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Biomedical Engineering

The Effect of α-Synuclein Phosphorylation on the Chaperone Activity of 14-3-3

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Abstract

Parkinson's disease is a neurodegenerative disease, which is believed to be caused by the aggregation of protein α-Synuclein into inclusion bodies in neurons. Cells are able to prevent the formation of cytotoxic aggregates by utilizing cellular protein quality control mechanisms. Protein quality control is regulated by chaperone proteins through the interaction with early aggregates, inhibiting further aggregation. The presence of chaperone protein 14-3-3τ was discovered in α-Syn inclusion bodies, suggesting its potential role in Parkinson's disease. It has been uncovered that 14-3-3τ specifically binds to phosphorylated serine motifs on proteins. Notably, approximately 90% of the α-Synuclein proteins is phosphorylated in Parkinson's disease. Building upon these findings, our aim was to investigate whether the chaperone activity of 14-3-3τ is guided by phosphorylation of α-Synuclein. We performed MicroScale Thermophoresis experiments to study the early oligomerization behaviour of α-Synuclein under various conditions. Our findings suggest that 14-3-3τ's chaperone functions are enhanced when α-Syn is phosphorylated. This link between phosphorylation and chaperone activity gives insights into the existing protection mechanisms in cells. Novel understanding of these mechanisms could have implications for the development of effective therapeutic strategies for Parkinson's disease.

Abstract

De ziekte van Parkinson is een degeneratieve hersenaandoening, die wordt veroorzaakt door de aggregatie van het eiwit α-Synucleïne tot insluitlichamen in neuronen. Cellen zijn in staat om de vorming van cytotoxische aggregaten te voorkomen door gebruik te maken van kwaliteitscontroles. De kwaliteitcontrole van eiwitten wordt gereguleerd door chaperonne-eiwitten, die interactie aangaan met vroegtijdige aggregaten en verdere aggregatie remmen. Chaperonne-eiwit 14-3-3τ is ontdekt in α-Synucleïne insluitsels, wat duidt op een mogelijke rol van 14-3-3τ in de ziekte van Parkinson. Het is aangetoond dat 14-3-3τ specifiek bindt aan gefosforyleerde serine motieven op eiwitten. Opmerkelijk is dat ongeveer 90% van de α-Synucleïne eiwitten gefosforyleerd is bij de ziekte van Parkinson. Voortbouwend op deze bevindingen, was het doel van dit onderzoek om te onderzoeken of de chaperonne-activiteit van 14-3-3τ wordt gestuurd door de fosforylering van α-Synucleïne. We hebben MicroScale Thermophoresis experimenten uitgevoerd om het vroegtijdige oligomerisatiegedrag van α-Synucleïne onder verschillende condities te bestuderen. Onze bevindingen geven aan dat 14-3-3τ's chaperonne functies worden versterkt wanneer α-Synucleïne is gefosforyleerd. Deze relatie tussen fosforylatie en chaperonne-activiteit geeft inzicht in de bestaande beschermingsmechanismen in cellen. Nieuwe kennis over deze mechanismen kan mogelijk bijdragen aan de ontwikkeling van effectieve therapeutische behandelmethoden voor de ziekte van Parkinson.

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Contents

1 Introduction

Parkinson's disease is a chronic neurodegenerative disease characterized by symptoms such as tremor and cognitive impairments. [1] These motor and neuropsychiatric symptoms progressively impact the daily activities of patients diagnosed with Parkinson's. While treatments are available to improve the quality of life of the individual, no definitive cure exists due to the complexity of the disease.

Existing research has shown that the symptoms related to motor dysfunctions are primarily caused by neuron cell death in the substantia nigra, which is a brain region responsible for dopaminergic processes that regulate motor control. [2] In the substantia nigra, Lewy bodies are detected in brain tissue from the majority of patients, which are believed to be related to the progression of the disease. [3] These inclusion bodies mainly consist of amyloid fibrils, which are formed by aggregation of the presynaptic protein α-Synuclein (α-Syn).

α-Syn is an intrinsically disordered protein (IDP) with low hydrophobicity and high net charge. Changes in its direct environment could lead to alterations of these properties, resulting in partial folding of the IDP. [4] The unstructured soluble α-Syn monomers can aggregate into oligomeric structures through a spontaneous process. Ultimately, these oligomers can form amyloid fibrils with β-sheet structures. [5]

Research on the cytotoxicity of these structures is still ongoing, as several studies suggest that oligomers are possibly more cytotoxic than the fibril structures. [6] However, the study of aggregate formation is complex as α-Syn can adopt distinct conformational forms at each aggregation state. Moreover, the early formed aggregates are low in abundance and have a transient nature. Cells possess protein quality control mechanisms against the cytotoxic effects of these aggregates. These processes involve chaperone proteins, which assist in refolding of misfolded proteins to prevent aggregate formation. Particularly in the brain, ubiquitous protein 14-3-3 has been revealed to perform chaperone functions. [7] 14-3-3 proteins are essentially involved in diverse cellular processes, such as signal transduction and stabilization of specific protein conformations, by binding to substrates. [8] Recent findings have revealed that 14-3-3 proteins are able to inhibit the aggregation of their interactors. [7] Their interaction with other proteins is based on the recognition of phosphoserine motifs. [9] However, its specific neurophysiological functions are still a topic of research.

Immunohistochemical studies have revealed that 14-3-3 is located in Lewy Bodies in Parkinson's disease, suggesting it is involved in Lewy body formation. [10] In fact, the 14-3-3τ isomer was found to prevent the formation of Wild-type (WT) α-Syn inclusion bodies, indicating that 14-3-3 possibly has neuroprotective functions. [11] This finding is supported by other studies that showed that α-Syn aggregation was delayed in the presence of 14-3-3τ, suggesting it stabilizes soluble α-Syn monomers. [12, 13]

It is not established how 14-3-3τ performs its exact chaperone function, apart from recognizing and interacting with phosphoserine motifs on substrates. [7] Interestingly, it was found that approximately 90% of the α-Syn is phosphorylated at Serine129 in Lewy bodies, contrasting the 4% phosphorylated α-Syn in physiological conditions. [14] This finding suggests the possible relevance of phosphorylation for the progression of Parkinson's.

Building upon these findings, this study is centered on investigating whether the chaperone activity of 14-3-3τ is guided by α-Syn phosphorylation. Novel understanding of protein 14-3-3τ's interaction with single phosphorylated α-Syn could give valuable insights into the cell's general protection mechanisms against cytotoxic α-Syn aggregates. Moreover, the inhibition of α-Syn aggregate formation is viewed as a potential therapeutic target for Parkinson's disease.

To study the role of phosphorylation on the chaperone activity of 14-3-3τ, it is important to detect and analyze the interaction of the protein with early single phosphorylated α-Syn aggregates. As it is possible for these oligomeric structures to be in different conformational states, the detection of oligomers can be challenging. Nonetheless, by using Microscale Thermophoresis (MST), we were able to detect and quantify early oligomerization processes, based on the different thermophoretic properties of the various α-Syn forms.

The first steps included establishing the early oligomerization behaviour of WT α-Syn with MST, in the absence and presence of protein 14-3-3τ. This was done by applying a polymerization model to the MST data, which describes the early oligomerization of α-Syn. [15] The WT α-Syn monomers appeared to form oligomers through the formation of an unstable nucleus. After reaching that energetic state, energetically favourable elongation into oligomers occurs. This nucleation-elongation mechanism is considered to be a

cooperative system.

We investigated the effect of 14-3-3τ on the WT α-Syn oligomerization behaviour by performing MST experiments with increasing concentrations of 14-3-3τ. The obtained results show that in the presence of 14-3-3τ, the system's cooperativity decreases. It is suggested that 14-3-3τ interacts with WT α-Syn and decreases its association propensity. Therefore, this finding demonstrates 14-3-3τ's chaperone function of inhibiting early WT α-Syn oligomerization.

To study the effect of phosphorylation on the oligomerization behaviour of WT α-Syn, MST was performed with WT α-Syn and single phosphorylated α-Syn at Serine129. The fraction of single phosphorylated was increased with every MST experiment. We observed that larger fractions cause a higher elongation propensity, indicating that the formed α-Syn oligomer populations most likely differ from each other. This finding became an additional factor to consider when studying the influence of phosphorylation on 14-3-3τ's inhibitory effect.

We separated the two factors, phosphorylation and differences in oligomer populations, by performing the 14-3-3τ experiments with single phosphorylated α-Syn at 30% and 100%. We found that the particular oligomer population caused by the 30% fraction inhibited 14-3-3τ's interaction with α-Syn. The results for 100% single phosphorylated α-Syn showed a transition from a cooperative to a non-cooperative system, similar to WT α-Syn. However, compared to WT α-Syn, the transition for single phosphorylated α-Syn occurred at a lower 14-3-3τ concentration. This suggests that 14-3-3τ has a greater higher affinity for phosphorylated α-Syn. Thus, our findings provide evidence that 14-3-3τ's chaperone activity is enhanced when α-Syn is single phosphorylated.

2 Materials and Methods

2.1 Microscale Thermophoresis

Microscale Thermophoresis (MST) is a technique used to study the interaction between two different biomolecules. Its principle is based on the detection of thermophoresis by locally inducing heat to samples containing fluorescently labelled molecules. The detected thermophoresis behaviour is influenced by the interaction between the two molecules. The measured change in fluorescence intensity allows for high sensitive detection and analysis of intermolecular binding. The sample volume enables testing samples in short supply at high concentrations, without requiring a large quantity. Several studies have demonstrated MST is a potent technique that can be used for a wide range of applications in research. [16]

In this study, MST is utilized to study early oligomerization of α-Syn instead of the interaction between two different molecules. Given the complexity of the different forms of α-Syn structures, scattering methods or other biophysical techniques have limitations in the sensitive detection of minor changes in oligomerization behaviour. Furthermore, detection of early α-Syn oligomers is challenging considering their transient nature and low abundance in α-Syn populations. Nonetheless, a study has revealed that it is possible for MST to detect the various α-Syn types, as oligomer intermediates have different thermophoretic properties compared to α-Syn monomers. [17]. Therefore, this technique was expected provide to valuable insights into the early oligomerization processes prior to aggregate formation, with high sensitivity. In this study, the primary interest lies in investigating whether the chaperone activity of 14-3-3τ is guided by α-Syn phosphorylation. To address this, MST experiments were conducted with various experimental conditions, such as the addition of chaperone protein 14-3-3τ, in order to analyze the α-Syn oligomerization behaviour.

To study α-Syn oligomerization, the MST samples consisted of a fixed concentration of fluorescently labelled α-Syn and an increasing concentration of non-labelled α-Syn. The samples were loaded into capillaries from the Monolith Capillary Kit (NanoTemper Technologies GmbH, Munich, Germany). By increasing the concentration of non-labelled α-Syn with every capillary, it was possible to analyze the changes caused by increasing the propensity of oligomer formation. All MST experiments were conducted utilizing the Monolith NT.115 (NanoTemper Technologies GmbH, Munich, Germany).

A typical MST measurement is divided into four distinct stages, illustrated in Figure 1. The measurement starts as the LED laser excites the fluorescent label, which lasts approximately five seconds. During this initial stage, the initial fluorescence is determined.

In the second stage, an infrared beam is directed onto a specific region within the capillary, locally increasing its temperature. The infrared has an emitting wavelength of 1480nm. The resulting temperature change alters the properties of the fluorophore, which leads to a substantial decrease in the fluorescence intensity within 100ms. [18] Figure 1 demonstrates this decline in fluorescence intensity in stage 2.

The third stage begins as thermophoresis starts to occur. The temperature gradient generated by the infrared laser results in thermophoretic movement of the molecules, until the effect of mass diffusion is equal to the effect of thermophoresis. At that moment a steady state is acquired, observed as the plateau starting around the 25-second mark in Figure 1. [18] The movement of α-Syn is monitored through detection of the fluorescence signal of the labelled α-Syn, which is captured by the same objective as the infrared laser.

In the fourth stage, as the IR is inactivated, the molecules move as a result of mass diffusion leading to a subsequent increase of the fluorescence intensity.

The fluorescence intensity of all 16 capillaries during these four stages is measured over time and plotted for every concentration, which results in an MST trace graph as shown in Figure 1. α-Syn oligomers have different thermophoresis properties than monomers, resulting in different MST traces. Since the higher concentration samples have a higher propensity of oligomer formation than lower α-Syn concentration samples, we obtain different MST traces at different α-Syn concentrations. This is illustrated in Figure 1, where the MST curve shifts downward for increasing concentrations. This change is quantified using the normalized fluorescence (F_{norm}), which is obtained by dividing the measured fluorescence obtained near the end of the IR laser activation duration by the initial fluorescence. The 16 concentrations of initial total α-Syn

are then plotted with their corresponding F_{norm} value. To acquire valuable information from the obtained F_{norm} data about the oligomerization behavior, the data was analyzed by using a model that describes early oligomerization.

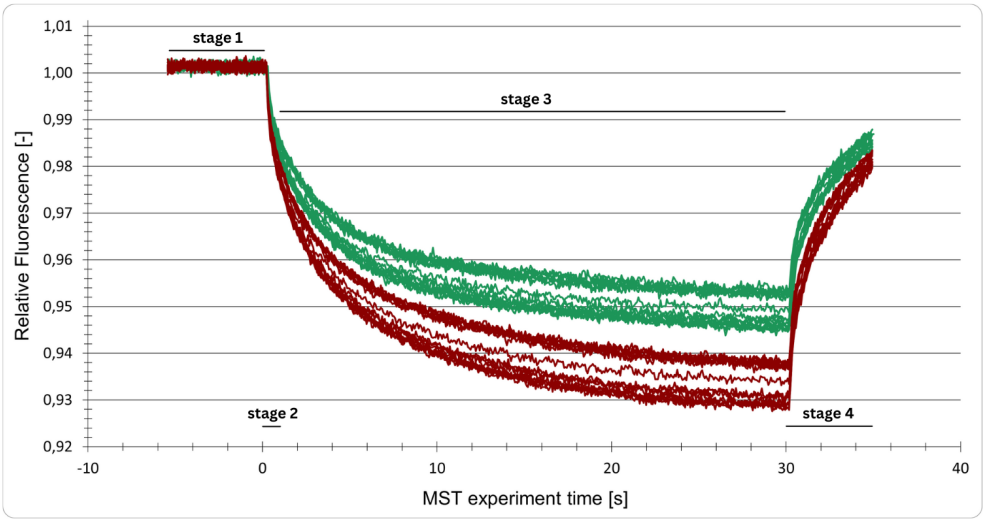

Figure 1: *Typical MST trace graphs of 16 capillaries. For each capillary, four stages are identified during one measurement. In stage 1, the initial fluorescence intensity is measured. The IR laser is activated in stage 2, causing a substantial decrease in intensity. The effect of thermophoresis is observed in stage 3. In stage 4, the increase of the intensity is a result of IR laser inactivation. An MST experiment can be performed with several MST powers, represented with the green and the red colored curves. Higher MST powers induce stronger temperature gradients and result in stronger thermophoresis effects.*

2.2 Oligomerization Model

Early oligomerization of α-Syn can be studied by using a polymerization model (Zhao et al., 2003), where oligomerization is described as sequential addition of α-Syn monomers. [15]

$$A_{i-1} + A_i \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} A_i \tag{1}$$

$$K_i = \frac{k_i}{k_{-i}} = \frac{[A_i]}{[A][A_{i-1}]} \tag{2}$$

Equation 1 illustrates the fundamental mechanism used for this model, which describes the polymerization process. The degree of polymerization is indicated with i. In Equation 2, K_i is the equilibrium constant, which is a ratio of the rate constants for the polymerization reaction. In a simplified polymerization model, the monomer addition steps are all equal: $K_i = K_{i+1} = K_{i+2} = K$. However, considering possible variations in the equilibrium constants, the cooperativity of a system is incorporated in the model. Cooperativity can be described as the formation of an unstable nucleus before energetically favourable elongation into higher-order structures through monomer addition. Nucleus formation requires a significant increase in the system's potential energy, making this process the rate-limiting step of a cooperative system. After successful surpassing of the energy barrier, elongation becomes energetically favorable. The presence of a nucleation step, and therefore the cooperativity of a system, can be evaluated using a nucleation factor σ. It relates the rate limiting nucleation step, simplified to a single nucleation step, to the elongation steps as shown in Equation 3.

$$\sigma = \frac{K_2}{K} \tag{3}$$

In a cooperative system, with an energetically disfavoured nucleation step, σ is smaller than 1. A σ value equal or larger than 1 describes an non-cooperative system, where the polymerization process occurs without nucleus formation. It is believed that early oligomerization of α-Syn follows a cooperative mechanism, with the formation of an unstable oligomer intermediate before forming higher-order structures. [4,19] Therefore, this specific model is utilized to describe the formation of α-Syn oligomers. With the nucleation factor σ, a function for the total α-Syn monomer concentration in a cooperative system can be derived. This is accomplished by first formulating a general function for the monomer concentration A_i, based on the equations for different equilibrium constants as described in Equation 1. [15] The final function in this model is shown in Equation 4, where $[A]$ is the concentration of α-Syn in monomeric form and c_t the total initial α-Syn monomer concentration.

$$c_t = [A] + \sum_{i=2}^{\infty} i\sigma K^{-i}(K[A])^i = (1-\sigma)[A] + \frac{\sigma[A]}{(1-K[A])^2} \tag{4}$$

Equation 4 can be rearranged to obtain an oligomerization plot where the $[A]/c_t$ is plotted as a function of c_t, shown in Figure 2.

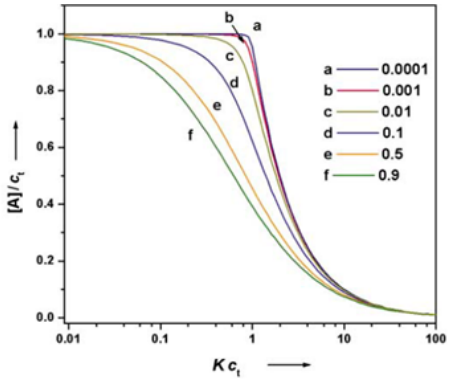

Figure 2: *Polymerization curves plotted for different values for σ, representing the monomer fraction as a function of the total initial α-Syn concentration. These nucleation factors represent the cooperativity of the system, where a value lower than 1 is indicative of a nucleation process. Curve a shows a highly cooperative system as σ is close to 0. [15]*

σ approaches 0 for highly cooperative systems, represented by oligomerization curve a in Figure 2. The plateau in the low total α-Syn concentrations demonstrates that α-Syn monomers remain in their monomer state as the association propensity is low at low total α-Syn concentrations. The total α-Syn concentration at which the association propensity is high enough to form a nucleus, is defined as the critical concentration. The sequential energetically favourable elongation of this nucleus, the oligomer formation, leads to a stark decrease in the monomer fraction. The relationship between K, a measure for the association propensity, and c_c is defined as shown in Equation 5. [15]

$$c_c = \frac{1}{K} \tag{5}$$

Higher values for σ corresponds to systems with a less cooperative mechanism, which is reflected in the oligomerization curves in Figure 2, as the transition in the fraction of monomers becomes more gradual. By analyzing the oligomerization curves and corresponding sigma value, it is possible to evaluate the cooperativity of a system with α-Syn monomers. Therefore, this model enables the study of the potential impact of various factors, such as 14-3-3τ and phosphorylation, on the early oligomerization behaviour of α-Syn. The combination of the highly sensitive MST technique and the oligomerization model allows for a comparative analysis of the early aggregation behaviour between WT α-Syn and single phosphorylated α-Syn, in the presence and absence of the chaperone protein 14-3-3τ.

2.3 MST Experimental Design

The oligomerization behaviour of α-Syn was studied in various conditions by performing different sets of MST experiments. First, the general oligomerization behaviour of WT α-Syn was established. Subsequently, we studied the impact of 14-3-3τ and phosphorylation on the oligomerization process. Finally, to examine whether phosphorylation of α-Syn has an effect on 14-3-3τ's chaperone activity, a comparative analysis was conducted. As mentioned in Section 2.1, the 16 MST glass capillaries contain an increasing concentration of total α-Syn to examine the changes induced by the increased propensity of oligomer formation along the capillaries. All MST samples for the capillaries were prepared by creating a 1:1 dilution series of non-labelled α-Syn and subsequently adding equal volumes of labelled α-Syn. All measurements were performed in a buffer consisting of 10mM Tris, 10mM NaCl and 0.1 mM EDTA, with a pH value of 7.4, which will be referred to as the EDTA buffer. The MST experiments were conducted directly after the sample preparation as described in Section 2.3, with 60% and 80% MST power and 21% blue filter LED power.

2.3.1 WT α-Syn Oligomerization and 14-3-3τ

To study the oligomerization behaviour of α-Syn, we first performed an MST experiment with α-Syn-A140C labelled with AlexaFluor488 (α-Syn-A140C-AF488) and increasing concentrations of WT α-Syn. The WT α-Syn stock was stored in 10mM Tris, at a concentration of 250μM. Prior to the experiments, the stock was re-buffered and diluted, resulting in 220μM α-Syn in the EDTA buffer. α-Syn-A140C-AF488, with a Degree of Labelling (DOL) of 1, was used as the labelled target for the MST experiments. It was stored at a concentration of 110μM in the EDTA buffer. For this experiment, the dye was diluted to a final concentration of 50nM in the MST samples.

For the experiment investigating the effect of 14-3-3τ on the WT α-Syn oligomer formation, WT α-Syn and α-Syn-A140C-AF488 were prepared as previously described. Protein 14-3-3τ was stored at a concentration of 200μM in 10mM Tris and 10mM NaCl with pH 7.4. Desalting and re-buffering this stock using the 7 kDa MWCO 2 mL ZebaSpin Column with the EDTA buffer, resulted in a protein concentration of 175μM. Concentrations of 0nM, 875nM, 3.5μM and 7μM of protein 14-3-3τ were used as experimental conditions in order to evaluate the protein's influence on the oligomerization. These conditions were investigated on the same dilution series of WT α-Syn. We added the 14-3-3τ solutions to the solution containing labelled α-Syn to ensure consistent concentrations across all 16 capillaries. The highest α-Syn concentration sample contained 110μM WT α-Syn and 50nM dye, which is identical to the first experiment.

Myoglobin (Sigma-Aldrich, M1882) was used as a control protein. The stock was prepared by dissolving it in the EDTA buffer, obtaining a concentration of 55μM. The α-Syn and the dye concentrations were identical to the previous experiments. To validate the results of the 14-3-3τ experiments, Myoglobin was also used at protein concentrations of 875nM, 3.5μM and 7μM as the experimental conditions.

2.3.2 Single Phosphorylated α-Syn Oligomerization and 14-3-3τ

The impact of phosphorylation on α-Syn oligomer formation was studied by using α-Syn singly phosphorylated at Serine129, α-Syn-P129Ser. The stock was stored at 163μM in 10mM Tris. After preparing the stock in the EDTA buffer, the resulting concentration was 145μM. We compared four different ratios of α-Syn-P129Ser and WT α-Syn, where α-Syn-P129Ser was present at 10%, 30%, 50% and 100%. These ratios were also consistently maintained in the total concentration of labelled α-Syn. The results obtained from the WT α-Syn experiment were used as the 0% condition for a comparative analysis. The WT-α-Syn and α-Syn-A140C-AF488 originated from the stock in Section 2.3.1. α-Syn-P129Ser-AF488 was stored at a concentration of 11μM in the EDTA buffer. After addition to the samples, the final dye concentration was 25nM. Different from the conjugation method of AlexaFluor488 to α-Syn-A140C (thiol-based), α-Syn-P129Ser was labelled using NHS ester modified AlexaFluor488, targeting the primary amines in the α-Syn chain.

Due to the different stock concentrations of WT α-Syn and α-Syn-P129Ser, the initial total α-Syn concentrations differed for 10%, 30% and 50% conditions. Specifically, the dilution series started at an initial

total α-Syn concentration of 209μM, 191μM and 175μM, respectively. It is worth noting that the DOL for α-Syn-P129Ser-AF488 was 0.41 instead of 1.0. To account for this, the DOL of α-Syn-A140C-AF488 was adjusted to 0.41 by adding WT α-Syn. The labelled mix was subsequently created according to the ratios and finally diluted to obtain a dye concentration of 25nM in all MST samples. For the experimental condition of 100% single phosphorylated α-Syn, a different α-Syn-P129Ser stock with concentration 169μM in 10mM Tris was used. Preparation with the EDTA buffer resulted in a concentration of 154μM, which was the starting concentration for the dilution series. α-Syn-P129Ser-AF488 was stored at a concentration of 109μM in the EDTA buffer. Having a DOL of 0.41, the AF488 concentration was 44.7μM. The final dye concentration of the samples in this experiment was 20nM. The LED power had to be adjusted with increasing percentage of single phosphorylated α-Syn to obtain a similar fluorescence intensity between 800 and 1100 units for every condition. Accordingly, the LED power settings for the experimental conditions were 45%, 62%, 70% and 95%, in the order of increasing fractions.

Based on the obtained results from the previous experiment in this section, it was decided to examine the effect of the 14-3-3τ protein on the α-Syn conditions with 30% and 100% single phosphorylated α-Syn. For the 30% condition, the α-Syn-P129Ser stock of 145 μM was used as the starting concentration for the dilution series. The WT-α-Syn and α-Syn-A140C-AF488 originated from the stock in Section 2.3.1. The labelled mixture was prepared according to the previously explained method, with the 109μM Alexa488 labelled α-Syn-P129Ser stock. In this experiment, the final dye concentration was 50nM concentration. The LED power was set to 30%. The 100% condition used the prepared Synuclein-P129Ser stock of 154μM as the starting concentration for the dilution series. The 109μM Alexa488 labelled α-Syn-P129Ser stock was used as the labelled target in this MST experiment. The final samples had a dye concentration of 20nM. For this MST experiment, the LED power was set to 95% . The 14-3-3τ protein originated from the prepared stock in Section 2.3.1 and were added according to the concentrations from the identical conditions of the 14-3-3τ experiment with WT α-Syn.

2.4 MST Data Analysis Method

The F_{norm} graph obtained from the MST data of all 16 capillaries was analyzed in Matlab using the polymerization model from Section 2.2. First, the mean was determined of the F_{norm} data obtained with 60% and 80% MST power. This F_{norm} data was normalized within the interval 5s-30s of the experiment to enable an estimation of the monomer fraction for all different concentrations. It is important to note that the normalized F_{norm} data does not represent an absolute monomer fraction, but rather an apparent monomer fraction, as it is likely that even at the highest concentration not all α-Syn monomers have formed oligomers. In addition, for this study, the underlying assumption is that the detected movement of fluorescently labelled α-Syn reflects the behaviour of the entire α-Syn population in the samples. Under this assumption, this method allows the study of aggregation behaviour by analyzing subtle changes in measured fluorescence caused by the formation of oligomers. The normalized F_{norm} data points were plotted with an error bar, determined by calculating the standard deviation using the MST data from different time intervals (early: 5s-6s, mid: 17s-18s and late: 29-30s) and from the used MST power (60% and 80%). This data was fitted using Equation 4 from the oligomerization model for a cooperative system. [15] The most important parameters for this model are σ and c_c. As explained in Section 2.2, σ is the nucleation factor and the critical concentration c_c is a threshold below which no oligomerization takes place. Various combinations of the parameters are tested using Matlab to ultimately obtain the best fit for the experimental data. This resulting oligomerization curve and the corresponding values for the two parameters give valuable information about the early aggregation behaviour of α-Syn. Comparative analysis of the obtained α-Syn curves under various experimental conditions provides insights to address 14-3-3τ's effect and α-Syn phosphorylation.

2.4.1 Removal of Outliers

The raw MST traces of the experiments were examined to identify any apparent outliers before fitting the F_{norm} data with the oligomerization model. The figure in Appendix A shows the MST traces of removed sam-

ples that thermophoretically behaved significantly different from the others. Possible causes of such outliers include various factors as inhomogeneities within the samples or unexpected higher-structure oligomers. [20]

In addition, to improve the accuracy of the fit and the subsequent assessment of a system's level of cooperativity, we removed the data point with the largest deviation from the best obtained fit. This removal process was specifically applied to data points for a total α-Syn concentration below 10×10^2 nM, as those points significantly determine and impact the overall shape of the oligomerization curve. Thus, we performed two rounds of fitting in total.

2.5 Aggregation Assay

The MST experiments give valuable insights into the early oligomerization behaviour of α-Syn. To study whether the 14-3-3τ and phosphorylation of α-Syn have an effect on the aggregation into high-structured fibrils, an aggregation assay with Thioflavin T (ThT) is performed. Ultimately, these results could potentially indicate a relationship between early α-Syn oligomerization behaviour and α-Syn aggregation into amyloid fibrils.

ThT is an amyloid dye that specifically interacts with the β-sheet structure of formed amyloid fibrils. ThT has an excitation and emission wavelength of 350 and 438nm, respectively. These wavelength maxima change to 450 and 482nm in the presence of amyloid fibrils. By measuring the fluoresence intensity over time, the formation of α-Syn fibrils can be detected. [21]

The aggregation of α-Syn was studied in the presence of increasing concentrations of 14-3-3τ: 0μM, 1μM, 5μM and 10μM. By increasing the amount of 14-3-3τ in this range, we expect to be able to determine the overall effect of the chaperone protein on α-Syn aggregation. The effect of 14-3-3τ was studied for 100% WT α-Syn and for α-Syn with 30% single phosphorylated α-Syn. The samples consisted of a monomer solution, 10 μM ThT and 0.02 w/v ‰ NaN_3 in the EDTA buffer from Section 2.3. The 14-3-3τ solutions were first prepared as described in Section 2.3.1 and subsequently added to these samples. The WT α-Syn samples involved 45μM WT and 5μM α-Syn-A140C-AF488, which were prepared according to Section 2.3.1. The samples involving the single phosphorylated fraction were 30μM WT α-Syn, 5μM α-Syn-A140C-AF488 and 15μM α-Syn-P129Ser, prepared as described in Section 2.3.2. The ThT assay was conducted in triplicates for all eight conditions using the Infinite 200Pro microplate reader (Tecan, Männedorf, Switzerland). The samples were pipetted into the Corning 96 Flat Bottom black Polystyrene microplate (Corning, New York, United States), which was incubated at 37°C with a shaking amplitude of 1.5mm. For the measurements, the emission wavelength of 485nm and an excitation wavelength of 445n were used. From the ThT assay data, the lag times were obtained by applying a threshold value equivalent to twice the initial fluorescence intensity. By comparing the lag times of various conditions, it is possible to assess the influence of 14-3-3τ and phosphorylation on α-Syn aggregation. Ultimately, a comparative analysis can assist in establishing whether the chaperone function of 14-3-3τ is guided by α-Syn phosphorylation.

3 Results and Discussion

3.1 WT α-Syn Oligomerization

The first MST experiment was performed with samples solely containing α-Syn to obtain insights on α-Syn aggregation before adding other factors, such as single phosphorylated α-Syn and 14-3-3τ.

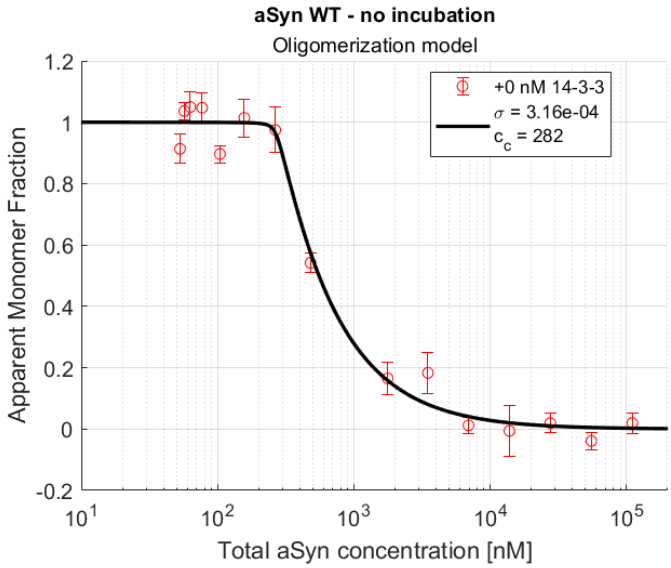

Figure 3: *Oligomerization curve of α-Syn WT with 110uM as the highest initial total α-Syn concentration, 50nM originating from the labelled α-Syn. The found value for σ was 3.16×10^{-04}, indicating a cooperative system, with a c_c value of 282nM.*

The MST data for WT α-Syn was analyzed as described in Section 2.4. The obtained oligomerization curve is shown in Figure 3. The corresponding sigma value of 3.16×10^{-04} is indicative of a cooperative system, as the nucleation factor is significantly smaller than 1. In this cooperative system, the formation of the unstable nucleus results in oligomer formation through energetically favoured elongation, which is evident starting from a c_c value of 282μM. This is in line with the reported required concentration of 200μM for WT α-Syn oligomer formation. [22] The observed oligomerization behaviour of WT α-Syn during this experiment provides evidence that it follows a cooperative system. [4, 19]

3.2 WT α-Syn and 14-3-3τ

The oligomerization of α-Syn in the presence of 14-3-3τ was studied using four conditions, by progressively increasing the concentration of 14-3-3τ: 875nM, 3.5μM and 7μM. The oligomerization curve for 0nM, solely WT α-Syn, can be seen in Figure 4A and shows cooperativity comparable to Figure 3. In both cases, the values for c_c were also found to be similar. For this experiment, the found σ value was 7.94×10^{-02}.

In the presence of the lowest 14-3-3τ concentration, 875nM, the aggregation curve in Figure 4B exhibits less cooperativity than in the absence of 14-3-3τ. Comparing this condition to the other two concentrations in Figure 4C and D, 3.5μM and 7μM, it appears that in the increasing presence of 14-3-3τ, the nucleation behaviour in α-Syn WT oligomerization becomes less pronounced and even completely disappears at 3.5μM and 7μM. The slope of the transition in monomer fraction gradually decreases with higher total α-Syn concentration in the increasing presence of 14-3-3τ. Upon examining the corresponding nucleation factors for the four conditions, an increase in the concentration of 14-3-3τ resulted in an increase of the σ value. This finding suggests that protein 14-3-3τ decreases the system's cooperativity by interacting with α-Syn.

As explained in Section 2.2, in a cooperative system, elongation steps are energetically favoured after the formation of a nucleus. In other words, a formed unstable nucleus is at a energetic state that promotes

subsequent elongation. A reduction in the cooperativity diminishes the likelihood of reaching this state necessary for energetically favourable elongation into early α-Syn oligomers. Consequently, the elongation process becomes less favourable, making the oligomerization system more stable. Therefore, a decreased level of cooperativity could imply that the system has become more stable, which could lead to a delay in the formation of early α-Syn oligomers. Thus, the results of this experiment demonstrate the potential ability of 14-3-3τ to delay oligomer formation by binding to α-Syn. This chaperone function has also been observed in other experimental research. [12]

The cooperative behaviour is completely eliminated when 3.5μM 14-3-3τ was added. The found σ value for this condition was 1.26×10^{0}, surpassing the threshold of 1.0. This indicates the oligomerization process, as explained in Section 2.2, can be described as a non-cooperative system. Increasing the 14-3-3τ concentration to 7μM did not significantly affect the cooperativity. It is possible that at a concentration of 3.5μM, the binding sites on α-Syn may have reached saturation. As a result, there are only limited binding sites of α-Syn available for additional 14-3-3τ molecules to interact with. Consequently. higher concentrations of 14-3-3τ do not lead to further changes in oligomerization behaviour. However, it should be emphasized that it is not possible to make definitive statements due to limited knowledge available about the specific interaction behaviour of 14-3-3τ.

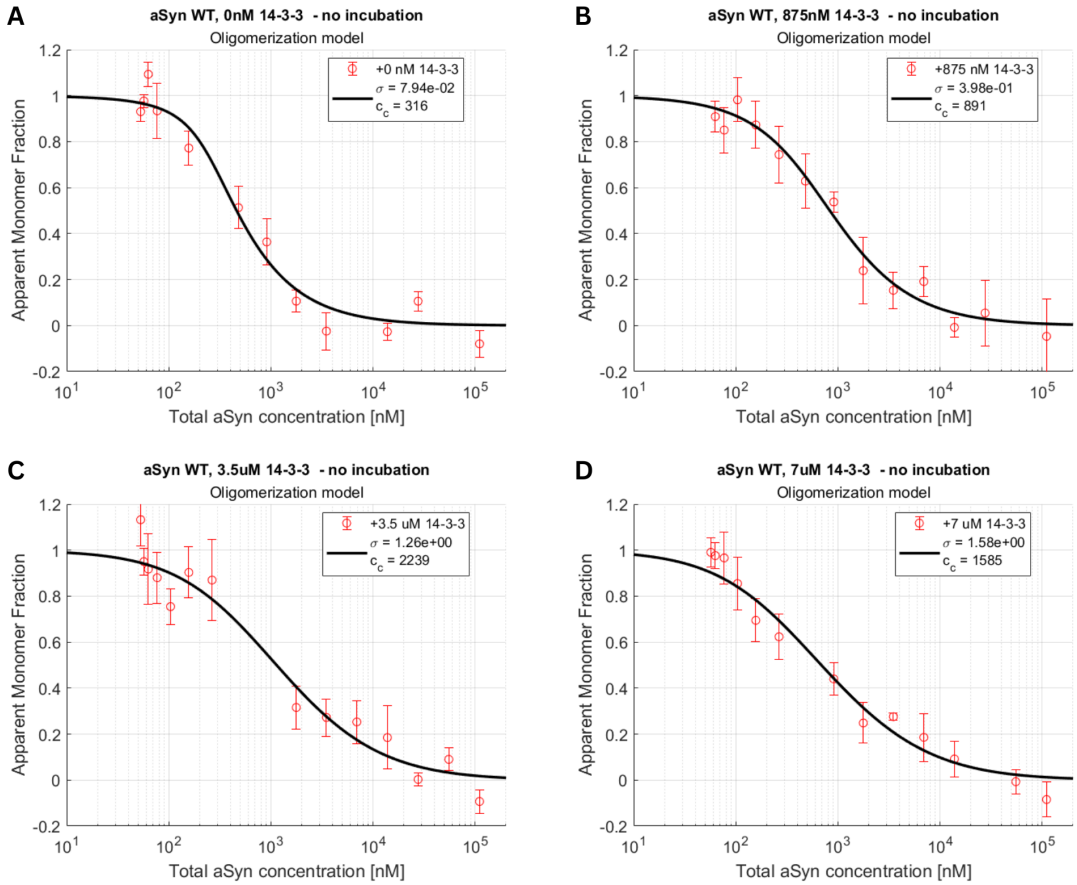

Figure 4: *Oligomerization curves of WT α-Syn, in the presence of 14-3-3τ. The highest initial total α-Syn concentration was 110μM, 50nM originating from the labelled α-Syn. The concentration of 14-3-3τ was increased for each condition: 0nM (**A**), 875nM (**B**), 3.5μM (**C**) and 7μM (**D**).*

There are noticeable differences between the 0nM condition and the WT α-Synuclein condition from the experiment in the previous section, which had a sigma value of 3.14×10^{-04}. In the MST results from this experiment, three outliers were identified and subsequently removed from the analysis, one originating from

capillary 10. These deviating behaviours could be attributed to the presence of larger formed oligomers or sample inhomogeneities. [20] The MST trace graph in Appendix A shows that the samples in those three capillaries only behaved differently as a response to a MST power of 60%. In addition, after removing these three outliers, we identified the data point from capillary 12 as an outlier during the fitting process. Considering that capillaries 10 and 12 substantially determine and affect the overall shape of the curve, the curves show more similarities than is reflected by the σ value alone. Therefore, despite the variability indicated by the σ value, the results of the two experiments share common characteristics.

3.2.1 Control Experiment

An additional MST experiment was conducted with a control protein to validate that the observed effect in the previous section was specifically caused by the chaperone protein 14-3-3τ. Selecting a suitable control protein is difficult for α-Syn, considering its high tendency to interact with a large variety of proteins. [23] It was decided to use protein Myoglobin, which is known to not alter the aggregation lag time of α-Syn. Based on this understanding, Myoglobin was not anticipated to influence early α-Syn oligomerization.

Increasing the concentration of Myoglobin did not alter the oligomerization behaviour of WT α-Syn. The curves show similar oligomerization behaviour, with nucleation factors in the same range of magnitude. This differs from the observed change in oligomerization behaviour of WT α-Syn in the presence of increasing protein 14-3-3τ concentrations. Section 3.2 shows the nucleation factor progressively increased as the chaperone concentration increased, reaching a value of 1.58×10^0 at 7μM. These findings provide evidence that the established effect from the previous experiment in this section is attributed to the 14-3-3τ protein. It is however crucial to note and emphasize that the starting control condition, without Myoglobin, shows a different oligomerization curve than the ones observed in Section 3.2. The detailed results for this control experiment are included in Appendix B. Despite the limitations of the findings for the control protein, they demonstrate that protein 14-3-3τ influences the oligomer formation significantly, whereas no change is observed in the presence of similar Myoglobin concentrations.

3.3 Single Phosphorylated α-Syn Oligomerzation

The overall effect of phosphorylation of α-Syn on the oligomerization was examined by using four experimental conditions with increasing percentages of α-Syn-P129Ser: 10%, 30% and 50% and 100%. The samples were prepared according to the experimental design in Section 2.3. The MST measurements were conducted directly after preparing the samples.

From the four obtained oligomerization models, shown in Figure 5, it can be observed that the phosphorylation of α-Syn influences the oligomerization behaviour. By increasing the fraction of single phosphorylated α-Syn in the samples, the value for c_c decreases. The c_c value for solely WT α-Syn, as found in Section 3.2, was 282nM. When the percentage of single phosphorylated α-Syn was increased in the experimental conditions, c_c gradually decreases to 200nM, 158nM and eventually 141nM. A decrease in the c_c value indicates a higher association propensity of α-Syn, as described by the relation in Formula 5 from Section 2.2. Given a higher value of K, phosphorylated α-Syn could lead to an increased and enlarged formation of oligomers at a given total α-Syn concentration. Interestingly, previous studies have shown that phosphorylation at Serine129 promoted the formation of α-Syn in oligomers in vitro. [14] In our experiments, the found higher K value associated with oligomerization of phosphorylated α-Syn could indicate a similar effect.

According to the oligomerization model, cooperative systems with higher elongation propensity are also characterized by a higher level of cooperativity, assuming that K_2 remains the same. This specific relationship is evident to some extent when comparing the results for the various fractions of single phosphorylated α-Syn. However, if K changes, it is unlikely that the value of K_2 remains consistent. One possible explanation for the change in K_2 is that the degree of phosphorylation of α-Syn profoundly affected the structural and interaction properties of the monomers. The resulting change in the stability of α-Syn, leading to a different K_2, could change its oligomerization behaviour. This suggests that the formed oligomers from different fractions of single phosphorylated α-Syn, differ from each other and from the condition with solely WT α-Syn. This has to be taken into account when interpreting other MST results involving single phosphorylated α-Syn. The

formation of a different oligomeric population is further supported by the F_{norm} data, shown in Appendix C, where the change in thermophoretic behaviour with increasing concentrations of α-Syn is stronger with increasing fractions of single phosphorylated α-Syn, suggesting the formation of more and possibly larger oligomers. This supports the previous hypothesis that larger fractions of single phosphorylated α-Syn cause a higher elongation propensity.

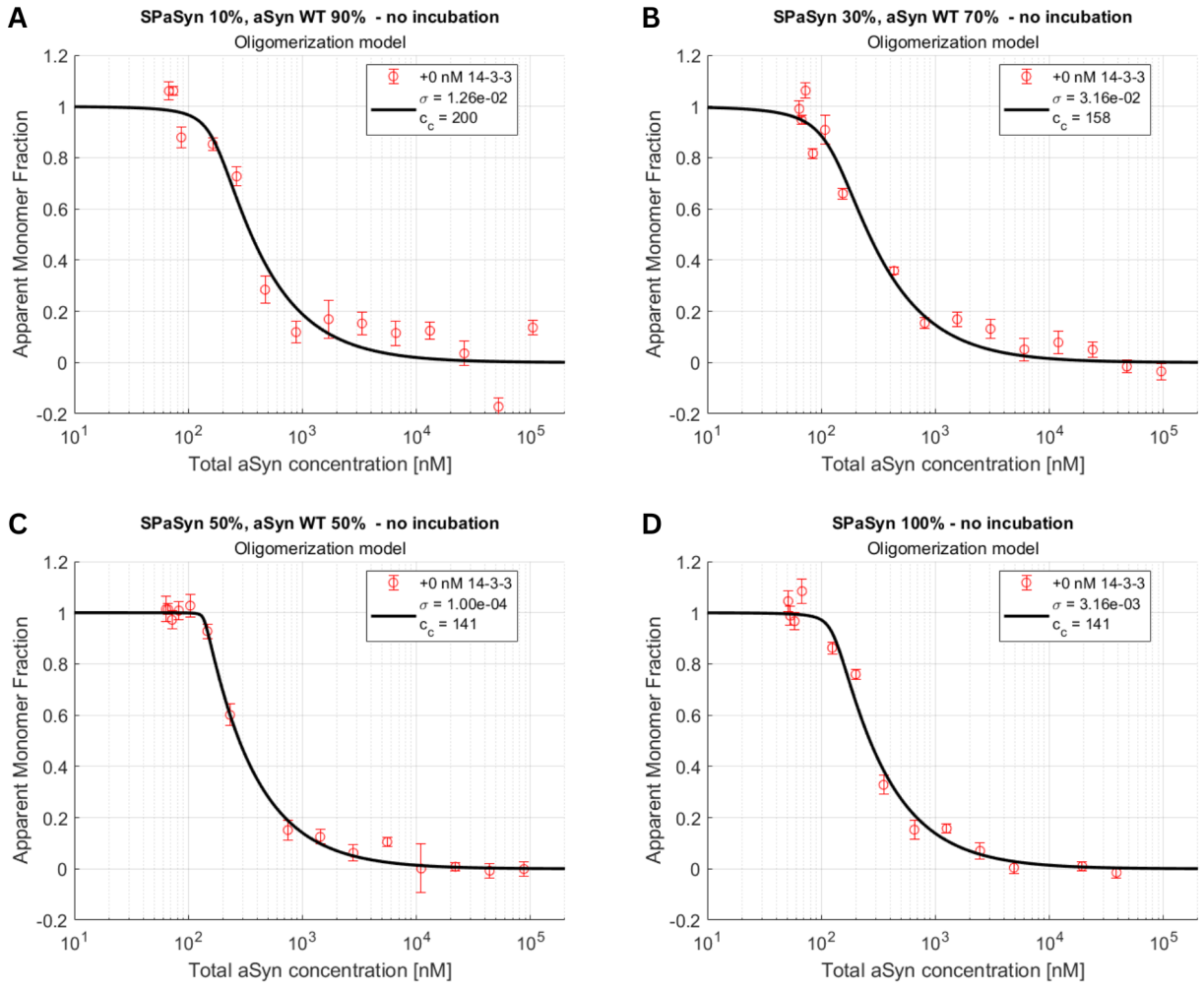

Figure 5: *Oligomerization curves of α-Syn, with different fractions of single phosphorylated α-Syn at Serine129. The fraction of single phosphorylated α-Syn was increased for each condition: 10% (**A**), 30% (**B**), 50% (**C**) and 100% (**D**) The highest initial total α-Syn concentration for each condition was 105μM, 95μM, 88μM and 77μM, respectively. The used dye concentration for the 100% condition was 20nM instead of 25nM.*

The possibility of having different oligomer populations at different ratios of single phosphorylated α-Syn complicates our ability to determine the influence of phosphorylation on 14-3-3τ's inhibitory effect on α-Syn oligomerization. Rather than solely looking at whether phosphorylation affects interactions with 14-3-3τ proteins, it is important to consider that the resulting oligomer population might also potentially play a role in affecting the interaction with 14-3-3τ. In order to minimize the possible confounding effects and to separate the two factors as much as possible, we decided to conduct the 14-3-3τ experiments with 30% and 100% single phosphorylated α-Syn. It is expected that the experimental condition with 100% single phosphorylated α-Syn reflects the effect of phosphorylation on 14-3-3τ most prominently. On the other hand, the condition with 30% is expected to relatively capture the effect of having a distinct population

the most. Compared to 10%, 30% is expected to reflect the effect of having a distinct population to a greater extent, as the fraction of phosphorylation is higher. Furthermore, the oligomerization curve for the 50% condition in Figure 5C remarkably shows insubstantial differences with the 100% condition in Figure 5D, considering the subtle differences between the σ and c_c values. Therefore, the choice for using 30% in the 14-3-3τ experiments is expected to embody the effect of having distinct populations most accurately. Identical to the experiment with α-Syn and 14-3-3τ in Section 3.2, three increasing concentrations of 14-3-3τ were studied in this experiment.

3.4 Single Phosphorylated α-Syn and 14-3-3τ

3.4.1 30% Single Phosphorylated α-Syn

The α-Syn aggregation curve for the MST results of 30% single phosphorylated α-Syn in the absence of 14-3-3τ is shown in Figure 6A. It is similar to the identical condition from Section 3.3 shown in Figure 5. While the nucleation factors are closely similar, there is a significant difference in the c_c value. The only difference between the two experiments was the usage of 50nM dye concentration in this experiment, instead of 25nM. The c_c value found in the experiment with 25nM dye was 158nM, whereas a 50nM dye concentration led to a value of 355nM. This finding indicates that the presence of AlexaFluor488 labelling possibly impacts the aggregation behaviour, which is further analyzed and discussed in Section 3.6.

The addition of 875nM 14-3-3τ resulted in a noticeably different value of the nucleation factor. However, after close examination of the curves shown in Figure 6A and B, the two curves do not differ significantly. The curves are plotted in one graph in Appendix D. Moreover, the data points for the 875nM condition have a larger error bar than the 0nM 14-3-3τ condition, particularly at the lowest α-Syn concentrations. These relatively large errors in the nucleation portion could have affected the overall trend of the curve and consequently affected the sigma value.

Following the addition of a higher concentration, 3.5μM of 14-3-3τ, the model for the α-Syn oligomerization remained unaltered. The found curve closely resembles the ones from the previous conditions and the corresponding σ has a value of 1.26×10^{-02}, which is similar to that of the 0nM condition.

In case of 7μM 14-3-3τ addition, there is a substantial change in the oligomerization behaviour as seen in Figure 6D. The curve suggests a reduced level of cooperativity of the α-Syn monomers, with a relatively more gradual decline in the monomer fraction. This change is comparable with stabilizing effect of 14-3-3τ on α-Syn oligomerization as established in Section 3.2. However, the results for 30% single phosphorylated α-Syn indicate that it is only at a concentration of 7μM where 14-3-3τ's chaperone activity is evident. Nevertheless, it is important to note that this system remained cooperative as σ did not exceed a threshold of 1.

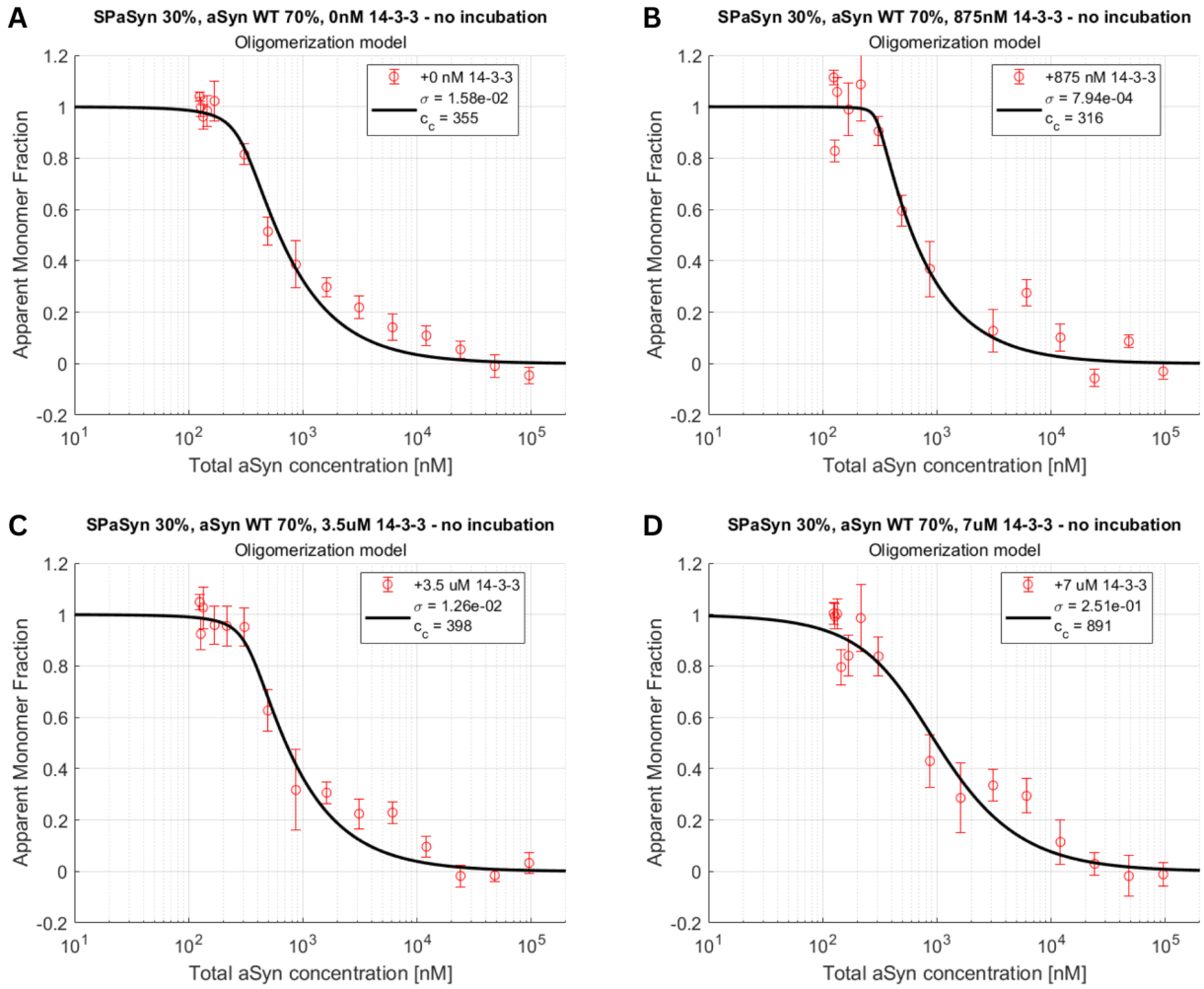

Figure 6: *Oligomerization curves of WT α-Syn, α-Syn, 30% single phosphorylated, in the presence of 14-3-3τ. The highest initial total α-Syn concentration was 95μM, 50nM originating from labelled α-Syn. The concentration of 14-3-3τ was increased for each condition: 0nM (**A**), 875nM (**B**), 3.5μM (**C**) and 7μM (**D**).*

Comparing these findings to those of Section 3.2, it is suggested that in the presence of single phosphorylated α-Syn, the influence of 14-3-3τ is less evident upon initial observation. However, upon analyzing the results in Section 3.3 it was suggested that the aggregate populations possibly significantly vary when modifying the amount of single phosphorylated α-Syn. The obtained results in this section provide evidence that having distinct oligomer populations influences the interaction with 14-3-3τ. This finding stresses the importance of experimenting with the 100% condition to study the effect of phosphorylation.

3.4.2 100% Single Phosphorylated α-Syn

In the absence of 14-3-3τ, single phosphorylated α-Syn is found to form oligomers according to a nucleation-elongation mechanism, like WT α-Syn, as a plateau is observed in Figure 7A. With the addition of 875nM 14-3-3τ, no nucleation was detected as its nucleation factor reaches a value of 1.00×10^{0}, indicating it follows a non-cooperative system. This finding is in line with the suggested stabilizing chaperone function of 14-3-3τ as described in Section 3.2. Contrary to the anticipated correlation based on the findings of the WT α-Syn experiment with 14-3-3τ, increasing the concentrations of 14-3-3τ does not result in enhanced stabilization effect of the chaperone protein. This reaffirms the recognized complexity of the interaction between α-Syn,

in particular, single phosphorylated α-Syn.

To further investigate this, conducting a series of MST experiments with various lower 14-3-3τ concentrations ranging from 0nM and 875nM could give valuable information on the concentration-dependent effects of 14-3-3τ. It is possible that the transition into a non-cooperative system occurs at lower concentrations than the measured condition of 875nM, which could be confirmed by the proposed additional experiment.

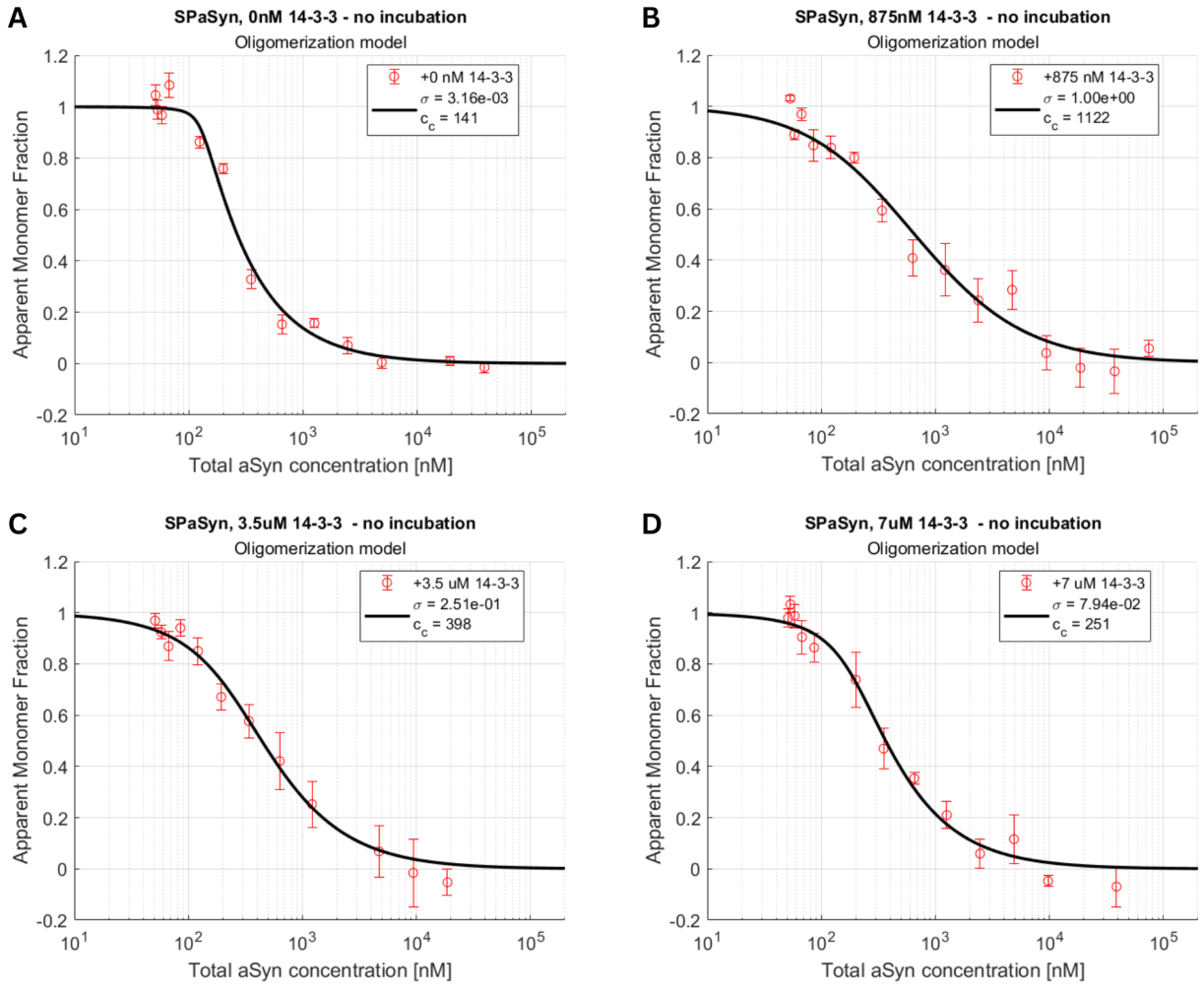

Figure 7: *Oligomerization curves of single phosphorylated α-Syn, in the presence of 14-3-3τ. The highest initial total α-Syn concentration was 77μM, 20nM originating from labelled α-Syn. The concentration of 14-3-3τ was increased for each condition: 0nM (**A**), 875nM (**B**), 3.5μM (**C**) and 7μM (**D**)*

The complete transition into a non-cooperative system, induced by the addition of 14-3-3τ, was only observed when 100% of the α-Syn was either non-phosphorylated or single phosphorylated at Serine129. The transition for the WT α-Syn system occurs at 3.5 μM, at which σ exceeds the threshold value of 1. In the case of the single phosphorylated α-Syn system, this is the case at a lower 14-3-3τ concentration, specifically at 875nM. This observation suggests that through single phosphorylation of α-Syn, 14-3-3τ is able to perform its stabilizing function at lower concentrations. Therefore, this finding indicates that 14-3-3τ potentially has a higher affinity with single phosphorylated α-Syn. It provides evidence that 14-3-3τ is able to perform its chaperone functions more effectively through α-Syn phosphorylation.

As the samples from the previous experiment contained WT α-Syn 70% besides single phosphorylated α-Syn, this confirms that 14-3-3τ performs its stabilizing function not only on the WT but also on the

single phosphorylated α-Syn. In particular, a reduced cooperativity through 14-3-3τ addition was found for 100% WT α-Syn and 100% single phosphorylated α-Syn at the lowest concentration of 875nM. For the 30%, this was only found at the highest 14-3-3τ concentration of 7μM. Therefore, the specific oligomer population caused by 30% single phosphorylated α-Syn, appears to inhibit 14-3-3τ's interaction with α-Syn. The disparity in these findings demonstrate the distinct effect of the two mentioned factors, phosphorylation and having different oligomer populations, on 14-3-3τ's interactions with α-Syn.

The F_{norm} data obtained from the MST experiments gives additional valuable information on the interaction of 14-3-3τ with solely single phosphorylated α-Syn. As the concentration of 14-3-3τ increases, the difference between F_{norm} values of the lowest and the highest concentrations reduces, which is shown in Appendix F. This could reflect the increased affinity of 14-3-3τ for single phosphorylated α-Syn, as previously established from the effect on the σ value at 875nM. The decrease in F_{norm} values at low α-Syn concentrations in the increasing presence of 14-3-3τ could indicate the increased affinity for the α-Syn monomers or the smallest formed α-Syn oligomers. This provides further explanation for the decrease in the σ value at a 14-3-3τ concentration of 875nM, as an increased level of affinity could have altered the observed oligomerization curve. The previously mentioned proposed MST experiment with various 14-3-3τ concentrations ranging from 0nM and 875nM could confirm the suggested elevated level of affinity, by focusing on the obtained F_{norm} data and the change in σ values.

Initially, identical to the previous experiment, a dye concentration of 50nM was used by adding Alexa labelled α-Syn-P129Ser, which had a DOL of 0.41. However, the MST results did not show a plateau at the lowest concentrations, suggesting that the total concentration of initial α-Syn was too high to detect one. This can be observed in the F_{norm} data shown in Appendix E. It was decided to prepare the samples with 20nM dye instead of 50nM dye in order to investigate the MST response at lower total α-Syn concentrations. A dye concentration of 10nM could not reach an optimal fluorescence intensity for all 16 capillaries between 200 and 1500. However, 10nM dye was not necessary since the results with 20nM dye show that the lower total α-Syn concentrations were sufficient low to obtain a plateau.

3.5 Time-dependent Effects

Up until now, we have chosen to disregard time-dependent effects by performing experiments directly after sample preparation. This decision was made to avoid complicating the studied system. However, considering the transient and dynamic nature of α-Syn, following its oligomerization behaviour over time could provide valuable information on the temporal changes. Ultimately, studying the combined effects multiple factors such as time, single phosphorylation and 14-3-3τ could provide a more realistic representation of the complex interactions.

It was established that the WT α-Syn follows a cooperative system for its oligomer formation. The oligomerization model can only be applied to describe the cooperativity of the oligomerization process of a system and does not take kinetics into account. While the model cannot directly explain the changes observed over time, it can still be used to determine relative changes in the formed oligomers as time progresses, resulting in changes in thermophoretic behaviour. Hence, we measured α-Syn oligomerization at six multiple time points ranging from t = 0s and t = 40h. The change in thermophoretic behaviour can be observed in the model as a decrease in the apparent monomer fraction for those samples at the lowest concentrations. This is observed in the oligomerization curve in Figure 8B for an incubation period of 30 minutes. The nucleation factors obtained for the time series can be used to determine the time point at which the populations of oligomers are no longer subject to change, plotted in Figure 8D. The curve and nucleation factor of the measurement after 40 hours are closely similar to the first incubation measurement. Based on these findings, it appears that the oligomer population is only changing in the first 30 minutes, after which it remains stable. Consequently, it was decided to measure all batches for a second time after an incubation period of 20 hours. However, none of the experiments with 14-3-3τ resulted in conclusive findings regarding potential time-dependent effects on the chaperone functions of protein 14-3-3τ.

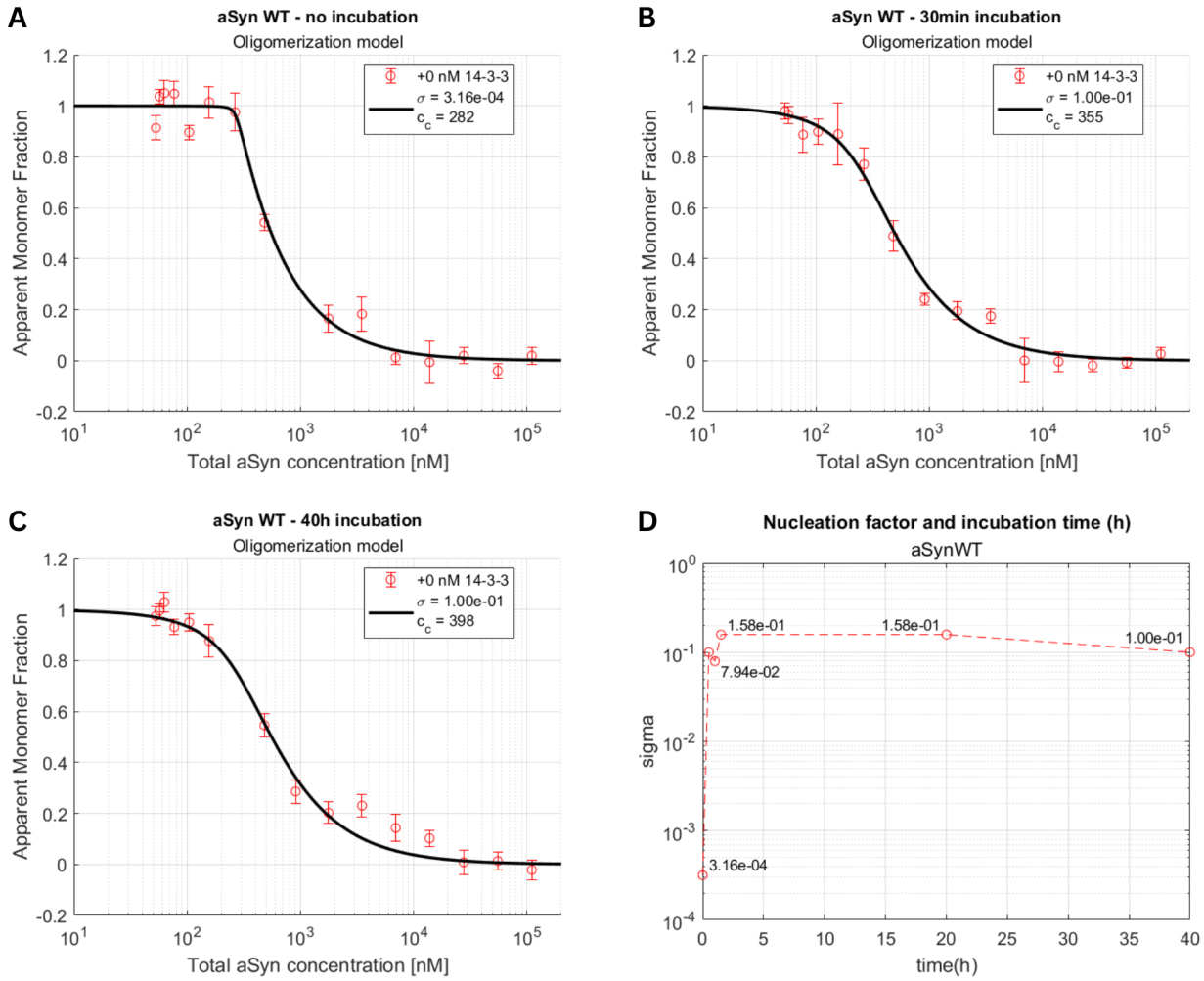

Figure 8: *Aggregation curves of α-Syn WT, starting at a α-Syn concentration of 110μM, with 50nM originating from the labelled α-Syn. Sufficient sample volumes were prepared for the 16 concentrations to enable multiple MST experiments over a period of incubation time. The remaining portions of the original samples were placed in the incubator after loading a portion into the capillaries for each experiment. The aggregation curve obtained from the first MST measurement, without incubation, is shown in graph* ***A****. Graph* ***B*** *and* ***C*** *show the results after an incubation period of 30min and 40h, respectively. in Figure* ***D*** *the nucleation factors are plotted for every performed experiment, with the specific incubation time.*

3.6 Influence of Labelling on the Oligomerization

Studies suggest that the labelling of α-Syn possibly affects the properties of the monomer and its oligomerization behaviour. [24] In this research, we used different dye concentrations of AlexaFluor488 for the same experimental condition. 100% single phosphorylated α-Syn oligomerization was studied with MST in three different experiments, each case involving a different dye concentration: 10nM, 20nM and 50nM. Comparison of the obtained F_{norm} values for the three dye concentration can provide insights into the influence of α-Syn labelling with AF488. To compare the trend in F_{norm} of the three cases, the F_{norm} values were adjusted relative to their lowest F_{norm} value and plotted in the same graph, shown in Figure 9A. As the three measurements were conducted with different LED powers, this correction method accounts for the resulting differences in absolute F_{norm} values and enables a focus on the relative changes.

In Figure 9A, the F_{norm} values differ at total α-Syn concentrations lower than 150nM for the three dye

concentrations, suggesting different thermophoretic behaviour of populations with identical total α-Syn concentrations. In particular, it appears that more α-Syn monomers were needed for oligomer formation when a larger fraction of the α-Syn is labelled. Therefore, the overall found differences in F_{norm} at these low concentrations indicate a difference in oligomerization behaviour between labelled and unlabelled phosphorylated α-Syn.

The MST experiment with 30% single phosphorylated α-Syn and 70% WT was performed in two different experiments. One measurement involved a dye concentration of 25nM, whereas the other one was performed with a dye concentration of 50nM. The referenced F_{norm} values for both cases are plotted in the same graph in Figure 9B. In this case, the dye concentration consisted of two differently labelled α-Syn. With both dye concentrations, a plateau is observed for the lowest concentrations. For the 25nM dye experiment, the c_c value was found to be around 158nM, whereas 50nM resulted in a value of 355nM, as mentioned in Section 3.4.1. Increasing the dye concentration resulted in a shift of the plateau to a higher concentration, supporting the previous finding where oligomerization occurs at higher α-Syn concentrations. It provides evidence to the revelation that more α-Syn monomers are necessary to form a oligomers when a larger number is labelled, indicating a difference in oligomerization behaviour between labelled and unlabelled α-Syn. While more research on direct comparison of the labels is needed, these findings suggest that the labelling of α-Syn possibly influences the oligomerization of the monomers. Therefore, this could affect interpretations of results of any experiment involving labelled α-Syn.

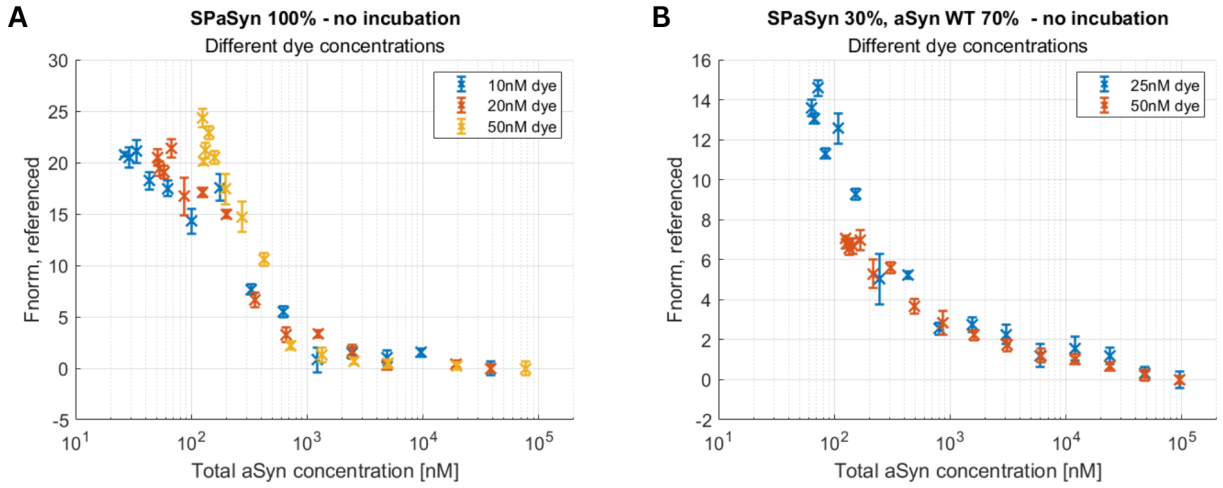

Figure 9: *F_{norm} data for **A**) single phosphorylated α-Syn and **B**) 30% single phosphorylated α-Syn and 70% WT α-Syn, with different dye concentrations. A discrepancy in F_{norm} values for the lowest total α-Syn concentrations can be observed in **A**. **B** illustrates the shift of the plateau to higher total α-Syn concentrations for higher dye concentrations. The F_{norm} data is referenced to the highest total α-Syn concentration.*

3.7 Aggregation Assay

The experimental conditions for this ThT assay involved 14-3-3τ at concentrations of: 0μM, 1μM, 5μM and 10μM. The lag times obtained from the WT α-Syn aggregation data for the various 14-3-3τ concentrations are plotted in Figure 10A. Figure 10B contains the lag times for the aggregation data of α-Syn where 30% was phosphorylated. The aggregation data is included in Appendix G. Based on the results for the 0μM condition, the presence of phosphorylated α-Syn appeared to decrease its lag time, suggesting that phosphorylation of α-Syn promotes fibril formation. This is in line with the finding from Section 3.3, where increasing fractions of single phosphorylated α-Syn were established to result in higher association propensity. It provides further evidence for the hypothesis that the presence of a phosphate group at Serine129 promotes oligomer and subsequent fibril formation. [14]

As apparent in Figure 10A, the addition of 14-3-3τ to WT α-Syn decreases the lag time, suggesting that

14-3-3τ enhances the propensity of fibril formation. This does not support the finding and inference from Section 3.2, where the observed decreasing level of cooperativity with increasing 14-3-3τ concentrations was thought to stabilize and subsequently delay early oligomer formation. These contrasting findings reinforce the established complexity of α-Syn aggregation into higher-structure fibrils, as most likely various other factors besides early oligomer behaviour are involved. Therefore, it is possible that a decreased level of cooperativity does not directly translate to delayed α-Syn aggregation. Furthermore, it is important to note that our results are also not in line with other literature, which reduces the overall reliability of the obtained results from this ThT assay. [11–13]

The lag times for 30% phosphorylated α-Syn did not show a clear trend, as observed in Figure 10B. Remarkably, the addition of 1μM 14-3-3τ resulted in a subtle increase in the lag time of the aggregation process. There is a possibility that this result shows that 14-3-3τ stabilizes early oligomers through binding to phosphorylated α-Syn sites, which was discussed in Section 3.4.2. However, it is worth emphasizing that the observed prolongation of the lag time is not significant. Therefore, even if there is an impact, it most likely cannot be considered as significant. Identical to the results from 3.4.2, no direct relationship was found between the concentration of added 14-3-3τ and the potential stabilization effect. Additional ThT assays with 14-3-3τ concentrations in the range of 0μM and 1μM could give more valuable insights into the dependency of 14-3-3τ's chaperone function on concentration, which is similar to the proposal made for additional MST experiments in Section 3.4.2. Furthermore, an additional ThT assay with solely single phosphorylated α-Syn and identical concentrations of 14-3-3τ could exclude the possibility of confounding effects.

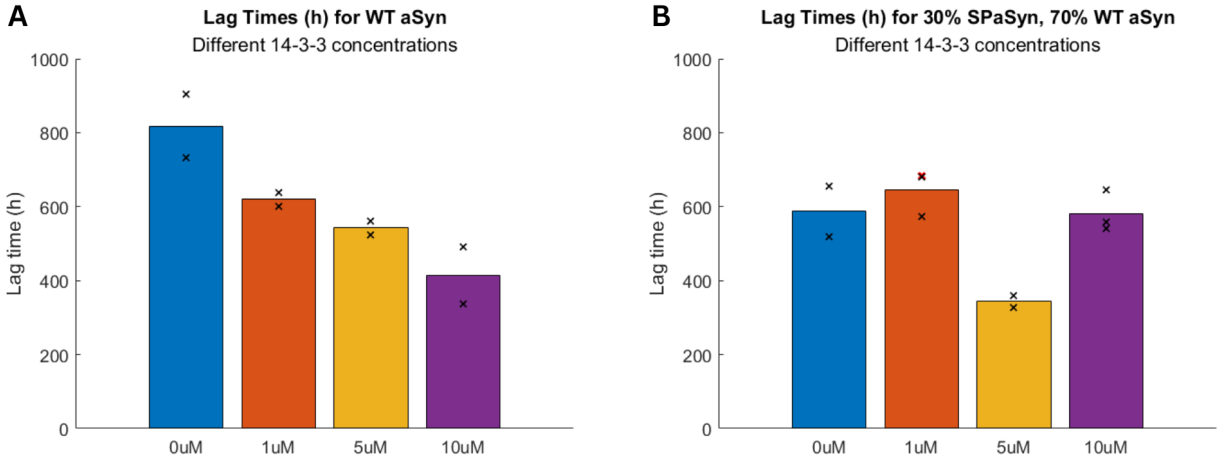

Figure 10: *Lag times (h) obtained from the aggregation data with various 14-3-3τ concentrations for* ***A****) WT α-Syn and* ***B****) 30% single phosphorylated α-Syn and 70% WT α-Syn. The 14-3-3τ concentration was increased for each condition: 0μM, 1μM, 5μM and 10μM. The bars in the graphs indicate the mean lag time of the replicates. The individual lag time is plotted with a cross marker for each replicate.*

To improve the accuracy of the lag time calculation and subsequent data analysis, we removed an outlier from the triplicates when the calculated standard deviation was larger than 100 hours. The variation in the observed lag times can be attributed to the nature of fibril formation, which is a spontaneous process susceptible to influences from external factors. However, identifying and excluding extreme values is required to enable a comparative analysis of the lag times. As a result, one outlier was removed from each condition, except for the 30% single phosphorylated α-Syn conditions with 1μM and 10μM 14-3-3τ.

It is important to note that within the measured time-frame, the fluorescence intensity did not show a plateau for every condition. This indicates that α-Syn was still in the exponential phase of the characteristic sigmoidal curve, which corresponds to unfinished fibril growth.

4 Conclusion

In this study, the effect of α-Syn phosphorylation on the chaperone activity of 14-3-3τ was studied by detecting early oligomer behaviour with MicroScale Thermophoresis. The oligomerization of WT α-Syn was found to follow a nucleation-elongation mechanism, with cooperativity of α-Syn monomers leading to the formation of a nucleus. 14-3-3τ was observed to decrease the cooperativity, inhibiting early WT α-Syn oligomer formation.

The study on the influence of phosphorylation on the interaction of 14-3-3τ became more intricate as the discovery was made that phosphorylation of different fractions of α-Syn results in the formation of distinct oligomer populations. To distinguish the effect of phophorylation from the effect of having different oligomer populations on 14-3-3τ, we decided to perform the 14-3-3τ experiments with 30% and with 100% single phosphorylated α-Syn. We discovered that the 30% single phosphorylated oligomer population possibly inhibits the chaperone activity of 14-3-3τ. Comparing the results of 100% single phosphorylated α-Syn with 100% WT α-Syn, we found a higher affinity of 14-3-3τ for single phosphorylated α-Syn. This provides valuable insights into the protein quality control involving 14-3-3τ in the context of α-Syn.

An additional experiment was proposed to further investigate the concentration dependent effects of 14-3-3τ in the presence of solely single phosphorylated α-Syn, as an increase in 14-3-3τ did not lead to stronger chaperone activity. Furthermore, we have discovered a potential affect of α-Syn labelling with AlexaFluor488 on the oligomerization behaviour of α-Syn, which needs to be considered for future research. Lastly, no definitive conclusions can be made from the aggregation assay, as the results are not in line with existing literature. Therefore, more research is needed to establish the relationship between early oligomerization and the aggregation into amyloid fibrils.

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Appendix A: MST Trace Outliers

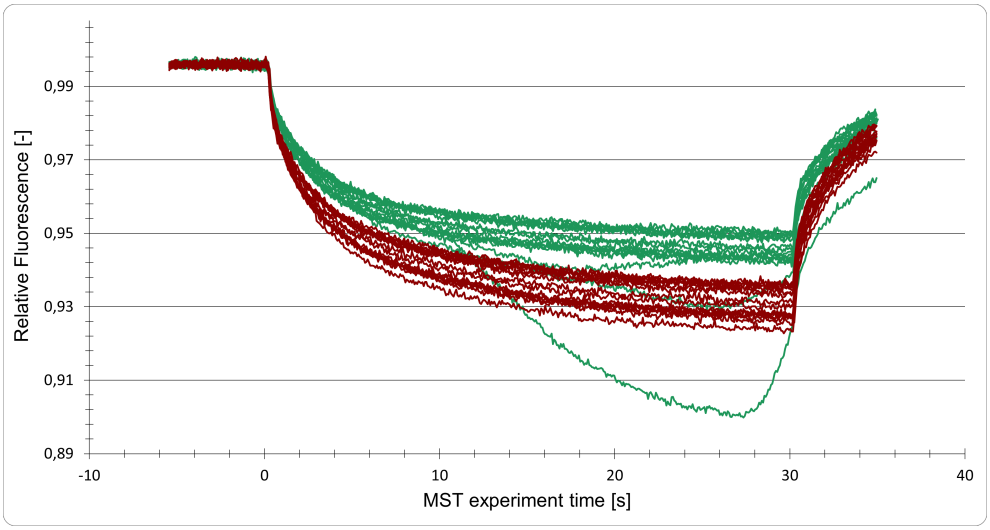

Figure 1: *MST trace outliers in capillaries #2, #5 and #10 found for 14-3-3τ the 0nM condition in the experiment with 14-3-3τ in section 3.2. The outliers were only observed and therefore removed for the 60% MST power measurement. Possible causes include are inhomogeneities or larger formed oligomers in the samples during the measurement.*

Appendix B: Control Experiment

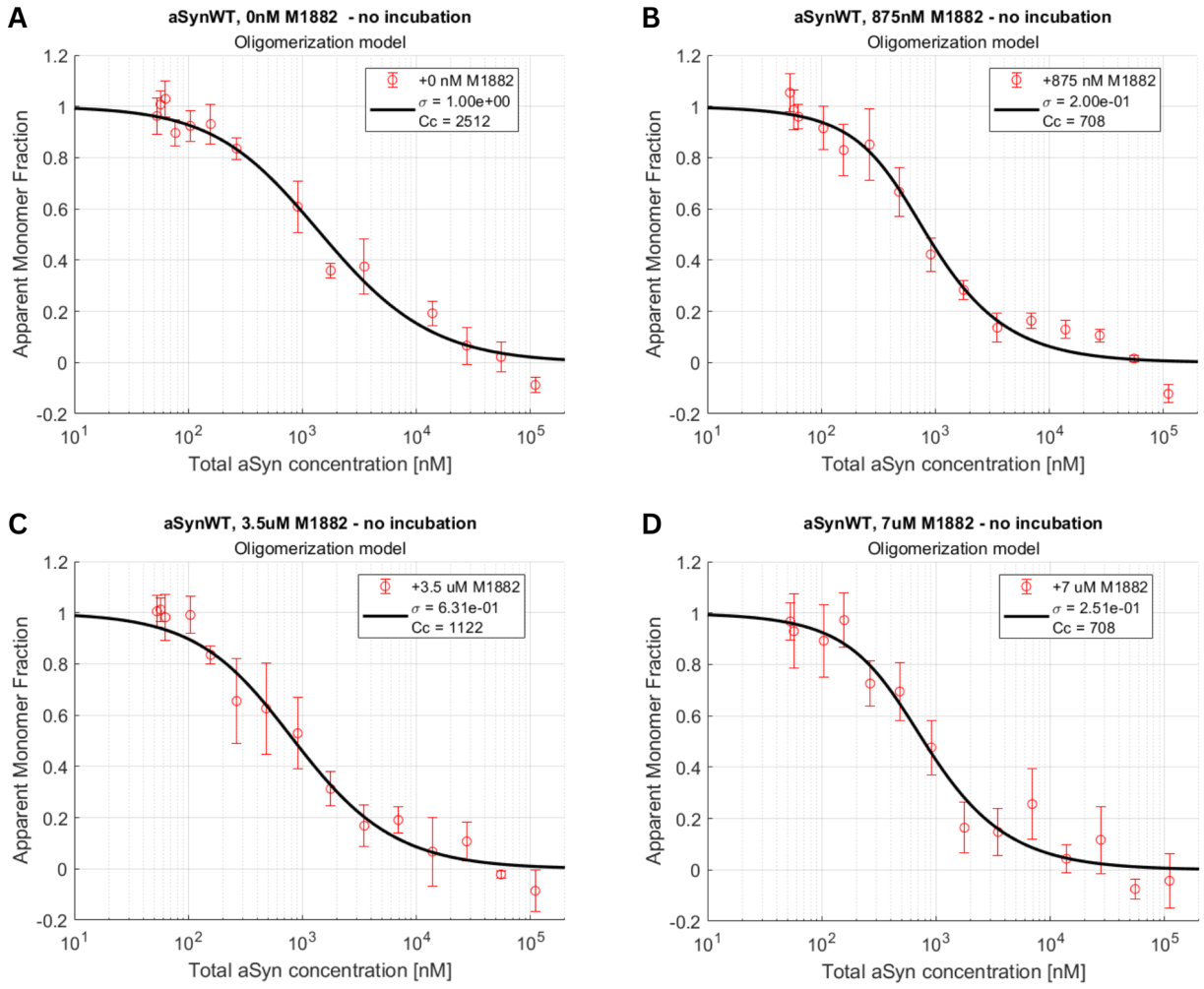

Figure 2: *Control experiment with Myoglobin at concentrations 0nM, 875nM, 3.5 μM and 7.0 μM.*

Appendix C: Fractions of Single Phosphorylated α-Syn

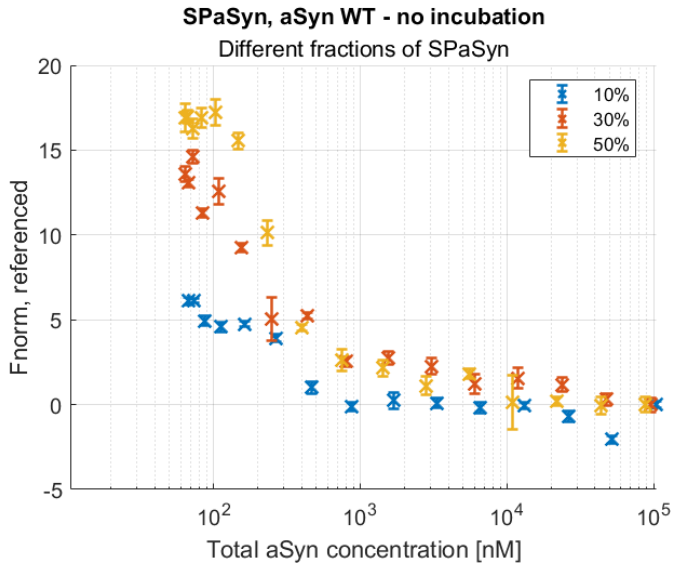

Figure 3: *F_{norm} data for different fractions of single phosphorylated α-Syn: 10%, 30% and 50%. Each fraction shows distinct change in thermophoretic behaviour with increasing concentrations of α-Syn. The F_{norm} data is referenced to the highest total α-Syn concentration, to enable better visualization of the relative differences between the data points.*

Appendix D: 0nM and 875nM (70% WT)

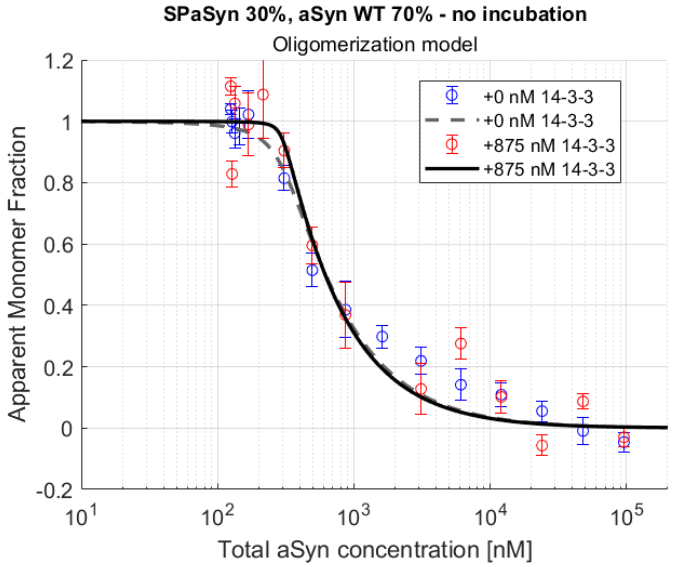

Figure 4: *Obtained oligomerization curves for 30% single phosphorylated and 70% WT α-Syn with conditions 0nM and 875nM 14-3-3τ. The curves show a high degree of similarity, despite the disparity in the σ values.*

Appendix E: α-Syn-P129Ser with 50nM Dye

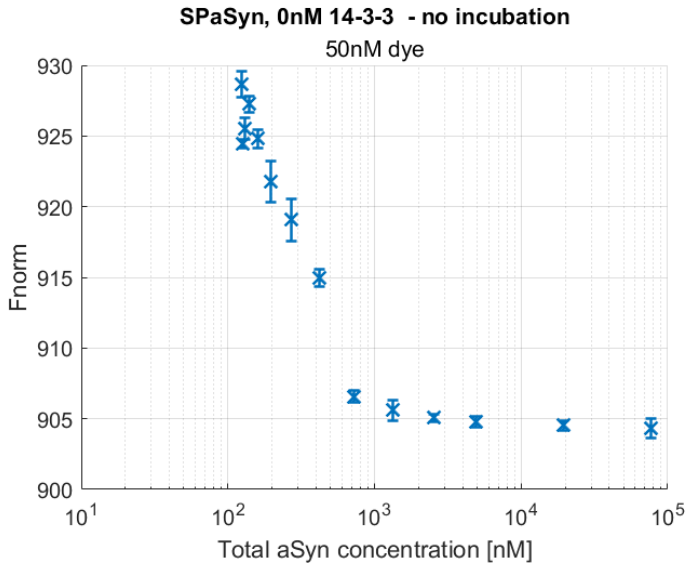

Figure 5: *The F_{norm} data points for 100 % single phosphorylated α-Syn, with a dye concentration of 50nM. At the lowest α-Syn concentrations from this experiment, no plateau is detected.*

Appendix F: α-Syn-P129Ser F_{norm} Data

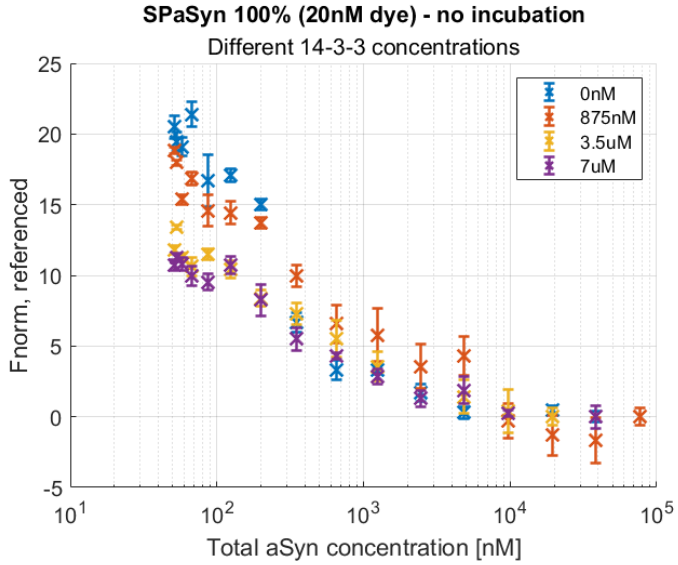

Figure 6: *By increasing the 14-3-3τ concentration, the difference between F_{norm} values of the lowest and highest total single phosphorylated α-Syn concentration reduces. As discussed in section 3.4.2, this could indicate 14-3-3τ has a role in the disassembly of α-Syn oligomers. The F_{norm} data is referenced to the highest total α-Syn concentration, to enable better visualization of the relative differences between the data points.*

Appendix G: Aggregation Assay Data

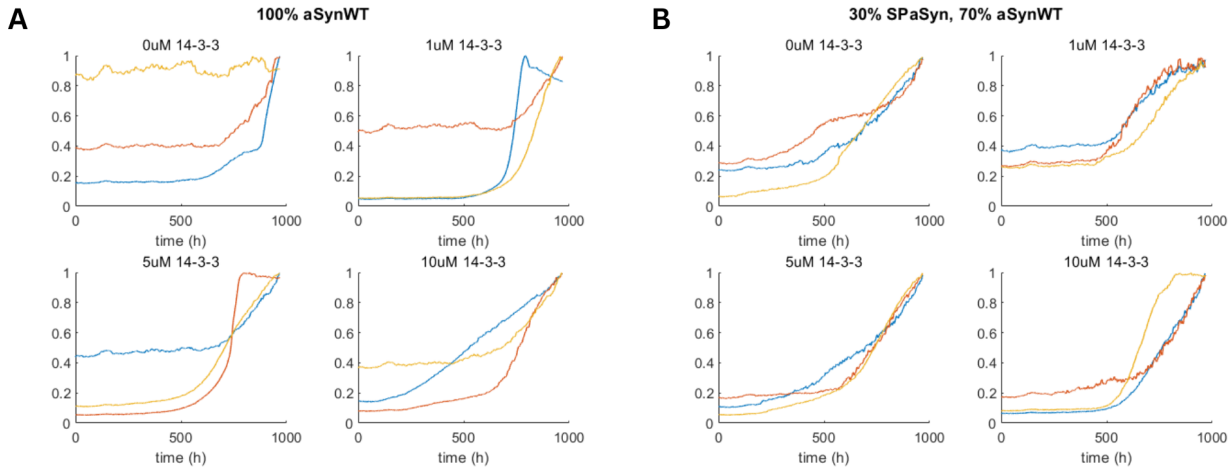

Figure 7: *Normalized aggregation data for* ***A****) WT α-Syn and* ***B****) 30% single phosphorylated α-Syn and 70% WT α-Syn with increasing concentrations of 14-3-3τ. Outliers can be identified in all graphs, except for the conditions 1μM and 10 μM shown in* ***B****.*