fluorescent impurities, quickly formed off-pathway aggregates or seeds that landed on the surface in an orientation disfavouring fibril elongation. I also observed fluorescent dots appearing during incubation, usually within hours after experiments started. They persisted on the surface after formation but didn’t grow into long fibrils, nor interact with the growing fibrils, suggesting they might be off-pathway oligomeric species. The off-pathway oligomers were previously observed during FL MoPrP aggregation in the bulk assay [44]; however, they were transient and were detected only before the formation of fibrils.

I didn’t observe fibril fragmentation or branching; the latter was an indicator for secondary nucleation. Dissociation was expected to occur; however, its rate would be much slower than elongation when the monomer concentration was well above the critical concentration. Fibril shortening wasn’t observed, supporting that dissociation was not profound or was slow compared to elongation.

Studying the seeding process in situ by TIRFM or AFM has been performed for multiple amyloidogenic proteins, such as Aβ40 [224], Aβ42 [120], α-Syn [131] and Sup35 [135, 136]. They have been discussed in Section 2.3.3. Similarly, elongation was the main process taking place. In some systems, oligomer formation [136], secondary nucleation [144] or fibril branching [201] were also observed.
Sang and co-workers [138] studied seeded aggregation of full-length MoPrP under the native condition on the coverslip. They observed elongation of seed particles and, strikingly, fragmentation. However, the fibrils they described only acquired lengths of a few pixels (less than 15), precluding a precise quantitative analysis of growth rates.

(2) Intermittent growth pattern was observed.

It was commonly observed that growth ceased for a period before continuing; some fibrils didn’t start growing until the experiment had run for a while. This intermittent growth was commonly observed for other protein amyloids [120, 131], possibly suggesting that the bound PrP species were trapped in an incorrect confirmation and unable to elongate [105]. Further elongation required the detachment of those species from the fibril ends or a structural rearrangement into a correct fold. The stall phases mentioned here were long-lived, ranging from hours to tens of hours, indicating a slow detachment or structural rearrangement. Since the time interval between acquisition was 15 min, stall time shorter than 15 min, or even a few time intervals for relatively slow-growing fibrils, couldn’t be identified. The short stalls would result in a decrease in elongation rate instead of identifiable stall phases.

Another possible cause for stalls was the interference of the glass surface. Growing fibril ends could stick to the coverslip surface during growth. This possibility could be tested by measuring stall phase percentages at varying solution conditions such as PrP or denaturant concentrations since surface hindrance would not depend on buffer conditions, which will be discussed in Section 6.5.

(3) Directionality of fibril growth

The initial dot-like seeds could grow unidirectionally (for example, fibril c in Figure 6-2 B) or bidirectionally (fibril a) into a single fibril, or grow into multiple fibrils forming a cluster (for example, the cluster pointed by arrow b). The bidirectional growth might suggest growth took place from two opposing ends of a fibril, or the initial seed was not isolated but a cluster of two particles with opposing growth directions. The exact situation couldn’t be determined due to the resolution limit of TIRF microscopy. Dots growing into clusters indicated
that they were made of multiple seed particles, as shown by TEM images in Figure 6-1 B. Detailed analysis for fibril directionality required quantitative analysis and will be presented in Section 6.4.

(4) Fibril elongation displayed heterogeneity in apparent rate, fibril brightness and morphology.

Variations in fibril elongation rate and fibril brightness were observed among different fibrils. For example, fibril a in Figure 6-2 B elongated faster than the short fibril pointed by b and acquired a dimmer brightness. A coarse correlation was seen between fibril brightness and growth rate: fast-growing fibrils were generally dimmer. A more accurate description will require quantitative determination of fibril rate and brightness.

![Figure 6-3](image-url)

**Figure 6-3.** Elongation of a unique type of fibril. The scale bar is 5 µm.

Another type of fibrils with unique elongation dynamics was identified from the growth stacks. They usually elongated with a very bright tip (but not always) and sometimes grew abruptly, likely caused by elongation in solution. The segments which had elongated for some time could become dim. Figure 6-3
shows an example of this fibril type. The abrupt growth was seen between time points 2.75 h and 3 h (one time interval). In the diffraction-limited image made from running z-projection of a growth stack, these fibrils usually acquired a bead-like morphology, which was resolved later by TAB imaging as helical structures.

The heterogeneity in fibril growth rate, brightness and morphology could result from different seed types, i.e. seed structures. This hypothesis can be verified by measuring fibril structures using high-resolution approaches such as TEM, which will be shown in Section 6.7.

(5) Change of fibril type during elongation was rarely seen.

An elongating fibril usually maintained its property such as growth rate and brightness during the entire growth phase. However, changes in growth properties during elongation were occasionally observed. An example is shown in Figure 6-4 A. The original seed pointed by a yellow arrow at 0 h first elongated into a bead-like structure, as seen next to the blue curve in the Z-projection image. Later in the elongation, this fibril changed its morphology into a straight fibril (shown next to the red line in the Z-projection image) and maintained this morphology afterwards. A kymograph was generated for this fibril and shown in Figure 6-4 B. Kymographs plot fibril growth in two dimensions. The y-axis represents time, while fibril growth is represented in the x-axis by a stack of multiple 1-pixel wide horizontal images, each of which is a straightened image along a line passing through the fibril axis. The kymograph was built in ImageJ for a drift-corrected, time-lapse image stack. It clearly showed that before ~23 h, the seed elongated relatively fast, with the tip brighter than the rest of the fibril; at ~23 h, the growth pattern changed, and the fibril elongated slowly with high intensity afterwards.

The structural switch was rare. For example, for fibrils elongating in a monomer solution with 1 μM PrP and 2 M GdnHCl, only 4 fibrils show a possible switch between the helical fibril type and one of the straight fibril types among > 200 growing fibril ends that were analysed. However, in a repeat experiment where > 400 fibril ends were analysed, none of them show an apparent switch.
Figure 6-4. Elongation of a fibril which changed its type. The experiment was conducted in 1.4 M GdnHCl using 1 µM PrP at 37°C. (A) Images of the fibril at increasing time points and a Z-projection of the growth stack from 0 h to 70 h. (B) Kymograph of the fibril. The horizontal scale bar represents 5 µm, and the vertical scale bar represents 10 h.

Figure 6-5. Elongation of a fibril on an existing one. (A) The experiment was conducted in 1.7 M GdnHCl using 1 µM PrP at 37°C. (B) The experiment was conducted in 2 M GdnHCl using 1 µM PrP at 37°C. The horizontal scale bar represents 2 µm, and the vertical scale bar represents 10 h.
In addition to the change in fibril morphology, a change in fibril brightness during elongation was occasionally observed as well. Changes in fibril morphology or brightness were rare, and most fibrils elongated with fidelity.

Another interesting and more commonly observed phenomenon is the elongation on an existing fibril. Two examples are presented in Figure 6-5.

6.4 Quantitative analysis for elongation data

6.4.1 Extracting kinetic information for a single fibril

Each fibril's lengths at sequential time points are needed to extract kinetic information. One strategy was to use the ImageJ ridge detection plugin to follow the axis of all fibrils in an ROI and report their lengths at all time points. However, the determination of the fibril axis was not always accurate. Fibril ends were occasionally not included, especially when the end regions were dim in brightness. This error led to a fluctuation in the extracted fibril length.

Another strategy was extracting the fibril end's position from a kymograph [193]. Figure 6-6 A shows examples of kymographs for fibrils a and b in Figure 6-2 B. Kymographs show the growth from small seeds into longer fibrils and present information on the growth directionality and the intermittent pattern.

![Figure 6-6](image)

**Figure 6-6.** Kinetic analysis for two fibrils. (A) Kymographs for fibrils a and b in Figure 6-2 B. (B) Length versus time traces for the two fibrils obtained by identifying the fibril end positions at increasing time points. (C) Length versus time trace after removing the stalls. The figure is adapted from [193].
The edges between the bright fibril region and the dark background represented the fibril end positions at sequential time points. A segmented line was drawn along the fibril edge by hand in ImageJ to extract edge positions. Here, the period when the fibril end position didn’t change was recognised as stalls. The determination of stall phases for the fast-growing fibrils were relatively easy. However, for the slower-growing fibrils, it was difficult to differentiate complete stalls from slow-growing phases. Thus, this step introduced some degree of personal bias. This step was also time-consuming.

I next explored other more automated methods utilising MATLAB scripts, which detected the edge in a kymograph by either an edge detection algorithm or a simple thresholding step. One difficulty in the following step was, again, differentiating slow growth phases from pause phases. Slow-growing fibrils might take several time intervals to extend the end to another pixel. This was often mistakenly recognised as a stall phase followed by a jump in the end position. Eventually, I decided to use the manual approach to follow end positions in kymographs. In the future, more objective criteria to define the stall phases are needed to reduce personal bias and to facilitate automated analysis.

The hand-drawn line of the fibril edge on the kymograph was then converted to a trace representing the elongated fibril length as a function of time. Figure 6-6 B plots two traces for the two fibrils shown in A. The apparent rate of each fibril was calculated by dividing the total elongated length by the total time.

The stall phases of each trace were removed to generate new traces shown in Figure 6-6 C. A ‘pause-free’ rate was obtained for each fibril as the ratio of the final length and the time spent in elongation phases. For each fibril, the growth rate remained nearly constant; however, pause-free rates between different fibrils showed heterogeneity. For example, there was a dramatic difference in pause-free rates of the two fibrils plotted here. Stall time percentage can be obtained as the ratio of stall time and total time.
6.4.2 Analysis for multiple fibrils in an experiment

Under each experimental condition, the acquired images were put through a workflow for quantitative analysis [193]. Image stacks taken for the same FOV with a 15 min interval were drift corrected by ImageJ plugins StackReg [188] and Image Stabilizer [189]. For each selected fibril, a segmented line was drawn along its axis to generate the kymograph using Reslice command in ImageJ. The fibril edge in the kymograph was selected by drawing a segmented line by hand, and the coordinates on that line were imported into MATLAB for further processing.

To minimise bias in the selection of fibrils, all fibrils which met the following criteria were selected: a) the contrast of the fibril was good enough to be distinguished from the background; b) the fibril grew from a single seed or a seed cluster with no more than 4 growing ends; c) the fibril didn’t intersect with other ones during growth; d) the fibril ends didn’t wobble on the surface or move out of focus; e) there was no abrupt growth > 9 pixels (990 nm) between two adjacent time points, as it could not be excluded that these fibrils had grown in solution and deposited between image acquisition. Usually, 3-4 stacks were used, and >70 fibrils that met the selection criteria were analysed for each experiment.

Here, I present the detailed analysis of the elongation assay conducted with 1 μM MoPrP in the standard buffer at 37 °C. 114 fibrils from 5 FOVs were analysed, and their elongation traces before and after removing stall phases were plotted in panels A and B of Figure 6-7, respectively. Distributions of fibril’s apparent rate and pause-free rate are shown in panels C and D, respectively. Strikingly, despite that all fibrils were seeded from the same seed population, fibril growth rates (Figure 6-7 B and D) did not follow a single distribution as previously observed for other amyloids [23]. Instead, growth rates could be grouped into two main clusters featuring slow or fast growth. A few fibrils adopted rates between the fast and slow fibrils (panel B), possibly representing a third, less populated group.
Figure 6-7. Kinetic analysis for the elongation assay conducted under the standard condition. (A) Elongated length versus time traces for 114 fibrils analysed. (B) Elongation traces after removing stall phases. (C) Distribution of apparent fibril growth rates. (D) Distribution of pause-free rates. (E) Distribution of stall phase percentages. The figure is adapted from [193].

Intermittent growth was a common feature, and stall phases accounted for a substantial fraction of time for most fibrils. The distribution of stall phase percentages (Figure 6-7 E) displays a significant variance among the fibril population, with an average of 74% and a standard deviation of 15%.

6.4.3 Correlation between fibril rate and brightness

Analysis of a large number of fibrils confirmed the previous observation that there was a correlation between elongation rate and fibril intensity [193].

I calculated the brightness of each fibril at the time point right before its last stall event when the fibril had reached its longest length and wasn’t
significantly affected by photobleaching. The mean brightness of the middle half of the fibril was extracted as the measured value.

Laser illumination over the whole FOV caused a Gaussian distribution of excitation intensity, which affected the fibril’s fluorescence intensity and needed to be corrected. An intensity correction factor was calculated for each fibril as the ratio of the highest background intensity of the FOV (the Gaussian peak value) and the background intensity at the fibril position. Here I assumed that fibril intensity was proportional to the background intensity. The actual fibril brightness was corrected by multiplying the measured value by this correction factor.

![Images](image1.png)

**Figure 6-8.** Identification of three fibril types. (A) Fibril growth traces with stall phases included. (B) Traces with stall phase removed. (C) Scatter plot of fibril brightness versus pause-free rate. (D) Grouping of fibrils into three major groups. The figure is adapted from [193].
Traces in Figure 6-7 A and B were replotted to show the intensity of each fibril, with each trace colour coded by the fibril’s corrected brightness (Figure 6-8 A and B). Figure 6-8 B shows that slow-growing fibrils usually adopted higher brightness than faster-growing ones. A scatter plot of fibril brightness versus pause-free rate is shown in Figure 6-8 C, revealing that bright fibrils were exclusively slow-growing and all fast fibrils were relatively dim. Fibrils could be put into three groups from their clustering in this scatter plot, represented by three colours in panel D. Slow-growing and bright fibrils are denoted as type I fibrils and coloured blue; slow-growing dim fibrils are denoted as type II fibrils and coloured orange; fast-growing dim fibrils are denoted as type III fibrils and coloured purple. A small portion of fibril growth traces did not fall into any group or displayed inconsistent growth rates during the experiment. These are shown as open circles in panel D.

I was interested in the intrinsic mechanism leading to the different properties of the three groups. I hypothesised that the three types of fibrils elongated from different types of seeds and adopted distinct structures, such as the number of strands or the internal packing within each strand. This theory can explain the variation in growth rate: for elongation of fibrils with different internal structures, monomeric PrP needed to go through varying levels of structural rearrangement; for fibrils with different numbers of strands, the number of PrP molecules required for elongating one layer was distinct. This hypothesis would also explain the variance in brightness since different dye-binding sites might be exposed by structurally different fibrils. The hypothesis needs to be verified by structural analysis of different types of fibrils using methods such as TEM or Circular Dichroism (CD), which will be presented in Section 6.7.

6.4.4 Directionality of fibril elongation

Seeds that grew into single fibrils elongated either bidirectionally or unidirectionally. For a quantitative study of the directionality, single fibrils from 9 FOVs were analysed, making 34 unidirectional fibrils and 23 bidirectional fibrils in total. It is worth noting that TIRF microscopy cannot differentiate a single seed or a seed cluster; it is possible that a seemingly bidirectional fibril
is growing from a seed cluster containing two particles with different growth directions. To reduce this possibility, fibrils were discarded if the two ends didn’t grow in opposing directions initially. However, fibrils with two ends at different brightness levels were included and analysed in Figure 6-9.

Figure 6-9. Analysis of growth directionality. (A) Scatter plot of fibrils’ pause-free rates of opposing ends. Blue, circular dots on the x-axis represent pause-free rates of unidirectional fibrils. Each diamond-shaped dot (orange or grey) represents a bidirectionally growing fibril with the pause-free rate of its forward end on the x-axis and reverse end on the y-axis. (B) Scatter plot of bidirectional fibrils’ pause-free rates of opposing tips, colour coded by the brightness of the forward ends. The inset shows a zoomed-in view of the slow-rate regime. (C) Scatter plot as in (B), with each dot colour coded by the brightness of the reverse ends. The colour bar represents brightness in arbitrary fluorescence units. The figure is adapted from [193].

Kinetic analysis was conducted for each growing end to obtain the growth rate and intensity.

Figure 6-9 A plots pause-free rates of the fast (forward) growing end versus the slow (reverse) end of each fibril in a scatter plot. Each dot represents a single fibril. Blue dots on the x-axis represent the unique rates of unidirectional fibrils, while diamonds represent rates at two ends for bidirectional fibrils. Interestingly, most bidirectional fibrils were highly directional, with the reverse growth much slower than the forward growth (red diamonds). These data
suggested that PrP fibrils were structurally distinct at opposing ends, leading to faster monomer conversion at one end over the other. Other bidirectional fibrils (marked by grey diamonds) had similar growth rates at two ends, most of which grew slowly. Since TIRFM cannot resolve single seeds and seed clusters, whether these fibrils were seeded by isolated seeds or clusters that happened to align in their growth axes couldn’t be determined.

Figure 6-9 B and C plot pause-free rates of bidirectional fibrils, colour-coded by the brightness of the forward end (panel B) or the reverse end (panel C). It confirmed our previous observation that fast-growing fibrils were always dim in intensity. For fibrils in the green rectangles, which were all dim in brightness, their forward ends displayed fast growth, putting them in the group of type III fibrils, while their reverse ends grew slowly, corresponding to type II fibrils. This observation suggests that type II and III fibrils likely represent the same fibril structure elongating from the reverse or forward ends, respectively.

Several fibrils with very high brightness were observed (the yellow symbols in the inset of panel C). Those fibrils always grew slowly and belonged to type I fibrils. The opposite end of those fibrils was typically dim and grew slightly faster, possibly suggesting that initial seeds consisted of two particles with different brightness levels. However, the very limited number of fibrils restricted any statistically meaningful conclusion.

6.5 Dependence of elongation kinetics on monomer concentration

Elongation assays and kinetic analyses were conducted under different PrP concentrations: 0.3, 0.5, 1, 2, 3, 5 and 10 µM. For quantitative analysis, the trace, elongation rate and brightness were identified for each selected fibril under each condition. Similar to the 1 µM standard case discussed above, fibrils under all other conditions can be grouped based on their clustering in the scatter plot of brightness versus pause-free rate (Figure 6-10). Only dim fibrils were observed at the lowest concentration (0.3 µM), which were appointed to be type III fibrils. At low PrP concentrations, type I and II fibrils grew so slowly that the growth rate determined might not be accurate. Due to
the fast-growing nature of type III fibrils, kinetic analysis for those fibrils were more reliable.

Figure 6-10. Grouping of fibrils under each PrP concentration. The figure is adapted from [193].

![Figure 6-10](image)

Figure 6-11. Dependence of three fibril types’ fractions on PrP<sub>C</sub> concentration. Total fibril numbers under each condition were the sum of type I, II and III fibrils, not including ungrouped fibrils. At 1 µM PrP<sub>C</sub>, fibrils in five FOVs were analysed, and standard deviations of fractions among those five FOVs were 12%, 13% and 14% for type I, II and III fibrils, respectively. The figure is adapted from [193].

Monomer concentration strongly affected the relative populations of type I, II and III fibrils (Figure 6-11). Type III fibrils accounted for over 80% at or below 0.5 µM, while nearly no bright type I fibrils were present. However, the fraction
of type III fibrils fell quickly with PrP concentration. Concurrently, the fraction of slow-growing type I and II fibrils increased, so similar fractions of all fibril types grew between 2–10 µM PrP\textsuperscript{C}. Thus, type III fibrils were favoured at lower monomer concentrations, while high PrP concentrations shifted the equilibrium towards slower-growing fibrils.

The distribution of pause-free rates and stall phase percentages for each fibril type are plotted in Figure 6-12 A and B, respectively. Pause-free rate distribution for each fibril group displays a single peak, possibly indicating that the grouped fibrils were indeed homogeneous, or at least a population of species displaying similar kinetic properties. A Gaussian function was fitted to these distributions (except type I fibrils at 0.5 µM), and the fitted mean and sigma were extracted.

The three fibril types exhibited remarkably different concentration dependencies of their pause-free elongation rates (Figure 6-12 C) and stall percentages (Figure 6-12 D).

For type III fibrils, pause-free rates increased nearly linearly with monomer concentration in the lower concentration range from 0.3 to 1 µM (Figure 6-12 E). Pause-free rates approached saturation in the middle concentration range (2 µM to 5 µM), then decreased at the highest concentration (10 µM), indicating an inhibition effect.

This observation suggests that at low PrP\textsuperscript{C} concentrations below 1 µM, fibril elongation could be considered a bimolecular reaction between fibril end (E) and monomer (M). A linear fit to pause-free rates against PrP concentration (Figure 6-12 E) yielded a slope of $13.1 \pm 0.6$ nm µM\textsuperscript{-1} min\textsuperscript{-1}, corresponding to a rate constant of $(4.5 \pm 0.2) \times 10^5$ M\textsuperscript{-1} s\textsuperscript{-1} for a bimolecular reaction. Here, I assumed that type III fibrils were single-strand with a layer-to-layer distance of 0.48 nm [26].
Figure 6-12. Dependence of elongation kinetics on PrP\textsuperscript{C} concentration. (A) Distribution of pause-free rates of type I, II and III fibrils under each PrP concentration. Each Red curve represents the Gaussian fit to the distribution. (B) Distribution of stall phase percentages of each fibril type. The red line is the mean of each histogram. (C) Dependence of pause-free rates of type I (top), type II (middle) or type III (bottom) fibrils on PrP\textsuperscript{C} concentration. Pause-free rates are the peak positions extracted from the corresponding Gaussian fits in (A), and error bars represent σ. The black curves in the top and bottom panels represent MM fits. In the bottom panel, data points at 3 µM and 10 µM were removed from the fitting. (D) Dependence of stall phase percentages of type I, II or III fibrils on PrP\textsuperscript{C} concentration. Stall percentages are the average value of fibrils of the specific type. Errors are standard deviations. (E) Linear fit to the pause-free rates of type III fibrils in the low concentration regime. The figure is adapted from [193].

At a higher concentration range between 2 µM and 5 µM, structural conversion from an attached monomer to a building block of the fibril becomes rate-limiting. This would be a saturatable process analogous to a Michaelis-Menten (MM) catalytic reaction scheme:

\[ E + M \xrightarrow{k_1} EM \xrightarrow{k_2} E \]  

(Equation 6.1)

As discussed in Section 2.3.1 (4), the pause-free rate \( r \) as a function of the substrate (monomer) concentration \( C_M \) can be described by:

\[ r = \frac{R_m C_M}{K_m + C_M} \]  

(Equation 6.2)

where \( R_m \) represents the maximum rate, the asymptotic rate at high substrate concentrations; \( K_m \) stands for a composite constant \( K_m = (k_{-1} + k_2)/k_1 \).

After fitting the kinetic equation 6.2 to the concentration dependence of type III fibrils (shown as the black curve in Figure 6-9 C; data points at 3 µM and 10 µM PrP concentrations were deleted), two constants were obtained: \( K_m = 1.4 \)
\[ \pm 0.5 \mu M, R_m = 24 \pm 5 \text{ nm/min.} \] The maximum rate \( R_m \) can be converted to \( k_2 \) by the following expression:

\[ k_2 = \frac{R_m}{0.48} = 0.84 \pm 0.18 \text{ s}^{-1}. \]

However, the MM-type mechanism failed to capture the inhibition of type III fibril growth at 10 \( \mu M \), the highest monomer concentration. It has been proposed that the unproductive binding of PrP monomers to the amyloid fibril ends could account for this inhibition [152]. Alternatively, PrP could form off-pathway assemblies at high protein concentrations, which bound at the fibril ends and prevented fibril growth.

For type I fibrils, the concentration dependence of pause-free rates can be described accurately by the saturable kinetics analogous to MM kinetics, with constants:

\[ K_m = 2.4 \pm 0.3 \mu M, R_m = 6.3 \pm 0.8 \text{ nm/min}, \] and

\[ k_2 = 0.44 \pm 0.05 \text{ s}^{-1}. \]

Type I fibrils were assumed to be double-strand, and the reason behind this assumption will be discussed in Section 6.7. The assumption only affects the conversion from \( R_m \) to \( k_2 \): \( k_2 = 2 \times \frac{R_m}{0.48} \), while the reaction mechanism (i.e. reaction order) was assumed to be unchanged, i.e. both strands elongated by the addition of single PrP monomers. \( k_2 \) calculated for type I fibrils was only half the value for type III fibrils, suggesting a slower structural conversion from the tip-bound molecule to a correct fold in the type I fibrils.

In contrast to types I and III, type II fibril growth showed a weak concentration dependence overall, suggesting that elongation was likely 0th order with respect to PrP concentration. This behaviour is compatible with the rate-limiting step being the slow structural conversion of PrP. Different types of fibrils having distinct concentration dependence points to distinct rate-limiting steps during fibril elongation.

Stall percentages didn’t show a profound concentration dependence for type II fibrils; however, for type I and III fibrils, stall percentages increased with PrP concentration before reaching a plateau in a concentration range from 1 \( \mu M \) to 10 \( \mu M \). For type III fibrils, the high stall percentage and the drop in elongation rate at high monomer concentrations indicated a mechanism inhibiting fibril growth. It is worth mentioning that the sampling time of our kinetic assay was 15 min. Thus, stall events < 15 min (or even several folds of 15 min due to the
uncertainty in the analysis) were not counted in stall percentage but were perceived as a reduction in elongation rate.

Here, the fibril stall percentage was affected by the solution condition, which was monomer concentration in this case. This fact argues against the hypothesis that the stalls are caused by steric inhibition by the coverslip surface since steric inhibition should be independent of monomer concentration.

6.6 Dependence of elongation kinetics on GdnHCl concentration

Denaturant concentration changes the relative abundance of different PrP^C species, i.e. natively folded PrP or unfolded structure. I analysed the dependence of elongation kinetics on denaturant (GdnHCl) concentration from 1.4 to 2.3 M at a fixed PrP concentration (1 µM). The chemical denaturation curve measured by CD by a lab member (Figure 6-13) reveals that the fraction of unfolded PrP increases from 0.9% at 1.4 M GdnHCl to 52% at 2.3 M GdnHCl, with the midpoint of unfolding at 2.3 ± 0.1 M GdnHCl.

![Chemical denaturation curve](image)

**Figure 6-13.** Chemical denaturation of monomeric MoPrP 91-231. (A) Dependence of ellipticity at 222 nm on GdnHCl concentration. (B) Fraction of unfolded PrP during chemical denaturation. The red curve is a fit of the two-state model (i.e. protein adopts either an unfolded or a native state with no folding intermediate). The figure is adapted from [193].
Figure 6-14. Kinetic analysis for the dependence of fibril elongation on GdnHCl concentration. (A) Scatter plots of fibril brightness versus pause-free rates showing the grouping of three fibril types at each condition. (B) The dependence of fibril type population on GdnHCl concentration. (C) Distributions of pause-free rates separated by fibril type with the fitted Gaussian curves. (D) Distributions of stall phase percentages, with the red line representing the mean of each distribution. (E) Dependence of pause-free rate on GdnHCl concentration. The mean and σ of Gaussian fits are shown. (F) Dependence of stall percentage on GdnHCl concentration. The mean and standard deviation of histograms are shown. The figure is adapted from [193].

Kinetic analyses were performed for experiments under different GdnHCl concentrations. Three fibril types were identified in each experiment, shown by different colours in Figure 6-14 A. GdnHCl concentration changed the relative abundance of each fibril type (Figure 6-14 B). Increasing GdnHCl concentration led to a smaller fraction of type I fibrils and a larger fraction of type III fibrils. It is worth noting that at 2.3 M GdnHCl, ends of some fast-growing fibrils occasionally detached from the surface. Those fibrils were
eliminated from rate analysis, which artificially depressed the fraction of type III fibrils. Since varying GdnHCl concentrations changed the concentration of native or unfolded PrP, this result suggests that type I or III fibrils might recruit or be inhibited by PrP in different states.

For each fibril type, the distributions of pause-free rates and stall percentages are plotted in panels C and D. Panels E and F summarise the dependence of pause-free rates and stall percentages of each fibril type on GdnHCl concentration.

Type III fibrils’ elongation rates increased with the unfolded PrP concentration, suggesting that this fibril type elongated by incorporating unfolded PrP molecules. In contrast, type I and II fibril elongation showed no or small dependence on GdnHCl concentration. This behaviour was compatible with incorporating native-state PrP or a partially folded intermediate, whose concentration changed weakly in the GdnHCl concentration range probed in the experiment.

6.7 Type I and III fibrils were structurally distinct

As discussed above, fibrils from different groups differ in kinetics and brightness and show distinct dependences on PrP concentration and PrP denaturation state. The kinetic analysis also supports that different fibrils might incorporate PrP monomers in unique folding states. These observations suggest that PrP fibrils of different types are structurally different. However, the resolution of TIRF imaging is limited by light diffraction and isn’t high enough to provide accurate structural information. I then validated this hypothesis by imaging methods with higher resolution, such as TEM or AFM, and experimental approaches that can reveal secondary structures of proteins, such as CD [193].

I chose to study the fibrils’ structures under two reaction conditions favouring the growth of type I or type III fibrils, respectively. Condition (a) was 1 µM monomer in the buffer containing 1 M GdnHCl, where ~70% elongated fibrils
were type I fibrils; condition (b) was 1 µM monomer in 2 M GdnHCl (the standard buffer), where ~60% fibrils belonged to type III fibrils.

A.

![TEM images](image)

B.

![TEM images](image)

C.

![Graphs](image)

**Figure 6-15.** Fibril elongation on TEM grids. (A) TEM images of fibrils elongated under condition (a) for 3 d. The scale bars represent 500 nm. The last panel shows a zoomed-in view of the fibril segment in the white box. (B) Fibrils after elongation under condition (b) for 2 d. (C) Width distribution for fibrils after elongating under condition (a) (left) or (b) (right). The figure is adapted from [193].

I performed fibril elongation experiments on TEM grids to visualise fibril structures. Diluted seeds were incubated on plasma-discharged Au TEM grids, allowing seed particles to adsorb onto the carbon film. The grids were then incubated in monomer solutions under conditions (a) or (b). After incubation, the grids were washed by water droplets and stained with NanoW.
TEM images were then acquired and shown in Figure 6-15 A and B. Both fibril clusters and single fibrils were observed on the two grids incubated under conditions (a) or (b). Interestingly, under condition (a) which favoured type I fibrils, the majority of elongated fibrils were made of multiple strands with a varied number of protofilaments; however, under condition (b) favouring type III fibrils, most fibrils were made of a single protofilament.

It was challenging to identify newly grown fibrils from the seeds with certainty. To ensure the analysed fibrils were elongated fibrils instead of seeds, I only analysed fibril segments that were longer than 500 nm since they were longer than the typical length of the initial seeds (Figure 6-1 B). Many of the fibrils were tangled, preventing accurate measurement of fibril lengths. Instead, I measured fibril widths and plotted their distribution in Figure 6-15 C. PrP fibrils grown under the two conditions had distinct distributions of fibril width. Condition (a) generated mainly 14–18 nm wide fibrils, while condition (b) favoured 6–8 nm wide fibrils. The two different fibril widths likely represented double-strand and single-strand fibrils, respectively. Single-strand fibrils observed under condition (b) also tended to be much longer than fibrils under condition (a). Recall from in situ elongation experiments that type I fibrils were the most prevalent species under condition (a), while type III fibrils dominated under condition (b), TEM analysis supports that type III fibrils were single-strand while type I fibrils were double-strand.

TEM images identified more fibril morphologies than the three types grouped from in situ elongation experiments. Under condition (b), I observed single-strand straight fibrils, single-strand curvy fibrils, multiple-strand fibrils with regular twisting, multiple-strand fibrils without twisting and helically coiled fibrils (Figure 6-15 B). In TIRF images, the resolution was limited, and the determination of fibril types was based on their growth rate and brightness. It was likely that fibrils grouped as one type in TIRF imaging contained fibrils of multiple structures.

TEM analysis established that type I or III fibrils differed in the number of protofilaments. I then probed whether they also differed in secondary
structures. Solutions enriched in type I or type III fibrils were prepared by two rounds of bulk seeding assays with agitation in conditions (a) or (b), respectively. 0.1% seed was added for each seeding assay. The end products were pelleted by ultracentrifugation. Supernatants and pellet resuspensions were subjected to CD measurement separately (Figure 6-16). Under both conditions, the spectra of the resuspended fibrils had minima between 218–225 nm, indicating the presence of β-sheets. However, spectra recorded under conditions (a) and (b) were distinct, with minima at ~225 nm under condition (a) and ~220 nm under condition (b), confirming that fibrils generated under the two conditions acquired distinct secondary structures.

![Figure 6-16. CD spectra of PrP$^C$ and PrP aggregates after two rounds of seeding in condition (a) (left) or condition (b) (right). The figure is adapted from [193].](image)

6.8 Fibril types retained structural characteristics under competitive growth conditions

Previous data showed that type I and III fibrils were different in terms of secondary structures and the number of protofilaments. Their formation was favoured under specific buffer conditions. I tested whether the elongated fibril structure was determined by the seed structure or by buffer condition.
A two-phase elongation experiment was designed to answer this question. In the first phase, surface-bound seeds were allowed to elongate under buffer condition (a) favouring type I fibrils for 71 h. Then, the buffer was switched to (b), and fibril growth was monitored for a further 88 h by TIRFM. In Figure 6-17 A, fibrils present at the end of the first phase are shown in red, and fibrils elongated during the second phase are shown in green. Correspondingly, fibrils from the first phase that persisted in the second phase appear yellow, while aggregates present in the first phase which detached during the second phase appear red.

Previous analysis has established that type I and III fibrils can be distinguished by brightness. After the first elongation phase, only bright fibrils were present (the yellow fibril segments pointed by bold arrows in Figure 6-17 A), which
were type I fibrils. In the second elongation phase, type I fibrils continued to grow (the green segments pointed by bold arrows); dim type III fibrils started to grow from dot-like seeds that had not extended in phase 1 or at a position where no apparent seed was observed (the fibril pointed by the thin arrow).

For quantitative analysis, I analysed the brightness of fibrils grown during phase 1 (group 1) or grown exclusively during phase 2 (group 2) and compared them to the brightness of fibrils elongated during phase 2 from fibrils grown in phase 1, denoted (1→2). Figure 6-17 B plots the brightness profiles of the three groups of fibrils, confirming that the bright type I fibrils after elongation in the first phase (Figure 6-17 B, group 1) continued to grow at this high brightness in the second phase (group (1→2)). Conversely, no dim type III fibrils grew from type I fibril seeds. Fibrils that only started to grow in the second phase were dimmer in brightness, corresponding to type III fibrils (group 2). These data indicated that elongating fibrils retained their fibril structures after switching buffer conditions; type III fibrils were not cross-seeded by type I fibrils and vice versa.

Since type I fibrils elongated under both conditions while type III fibrils could only grow under condition (b), the change in fibril fractions under those two conditions was explained by different numbers of growing type III fibrils.

Overall, our data suggest that fibril type and structure are templated by the seed type rather than determined by buffer conditions; buffer conditions shift the equilibrium between fibril types so that type I fibrils could outcompete type III in condition (a) and vice versa.

6.9 Temperature dependence of elongation kinetics

Elongation experiments were carried out at five temperatures (27, 30, 34, 37 and 40°C) at a fixed solution condition (2 M GdnHCl, 1 µM PrP⁴⁰) to determine the activation energies for elongation. The fraction of unfolded protein varied from 5% at 27°C to 40% at 40°C (Figure 6-18).
Figure 6-18. Thermal denaturation of monomeric MoPrP 91-231. (A) PrP\textsuperscript{C} thermal denaturation during thermal unfolding (blue dots) and refolding (orange dots). (B) Fraction of unfolded PrP\textsuperscript{C} during thermal unfolding. The black curve represents a fit of the two-state model. The figure is adapted from [193].

Figure 6-19. Temperature dependence of fibril pause-free rate. (A) Pause-free rates of type I (blue), II (orange), or III (purple) fibrils under different temperatures. (B) Arrhenius plot for type III fibrils. The straight line is a linear fit to the five data points. The figure is adapted from [193].

For the experiment conducted at each temperature, the grouping of three fibril types was conducted. The mean rate was obtained for each fibril type by a Gaussian fit to the pause-free rate distribution; the average and standard deviation of stall phase percentage were also calculated. Figure 6-19 A plots how the pause-free rates of three fibril types change with temperature. Pause-
free rates for type I and II fibrils remained almost unchanged within this temperature range, suggesting the rate-limiting step was not temperature-dependent. In contrast, pause-free rates for type III fibrils had a strong temperature dependence, which followed the Arrhenius equation with activation energy (Ea) of 70 ± 2 kJ/mol. This is comparable to the literature value of ~50 kJ/mol under conditions when the unfolded monomer is abundant [151].

Buell and co-workers [149] reviewed elongation studies of multiple amyloid systems and compared thermodynamic parameters. Taking the transition state theory, activation enthalpy $\Delta H^\ddagger$, activation free energy $\Delta G^\ddagger$, and activation entropy $\Delta S^\ddagger$ could be estimated. In this work, activation enthalpy $\Delta H^\ddagger$ roughly equals Ea to be 70 ± 2 kJ/mol.

$\Delta G^\ddagger$ could be calculated by:

$$k = \Gamma \exp\left(-\frac{\Delta G^\ddagger}{RT}\right)$$

Equation 6.2

where $\Gamma$ is the diffusive pre-factor. I used the same simplification as in [105] and took $\Gamma$ as the upper limit, $1.8 \times 10^9$ M$^{-1}$s$^{-1}$. Here, I used the rate constant for type III fibrils in the linear phase ($(4.5 \pm 0.2) \times 10^8$ M$^{-1}$s$^{-1}$, Section 6.5) and calculated $\Delta G^\ddagger$ to be $21.4 \pm 0.1$ kJ/mol at 37°C.

$\Delta S^\ddagger$ can then be calculated:

$$\Delta S^\ddagger = \frac{1}{T}(\Delta H^\ddagger - \Delta G^\ddagger) = 0.16 \pm 0.01 \text{ kJ/mol/K}$$

Consistent with other amyloid systems, activation enthalpy for PrP elongation was unfavourable, while activation entropy was favourable [149]. The activation energy was roughly the same as short, unfolded peptide Aβ42 or intrinsically unfolded α-Syn [149], which might suggest the involvement of unfolded PrP$^{\text{C}}$ during the elongation of type III fibrils.
6.10 Discussion

(1) Single-molecule measurements reveal structural heterogeneity of recombinant PrP fibrils

TIRFM and TEM imaging of elongated PrP fibrils revealed the presence of multiple fibril types: straight fibrils with different brightness and rates in TIRFM images (Figure 6-2); fibrils with different numbers of strands and twisting patterns in TEM images (Figure 6-15 A and B). Different fibril types also acquired unique secondary structures (Figure 6-16). Taking the assumption that the fibril structure was determined by the seed, multiple fibril types after elongation suggested a seed heterogeneity, which resembled the ‘prion quasi-species’ concept in prion diseases – a ‘cloud’ of different misfolded PrP species co-existed [30].

Amyloid polymorphs have been revealed by structural analysis for both in vitro generated amyloid fibrils and ex vivo authentic fibrils, as have been reviewed in Section 1.3.

For fibrils formed in vitro, the polymorphism could result from different incubation conditions. For example, Aβ40 fibril morphology was affected by the level of agitation [101], the type of salt ions [225] and the presence of cofactors [226]. Full-length hamster PrP could form structurally different amyloid fibrils under different shaking conditions [227]. Multiple fibril structures could be generated under the same condition, from different test tubes [195] or even within one sample [116], similar to the observation in this study. Our study on PrP seeded aggregation revealed the presence of structurally different PrP fibrils, which co-existed under homogeneous assay conditions.

However, patient-derived authentic amyloid fibrils were generally homogeneous within individual patients but displayed polymorphic structures corresponding to different disease phenotypes [97, 228-230]. The structures of authentic fibrils were often, but not always, found to be different from fibrils formed in vitro. For example, atomic models built for authentic Aβ42 fibrils by cryo-EM revealed two major fibril types [97]; fibrils from every patient contained solely or dominantly one type of structure. These fibril structures
differed from Aβ42 fibrils formed in vitro [97]. Similarly, the structures of tau filaments from one type of tauopathies were usually conserved, while they were distinct among different diseases. Strikingly, two types of authentic, patient-derived fibrils, AD PHF (paired helical filaments from Alzheimer’s disease) and type II filament of CTE (chronic traumatic encephalopathy), can be formed in vitro utilising recombinant tau under specific reaction conditions [231].

Recently, atomic models were built for three prion strains, showing that all strains shared a similar dual lobe architecture spanning residues 94/95–225/227/229. The atomic models revealed structural heterogeneity mainly (1) between RML [26] and ME7 [27] strains, which propagated in the same species (mice), and (2) among strains propagating in different species. When comparing RML and ME7 strains, the two fibrils differed in internal side-chain packings, spacings between the two lobes and levels of glycosylation. For 263K [28] which propagated in hamsters, the PrP sequence was slightly different from that in RML and ME7, and the fibril structures again differed from the other two strains in their internal fold, inter-lobe distance and levels of glycosylation.

Structural heterogeneity was also observed within each strain (RML or ME7), related to the number of strands making up the fibril. Interestingly, RML double-strand fibrils seemed to exist in two distinct conformers in which the inter-strand interaction led to different overall fibril symmetries [26]. It is possible that other PrP assemblies also exist in the strain, but they are relatively unstable or less-populated, so that they are lost during purification from brain homogenates or unable to be identified by cryo-EM.

The structures of all three prion fibrils are remarkably different from amyloid fibrils formed in vitro by recombinant full-length human PrP [84]. The recombinant fibrils had a much smaller ordered core spanning from residue 170 to 229 and were composed of two intertwining protofilaments. There was no glycosylation since recombinant PrP didn’t involve post-translational modifications.
Overall, *in situ* TIRF imaging and high-resolution TEM measurement revealed that recombinant fibrils existed in different types with unique growth rates, ThT fluorescence intensities and morphologies. The discrepancies likely correspond to different numbers of strands and amino acid packings despite identical amino acid sequences. Structural polymorphism, a common feature of amyloid fibrils, is proven valid for recombinant PrP fibrils generated in this study.

(2) Structural heterogeneity corresponds to dynamic heterogeneity

Single-molecule measurements on PrP seeded elongation using TIRFM revealed three main groups of fibrils based on their brightness and kinetic profiles (Figure 6-8). Compared to high-resolution TEM images of fibrils grown on TEM grids (Figure 6-15), the fast-growing, dim type III fibrils in TIRF measurements were likely single-strand fibrils, while the slow-growing and bright type I fibrils likely corresponded to double-strand fibrils. Thus, fibrils displaying distinct dynamic properties were linked to their specific structures.

Why do structurally different fibrils have distinct elongation rates? The two types of fibrils might have distinct sizes of their ordered cores. When more residues are involved in the core structure, each PrP molecule may take longer to adopt the fibril fold. Another hypothesis is that the monomers incorporated into different fibrils are in distinct states (native, unfolded, or intermediate states), and structural conversion from different states has different rates. The earlier discussion (Section 6.6) proposed that type III fibrils recruit unfolded monomers, possibly leading to a faster conversion than type I fibrils recruiting native monomers or folding intermediates, since they need to (partially) unfold before being incorporated into type I fibrils. Finally, the reaction order with respect to PrP monomer may be different for type I or type III fibrils. Since type I fibrils are made of two strands, it is reasonable to speculate that elongation requires the simultaneous binding of two monomers or a dimer to the fibril ends, making the reaction order with respect to monomer two. For example, bulk seeding assays of ΔN6 [232], an N-terminal truncated variant of β2m, showed a higher than first order relationship between elongation rate and monomer concentration. They proposed that ΔN6 fibrils elongated by hexamer
addition. This hypothesis will be further investigated in the following discussion.

Single-molecule measurements performed here could track individual fibrils, study the kinetics of each fibril type and reveal the competing growth between fibril types. The information on structural and dynamic heterogeneity was hidden in bulk aggregation assays.

(3) Growth mainly templates seed structure

As seen from the sequential seeding experiment (Figure 6-17 A), type I fibrils maintained their properties such as brightness and relatively slow rate under different buffer conditions; in contrast, type III fibrils were able to elongate only in the suitable buffer. Changes in fibril type during elongation were rarely observed (Figure 6-4). Thus, fibril type is an intrinsic property defined by the seed structure rather than the solution condition.

Structural templating is common for amyloid elongation, as seen in the review by Alijanvand and co-workers [233]. The newly added monomer adopting the same structure as seeds was associated with a low free energy barrier, even though the seed structure might not be the most stable under the specific condition. An example came from cryo-EM structures determined for authentic SAA1.1 seeds and elongated fibrils, which showed that 98% of the elongated SAA1.1 fibrils took the same structure as the authentic seeds [150].

However, elongated fibril structure doesn’t always faithfully follow the template of the seed. A small number of fibrils changed their morphology during growth in our study (Section 6.3 (5)). As was discussed in Section 2.3.3, in situ Aβ42 fibril elongation observed by AFM [119] revealed occasional switches in fibril morphology, whose frequency increased when KCl replaced NaCl in the buffer. Helical fibrils of α-Syn E46K occasionally grew into 'striated' segments in buffer at a different pH value [132]. Hypothetically, the monomer at the fibril end could adopt a limited number of possible conformations which are separated by low free energy barriers, so that these conformations could interconvert under suitable conditions. The frequency of the transformation is affected by buffer conditions, such as the type of salt, which alters the strength of ionic interactions.
The elongation of wild-type α-Syn fibrils on a mica surface revealed that, for seed fibrils containing multiple strands, the elongated segments were always [130] or sometimes [132] thinner filaments. One or several strands were lost when the seeds started to grow, which was likely caused by steric hindrance by the mica surface, and suggested that protofilaments can grow as an entity. In contrast, I didn’t observe the loss of a strand during the growth of double-strand type I fibrils. This can be explained by the higher stability of the double-strand PrP fibrils, the inability of the single fibril strand of type I fibrils to exist alone, or the lack of overhanging single-strand ends.

(4) Growth of different types of fibrils is favoured under specific conditions and possibly involves different precursors and mechanisms

Figures 6-11 and 6-14 B suggest that type I fibril growth was favoured at higher PrP concentrations and low GdnHCl concentrations. In contrast, type III fibril growth was favoured at low PrP concentrations and high GdnHCl concentrations.

In the monomer concentration series, no type I fibrils elongated at the lowest PrP concentration (0.3 µM), possibly due to its critical concentration being higher than 0.3 µM. Only 5 of 81 fibrils elongated at 0.5 µM PrP were categorised as type I fibrils with a low elongation rate of 1 nm/min (mean value; Figure 6-12 A). The fraction of type I fibrils increased with PrP concentration before reaching a plateau above 2 µM (Figure 6-11).

Several hypotheses were brought up to explain the low fraction of type I fibrils in the low concentration regime. Part of the type I seeds didn’t elongate obviously, possibly due to (a) the nonnegligible monomer dissociation, (b) fibril ends being trapped in a long stall phase or (b) their growth rate being too slow to be observed. Alternatively, type I seeds might grow into type III fibrils at low PrP concentrations. This hypothesis is unlikely, though, as our experimental findings revealed that growth was almost always faithfully templated by the structure of the seeds (Figure 6-17).

With increasing PrP concentrations above 2 µM, the fraction of type I fibrils was maintained at a maximum, suggesting that nearly all type I seeds elongated under these conditions.
As discussed in Section 6.8, the fraction of type I fibrils generally decreased with GdnHCl concentration, which was caused by the increasing absolute number of type III fibrils with GdnHCl concentration.

In the GdnHCl concentration series, both rates and stall percentages of type I fibrils didn't vary significantly with GdnHCl concentration. Therefore, it is unlikely that the unfolded PrP is the direct precursor for elongation, considering two facts: (1) the unfolded PrP concentration varied substantially within this GdnHCl concentration range (0.8 – 52%), and (2) the low concentration of unfolded PrP at the lowest GdnHCl concentration is not likely to cause saturation in growth rate.

However, the concentration of native-state PrP shows a much smaller change: 99% at 1.4 M and 48% at 2.3 M GdnHCl. Considering that the elongation rate also didn't change significantly in this GdnHCl concentration range, the data could suggest that type I fibrils recruited native-state monomers.

An unfolding study using GdnHCl [234] supported a two-state mechanism for PrP unfolding, with no significantly populated folding intermediates [234]. However, another study [235] revealed perturbation of native-state HuPrP 91-231 structures under low denaturant concentration, including conformation changes of C-termini of helices II and III and the loop connecting the two helices, and the occasional detachment of residues 125-146 from the core region [235]. Those folding intermediates likely acquired a less stable folded core and were associated with a lower free energy barrier when incorporated into the fibril. Thus, we couldn't rule out the possibility that type I fibrils recruit a PrP folding intermediate.

Based on TEM images (Figure 6-15), type I fibrils are assumed to contain two strands. Therefore, it is possible that simultaneous binding of two monomers or a dimer at the two strands' tips is required for elongation. Therefore, I tested whether the elongation reaction is first-order or second-order with respect to monomers; the latter would be the case for the simultaneous addition of two monomers or a dimer.
Figure 6-20. Pause-free rates of type I fibrils as a function of PrP concentration. The black curve represents a fitting of the MM-type kinetics (Equation 6.2), $R^2 = 0.96$; the yellow curve represents a fitting of Equation 6.3, $R^2 = 0.88$.

To test which model better describes the elongation mechanisms of type I fibrils, I fitted the equations describing the two different models to the elongation rates at different monomer concentrations. A MM-type saturable mechanism (Equation 6.2) can usually describe the kinetics of the first-order model. This equation captured the concentration dependence well ($R^2 = 0.96$, Figure 6-20, black curve). Alternatively, if the simultaneous binding of two monomers or a dimer to the fibril end was required for a structural conversion (order = 2), the concentration dependence of pause-free rates would be described by the equation:

$$r = \frac{R_m [M]^2}{K_m + [M]^2} \quad \text{Equation 6.3}$$

A fit of this equation to the data is plotted as the yellow curve in Figure 6-20. Although this fit ($R^2 = 0.88$) was not as good as using Equation 6.2, it could also follow the trend of the data. Thus, this possibility couldn’t be ruled out. As discussed, a similar elongation mechanism was shown in literature for the elongation of $\Delta N6$ [232], stating that elongation occurred by oligomer addition.

Overall, type I fibrils might elongate with first- or second-order kinetics with respect to the precursor at low monomer concentrations, while structural...
conversion became rate-limiting at high PrP concentrations. The precursor could be native-state PrP or folding intermediates.

For type III fibrils, their fraction decreased with PrP concentration and plateaued at ~35%, concurrent with the fraction of type I fibrils increasing with PrP concentration before plateau (Figure 6-11). Three hypotheses could account for this observation: (a) the absolute number of type III fibrils remained constant at different PrP concentrations, while the increasing number of type I fibrils caused changes in fractions of type I and type III fibrils, respectively. However, this theory was difficult to validate, as the absolute number of fibrils was also affected by seed density, which was difficult to control. (b) An increasing concentration of elongation incompetent PrP species at high total PrP concentrations could lead to an increasing percentage of type III seeds being blocked for the entire experiment. However, the time for a single stall event rarely exceeded 30 h, arguing against this hypothesis. (c) Type I and type III fibrils competed for the monomer substrate, and type III fibrils outcompeted only at low PrP concentrations. This theory was unlikely as a low number of seed particles and a large reservoir of monomers ensured that monomers were not depleted during the experiment, which was supported by the near-constant pause-free rates of each fibril over the whole period of the experiment.

The fraction of type III fibrils generally increased with GdnHCl concentration (Figure 6-14B). As shown in Figure 6-17A, type III seeds couldn’t elongate in 1 M GdnHCl; however, they started to grow in 2 M GdnHCl. The deficiency of type III seeds to grow at low GdnHCl concentrations can be explained by the very small concentration of the substrate (0.1% PrP is unfolded at 1 M GdnHCl) or a higher binding frequency of species that cannot elongate.

The elongation rate of type III fibrils increased with PrP concentration in the 0.3–2 μM range at 2 M GdnHCl concentration. It also increased with GdnHCl concentration at a constant PrP concentration (1 μM). I hypothesised that type III fibrils recruited unfolded monomers. To confirm, the concentrations of unfolded monomers were calculated for both data sets (at varying PrP
concentrations or GdnHCl concentrations), and pause-free rates are plotted as a function of unfolded PrP concentration (Figure 6-21). Data for the concentration series and the GdnHCl series fell into the same trend, supporting the hypothesis.

![Plot of pause-free rates vs. PrP concentration](image)

**Figure 6-21.** Dependence of pause-free rates of type III fibrils on unfolded PrP concentration. The figure is revised from [193].

Inhibition of type III fibril growth was observed at high PrP monomer concentrations above 2 µM, as seen in Figure 6-12 C. As discussed in Section 6.5, the inhibition effect could have two possible causes: (1) unproductive binding events of native-state PrP monomers onto fibril ends, which were previously shown to account for the decrease in elongation rate of HuPrP at high PrP concentrations [152]; (2) off-pathway assemblies formed at high protein concentrations acting as an inhibitor. When the unproductive binding events lasted for several intervals, they would be observed as stall phases. However, if the binding lasted for a shorter time (for example, less than the measurement interval of 15 min), it could lead to a reduction in the measured elongation rate. The slightly increasing stall percentages (Figure 6-12 D) and the reducing pause-free rates (Figure 6-12 C) at PrP concentration ranging from 2 to 10 µM suggested an increasing frequency of both long-lived and short-lived unproductive binding events with PrP concentration. These results are compatible with the fibril elongation model from Honda et al. [152], as discussed below.
The presence and propagation of multiple fibril types are analogous to the 'quasi-species' model

As discussed in Section 1.1.2, the genetic quasi-species model [31] describes self-replicating DNA or RNA species, which contain individuals with slightly different sequences caused by imperfect replication. The population's mean sequence shifts when the environment changes.

Here, I show that structurally different PrP fibrils co-exist in a seemingly homogeneous sample, which show similarities to the 'quasi-species' concept. Although only elongation is observed in the surface-based elongation experiments shown in this chapter, kinetic analysis for bulk seeding assays in Chapter 5 emphasises the critical role of secondary processes to generate new aggregates under agitating conditions. Thus, PrP amyloid fibrils can self-replicate by the repeating cycles of elongation and secondary processes. One discrepancy between PrP fibrils and the conventional quasi-species model is that, different fibril types mainly arise from the formation of structurally heterogeneous nuclei instead of by mutation during replication. However, the rare change in fibril type during elongation was also observed (Figure 6-4), resembling 'mutation' seen in DNA or RNA replication. The buffer conditions shift the relative population of different fibril types instead of changing the structure of individual fibril (Figure 6-17). This change is likely due to the higher fitness of one type of amyloid structures over the others in the new environment, owing to their higher stability or faster replication.

By studying prion propagation in cell culture, Li and co-workers [236] showed that prions are subject to 'mutation', thus acquiring structural heterogeneity; distinct sub-populations are selectively amplified under selection pressure. Their findings are compatible with the quasi-species concept.

Compare PrP elongation kinetics with PrP seeding experiments in literature (Section 2.3.4)

Honda and co-workers [152] studied seeded elongation of FL HuPrP under systematically varied conditions by ThT assays in bulk. They concluded that PrP in its unfolded state was the direct substrate for elongation, while the native monomer acted as an inhibitor for elongation, either by binding to the
fibril (noncompetitive inhibition) with or without the attached unfolded monomer, or by binding to a complex of the unfolded monomer attached fibril (uncompetitive inhibition).

At high GdnHCl concentrations (> 2.3 M) when unfolded PrP dominated, they observed a similar MM-type saturation kinetics with respect to PrP concentration, as I have determined in the single-molecule experiments for type I fibrils and for type III fibrils within a low to medium PrP concentration range. Their fitting of the MM-type kinetics and extrapolating to 2 M GdnHCl concentration led to a k₂ of ~1 s⁻¹. k₂/Kₘ was found to be a parameter affected by GdnHCl concentration but constant for protein mutants and under different pH conditions. The value they obtained at 2 M GdnHCl was slightly less than 10⁵ M⁻¹s⁻¹. Extrapolation of this parameter to 0 GdnHCl concentration resulted in a value close to the diffusion limit. Thus, they concluded that the initial binding rate was fast, approaching the diffusion limit, while the subsequent structural conversion was slow. In our study, the k₂ and k₂/Kₘ were comparable to the values obtained by them [152]: k₂ = 0.44 ± 0.05 s⁻¹, k₂/Kₘ = 1.8x10⁵ M⁻¹s⁻¹ for type I fibrils; k₂ = 0.84 ± 0.18 s⁻¹, k₂/Kₘ = 6x10⁵ M⁻¹s⁻¹ for type III fibrils. Here, the rate constants were calculated using the total monomer concentration without further considering which species, native or unfolded PrP, was involved. This assumption was used in [152] as well.

It should be noted, though, that their mechanism is only compatible with the elongation of type III fibrils. In my experiments, type I and II fibrils lacked the specific dependence of elongation rates on GdnHCl concentration, which would be expected from the incorporation of native PrP monomer or an intermediate.

At lower GdnHCl concentrations, they observed an inhibitory effect at high PrP concentrations, similar to what I observed for type III fibrils. This inhibition was attributed to the binding of elongation incompetent, native PrP onto fibril ends.

Our single-molecule assay further revealed the existence of different fibril types, which reacted differently under varying conditions. This information couldn’t be revealed by bulk measurements.
Sang and co-workers [138] studied the elongation of full-length MoPrP under native conditions (without GdnHCl) and derived an elongation rate constant of \( \sim 10^4 \text{ M}^{-1} \text{s}^{-1} \). This rate constant was significantly smaller than the value obtained in this study for type III fibrils in the low concentration regime, \((4.5 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{s}^{-1}\). This was expected, as the partially denaturing condition used in my study promoted PrP\(^C\) unfolding and reduced the energy barrier of elongation [138]. Interestingly, they observed fibril fragmentation and argued that fragmentation was essential for prion propagation \textit{in vivo}. However, fragmentation was never observed in the surface elongation assays I conducted. The discrepancy might be explained by different buffer conditions and fibril properties involved in the two studies. Besides, the quiescent condition and the surface attachment of the fibrils might disfavour fragmentation. In contrast, secondary processes played an important role in both spontaneous aggregation assays and seeding assays in bulk assays with agitation (Chapter 6).

In summary, I observed \textit{in situ} elongation of MoPrP 91-231 seeds by TIRFM. Single-fibril kinetic analysis determined that at least three structurally different PrP fibril types were growing in competition from a seemingly homogenous pool of seeds. They likely recruited different PrP species and elongated with distinct mechanisms. While growth conditions (PrP\(^C\) or GdnHCl concentration) could shift the equilibrium between fibril types, fibrils mostly elongated faithfully without apparent cross-seeding by distinct mechanisms.
VII. Super-resolution imaging of amyloid fibrils

7.1 TAB imaging reveals different structures of PrP fibrils

Transient amyloid binding (TAB) imaging technique, based on single-molecule localisation microscopy (SMLM), is used to image amyloid fibrils at an improved resolution compared to conventional fluorescence microscopy. Amyloid-binding dyes are added to the solution, transiently generating strong fluorescence emission upon binding to surface-attached amyloid structures. Under suitable conditions, isolated blinking events are detected in single image frames, and their central positions are identified, allowing a reconstruction of a super-resolution image from thousands of frames. The large reservoir of dye molecules makes long-term observation possible.

Previous work in our lab in collaboration with Dr. Matthew Lew established the application of this technique in imaging multiple amyloid fibrils such as Aβ40, Aβ42, α-Syn, tau and light chain [158]. Here, I utilised the TAB technique to image amyloid fibrils formed by MoPrP 91-231.

In the previous chapters, multiple types of elongated MoPrP 91-231 fibrils were observed. I tested whether TAB imaging could provide enough structural information to differentiate them. To generate long fibrils for TAB imaging, I performed seed elongation assays on the coverslip surface. 50 nM NR was present in the buffer to monitor elongation kinetics during growth by TIRF imaging and perform TAB imaging at the end.

The dye used for TAB imaging should meet the following requirements. (a) a high photon output when excited so that each blinking event can be localised with high precision; (b) a high binding affinity and specificity to ensure satisfactory blinking events and long-term observation.

Three dyes were tested: Thioflavin T (ThT), Nile red (NR) and Nile blue (NB). ThT was utilised in [158] to image multiple amyloid fibrils. However, photobleaching during the imaging of PrP fibrils limits the number of photons collected per blinking event and the long-term observation. NB and NR were good candidates to image PrP fibrils. Well-isolated blinking events were observed at a low dye concentration (50 nM to 100 nM) and a high laser power.
Photobleaching was not pronounced, allowing enough blinking events to be generated for high-resolution image reconstruction.

TAB imaging can be performed in the same buffer used for PrP aggregation and elongation without adding reducing or oxygen-scavenging agents. As a result, it can be implemented into our elongation assay and potentially track the elongation process with high local precision.

### 7.1.1 TAB imaging reconstructs fibrils with enhanced resolution

I obtained high-quality TAB data using NR at 50 nM and the highest laser power (high-intensity mode, 150 mW of 561 nm laser) at 20 ms exposure time. Several isolated blinking events on fibrils were observed in each frame, as shown in Figure 7-1 A-C. However, blinking events that were close to each other were occasionally observed. Usually, ~10,000 frames were taken for each region of interest (ROI).

![Figure 7-1](image)

**Figure 7-1.** Selected frames during TAB acquisition (A-C) and the diffraction-limited image of the same region (D). The diffraction-limited image was built from the image stack by integrating the frames using the Z-stack command in ImageJ. The scale bar represents 5 μm.
Figure 7-2. TAB reconstruction and fibril FWHM measurements. (A) Reconstructed TAB image for the same ROI in Figure 7-1. The scale bar represents 5 µm. The colour scale represents localisations/bin. The figure is adapted from [193]. (B) Profile (dots) of three cross-sections of fibril a and the corresponding fitted Gaussian curves (black curves). The intensity profile along the fibril cross-section was obtained by the ImageJ ‘plot profile’ command. The dashed line represents the FWHM of the fibril. All points and curves were shifted along the x-axis to make the fitted Gaussian peak position at 0 nm. (C) Profile of three cross-sections of fibril b, fitted Gaussian curves and FWHM.

For super-resolution image reconstruction, the centre of each blinking event was calculated by fitting a point spread function (PSF) model of integrated Gaussian to its image using the ImageJ ThunderSTORM plugin [190]. Localisations were eliminated if the fitted PSF width was not reasonable (σ < 80 nm or σ > 240 nm) or the detected photon number was small (< 70). Figure 7-2 A displays the TAB reconstruction of the same ROI as in Figure 7-1, plotted as a histogram showing the number of blinking events falling into each bin.

Compared to the diffraction-limited image (Figure 7-1 D), fibrils in the TAB reconstruction (Figure 7-2) had a smaller apparent width. A helical fibril
In Figure 7-2 B and C, I analysed the apparent widths of two fibrils, (a) and (b). These fibrils likely represent fibril types I and III, as detailed below in Section 7.1.2. Three cross-sections were selected on each fibril, whose intensity profile could be captured by a Gaussian function

\[ I = a e^{-\frac{(x-b)^2}{2\sigma^2}}. \]

The apparent width of fibrils was quantified by the full width at half maximum (FWHM) of the intensity profile, obtained by equation

\[ \text{FWHM} = 2\sigma \cdot \sqrt{2\ln 2}. \]

FWHM was 43 ± 1 nm for fibril (a) and 61 ± 10 nm for fibril (b). The resolution obtained by analysing the FWHM of multiple fibrils is 48 ± 7 nm, showing a ~5 fold improvement compared to the resolution of conventional fluorescence microscopy (~250 nm).

Typical amyloid fibril width measured by TEM is 8–20 nm, much smaller than the FWHM obtained here. The reason is that FWHM in super-resolution images isn’t a direct representation of fibril width; instead, it takes a larger value caused by the localisation uncertainty of each localisation event. The localisation precision was calculated from emitted photons. I observed a larger FWHM for fibril (b) than fibril (a), which could be explained by a higher degree of uncertainty of localisation events on fibril (b) or by a larger physical width. The latter hypothesis is unlikely, though, as Section 7.1.2 will show that fibril a is likely a type I fibril, and fibril b is likely a type III fibril. TEM images (Figure 6-15) show that type I fibrils, with a typical width of 14-18 nm, are wider than type III fibrils, typically 6-8 nm wide.

In addition to a small difference in FWHM, the two fibrils differed in the value of localisations/bin, i.e. fibril brightness, with a ~10-fold difference. Fibril (a) bound dye molecules at a much higher frequency, suggesting a larger dye-binding surface or higher dye-binding affinity. This led to a hypothesis that fibrils (a) and (b) have different numbers of strands or surfaces exposed to the solution.
Figure 7-3. Diffraction-limited image (A) and TAB reconstruction (B) of a new ROI. The region indicated by a yellow rectangle is enlarged and shown next to it. The scale bar is 5 µm.

Figure 7-4. Three cross-sections (A) and the corresponding intensity profiles (B) of the splitting fibril. In B, the plots of brightness profiles were shifted so that the peak for the dim fibril was at 0 nm.
Here, I present one more example of TAB reconstruction showing that this technique can resolve structures with a distance less than the diffraction limit. Figure 7-3 B displays a region with one fibril splitting into two. Due to the relatively low resolution, the split is not visible in the diffraction-limit image (Figure 7-3 A).

The intensity profiles of the splitted fibrils are shown in Figure 7-4. A two-peak Gaussian function was fitted to each brightness profile (panel B), yielding a peak-to-peak distance varying from 187 nm to 125 nm at the three cross-sections. The number of localisations per bin for the dim fibril was small, but the peak position could still be obtained.

Overall, TAB technique was applied to image MoPrP 91-231 fibrils with improved spatial resolution. It resolved fine structures which diffraction-limited images couldn’t reveal. It also resolved closely spaced structures revealing the presence of multi-strand fibrils.

7.1.2 Multiple types of fibrils were identified and can be linked to fibril types in kinetic studies

(1) Bright and dim fibrils correspond to type I and III fibrils, respectively

Previous TAB images readily showed the presence of at least three kinds of fibrils: very bright, straight fibrils (for example, fibril (a) in Figure 7-2 A); dim fibrils (fibril (b)); and dim, helical fibrils (pointed by the yellow arrow in Figure 7-2 A).

Interestingly, kinetic measurements also revealed the presence of three fibril types by their pause-free elongation rates and fibril brightness (Chapter 6). Type I fibrils grew slowly with high brightness, type II fibrils were slow-growing and dim, and type III fibrils were fast-growing and dim. Combined with TEM structural imaging for elongated fibrils, type I and III fibrils likely represented double-strand or single-strand fibrils, respectively.

To map fibrils in TAB images to different fibril types identified from kinetic studies, fibril elongation was observed in situ under TAB imaging conditions to
monitor growth rates and brightness so that fibril types could be determined. TAB imaging was then performed at the end time point to resolve the structures of the elongated fibrils.

However, the low dye concentration suitable for TAB imaging led to a low signal-to-noise ratio in TIRF images acquired for kinetic analysis. I used low laser power (5%) and high exposure time (1s) to improve image quality during TIRF acquisition but only achieved limited improvement.

**Figure 7-5.** Observation of MoPrP 91-231 seed growth. (A) Images of the elongating fibrils at three time points. 50 nM NR was used in the monomer solution. The scale bar represents 5 μm. (B) Kymograph of fibril (a). The horizontal scale bar represents 5 μm, and the vertical scale bar represents 5 h. (C) Diffraction-limited image of the same ROI obtained by performing Z-project of a TAB stack (3057 frames) acquired at the end time point.

Images of an ROI at increasing time points are shown as an example in Figure 7-5. Panel A shows the elongation of a fibril (fibril (a), yellow arrow) with time. The Kymograph (panel B) shows a steady and slow growth pattern with a pause-free rate of ~1.8 nm/min. Panel C is a diffraction-limited image of the same ROI at the end time point, displaying all fibrils present in this region. It indicated the presence of several fibrils other than fibril (a). These fibrils were dimmer and couldn’t be reliably observed in the growth stack.
I attributed fibril (a) to a type I fibril due to its high brightness and slow rate. The dim fibrils were visible in the Z-projection (Figure 7-5 C) but weren't captured in single-frame TIRF images (Figure 7-5 A). They could be type II or III fibrils. Considering our hypothesis that type II and III fibrils elongated from different ends of seeds with unique rates, these dim fibrils are more likely type III fibrils as their lengths indicated higher growth rates than type I or II fibrils.

![Images of fibrils](227)

**Figure 7-6.** TAB images of type III fibrils. The scale bars represent 1 µm. The arrows in A and B point to several dips of the curvy fibrils.

When zooming in to type III fibrils, most were curvy with a very small radius and a somewhat regular, ~600–700 nm pitch length (Figure 7-6 A and B), while some were relatively straight (panel C). The curvy fibrils were also observed for fibrils grown on TEM grids, but fibrils on TEM grids acquired a much smaller pitch length (~150–400 nm), suggesting that the pitch length may be influenced by the surface substrate. A periodic change in fibril brightness was commonly seen for both curvy and straight fibrils. It likely reflected a twisting sub-structure of the fibril since twisted fibrils exposed different surfaces to the solvent along the fibril axis, possibly causing a varied dye-binding frequency.
(2) Helical fibrils are seen in TAB reconstruction

A.

<table>
<thead>
<tr>
<th>Time</th>
<th>Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td><img src="image1.png" alt="Image" /></td>
</tr>
<tr>
<td>10.25h</td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>12h</td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>12.25h</td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

B.

![Image](image5.png)

**Figure 7-7.** Elongation of fibrils in the same ROI as in Figure 7-3. (A) TIRF images acquired at different time points during elongation. The scale bar represents 5 µm. (B) TAB reconstruction of the image stack taken at the end time point. The figure is adapted from [193].

One more fibril type shown in TAB reconstruction is helical, as seen in Figures 7-2 A and 7-3 B. They weren’t resolved and appeared as bead-like fibres in diffraction-limited images (Figure 7-3 A). Using the fibril in Figure 7-3 as an example, I traced it back to the growth stack to find its kinetics (Figure 7-7 A): the fibril was relatively bright, and its growth mode changed at different time points. The growth modes included: (1) steady growth on the surface, (2) the
fibril end moving on the surface or in solution, as shown between 10.25 h (when the growing end pointed to left) and 12 h (when the growing end pointed to right), (3) partial detachment of the fibril from the surface, or an abrupt growth on the surface between two adjacent time points (for example, between 12 h and 12.25 h) caused by growth in solution. These elongation patterns suggested that elongation of this fibril took place both in solution and on the surface, unlike the type I and III fibrils which mainly grew along the coverslip surface. These fibrils were denoted as type H (helical) fibrils, which have distinct kinetics and structural properties compared to the other fibril types.

Another example of type H fibril is shown in Figure 6-3.

I next explored whether type H fibrils could be identified directly from a growth stack of TIRF images. Some type H fibrils acquired a high brightness or displayed an abrupt growth; however, they didn’t always share the above properties. Sometimes the growing tip was very bright while the segment which elongated at the earlier time became dim. They could also grow entirely on the surface during acquisition. With careful observation, I used the following property to identify a type H fibril from the growth stack: (a) fibrils grew with an extremely bright tip, or (b) fibrils remained bright and grew with a moving end or occasional abrupt growth, or (c) fibrils displayed bead-like structures in TIRF images took at low laser power and longer exposure time.

In the reconstruction (Figures 7-3 B and 7-7 B), type H fibrils had a regular periodicity of 574 ± 12 nm. In the diffraction-limited image, the repeat length could also be obtained by the displacement of beads.

I observed type H fibrils in previous elongation assays but didn’t do kinetic analysis due to the presence of abrupt growth events. Type H fibrils were not observed by TEM for fibrils elongated on TEM grids, suggesting a possible involvement of surface on seed attachment or fibril structure. Helical fibrils might be more difficult to attach to the carbon film of TEM grids than glass coverslips; the surface of TEM grids might disfavour the growth of helical fibrils or change their morphology. Alternatively, helical fibrils might grow on TEM grids but were washed away in the subsequent staining and washing steps.
7.1.3 Brightness profile along the fibril axis

TAB images showed periodic changes in brightness along the fibril axis (Figure 7-6), which may reflect structural features. To quantitatively analyse the periodic change, I plotted the intensity profile along the fibril axis using several ImageJ-based methods: (1) using the ‘plot profile’ command to draw segmented lines along the fibril axis to plot its intensity profile. The line width can be adjusted to cover the whole width of the fibril, and the profile shows how the mean value covered by this line changes along it. However, using a thin line introduces sampling error, while a wider line covers the fibril body and surrounding pixels whose brightness is 0. (2) Straightening the fibril in ImageJ based on a drawn fibril axis. The image of the straightened fibril is a matrix and can be imported into MATLAB for post-processing to eliminate pixels with zero intensities. However, interpolation is conducted during straightening, which might introduce error.

To avoid the problems, I developed a MATLAB script to straighten the fibril and calculate the brightness profiles automatically. The fibril axis is found by fitting a polynomial or spline function to all localisations’ coordinates; straightening is then conducted for the fibril, using the fitted fibril axis as a reference. The pixel intensity (localisations/bin) of the straightened fibril can be calculated directly by counting localisations that fall into the pixel, thus eliminating any error caused by interpolation. The detailed logic of the script is described below.

(1) Fibril straightening [237]. A single fibril or fibril segment is selected. (x,y) coordinates of all localisations on it are divided by the pixel size of the reconstruction (20 nm) and scatter plotted.

The first step is to fit the fibril axis. The fitted polynomial or spline curve is denoted \( y = f(x) \) and superposed onto the scatter plot (Figure 7-8). A polynomial fit with an order up to 8 works well for fibrils with small numbers of curvatures, such as most type I fibrils. However, for some curvy type III fibrils, polynomial fit only captures the major trend (Figure 7-8 A) but can’t track each curved segments (Figure 7-8 B, segments pointed by black arrows are not
well fitted). Increasing the order up to 11 doesn’t satisfactorily increase fitting quality.

A. Polynomial fit, n = 8

![Polynomial fit, n = 8](image)

B. Polynomial fit, n = 8

![Polynomial fit, n = 8](image)

C. Spline fit, l = 20, k = 4

![Spline fit, l = 20, k = 4](image)

D. Spline fit, l = 40, k = 4

![Spline fit, l = 40, k = 4](image)

**Figure 7-8.** Attempts to find the fibril axis by different fitting methods. (A) Scatter plot of all localisations of a typical fibril and the fitted fibril axis by a polynomial fit. (B) A zoomed-in region of (A). The black arrows indicate the
curvy regions on the fibril, which are poorly traced by the axis. (C) and (D) show the same zoomed-in region, with the fibril axis obtained by a spline fit with parameters indicated in each plot. The yellow and purple arrows indicate regions that are better traced by the fitted axis compared to the previous panel.

I next fit a spline curve (a piecewise-polynomial function) to the localisations. Two parameters are user-defined: \( l \) is the number of polynomial pieces, and \( k \) is the order of each piece which is set as 4. A relatively small \( l \) (20, for example) yields nice results for fibrils that don’t contain many curvatures. A larger \( l \) is required for fibrils with multiple curvatures. Figure 7-8 C shows the fibril axis using a spline fit with \( l = 20 \). The curvatures pointed by the yellow arrows show better fits compared to the polynomial fit (panel B), while the one pointed by the black arrow still requires improvement. This curvature is better captured in panel D where \( l \) is increased to 40. To quantify the goodness of the fitting, I calculated the mean distance from each localisation to the fibril axis, which was 1.07 in a polynomial fit (Figure 7-8 A and B), 0.97 in a spline fit with \( l = 20 \) (panel C), and 0.94 in a spline fit with \( l = 40 \) (panel D). The smallest mean distance in the last case suggests the best fitting results among the three tests. Further increasing the \( l \) value didn’t yield a smaller mean distance.

In the following analysis, spline fits are applied to find fibril axes. For long curvy fibrils, \( l \) is set to be 40; for short or straight fibrils, \( l \) is 10 or 20 to avoid overfitting.

The fitting at two fibril ends is sometimes inaccurate, so localisations in small segments at two end regions (usually 10–15 pixels in length) are discarded.

This fitting method requires that the fitted fibril axis is a function: for each \( x \) value, there is only one corresponding \( y \) value. Usually, this requirement can’t be satisfied for fibrils that align nearly vertically. A simple way to deal with it is to interchange each localisation’s \( x \) and \( y \) values to make the fibril horizontal.

(2) Locating each localisation \((x_i, y_i)\) from the Cartesian coordinate system into the fibril axis-based coordinate system. A point \( O \) on the fibril axis at the left end is selected as the origin of the converted coordinate system. For each
localisation, an orthogonal projection onto the fibril axis is made, and the coordinates of the projection point \( P_i (x_{Pi}, y_{Pi}) \) are calculated by numerically solving the following equations:

\[
\begin{cases}
  y_{Pi} = f(x_{Pi}) \\
  f'(x_{Pi}) \cdot \frac{y_i - y_{Pi}}{x_i - x_{Pi}} = -1
\end{cases}
\]

where \( f \) is the fitted function to the fibril axis and \( f' \) is its first-order derivative.

The fitted spline function has \( k-2 \) continuous derivatives, i.e. 2 continuous derivatives when \( k = 4 \), which ensures the first-order derivative is continuous.

The localisation’s coordinates in the new fibril-axis-based coordinate system are denoted as \( (x'_i, y'_i) \). \( x'_i \) refers to the distance along the fibril axis from the origin \( O \) to the projected point \( P_i \); \( |y'_i| \) refers to the perpendicular distance from the localisation to the axis, i.e. distance from \( (x_i, y_i) \) to \( P_i (x_{Pi}, y_{Pi}) \). \( y'_i \) is set positive if \( y_i > y_{Pi} \), and negative if \( y_i < y_{Pi} \).

**Figure 7-9.** Determination of localisations’ coordinates in the new coordinate system. The purple curve is the fitted fibril axis; the yellow crosses on it represent the pixel edges in the direction of the fibril axis. Blue dots are localisations. For each of the four localisations shown as examples, a line connecting it and the projection on the fibril axis is shown. The length of this line determines \( y'_i \). The length from the origin to the intersection point along the fibril axis is \( x'_i \).
An illustration of this step is shown in Figure 7-9. Once the coordinates are determined for each localisation, the pixel location is determined by rounding up the $x'$ and $y'$ values to the nearest integer and adding a constant to make $y'$ positive.

The pixelised image of the straightened fibril is generated by plotting a 2-D histogram of all localisations’ coordinates in the new system, with the colour indicating the brightness (localisations/bin) at each pixel. An example of the straightened fibril is shown in Figure 7-9 B.

Seven fibrils from the two mentioned ROIs are selected for analysis, including five type III and two type I fibrils. Each fibril is straightened using the method described above. Brightness at each axial position (i.e. at the fibril cross-section) is calculated by taking the sum of all pixels at this axial position. The brightness profile is plotted against the axial position. To analyse if periodicity exists, the brightness profile is put through fast Fourier transform (FFT) using MATLAB [238]. The amplitude of FFT analysis is plotted against the periodicity $1/F$, which is reciprocal of the periodic frequency ($F$).

Figure 7-10 shows an example of the analysis process. The fibril segment to be analysed is fibril 1 in panel A, a type III fibril. Its straightened form is shown in panel B. Panel C shows the intensity profile along the fibril axis (top) and the FFT plot (bottom). The intensity profile is very noisy, but FFT could still extract several peaks. In panel D, the brightness profile is smoothed using MATLAB's 'smooth' function to reduce noise. The trace shows at least two repeating patterns with different periodicities. FFT of the smoothed data is similar to that of the unsmoothed curve in the low-frequency domain (large periodicity $1/F$), while small peaks in the high-frequency range are largely eliminated due to the smoothing step. Peaks in the FFT curve show the presence of three main periodicities at $\sim 300$ nm, $610$ nm and $790$ nm, together with other small peaks. The selected fibril segment was slightly curvy with a pitch length of $\sim 600$–$700$ nm; thus, the $610$ nm peak in FFT likely represents the pitch length.
Figure 7-10. Analysis of the intensity along the fibril axis. (A) Selection and labelling of seven fibril segments. Fibrils in the orange rectangles are type III fibrils, and those in the green rectangles are type I fibrils. The left figure is rotated from Figure 7-2 A to make the selected fibrils lie nearly horizontally. The scale bar represents 5 µm. (B) Image of the straightened fibril 1. The
Colour bar represents brightness (localisations/bin). The scale bar represents 1 µm. (C) The brightness profile along the fibril axis (top) and the FFT plot (bottom). (D) The brightness profile using the smoothed brightness data and the FFT plot.
B

Fibril 5

![Image of Fibril 5](image1)

![Graph of Fibril 5](image2)

Fibril 6

![Image of Fibril 6](image3)

![Graph of Fibril 6](image4)

C.

![Graphs of Periodicity Peak](image5)

Figure 7-11. TAB images of the straightened fibrils and their brightness profiles. (A) Straightened images (left) of four type III fibrils, and the smoothed intensity profiles with FFT curves (right). (B) Straightened images and brightness analysis of two type I fibrils. (C) Distributions of periodicity peak
locations (1/F) for the five type III fibrils (left panel) and the two type I fibrils (right panel).

The same analysis was conducted for other fibril segments shown in Figure 7-10 A, which are presented in Figure 7-11.

For type III fibrils, periodicities displayed two peaks at ~300 nm and 600–700 nm (Figure 7-11 C, left). The larger periodicity likely reflected the fibrils’ curved shape, and the smaller value could relate to other regular structural features that were not directly observed by TAB imaging.

Type I fibrils were brighter than type III fibrils. The straightened fibril 5 displayed an alternate change in fibril brightness, while FFT detected multiple peaks without a dominating one. This suggested that the change in fibril brightness wasn’t regular. Consistently, the distribution of periodicities for the two type I fibrils (Figure 7-11 C, right) doesn’t show an apparent clustering.

In summary, TAB reconstruction generates images of elongated MoPrP fibrils at a high resolution. It enables the distinguishing of type I (bright), III (dim, straight or curvy) and type H fibrils (helical fibrils). Fibril brightness profiles provide additional information, such as the periodic change, which likely relates to the fibril’s morphology.

7.2 p-TAB imaging of elongated MoPrP fibrils reveals different dye-binding orientations

TAB microscopy could readily resolve finer structures of MoPrP 91-231 fibrils than conventional fluorescence microscopy, as seen in the helical structures of type H fibrils (Figure 7-3 B). TAB could also reveal different fibril types, for example, type I and III fibrils were mainly differentiated by their brightness. However, the resolution of TAB imaging is limited by the localisation precision of each localisation event. TAB imaging couldn’t resolve fibrils’ fine structures, such as the actual width, fibril helicity and the number of protofilaments. For example, TEM images revealed that type I fibrils are double-strand and ~16–
18 nm wide, and type III fibrils are single-strand and ~6–8 nm wide. However, TAB reconstruction revealed wider FWHMs, 43 ± 1 nm for type I fibrils and 61 ± 10 nm for type III fibrils. The information about the number of strands cannot be obtained.

The polarised-TAB (p-TAB) technique was developed to help elucidate fibrils’ fine structures. It analyses the polarisation of each fluorescence burst, generates information on the orientation of dye molecules and leads to a guess of the organisations of fibril strands.

7.2.1 Theory behind p-TAB imaging

Amyloid dyes bind to the amyloid fibril surface at a preferred orientation. For example, ThT preferentially orients parallel to the long axis of fibrillary amyloid [168, 169]. The emission fluorescence is polarised, and the polarisation is determined by the orientation of dye molecules. ThT was found to emit photons through the emission dipole moment which was parallel to its long axis. p-TAB technique analyses the polarisation of emission fluorescence, to estimate the orientation of dye molecules and the local orientation of the fibril or protofilament.

Using a polarisation beam splitter (PBS), emission fluorescence (emitted photons) from each single and well-isolated dye molecule is split into two orthogonally polarised channels and mapped onto separated regions of an sCMOS camera. The two channels are referred to as the x and y channels, respectively. A linear dichroism (LD) value is calculated for each blinking event using photons detected in both channels:

\[ LD = \frac{I_x - I_y}{I_x + I_y} \]

where \( I_x \) and \( I_y \) represent photons in the x and y channels, respectively. The LD value is used to quantify dye orientation and thus is a direct indicator of fibril axis orientation. Here, the axis refers to that of the fibril or protofilament where the dye binds.
However, the bound dye molecules display motion instability, i.e. ‘wobble’ [172, 173]. The wobble angle could be treated as the variation in the orientation of a bound dye molecule. In this study, I focused on the orientation of molecules and didn’t analyse their wobble.

Technologies similar to p-TAB were previously utilised for super-resolution structural imaging on insulin fibrils [172]. Based on the orientation of bound ThT molecules, a morphology model was built that a mature fibril consisted of two intertwining protofibrils, consistent with TEM results.

Recently, p-TAB imaging was conducted on amyloid fibrils formed by Aβ42 using NR as the dye [173]. NR’s binding mode and orientation on the amyloid fibril surface were not as thoroughly investigated as ThT. This orientational analysis revealed that NR bound to amyloid fibrils with a preference to align the axis of the dye molecule to the backbone of amyloid fibrils, similar to ThT. This alignment was profound for thin fibrils, while NR binding to thick fibril bundles was more disordered.

7.2.2 Image acquisition and analysis procedure for p-TAB

p-TAB stacks were acquired on the same microscope used for TAB imaging. A PBS was inserted in the emission pathway to decompose the fluorescence into orthogonally polarised x and y channels.

Stacks were acquired for an ROI covering the structures in both channels at high laser power and low exposure time (20 ms), the same as TAB imaging. The acquired stacks were analysed in ImageJ using the ThunderSTORM plugin, generating a list of all localisations. Localisations were deleted if (1) fitted sigma values were not reasonable: \( \sigma < 80 \text{ nm} \) or \( \sigma > 240 \text{ nm} \); or (2) less than 60 photons were detected. Post-processing was conducted using a MATLAB script, including two-channel registration, pairing localisations in two channels, calculating the LD value of each blinking event and pixel, and generating an LD map. The script was adapted from Dr. Matthew Lew’s lab with minor changes to adapt to our system, and the logic was explained in the Method (Chapter 4 and Figure 4.7).
LD for every single emitter was calculated using photons detected in x and y channels by Equation 7.1. LD is in the range of [-1,1], where LD = 1 means fluorescence is detected only in the x channel, i.e. fluorescence is horizontally polarised. LD = -1 means fluorescence is detected only in the y channel and is vertically polarised; LD = 0 means it has the same amplitude in horizontally and vertically polarised directions.

An LD map was then built for the ROI with a pixel size of 20 nm. LD of each pixel was identified using the median LD of all single emitters in that pixel. The generated reconstruction pictured both the number of localisations (brightness) and the median LD (colour) of each pixel.

Figure 7-12 A shows the LD map of one ROI. LD value was affected by fibril backbone orientation, as seen when comparing a vertically orientated fibril (the blue arrow) and a nearly horizontal fibril (the orange arrow) with a fibril segment oriented at ~45° (the green arrow). As a result, it was difficult to compare LD for fibrils with different orientations. Most fibrils were not straight morphologically but with curvatures or acquiring helical structures; the structural features led to changing fibril directions and consequently, changing LD values along the fibril. Usually, a periodic change of LD was observed along the curvy fibrils, as shown in Figure 7-12 B.

Variability in LD value was also observed within the straight fibril segments in both directions along the fibril axis and across cross-sections, suggesting a more complex fibril substructure, such as multiple fibril strands. For example, the zoomed-in region in Figure 7-12 A includes a fibril which was relatively straight, while the LD value changed along the fibril axis showing alternate green and yellow patterns.

The change in LD value originating from fibril backbone orientation could mask changes caused by intrinsic structures and need to be corrected.
Figure 7-12. LD map of an ROI. (A) LD map. The scale bar represents 5 µm. The colour bar indicates the LD value and brightness (localisations/bin). The region inside the small rectangle is enlarged and shown. (B) Two examples showing the periodic change in LD value along the slightly curvy fibrils. The scale bar is 1 µm.

To track how LD changes along the fibril, the mean LD value across the fibril cross-section is calculated at each axial position, and the profile is plotted. The mean LD at an axial position is the average LD value of all localisations at this cross-section. This step doesn’t eliminate the effect of fibril direction.
The next step is to correct the variation in LD values caused by the change in fibril backbone orientation.

LD value of each localisation is calculated based on the azimuth angle of the dye molecule in the Cartesian coordinate system. Here I calculate a ‘revised’ LD to represent the angle between the dye molecule axis and the local orientation of the fibril axis. In this way, the revised LD doesn’t depend on the orientation of the fibril axis and is a direct representation of how dye molecules lying on a fibril. Detailed steps are described below.

The fibril axis is first identified by fitting a polynomial or a spline function to localisations, as discussed for TAB data processing.

For each dye molecule that generated a single fluorescence burst, its azimuth angle (orientation) is calculated from its intensity in the x and y channels. For
emission fluorescence with an amplitude $E_0$ and a polarisation angle $\beta$ to the horizontal direction, the decomposed, horizontally polarised light has an amplitude of $E_0 \cdot \cos(\beta)$, while the light polarised vertically has an amplitude of $E_0 \cdot \sin(\beta)$. Since light intensity (as in photons) is proportional to the square of the amplitude, photons collected in the $x$ channel (the horizontally polarised channel) or the $y$ channel (the vertically polarised channel) could be calculated by:

$$I_x = C \cdot E_0^2 \cdot \cos^2 \beta$$

$$I_y = C \cdot E_0^2 \cdot \sin^2 \beta$$

where $C$ is a constant. LD can be expressed by the following equation:

$$LD = \frac{I_x - I_y}{I_x + I_y} = 1 - 2 \sin^2 \beta$$

Thus, $\beta$ of every single emitter can be calculated based on photons detected in both channels, $I_x$ and $I_y$, or the LD value:

$$\beta = \pm \sin^{-1} \left( \frac{1 - LD}{2} \right), \quad \beta \in \left( -\frac{\pi}{2}, \frac{\pi}{2} \right]$$

The sign of $\beta$ couldn’t be determined, as the polarised lights with opposite azimuth angles produce the same $I_x$ and $I_y$. For every single emitter, there are two possibilities of how it orientates which are mirrored over the x-axis (or the y-axis).

Fibril orientation at the position of each blinking event can be unambiguously determined. The azimuth angle of the fibril axis, $\alpha$, is the slope of the fibril at the projection point:

$$\alpha = \tan^{-1} f'(x_p) \in \left( -\frac{\pi}{2}, \frac{\pi}{2} \right]$$
Figure 7-14. Determination of $\theta$. The fibril axis is represented by the blue curve. One blinking event locates at the green dot, and the projection point on the fibril axis, $P$, is found, where the orientation of the fibril axis is determined (the yellow line). $\alpha$ is the azimuth angle of this line. The azimuth angle of the dye molecule $|\beta|$ is obtained by its LD. $\beta$ could take two values represented by the two purple lines passing through the green dot. The $\beta$ angle, which results in the smaller of the two possible angles $\theta$, is selected. $\theta$ is the difference between $|\alpha|$ and $|\beta|$.

Finally, I am to calculate the angle between the fibril axis and the dye molecule, $\theta$. Straightforwardly, it is the difference between the azimuth angle of the fibril axis $\alpha$ and that of the dye molecule $\beta$. Since $\beta$ could take two possible values, it is impossible to determine $\theta$ without any new information. Here, I manually select the $\beta$ value allowing the angle $\theta$ to be smaller. $\theta$ is calculated to be $|\alpha| - |\beta|$. This assumption implies that if the slope of the fibril axis is negative ($\alpha < 0$), $\beta$ would take a negative value; if $\alpha > 0$, $\beta$ should be positive as well. If the local fibril axis lies horizontally, the sign of $\beta$ couldn’t be determined. However, this assumption is not always valid and set a range for $\theta$: for a fibril axis that has an azimuth angle $\alpha$, the largest $\theta$ is the larger value of $|\alpha|$ and $\frac{\pi}{2} - |\alpha|$. An illustration of determining the three angles is shown in Figure 7-14.

Two segments of type I fibrils are plotted in Figure 7-15. The sticks show the chosen orientation (azimuth angle $\beta$) for all dye molecules whose blinkings can be paired in $x$ and $y$ channels. The orientation of each dye molecule generally followed the fibril axis, with a typically small angle in between. Interestingly, in several regions, sticks with slightly different azimuth angles overlapped. For example, in the yellow rectangle in Figure 7-15 A, the dye molecules can be
categorised into two groups based on their azimuth angles: one group acquires a green colour, and the other appears yellow. This might suggest the existence of two intertwining protofilaments, and dye molecules in each group bind to a protofilament. It could also suggest a twisting structure of a single filament.

**Figure 7-15.** Segments of two type I fibrils with each localisation’s orientation plotted as a stick colour-coded by its LD value. The scale bar represents 100 nm. Only paired localisations are chosen; blinking events detected only in the x or y channel are not shown. The yellow box highlights a region in which blinking events with different azimuth angles overlap.

The determination of \( \theta \) allows orthogonal decomposition of a burst of fluorescence in two new directions: parallel to the fibril axis (\( x' \)) or perpendicular to the fibril axis (\( y' \)). Light polarised in the direction of the fibril axis has an amplitude of \( E_0 \cdot \cos(\theta) \), and light in the direction perpendicular to the fibril axis has an amplitude of \( E_0 \cdot \sin(\theta) \). Similar to the previous discussion, the intensity of light in the two polarised directions is described by:

\[
I_{x'} = C \cdot E_0^2 \cdot \cos^2 \theta
\]
\[ I_{y'} = C \cdot E_0^2 \cdot \sin^2 \theta \]

The corrected LD' is calculated by:
\[ LD' = \frac{I_{x'} - I_{y'}}{I_{x'} + I_{y'}} = 1 - 2 \sin^2 \theta \]

Mathematically, LD' is in the range of [-1,1]. LD' is 1 when the polarisation of emission light is entirely along the fibril axis, and it is -1 when the emission polarisation is perpendicular to the fibril axis. However, since the largest value of \( \theta \) can’t reach \( \frac{\pi}{2} \) except for a horizontal or vertical fibril segment, LD' rarely takes the value of -1.

In this way, a revised LD is calculated for each blinking event. Then, all blinking events are put into the fibril-axis-based coordinate system using the procedure described for TAB processing (i.e. fibril straightening). The revised LD for each pixel is obtained using the median LD' of localisations in the pixel. A revised LD map is then generated for the straightened fibril.

The mean LD' at an axis position is calculated by averaging LD' for all localisations in pixels (with a pixel-size of 20 nm) on that cross-section. How the mean LD' changes along the fibril axis is then analysed by conducting FFT of the LD' profile [238].

I conducted the above analysis on several elongated MoPrP 91-231 fibrils and presented the results grouped by fibril type.

7.2.3 LD analysis for MoPrP 91-231 fibrils

(1) Type III fibrils with curvy structures

Type III fibrils are typically long and are dimmer (smaller localisations/bin) than type I fibrils. A subgroup has a curvy structure, leading to a change in pixel LD value caused by varying fibril orientation (Figures 7-12 B, 7-16 B).
Using the fibril in Figure 7-16 B as an example, I first straightened it and plotted the LD map without correction (Figure 7-17 A and C, top panels). In panel A, the fibril axis was obtained by a polynomial fit, which followed the overall fibril trend but didn’t track each curvy shape. As a result, the straightened fibril (Figure 7-17 A, top) displays a wavy shape. In panel C, the fibril axis was found by a spline fit so that each curvature was nicely followed, yielding a perfectly straight fibril. This fibril had a small azimuth angle (17° to 22°), and LD of the binding molecules approached the higher limit. An alternate change in LD (i.e., the colour in the raw LD map) was observed along the fibril, and the yellow
arrows in panels A and C point to a few identifiable regions with smaller LDs than the surrounding regions. This could be explained by the changing orientation of these regions.

The revised LD (LD’) maps are pictured in the bottom panels of Figure 7-17 A and C for the two situations. A periodic pattern was observed in panel C, where the change caused by fibril axis orientation was better corrected. Here LD’ of each pixel rarely reached the higher limit (one), suggesting that many dye molecules didn’t completely align with the fibril. Green arrows in panel C pointed to regions with a smaller LD’ than the surrounding regions, indicating dye molecules bound to these regions had a larger angle to the fibril axis. The periodic change suggested a possible twisting sub-structure of this fibril.

Panels B and D show the LD (top) and revised LD (bottom) profiles on the left and the corresponding FFT spectra on the right. The difference between B and D was, again, the method used to identify the fibril axis, a polynomial fit or a spline fit, respectively.

LD profiles in both panels B and D were very similar, exhibiting a marked periodic change, likely caused by the periodic curvature of the fibril. The corresponding FFT spectra showed one major peak at ~740 nm. Other peaks are present whose signal was largely masked by the ~740 nm peak.

A. Fibril 2
Figure 7-17. p-TAB analysis for fibril 2 in Figure 7-16 B. (A) LD map of the straightened fibril with the adjusted colour scale (top) and the revised LD map of the same fibril (bottom). The fibril axis was obtained by a polynomial fit. For the colour bar, the colour represents the LD value, while the darkness represents the number of localisations per bin. (B) The LD profile along the fibril axis (the horizontal direction in panel (A)) after smoothing (top left) and the revised LD profile (bottom left) together with their FFT spectra (right
panels). (C) LD map and revised LD map as in (A), with the fibril axis obtained by a spline fit. (D) LD profile (top left) and revised LD profile (bottom left) along the fibril axis in panel (C) and their FFT spectra (right panels).

After correcting for the fibril axis direction, revised LD profiles were different in panels B and D. In panel B, there was still one major peak at ~740 nm (737 nm) and other peaks with a much smaller amplitude, similar to the corresponding LD profile before correcting. This was likely caused by the remaining helical structure after a partial straightening. In panel D, in addition to the 740 nm peak, peaks at other periodicities acquired a higher amplitude than in panel B. The 740 nm peak was still present even though the fibril axis direction has been corrected; this could suggest a structural feature, such as fibril twisting, with a 740 nm periodicity, in addition to the residue influence of the fibril axis direction. Other major peaks were found at 493 nm, 592 nm, 888 nm and 1110 nm, possibly representing structural properties of the fibril, such as fibril twisting.

For the other two fibrils in this group (fibril 1 in Figure 7-16 A and fibril 3 in C), the LD and LD’ profiles are plotted in Figures 7-18 A and B, respectively. For uncorrected LD, the periodicity peak at ~700 nm mentioned above was also dominant for these two fibrils, while in FFT of revised LD other peaks were amplified. Figure 7-19 plots the periodicity peak values for the three fibrils as a histogram. The peaks were located within a large range, while a few values appeared with higher frequency, suggesting they were typical for this fibril type.

Overall, for curved type III fibrils, fibril LD was affected by fibril orientation (curvature) which caused one major peak at ~700 nm in FFT. The revised LD map was supposed to correct for fibril orientation, but the FFT spectrum still exhibited this peak. Other peaks with smaller periodicity values were amplified after correcting for fibril backbone orientation, many of which were shared by different fibrils, likely suggesting common structural properties of this fibril group.
A. Fibril 1

![Graphs A. Fibril 1](image)

B. Fibril 3

![Graphs B. Fibril 3](image)

**Figure 7-18.** LD profiles of two type III fibrils and their FFT spectra. In each subplot, top panels represent the fibril’s LD profile (left) and FFT spectrum (right); bottom panels represent the corrected LD profile and FFT spectrum.

**Figure 7-19.** Distribution of peak locations of the three fibrils shown in Figure 7-16.
(2) Type III fibrils without a regular curvature

A.

![Image](https://example.com/image.png)

B.

![Graphs](https://example.com/graphs.png)

**Figure 7-20.** LD maps and FFT spectra of a straight type III fibril before and after correction. (A) LD map of the fibril. The scale bar represents 1 µm. (B) top panels: the fibril’s LD profile (left) and FFT spectrum (right); bottom panels: the corrected LD profile and FFT spectrum.

This fibril was not regular and didn’t have a periodic pattern like the previous three fibrils. The same analysis was conducted, which revealed a major peak at 382 nm and other smaller peaks in the LD profile. The revised LD profile displayed two more major peaks at 306 nm and 510 nm. Other straight, type III fibrils were shorter and were not analysed.
(3) Type I fibrils

Figure 7-21. LD analysis for a type I fibril. (A) LD map of the fibril. The scale bar represents 1 μm. (B) LD map for the straightened fibril. (C) The revised LD map. (D) LD profile along the fibril axis (left) and its FFT spectrum (right). (E) The revised LD profile with FFT.

I then analysed several bright, straight and relatively short type I fibrils. For the fibril shown in Figure 7-21 A, the fibril axis changed its direction and was analysed in three sections. The middle section had a larger slope than the other two, which was reflected by a decrease followed by an increase in the LD profile (panel D left). This change over a large fibril length is converted to a peak at a large 1/F value in FFT (panel D right, the peak beyond the maximal limit of the x-axis). A small peak was located at 180 nm, presenting a periodic change in the LD profile and indicating an intrinsic alteration in fibril arrangement with a ~180 nm periodicity.
In the revised LD plot (panel C), the LD change caused by the fibril axis direction was corrected. From the LD’ profile (panel E left), a periodic pattern was recognised with a periodicity of 432 nm (panel E right). Other peaks with smaller amplitude were also visible in the FFT spectrum. At 180 nm where a peak showed in panel D, a shoulder was seen next to the 216 nm peak. Multiple other peaks in the low 1/F range likely corresponded to the small fluctuations shown in the revised LD profile (panel E left).

The same procedure was conducted for four other type I fibrils, as shown in Figure 7-22. The LD or LD’ maps didn’t show obvious periodicities, but one or several peaks were identified in FFT spectra. The periodicity peak positions were plotted as a histogram for the 5 fibrils (Figure 7-23), revealing that most of the periodicities were in the range of 100–500 nm. More precisely, the peaks at ~420 nm and between 200 nm to 300 nm were present for all fibrils, suggesting a common feature with ~420 nm or 200–300 nm repeat length for all type I fibrils.

A. Fibril 2
B. Fibril 3

C. Fibril 4
Figure 7-22. LD analysis for four type I fibrils. For LD maps: original LD map (left), straightened LD map (middle in panel B or top right in other panels), straightened LD’ (right in B or bottom right in other panels). For plots: LD profile (top left), FFT of LD profile (top right), LD’ profile (bottom left), FFT of LD’ profile (bottom right).

Figure 7-23. Distribution of the periodicity peak positions for the 5 type I fibrils discussed.
7.3 Discussion

(1) TAB imaging resolved PrP fibril structures at an enhanced resolution and confirmed structural heterogeneity

Image reconstruction super-resolution microscopy allows the reconstruction of high-resolution structural images, overcoming the resolution limit caused by light diffraction. One group of this technique utilises dynamic, stochastic binding and unbinding/photobleaching of fluorophores onto the structures of interest, leading to transient blinking events whose centre positions can be accurately determined. The initially developed techniques were PAINT (points accumulation for imaging in nanoscale topography) [164] and BALM (binding-activated localisation microscopy) [157], in which the transient probe is only activated upon binding. Ries and co-workers [167] applied BALM to image α-Syn fibrils using p-FTAA as the fluorophore, reaching a nano-scale resolution of 14 nm. In this application, as in another super-resolution imaging with fibril-bound ThT [172], the binding of the probe was irreversible. The TAB technique developed in our lab in cooperation with Dr. Matthew Lew [158] combines both features and originally utilised ThT as the binding-activated probe, which bound transiently to amyloid fibrils formed by multiple amyloidogenic proteins. TAB offers the advantage of stable, long-term observation using transient, amyloid-specific dyes. Despite being slightly different imaging modes, TAB, BALM and PAINT overlap in the application and the terms are often used interchangeably by different laboratories.

Here, I applied TAB to image amyloid fibrils formed by MoPrP 91-231. TAB imaging using NR as the fluorophore was able to generate a reconstruction with higher resolution (~50 nm) than in TIRF images (Figures 7-2 and 7-3). Further improving the resolution could be achieved by utilising more suitable fluorescent dyes or fixing the fibrils onto the coverslip surface.

The conventional fluorescent dye used in TAB, ThT, photobleached fast during PrP imaging; another two dye molecules, NR and NB, were tested and exhibited better photochemistry. However, NR and NB don’t specifically bind
to amyloid fibrils; they also bind to the cellular membranes, limiting their applications in cellular imaging. On the other hand, the LCO dyes, such as p-FTAA [167] are highly specific to amyloid fibrils; however, the rates of unbinding of photobleached molecules is very slow, leading to a saturation of binding sites with time which limits the long-term observation.

TAB imaging revealed unique structural features of different PrP fibrils: type I and type III fibrils were different in their brightness (number of localisations/bin), with type I fibrils brighter than type III fibrils (Figures 7-2 and 7-5). Type I fibrils were typically straight and exhibited an alternate change in brightness along its axis, while the change was not regular. Type III fibrils acquired different lengths and could be straight or curvy. The brightness profile along the fibril showed periodic changes. One periodicity peak for curvy fibrils was located at 600-800 nm, coincident with the periodicity of their curvy morphology. One more identified periodicity peak in fibril brightness was located near 300 nm, possibly suggesting a twisting substructure of type III fibrils. TAB imaging also revealed a unique fibril morphology: the helical fibrils with a regular repeat length of 574 ± 12 nm (Figure 7-7 B). The new information obtained by TAB imaging shows its advantages over diffraction-limited, conventional fluorescence microscopy.

In literature, PrP could misfold and aggregate into amyloid fibrils acquiring different morphologies, such as ribbons [239], twisted straight fibrils with different numbers of strands and twisting modes [227], and untwisted but curvy fibrils which didn’t show a regular repeat length [227]. Type I fibrils observed in this study might resemble the twisted fibrils in [227]. The curvy type III fibrils could resemble the curvy fibrils in [227], although the periodicity in my work was larger.

The curvy morphology was also observed for other amyloid fibrils. Most ex vivo lysozyme D67H fibrils [240] adopted the curvy (‘wavy’) morphology with a periodicity of ~300 nm, and they also acquired an intrinsic fibril twisting. Cryo-EM measurements on hairpin-induced tau filaments [102] showed the coexistence of multiple fibril morphologies in a sample. Some fibrils adopted the twisted, curvy morphology (the ‘snake-shaped’ fibrils) with a crossover
distance of 65 nm. Some less common ‘hose’ type fibrils were less regular and had wider curvatures in the range of hundreds of nanometres. These results suggest that multiple proteins could adopt the curvy morphology; however, their repeat length is usually smaller than curvy type III fibrils in this work (~600-800 nm).

The helical fibrils might be a type of curvy fibrils with a larger radius and more consistent repeat length. Similar morphology was seen for α-Syn fibrils [241] with a somewhat irregular repeat length of ~600-700 nm. This value is similar to the regular repeat length (574 ± 12 nm) obtained here.

(2) p-TAB imaging estimates dye orientation and reveals twisting fibril sub-structure

p-TAB imaging further analyses the polarisation property of each fluorescent burst. The emission polarisation of each dye molecule reflects its emission dipole moment and, thus, its orientation. By splitting the emission fluorescence into two orthogonally polarised channels and using sophisticated analysis methods, the orientation and wobble of each fluorescent dye molecule (ThT [172] or NR [173]) have been obtained in literature, revealing the underlying fibril backbone orientation and the flexibility of dye binding events.

Here, I used a simplified analysis method to extract each dye molecule’s orientation from its intensity measured in the x and y polarised channels (equivalent to the LD value) by assuming: (a) the molecule didn’t wobble (rotate) during an acquisition duration, and (b) the angle between the axis of the dye molecule and the local fibril backbone takes a smaller value.

Under these assumptions, the orientation of each fluorescent dye molecule can be assigned. Its orientation followed roughly the orientation of the local fibril axis with normally small angles (Figure 7-15). An overlapping of dye molecules with different azimuth angles was also observed (Figure 7-15 A, yellow rectangle), possibly suggesting a local fibril twist. Both the dye orientation and the LD value of a blinking event were affected by the fibril backbone orientation. This effect was the most pronounced for the curvy type III fibrils (Figure 7-12 B), where an alternate change in colours along the fibril was seen in the LD map.
Measures were taken to correct the changes caused by the fibril backbone orientation. A revised LD (LD’) value was calculated for each blinking event for a straightened fibril segment, representing the angle between the dye molecule and the fibril backbone. For type III fibrils, analysis of LD’ along the fibril axis revealed one periodicity at ~800-900 nm (Figure 7-19), coincident with the curvy morphology. Other periodicity peaks were mainly located in the 200-500 nm range. This result is consistent with the periodicities identified from TAB images. For type I fibrils, multiple peaks were identified in the 100-500 nm range, possibly indicating a twisting structure with irregular pitch lengths. This hypothesis is consistent with the structural models of type I fibrils that they are made of two intertwining protofilament strands. In the future, we could analyse the LD profiles of more fibrils to identify whether a dominating peak exists for each fibril type.

However, the binding of dye molecules on the amyloid surface is not tight but with some degree of flexibility, allowing the molecules to wobble quickly within one fluorescent burst or within the exposure time used for image acquisition (20 ms). Shaban and co-workers [172] estimated the wobbling angle of ThT on insulin fibrils to be ~60°. The wobble was corrected in their study to determine the average dye orientation of each blinking event.

Using a polarised standard PSF, our collaborators [173] successfully obtained the position and orientation of each blinking event in a three-dimensional space. The orientation of the NR molecule was almost parallel to the backbone of some thin Aβ 42 fibrils. However, for thick fibrils and other thin fibrils, they observed a significant wobbling of a single dye molecule during blinking and a large variation in the orientation of dye molecules binding onto the same location (pixel) of the fibril. This can be explained by more disordered β-sheet assembly orientations due to fibril twisting and a larger number of binding sites on these fibrils. However, our study didn’t consider the wobbling of each dye molecule and was thus unable to provide accurate information on the dye orientations.
In this chapter, TAB and p-TAB imaging generated high-resolution structural images of amyloid fibrils. They confirmed the structural heterogeneity based on the reconstructed fibril morphology (straight, curvy or helical fibrils), brightness and periodic change in brightness or linear dichroism along the fibril axis. Here, fibrils with different brightness in TAB imaging are linked with different numbers of strands; the periodic change likely reflects repeating structural units along the fibril axis, such as twisting, which could have a regular or irregular pitch length.
Chapter VIII. Elongation of authentic prion rods

8.1 Both RML and ME7 fibrils can grow on the coverslip surface

I utilised the established methods to study whether authentic prion fibrils can elongate in vitro. I observed elongation under our experimental conditions in the presence of MoPrP 91-231 monomers. Next, I analysed elongation kinetics and compared elongation properties between two stains, RML and ME7, and between authentic prions and synthetic fibrils.

Figure 8-1. Elongation of RML seeds. The monomer solution contains 10 μM PrP<sup>C</sup>, 50 mM NaP pH 7.4, 300 mM NaCl, 2 M GdnHCl and 875 nM NB. (A) The images of one ROI at three time points showing the elongation of two fibrils, a and b. The scale bar represents 5 μm. (B) The kymograph of fibril (a). The left panel shows the kymograph along the fibril axis at 40 h, and the right panel shows that along the fibril axis at 80 h. (C) The kymograph of fibril (b). The figure is adapted from [193].
RML and ME7 prion rods were purified from infected mouse brains as previously described [185, 242]. No PK treatment was performed during the purification process. However, endogenous proteases led to partial digestion of the fibrils; thus, the purified fibrils contained both full-length and truncated PrP. Seeds were adsorbed onto the coverslip of an 8-well chamber, and a 200 µL solution containing recombinant MoPrP 91-231 monomers (10 µM) with 2 M GdnHCl was added. The chamber was then sealed and imaged by TIRFM over an extended period at 37 °C.

Elongation of purified RML fibrils was observed (Figure 8-1). The two fibrils (a and b) had different growth properties. Fibril (a) elongated unidirectionally at a higher rate; its growing end moved on the coverslip surface; the kymograph in Figure 8-1 B shows at least two stall phases. Fibril (b) elongated bidirectionally with a much lower rate at both ends.

However, most particles visible at the initial time point didn’t elongate. Since the purity of the isolated prion fibrils was over 90%, those particles were not likely proteinaceous impurities. They could be prion fibrils whose growing ends were damaged in the multi-step purification process or trapped in a long stall phase. Alternatively, these fibrils might land on the coverslip surface in an orientation preventing elongation.

I performed quantitative elongation analysis for 12 fibrils and plotted the growth traces with or without stall phases in Figure 8-2 A and B, respectively. A large variance existed in fibrils’ final length. Panel B shows that pause-free rates within a fibril were nearly constant, similar to fibrils grown from synthetic seeds; different fibrils displayed diversity in growth rates but no clear clustering that suggested distinct fibril structures. Traces in Figure 8-2 B are colour-coded by fibril brightness; however, there was no clear correlation between elongation rate/fibril length and fibril brightness. This observation was in contrast to the elongation of recombinant seeds, where a strong correlation was observed. Overall, I couldn’t identify distinct fibril types from fibril rates and brightness as observed for the recombinant seeds.
Figure 8-2. Kinetic analysis for RML fibrils. (A) Length versus time traces for 12 elongated fibrils. (B) Traces after removing the stall phases. The traces in (A) and (B) are colour coded by fibril brightness as indicated by the colour bar. (C) Scatter plot of stall percentage (calculated after removing the last stall phase) versus pause-free rate. (D) The overlay of the traces without stall phases for RML fibrils (the black traces) and recombinant fibrils elongated under the same condition. Traces for recombinant fibrils are colour coded by their groups. The figure is adapted from [193].

Figure 8-2 C analyses stall phase percentages for the selected fibrils and plots them against elongation rates. Figure 8-2 A shows that some elongation-competent fibrils ceased growing and remained in the stall phase until the end of the experiment. This last stall phase could last for a very long time, thus leading to a high stall phase percentage. This stall phase was removed from the analysis shown in Figure 8-2 C. Interestingly, two clusters were identified in this plot, possibly suggesting the existence of multiple fibril types in RML seeds.
The pause-free rate for RML elongation is 0.4–1.7 nm/min, slower than all three types of synthetic fibrils growing under the same condition, as shown in Figure 8-2 D.

![Image](image.png)

Figure 8-3. Elongation of ME7 fibrils. 62 nM NB was present for imaging. The scale bar represents 5 µm.

The elongation of ME7 fibrils was studied under the same condition as RML. Elongation was observed for a small fraction of the fluorescent particles; Figure 8-3 shows two seeds elongating into fibrils at 93 h. Elongated ME7 fibrils were always short and usually no longer than 2 µm at 93 h. On the contrary, RML elongated into both short and long fibrils, with the longest fibril exceeding 9 µm in length. No kinetic analysis was performed for ME7 elongation due to the small number of elongating fibrils, the slow growth rate and the low signal-to-noise ratio. I speculate that ME7 grew at a lower rate than RML or with a higher stall percentage based on the shorter final length.

To study fibril morphology after elongation, elongation experiments were conducted on TEM grids with surface-attached RML or ME7 seeds. After incubating TEM grids in PrP\(^\text{C}\) solution for around 4 days, the grids were washed, stained and imaged. Figure 8-4 A shows abundant elongated RML fibrils on the grid. Both long, thin fibrils and short segments were observed. Different fibril morphologies existed: fibril (a) consists of several ‘kinks’, fibril (b) is twisted, and fibril c is straight. Fibril widths were then measured for fibrils longer than 500 nm, ensuring that only elongated fibrils were analysed and not
the initial seeds. The distribution of fibril width (Figure 8-4 C) shows that most elongated RML fibrils were 12-16 nm wide.

However, it was difficult to find fibrils on the ME7 seeded grid other than short clusters, which were likely initial seeds. Figure 8-4 B shows the only long fibril found after elongation, with a width of ~18 nm. Consistently, it was difficult to find elongated ME7 fibrils on the coverslip in the elongation assay (Figure 8-3).

**Figure 8-4.** Elongation of RML and ME7 fibrils on TEM grids. (A) TEM images of elongated RML fibrils. (B) TEM image of an elongated ME7 fibril. (C) Width distribution of elongated fibrils. The figure is adapted from [193].
8.2 Discussion

The elongation of RML and ME7 prion rods was directly observed by TIRFM in situ, allowing the kinetics to be extracted.

For RML fibrils, growth rates and fibril brightness displayed heterogeneity, likely suggesting either structural heterogeneity of seed fibrils or different seed orientations on the coverslip leading to the exposure of fibril ends with different interacting surfaces. The TEM images taken for the elongated RML fibrils indeed displayed three different fibril morphologies (Figure 8-4 A): a long fibril with several kinks, a twisted fibril and a straight fibril. However, these fibril morphologies differed from the RML fibril structures [26] built by cryo-EM: 90% were single-strand twisted fibrils with a 135 nm half-pitch length, and 10% were double-strand fibrils. This might suggest that multiple minor morphologies exist in the prion seeds at a low fraction, which weren't identified by cryo-EM measurements but were able to elongate under specific conditions. This supports the concept of 'prion quasi-species' [30, 236], stating that each strain contains structural isomorphs, which co-exist and reach equilibrium.

Another possible explanation is that the elongated prion fibrils could adopt morphologies different from the prion seeds. This might take place due to the following reasons: (1) the prion seeds are glycosylated, while the elongated segments are not since recombinant monomers were used for elongation. Thus, the elongated fibril segment might acquire a different fibril structure for higher stability; (2) MoPrP 91-231 monomers were used, which might be different from the building blocks in prion seeds, and (3) the experiment conditions used for elongation were artificially chosen, which were markedly different from the environment in vivo where authentic prions have formed.

The possibility that elongated fibrils might take different morphologies from the prion seeds is in contrast to the properties of synthetic fibrils, which mainly retained seed structures during growth. Again, this might be explained by the complex structural features and unique formation conditions of authentic fibrils.
The growth rates for both RML and ME7 strains were slower than the synthetic seeds under the same elongation conditions (Figure 8-2 D). This could result from the larger number of amino acid residues involved in the ordered core region of the prion fibrils than the synthetic fibrils. [26] and [27] indicated that the PK resistant regions for both RML and ME7 strains involved residues 89 to the C terminus, while a much smaller PK resistant core [243] was found for MoPrP 89-230 fibrils formed in vitro under partially denaturing conditions, involving residues 138/141–230, 152/153–230, or 162–230. Cryo-EM measurements indicated that the ordered β-sheet core spanned residues 94-225 for RML [26] and 94-229 for ME7 [27]. Even though there hasn’t been an atomic model for recombinant MoPrP 91-231 fibril, the amyloid fibrils formed by full-length human PrP contained residues 170-229 in the core region [84]. The larger number of residues in the fibril core of prions suggests that a longer time is needed for each adding monomer to adopt the same fold as in fibrils, leading to a slower elongation rate.

Atomic models for the purified RML [26] and ME7 [27] fibrils show many similarities in the internal packing of each PrP subunit (Figure 1-5). The N and C terminal lobes of both strains acquire a double-hairpin and single-hairpin structure, respectively, with small discrepancies. The main difference lies in the angle between the two lobes, which is smaller in ME7 strain. Even though the structural difference is small, the elongation kinetics are markedly different between the two strains, suggesting that the structural basis for the different growth properties is not clear and is a subject of further studies.
Chapter IX. Conclusion and future directions

9.1 Conclusion

Prion diseases are associated with the infectious amyloid species, PrP\textsuperscript{Sc}, which can self-propagate by recruiting and transforming PrP\textsuperscript{C} into the misfolded form. To gain insights into the mechanism and kinetics of the transformation process, I studied the seeded growth process \textit{in situ} on a single-particle level, for both synthetic seeds and authentic prion fibrils. The data confirmed that synthetic amyloid fibrils and prion fibrils displayed structural polymorphism and dynamic heterogeneity, supporting the ‘prion quasi-species’ hypothesis. Novel super-resolution techniques were then applied to extract the unique microscopic features of different types of fibrils.

Spontaneous and seeded aggregation of MoPrP 23-231 and MoPrP 91-231 were studied by bulk ThT assays, revealing that secondary processes, such as secondary nucleation or fragmentation, played a vital role in the aggregation. The evidence includes: (1) the rapid initial increase in fluorescence (after the lag phase) during spontaneous aggregations and (2) the sigmoidal shape of seeding assay kinetics at multiple, even very small, seed concentrations. The kinetics of spontaneous aggregation assays displayed a large degree of heterogeneity in terms of lag time and final fluorescence.

Elongation of seed particles, either MoPrP 91-231 fibrils or prion fibrils (RML or ME7), was observed by elongation experiments \textit{in situ}, while no other processes, such as fragmentation or nucleation, were observed. Elongation was interrupted by stalls phases which likely represented unproductive binding events of PrP species on fibril ends.

Synthetic amyloid fibrils formed by MoPrP 91-231 were morphologically polymorphic. In the elongation experiments, the structurally different seeds displayed unique dynamic properties, recruited PrP at different folding states, and elongated with distinct mechanisms. The double-strand type I fibrils elongated with a slow rate and high brightness. Type II and type III fibrils were
dim, single-strand fibrils elongating from the reverse (slow) and forward (fast) ends, respectively. Type I fibril growth followed the Michaelis-Menten-type mechanism, involving PrP monomers in the native or intermediate state. Type II fibril growth didn’t show a strong dependence on PrP concentration, suggesting a structural rearrangement step was rate-limiting. Type III fibril growth recruited unfolded PrP. Their growth followed the Michaelis-Menten-type mechanism at low PrP concentrations but was inhibited at high concentrations by the blocking of fibril ends with unproductive species. More fibril morphologies were identified from TEM and TAB images.

Fibrils of different types usually maintained their structural properties during growth, even under a disfavouring condition. Switch of fibril type was only occasionally observed with a frequency of < 1%. Environmental conditions shifted the equilibrium between different fibril types while didn’t alter fibril structure.

The above results support the theory of prion quasi-species, stating that structurally distinct different PrP fibrils co-exist in an aggregated sample. They proliferate by elongation (Chapter 6) and secondary processes (Chapter 5). The rare change in fibril type during elongation resembles ‘mutation’ seen in DNA or RNA replication. The environmental (buffer) conditions shift the relative population of different fibril types instead of changing the structure of individual fibril.

The elongation of RML and ME7 fibrils was observed in a pilot experiment, displaying slower rates compared to recombinant fibrils and heterogeneous growth properties among fibrils in one strain and between the two strains.

Imaging PrP fibrils using TAB overcomes the resolution limit caused by light diffraction and reaches a resolution of ~50 nm. TAB technique could resolve closely located fibrils, such as a splitting multi-strand fibril. TAB could distinguish type I (bright), III (dim, straight or curvy) and the helical type H fibrils. Several fibrils displayed a periodic change in brightness profiles along the fibril axis, which might result from a twisting sub-structure of the fibril.

Polarised-TAB technique estimated the orientation of single dye molecules. After correcting for the fibril backbone orientation, the revised LD map
displayed repeating polarisation patterns along the fibril axis, corresponding to structural features such as fibril twisting.

9.2 Future directions

The first area to improve is the heterogeneity in aggregation kinetics regarding lag time and fluorescence amplitude in bulk assays. In order to study the aggregation mechanism and the effect of various factors on the aggregation process, it is essential to obtain kinetic data with high quality and reproducibility. As discussed in Chapter 5, the most promising approach is to ensure that PrP used for the assay is pure and monomeric, which may be achieved by purifying the monomer stock by SEC immediately before starting the aggregation assays.

One crucial question in the field of prion diseases is the propagation mechanism of authentic, disease-related prion fibrils. Answering this question can help identify critical processes during prion elongation and disease development, which is vital to developing therapeutic strategies. The first strategy to answer this question can be to study the elongation kinetics of purified prion fibrils, using the experimental setup built here to observe in situ amyloid elongation. One difficulty is the slow dynamic of prion fibril elongation, as shown in this study. Thus, tests on experimental conditions are needed to find a suitable one that accelerates the elongation process. The suitable experimental condition could involve increasing temperature, changing buffer pH or adding cofactors. Future research will build on my thesis research to elucidate the elongation mechanism of authentic, infectious prion fibrils and to identify the substrates involved. Furthermore, the same experimental setup can also be used to reveal possible drugs and their effective mechanisms.

However, the somewhat artificial experimental conditions of the fibril elongation assay might alter the properties of prion fibrils and mechanisms of prion propagation. For example, the morphologies of the elongated RML fibrils might be altered, as shown here. Thus, a future direction will be to study the propagation under more natural conditions in the cellular environment. The
difficulty here is finding a suitable dye specific to amyloid fibrils and non-invasive to live cells. The development of highly specific amyloid dyes could extend the TAB microscopy technology into the cellular environment. Alternatively, substrate proteins could be labelled in vivo via the incorporation of unnatural amino acids. These future studies could provide information on the formation sites of prion fibrils in vivo, the elongation mechanisms in cells and how the cellular machinery reacts to the prion fibrils.

TAB and p-TAB imaging can resolve finer fibril structures at an enhanced resolution. These techniques, especially p-TAB, have the potential to image oligomeric species to provide structural information, such as the arrangement of β-sheet assemblies within an oligomer. Future studies will aim to image oligomeric species on the pathway to fibril formation and mature fibrils using p-TAB, to study the complete process of de novo aggregation rather than just elongation. The difficulties are accurately determining the localisation of dye binding events and the orientation of dye molecules as well as their wobbles. To overcome the difficulties, dyes with high photon yields should be developed, and an analysis script which can accurately reveal dye orientation and wobble is needed. Promising analytical approaches have recently been developed [244]. If successful, this setup can image the dynamic process of amyloid formation, reveal the critical oligomeric species involved and help elucidate the aggregation mechanism of amyloidogenic proteins.
References


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