University of Twente

Bachelor Thesis

A cell model to study the effect of SARS-CoV-2 infections on α-Synuclein aggregation

Visscher, L.J. (s2487764)

Supervisors:
K.A. van Leijenhorst - Groener
S.A. Semerdhiev

Nanobiophysics
Faculty Science and Technology
University of Twente
Netherlands
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Abstract

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. It is estimated that over 1.1 million individuals suffer from PD worldwide. It is known that α-Synuclein is vital in the pathology of PD. Especially the aggregation of α-Synuclein into amyloid fibrils. The aggregation of α-Synuclein disrupts the healthy mitochondrial activity starting a vicious cycle that causes an increase in neural death. It is suspected that viruses could cause the initial aggregation of α-Synuclein into amyloid fibrils. This is based on the increase in PD patients after the Spanish flu or influenza virus (H1N1) pandemic. The SARS-CoV-2 virus shares multiple similarities to the H1N1 virus, and the symptoms caused by the SARS-CoV-2 are akin to the symptoms of PD. This led to speculation about whether SARS-CoV-2 could cause post-infectious parkinsonism. This research paper looked at the nucleocapsid protein (N-protein) of SARS-CoV-2 and its ability to aggregate α-Synuclein in SH-SY5Y cells. A microscale thermophoresis (MST) measurement showed that the N-protein does bind to α-Synuclein and a ThT aggregation assay indicated that the N-protein increases the rate at which α-Synuclein aggregates. The N-protein was introduced to the SH-SY5Y cells with transfection. However, after multiple attempts, no conclusions could be made as either the cell count or efficiency of transfection was too low.

Samenvatting

De ziekte van Parkinson (PD) is de meest voorkomende neurodegeneratieve bewegingsstoornis. Naar schatting lijden wereldwijd meer dan 1,1 miljoen mensen aan de ziekte van Parkinson. Het is bekend dat α-Synuclein essentieel is in de pathologie van PD. Vooral de aggregatie van α-Synucleïne tot amyloïde fibrillen. De aggregatie van α-Synucleïne verstoort de gezonde mitochondriale activiteit waardoor een vicieuze cirkel ontstaat die een toename van neurale dood veroorzaakt. Vermoed wordt dat virussen de initiële aggregatie van α-Synucleïne tot amyloïde fibrillen kunnen veroorzaken. Dit is gebaseerd op de toename van PD-patiënten na de pandemie van de Spaanse griep of het griepvirus (H1N1). Het SARS-CoV-2-virus vertoont meerdere overeenkomsten met het H1N1-virus en de symptomen veroorzaakt door SARS-CoV-2 lijken op de symptomen van PD. Dit leidde tot speculaties over de vraag of SARS-CoV-2 postinfectieus parkinsonisme zou kunnen veroorzaken. In dit onderzoeksartikel werd gekeken naar het nucleocapside-eiwit (N-eiwit) van SARS-CoV-2 en het vermogen ervan om α-Synucleïne in SH-SY5Y-cellen te aggregeren. Een microschaal thermoforese (MST) meting toonde aan dat het N-eiwit bindt aan α-Synucleïne en een ThT-aggregatietest gaf aan dat het N-eiwit de snelheid verhoogt waarmee α-Synucleïne aggregeert. Het N-eiwit werd met transfectie in de SH-SY5Y-cellen geïntroduceerd. Na meerdere pogingen konden er echter geen conclusies worden getrokken omdat het aantal cellen of de efficiëntie transfectie te laag was.

Contents

1 Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. The disease causes motor symptoms such as tremors, bradykinesia and postural instability. PD also has non-motor symptoms including but not limited to depression, memory impairment and sleep disorders. [1] PD has a severe impact on the quality of life and the incidence of the disease is increasing. In 2019 it was estimated that over 1.1 million individuals suffer from PD. [2]

α-Synuclein is pivotal in the pathology of PD. α-Synuclein is an intrinsically disordered protein (IDP). This means that, in solution, it has no stable tertiary structures. [3] The function of α-Synuclein remains unclear. [4] However, it has been proven that α-Synuclein has an affect on the mitochondrial activity. With PD there is an abnormal accumulation and aggregation of α-Synuclein into amyloid fibrils. This causes interruption of normal mitochondrial activity which leads to abnormal mitochondrial morphology and accumulation. It also causes a decreased basal mitochondrial oxygen consumption rate. This leads to oxidative stress which diminishes the ubiquitin-proteasome system. This in turn causes a build-up of abnormal proteins which includes aggregated α-Synuclein. That starts a vicious cycle in which oxidative stress and neural death increase. [5]

The initial aggregation of α-Synuclein into amyloid fibrils could be caused by a virus infection. Literature shows that most influenza viruses or specific parts of the viruses are able to aggregate α-Synuclein. For instance, a study by Marreiros et al. has shown that the Spanish flu or influenza virus (H1N1) causes α-Synuclein to aggregate in Rag knockout mice in vivo. [6] In the case of influenza, there can be a large delay between the contracting of the virus and the diagnosis of PD. This delay can be as large as ten years. [7] Though nothing definitive has been proven, these findings do suggest that there is a link been virus infection and α-Synuclein aggregation resulting in the development of PD.

The possible ability of viruses to induce α-Synuclein aggregation and cause PD has recently become extremely relevant with the SARS-CoV-2 pandemic. Though SARS-CoV-2 and H1N1 are different viruses they do share similarities in their pathophysiological mechanisms. Both cause proteostasis and mitochondrial dysfunction. [8, 9] Therefore, it is plausible that SARS-CoV-2 also has the ability to cause post-infectious parkinsonism.

In addition, even though SARS-CoV-2 is considered a respiratory pathogen, virus particles have been found in the brains of SARS-CoV-2 patients. [10] Patients can have multiple neurological symptoms. These symptoms range from a headache to delirium and depression. [11] A hallmark symptom of SARS-CoV-2 infection is hyposmia with 65% of patients experiencing this. [12] Hyposima is also one of the first premotor symptoms of PD. With SARS-CoV-2 neurological symptoms, the similarities between the symptoms of SARS-CoV-2 and the early onset symptoms of PD and the similarities between the H1N1 virus, it is plausible that SARS-CoV-2 is another virus that can cause post-infectious parkinsonism.

This possible link has led to an investigation into the ability of the proteins from the SARS-CoV-2 virus to accelerate the aggregation of α-Synuclein into amyloid fibrils. Research by Semerdzhiev et al. specifically looked at the SARS-Cov-2 spike protein (S-protein) and the SARS-Cov-2 nucleocapsid protein (N-protein). The results show that the S-protein has no effect on the aggregation of α-Synuclein. The presence of the N-protein, on the other hand, shows a significant decrease in the lag time of the aggregation. One of the reasons N-protein has an effect on the aggregation of α-Synuclein is a result of electrostatics, α-Synuclein has a net charge of -9e where the N-protein has a net charge of +24e. [13] The test tube experiment also showed that the N-protein causes the fibril formation to proceed faster and in an abnormal 2-step process.

After the initial results showed the decreases in lag time Semerdzhiev et al. use microinjections to add N-protein to SH-SY5Y cells. SH-SY5Y cells express α-Synuclein and are often used for in vitro PD studies. [14] Furthermore, the presence of N-protein causes a disturbance to the α-Synuclein proteostasis, leading to less vesicle bound α-Synuclein. [13] This begs the question, if the N-protein was added with stable transfection, thereby foregoing the microinjections and simulating an in vitro test that is closer to the in vivo situation with patients who are infected with SARS-CoV-2, would the effects of the N-protein on α-Synuclein still be the same? The expectation is that the results will be comparable to the results of Semerdzhiev et al. as the different method of introducing the N-protein into the SH-SY5Y cells should not change the effect of the N-protein on α-Synuclein aggregation.

2 Materials and Methods

2.1 Test tube experiments

2.1.1 Microscale Thermophoresis

First, it needed to be confirmed that N-protein does bind to α-Synuclein. To investigate the binding efficiency between α-Synuclein and the N-protein a Microscale Thermophoresis (MST) was done. MST uses an infrared laser to induce a local increase in temperature. This temperature increase causes a change in the spatial concentration distribution. This spatial concentration distribution is dependent on whether or not the ligand is bound to the other protein present. Because one of the proteins is fluorescently labelled, the local change in concentration can be monitored and measured. An MST measurement is performed with multiple concentrations of one protein while the concentration of the fluorescent protein is kept constant. This means that a binding curve over the different concentrations can be made from the measured fluorescence. [15]

The MST measurement was performed with the Monolith Nt.115 (NanoTemperTechnologies GmbH, Germany) MST system. The buffer used in the MST measurements contained; 20mM Tris (Sigma-Aldrich, UK), pH = 7.4, 0,02 wt% Sodium Cloride, 10 mM NaCl (Sigma-Aldrich, USA). Since an MST assay needs one of the binding partners to be labelled, labelled N-protein was used at a constant concentration. The concentration used was determined by first performing a capillary scan. The fluorescence intensity of different concentrations of labelled N-protein between 5400 and 10 mM were measured. The fluorescence intensity should be between 200-1000, this was done with the LED power at 80% to keep the needed concentration of N-protein as low as possible. Then 16 conditions of a 1:1 dilution series of α-synuclein were made with a starting concentration of 125 mM and a lowest concentration of 8 nM. The dilutions were put into capillaries (Standard treated, NanoTemper Technologies GmbH, Germany) and measured at 37°C with a LED power of 80%. The measurement started 5 seconds before the MST infrared laser was turned on for 30 seconds and ended 5 seconds after. There was a 25-second delay between measuring the capillaries. Three measurements were done, each with a different MST infrared laser power (MST power), the intensities used were; 20%,40% and 80% power. The raw data was then analysed with MO. Affinity Analysis 2.1 software (NanoTemper MOAA). The MO. Affinity Analysis 2.1 was also used to make a Kd fit model.

The Kd fit model describes the binding between molecules with a 1:1 stoichiometry according to the law of mass action. It also calculates the dissociation constant (Kd) value. The Kd is the concentration of ligand, in this case α-Synuclein, where half of the target molecules are bound to the ligand. The lower this concentration the higher the binding affinity.

2.1.2 Preparation labelled N-protein

The purified N-protein was labelled using AlexaFluor 488 NHS - ester (Thermo Fisher Scientific, USA) targeting the accessible amino groups of the protein. 40 µM N-protein in 20 mM Hepes, pH = 8, 100 mM NaCl was incubated with 3-fold excess of AlexaFluor 488 NHS at room temperature, under gentle agitation, for 2 hours in the dark. The reaction mixture was then transferred to 2 ml ZebaSpin MWCO 7 kD desalting column (Thermo Fisher Scientific, USA) to separate the labelled protein from the free dye. The columns were pre-equilibrated by washing them with 20 mM Hepes, pH = 8, 100 mM for three consecutive times by spinning them at 1000 G for 2 minutes for each washing step. The reaction mixture was spun down for 2 minutes at 1000 G and the eluate containing the labelled protein was collected for further characterization. The degree of labelling (DOL) of 1 was determined by means of UV-Vis absorbance spectroscopy.

2.1.3 Thioflavin T aggregation assay

It was necessary to confirm that the N-protein has an effect on the aggregation of α-Synuclein. Therefore, a Thioflavin T aggregation assay was performed to study the effect of the N-protein on the aggregation α-Synuclein. Thioflavin T (ThT) is a fluorescent dye whose emission increases when bound to the β-sheet structure of amyloid fibrils. Therefore, the aggregation of α-Synuclein into amyloid fibrils can be followed over time by looking at the increase of fluorescence. [16]

The aggregation assay was done on a 96-well half-area clear flat-bottom polystyrene NBS (low bind) microplate (3881, Corning, US). The protocol from Wördehoff et al. was used to prepare the samples. [16] In the aggregation array four different conditions in triplicates were used; only α-Synuclein, α-Synuclein and the N-protein, α-Synuclein and GFP and α-Synuclein, GFP and the N-protein in one. The conditions to test the effect of GFP on the aggregation of α-Synuclein with and without the N-protein were included because N-protein tagged with GFP will be added to the SH-SY5Y cells. Therefore, it was necessary to know if GFP has an effect on the aggregation before adding GFP-tagged N-protein to the cells. The Thioflavin T aggregation assay was also used to determine the effect of cell lysate on the aggregation of α-Synuclein in the presence of the N-protein. Three different concentrations of cell lysate were used; 73%, 63% and 53%. Each condition used a buffer of 20mM Tris (Sigma-Aldrich, UK), pH = 7.4, 0,02 wt% Sodium Cloride, 5μM ThT (Fluka, Sigma-Aldrich, UK), 10 mM NaCl (Sigma-Aldrich, USA). 1μM GFP for the conditions with GFP. The N-protein had a concentration of 1μM. Lastly, the α-Synuclein was added with a concentration of 50μM. The α-Synuclein was recombinantly produced following the protocol as described by Semerdzhiev et al. [13] To follow the aggregation, the intensity of the ThT fluorescence was measured with a plate reader (Infinite 200 Pro, Tecan Ltd., Switzerland) at 37°C with 10 minutes of orbital shaking between the measurements. The samples were measured with 5 flashes. The ThT dye was excited at 445 nm and the fluorescence signal was measured at 485 nm. The data collected by the plate reader was then analysed with MATLAB. The time that is measured before the first aggregation is seen is referred to as the lagtime. The lagtime was determined by MATLAB by finding the first value that is higher than 3 times the initial value.

2.2 In vitro expermiments with SH-SY5Y Cells

2.2.1 Cell culture

The SH-SY5Y cells (ATCC, USA) were cultured in a medium consisting of DMEM-F12, 10% FBS, 1% Pen/Strep and 1% MEM-non-essential amino acids (Gibco, Invitrogen, USA) and 10 mM HEPES pH 7.4 made by Utwente. T25 and T75 culture flasks were used to culture the cells.

2.2.2 Optaining Cell lysate

The lysate was made from T75 culture flask with a confluence of 95% with SH-SY5Y cells. The cells were washed twice with PBS (10010023, Gibco, Thermo Fisher). The cells were detached from the T75 flask with trypsin (25200056, Gibco, Thermo Fisher) and kept in a cells solution with 10ml 10% FBS medium. The cells were counted with EVE™ Automated Cell Counter (NanoEtek). After which the cells were centrifuged at 300 G for 5 minutes. The cells were then dissolved in 10 ml PBS with protease inhibitor(4693132001, Roche). Then after another 5 minutes in the centrifuge, the cells were dissolved in 5 ml PBS with protease inhibitor. The cells were then lysed with a tip sonicator at power 1 for one second three times. The cells were counted again with the EVE™ Automated Cell Counter to determine the amount of cells that have been lysed. The sonification step was repeated until 75% of the cells have been destroyed. The lysate was stored at -80°C.

2.2.3 Transfection

There are multiple types of transfection. With stable transfection, the foreign DNA is introduced into the genome of the cell with a plasmid. Since the foreign DNA is now part of the cell's genome, the descendants of the cell will also contain the foreign DNA. This way the SH-SY5Y cells will be able to produce the N-protein on their own. Moreover, to be able to separate the cells where transfection has successfully taken place from those where it has not, antibiotic resistance for one specific antibiotic is included in the genes of the plasmid. By adding this specific antibiotic to the medium, only the cells where the plasmid has become part of the genome will survive. [17]

Transient transfection, on the other hand, does not transfer to the next generation of cells. With transient transfection, the foreign gene does not become part of the genome. This also means that the foreign gene

is not limited to DNA as mRNA can also be used to stimulate gene expression. Transient transfection is temporary as the genetic material can be lost by cell division or other environmental factors. [17, 18]

The transfection was done on a µ-Slide 8 well high Collagen IV plate (80802, Ibidi) using Lipofectamine™ 3000 Transfection Reagent(L3000001 Invitrogen). The corresponding protocol for a 48-well plate was followed since the working volume per well of a 48-well plate is the same as for the µ-Slide 8 well high plate. Lipofectamine™ 3000 reagent was added to the Opti-MEM™ Medium (31985-062, Gibco, Termofisher). In another tube, DNA was added to Opti-MEM™ Medium and P3000™ Reagent. These two solutions were mixed in a 1:1 ratio and then 25 µL was added to the wells. Two different transfections were done, one with N-protein(153201, Addgene) and one with N-protein labelled with EGFP (185451, Addgene). To check the effectiveness of the transfection pEGFP-N-H_2B was added to one well.

2.2.4 Differentiation

Differentiated SH-SY5Y cells produce more α-Synuclein. [19] Therefore it would be interesting to see whether an overexpression of α-Synuclein causes the interaction between the N-protein and α-Synuclein to change.

The cells were cultured in 1% FBS medium with 10µM retinoic acid for 7 days. One row of the 8-well plate was differentiated so it could be compared to the undifferentiated SH-SY5Y cells of the other row.

2.2.5 Immunocytochemistry (ICC) Staining

The cells were fixed with 3,7% PFA and washed with PBS(10010023, Gibco, Thermo Fisher). The cells were then permeabilized with a mixture of 0,3% saponin (47036, Fluka, Biochemika), 0,1% BSA (A7906, Sigma-Aldrich) in PBS. 50 mM NH_4CL (12125-02-9, Honeywell Fluka) was used to quench the auto-fluorescent signals. The nonspecific binding sites were blocked with Goat Serum Dilution Buffer(16% Goat Serum, 0.3% saponin and 0.3M NaCL). For the well with N-protein transfection two primary antibodies were used, SARS-CoV-2 Nucleocapsid Protein Mouse mAb (33717S, Cell Signaling Technology) and rabbit α-Synuclein C-Tail Antibody (sc-7011-R, Santa Cruz Biotechnology). For the well with N-protein labelled with EGFP only the rabbit α-synuclein Antibody was added. As secondary antibodies Goat anti-Mouse Alexa Fluor™ 488 (A11029, Invitrogen, Thermo Fisher Scientific) and Goat anti-Rabbit Alexa Fluor™ 647(A-21246 Invitrogen, Thermo Fisher Scientific). The secondary antibodies were also added to the transfection control to test for nonspecific binding. DAPI (D3571, Invitrogen, Thermo Fisher Scientific) (nuclear staining) was added to the wells that contain the N-protein. For each step, a working volume of 100 µL was used. The samples were kept covered in PBS in a humid box before being examined under a laser-scanning confocal microscope. (MicroTime 200 PicioQuant, Germany)

2.2.6 Stable cell line

To create a stable cell line, the wells with N-protein and N-EGFP were transferred, 24 hours after the transfection, to large dishes (57cm^2) and cultured with conditioned medium (1 part used medium and 2 parts fresh). After 24 hours, the selection antibiotics were added. Zeocin (ant-zn-05, InvivoGen) for the dish with N-protein cells and Puromycin (A11138-03, Gibco, Invitrogen) for the dish with N-EGFP cells. After two weeks the N-EGFP cells were sorted with fluorescence cell sorting. Sadly, due to time constraints, it was not possible to use these cells for viability assays.

3 Results

3.1 Microscale Thermophoresis

To confirm that the N-protein binds to α-Synuclein a MST was done. First, a capillary scan was done with different concentrations of labelled N-protein to determine the needed amount to meet the recommended fluorescence intensity. This scan showed that 125 nM labelled N-protein is the lowest concentration of labelled N-protein that is within the boundaries of the recommended fluorescence intensity. The lowest concentration was used because the higher the concentration N-protein the more likely aggregation would take place. Aggregation would interfere with the MST results as the objective of the MST measurement was to determine the binding between the N-protein and α-Synuclein

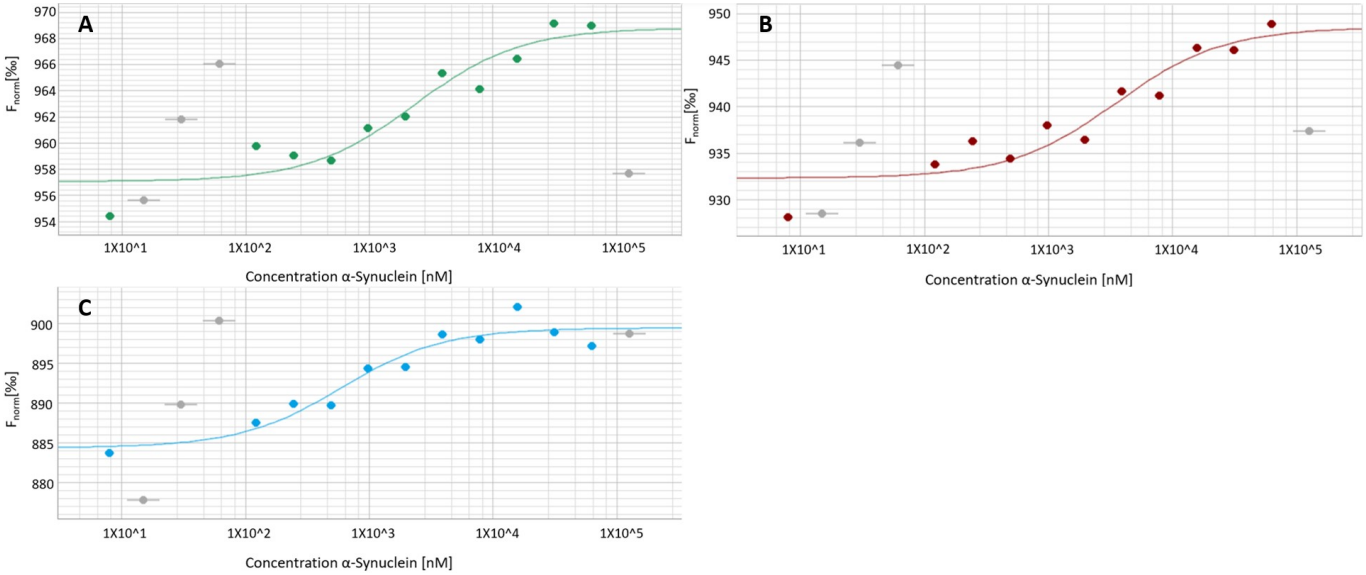

Figure 1: *Binding curves showing the interaction between α-Synuclein and labelled N-protein. The MST data points are the dots present in the graph. A (green) is the binding curve for 20% MST power, B (red) is the binding curve for 40% MST power and C (blue) is the binding curve for 80% MST power. The curve is a Kd fit that is a visible aid to see a possible binding curve of the data points.*

Figure 1 shows the binding curves of α-Synuclein with the labelled N-protein with the different intensities of MST power. The fluorescence of the 15 nM measurement had a deviation of more than 10% of the average and was therefore removed from the fit. The data for 30, 61 and 125 000 nM were also removed as those points were clear outliers.

In figure 1 all synuclein is bound and all synuclein is unbound in the high and low plateaus respectively. This means that an increase in α-Synuclein causes more binding with the N-protein. Figure 1C where an MST power of 80% was used is the only graph with clear platueaus. Here, the upper plateau starts at 5 µM ($5*10^3$ nM). The start of the lower plateau is unclear due to the invalid data points around the start of the lower plateau. The data points themselves follow in figure 1 A and B a linear trend instead of a binding curve. In figure 1 C the data points do show a curve.

Table 1: *The parameters used to make the Kd fit shown in figure 1. The parameters were chosen by MO. Affinity Analysis 2.1 software with the exception of the N-protein concentration as this was a fixed value. The Kd confidence and the Std. Error of Regression shows the quality of the Kd fit.*

MST Intensity	20	40	80
Kd (nM)	2262	3459	532
Kd Confidence	1174	1961	230
Conc. N-protein (nM)	125	125	125
Std. Error of Regression	1.59	2.33	1.71

Table 1 shows the data used to form the Kd fit. There is a high deviation for the Kd values between the different MST powers. The measurement with 80% MST power has both the lowest Kd confidence and a low Std. error of regression. This means that the curve shown in figure 1 C is the best-fitting curve of the three graphs.

3.2 Thioflavin T aggregation assay

3.2.1 Effect of the N-protein on α-Synuclein aggregation

To observe the effect of the N-protein on α-Synuclein aggregation a ThT aggregation assay was performed. As seen in figure 2, the N-protein significantly reduces the lag-time of α-Synuclein aggregation. Furthermore, the conditions with the N-protein have a first and second plateau whereas the conditions without only show one plateau. This is not visible in figure 2 as the second plateau was not relevant to comparing the lagtime between the four conditions. For the condition where only GFP is added to the α-Synuclein, no aggregation was measured within the 1100 hours measured. For the sample with only α-Synuclein one of the triplicates conditions was removed due to the large divination compared to the other two measurements. Even with this measurement removed the table 2 shows that the standard deviation is still significantly higher than the lagtime of the first plateau of the conditions with the N-protein. The lagtime in table 2 is determined with MATLAB with the exception of the condition where both the N-protein and GFP are present. Due to the higher starting value, this method used to determine the lagtime was not sensitive enough to detect the first plateau as the percentile difference between the starting value and the first plateau is lower than for the other conditions. Therefore, the lagtime was determined manually by looking at the increase in the gradient of the curve. Table 2 also shows that in the conditions where the N-protein is present the lag time is within the first 20 hours.

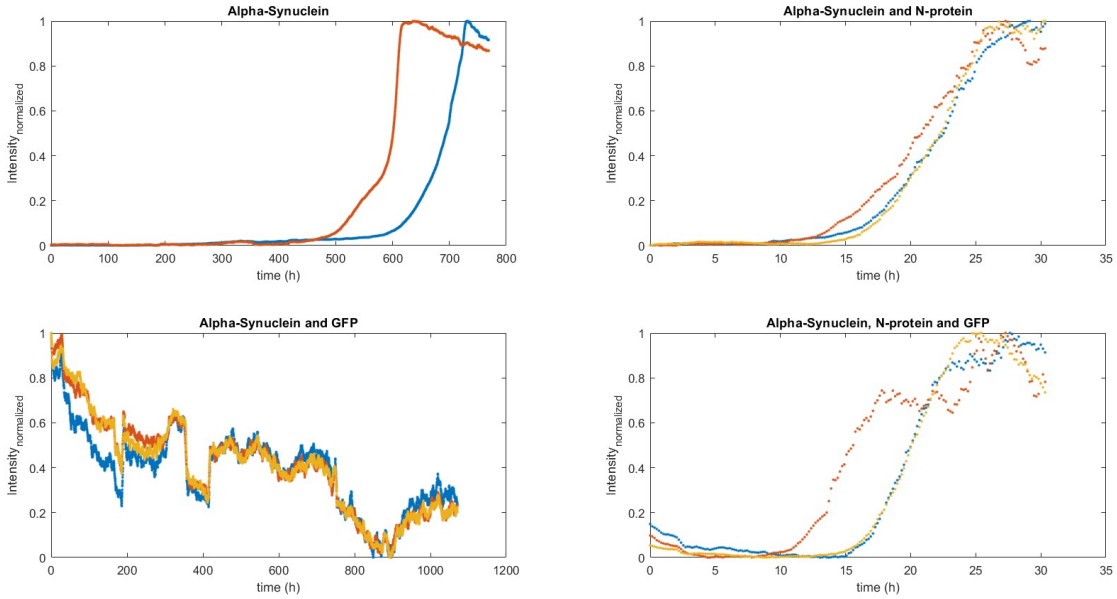

Figure 2: *The aggregation of α-Synuclein with multiple conditions. In all conditions 50 µM α-Synuclein was present. The conditions on the right contain 1 µM N-protein and for the bottom row, the GFP has a concentration of 1µM. One graph shows the triplicates of the same conditions. The graphs were normalized to the first plateau. A moving average was taken over 35 points.*

Table 2: *The average lagtime for the first plateau of the 3 measurements of the same condition and their standard deviation. The lagtime was determined by letting MATLAB find the first time when the intensity is three times the initial intensity. The lagtime of the condition where both N-protein and GFP are present was manually determined.*

	Lagtime (h) ± SD
Only Alpha-Synuclein	486 ± 36
N-protein	17.5 ± 1.4
GFP	>1100
N-protein and GFP	13.0 ± 2.2

3.2.2 The effect of cell lysate

To make the test tube experiment closer to the in vitro environment, cell lysate was added to the buffer of the ThT aggregation assay. Figure 3 shows the aggregation of α-Synuclein in the presence of N-protein with different concentrations of cell lysate. All conditions started to aggregate within the measured 500 hours. Even though, not all triplicates reached a plateau in the measured time. The conditions where cell lysate was added only have one plateau whereas the condition without cell lysate has two plateaus. The presence of two plateaus is customary when the N-protein is added to α-Synculein. [13]. Table 3 shows no clear correlation between the different percentages of lysate and the lagtime. On the other hand, table 3 does indicate that adding cell lysate to the buffer significantly increases the lagtime. However, the lagtime is still shorter than the lagtime of α-Synuclein without the N-protein, which can be seen in table 2. When comparing the tables 2 and 3 there is also a difference in lagtime between the measurements where the N-protein is added without cell lysate and GFP. These measurements should have the same lagtime since they are the same condition however, the difference between the lagtimes falls outside the standard deviation.

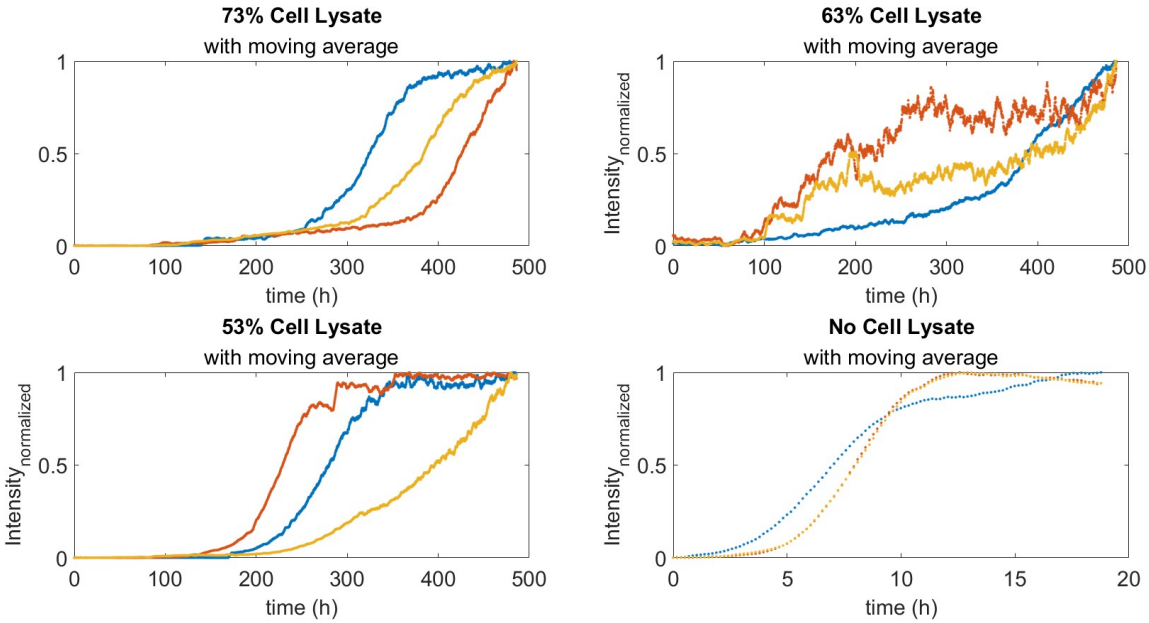

Figure 3: *The aggregation of α-Synuclein in the presence of the N-protein with multiple quantities of cell lysate. In all conditions 50 µM α-Synuclein and 1 µM N-protein were present. was present and the aggregation was followed with 20 mM ThT buffer. One graph shows the triplicates of the same conditions. The graphs were normalized to the first plateau. A moving average was taken over 35 points.*

Table 3: *The average lagtime for the first plateau of the 3 measurements of the same condition and their standard deviation. The lagtime was determined by letting MATLAB find the first time when the intensity is three times the initial intensity.*

	Lagtime (h) ± SD
73% Lysate	112 ± 24
63% Lysate	170 ± 23
53% Lysate	129 ± 38
No Lysate	4.0 ± 1.0

3.3 ICC Staining

The ICC staining was done to see the effect of the N-protein on the aggregation of α-Synuclein in SH-SY5Y cells. The first staining was used to determine the difference in the efficiency of the transfection if the transfection factors were kept on the cells for 48 or 72 hours. However, the samples were kept dry for multiple days before they were examined with the laser-scanning confocal microscope. Despite the fact that before the staining the wells had a high confluency, when examined it was found that only a dozen cells were still present. Nonetheless, figure 4 shows a clear increase in α-Synuclein present in the 72-hour sample compared to the 48-hour sample. Therefore, in further experiments, the transfection factors were kept on the cells for 72 hours if possible.

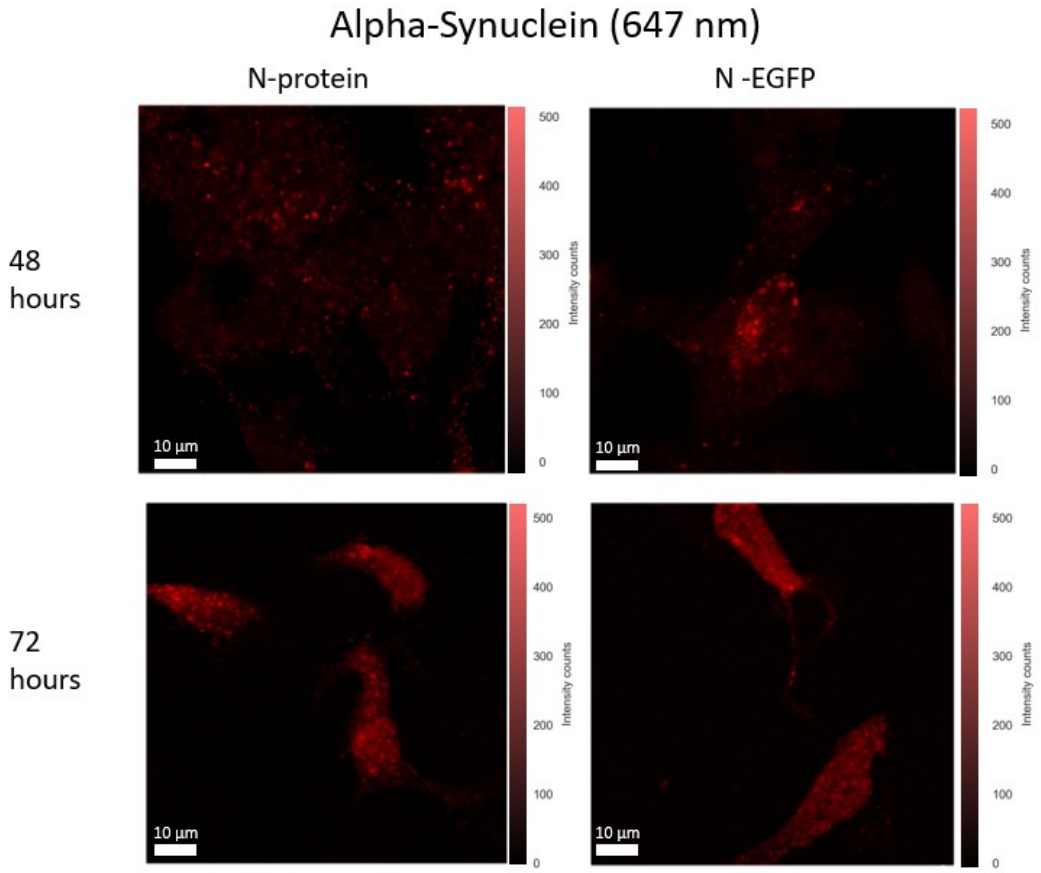

Figure 4: *SH-SY5Y cells after ICC staining with rabbit α-synuclein C-Tail Antibody and Alexa Fluor™ 647 48 and 72 hours after the start of transfection. The sample was kept dry for multiple days before the images were taken.*

Due to the low cell count the experiment was repeated to increase the number of cells available for analysis. This time the cells were covered with PBS after the staining. Once again, very few cells were present when viewed under the laser-scanning confocal microscope even though the wells had a confluency of 100% before the staining. Figure 5 shows a few of the dozen cells still present within the wells. Figure 5 A and C show the presence of the N-protein in the cells. Figure 5 B and D show the α-Synuclein in red. There are clear high-intensity spots which indicate a clustering of α-Synuclein. Most high-intensity spots coincide with where the N-protein is the most concentrated. However, not all of α-Synuclein clusters are at places where the N-protein concentration is high. To determine whether there is a correlation between the clustering of α-Synculein and the N-protein, more cells are necessary.

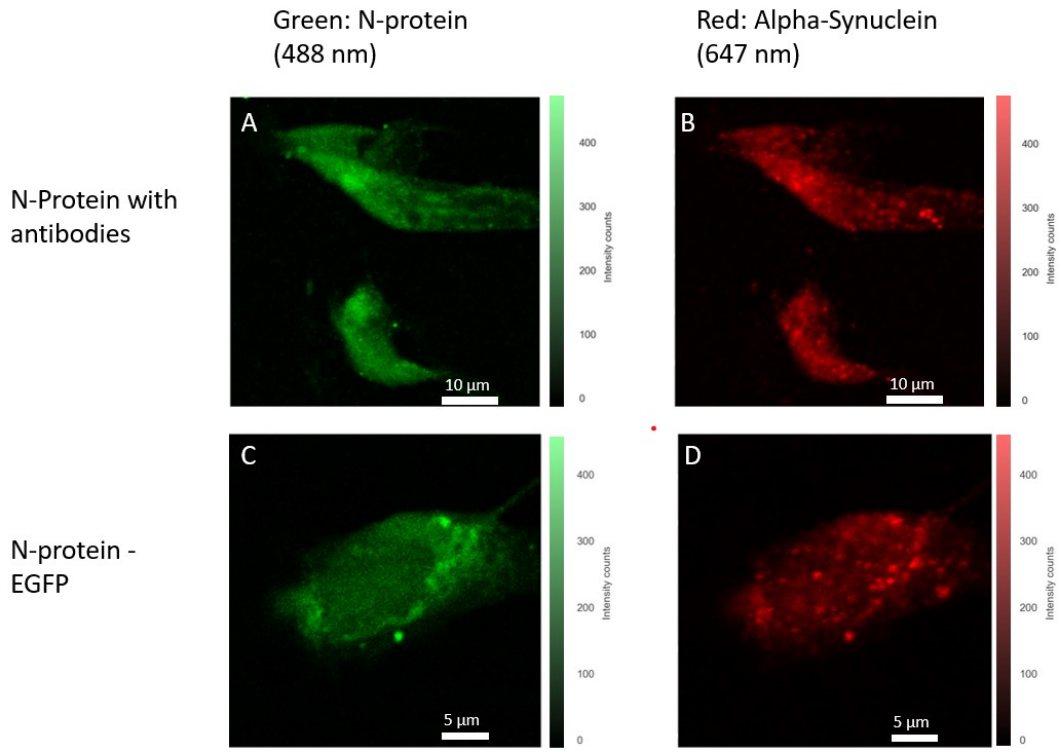

Figure 5: *SH-SY5Y cells after ICC staining with rabbit α-synuclein C-Tail Antibody and Alexa Fluor™ 647. A and B were also stained with SARS-CoV-2 Nucleocapsid Protein Mouse mAb and Alexa Fluor™ 488*

Since there were still only a few cells in the wells it was concluded that the main factor that caused the low cell count was not the cells being kept dry after the staining. So for the next batch, multiple adaptations to the protocol were implemented to decrease the change of cell loss during the staining process. First of all, a fresh stock of formaldehyde solution was used and the PFA was kept on the cells for 30 minutes instead of 15. In each step, 200 µL was added to each well instead of 100 µL to ensure the cells were completely covered. Lastly, instead of using a vacuum aspiration system, the solutions were removed with a micropipette to ensure minimum cell loss. To also test whether the collagen coating of the wells had any negative effect on the cell count, the next staining was done on two plates, one with coating and one without.

After the staining 7 of the 16 wells still had an acceptable amount of cells left. All 7 wells were wells where differentiation had taken place. These wells had a lower confluency than the wells with undifferentiated SH-SY5Y cells. However, in all the wells the confluency was very high before the staining causing multiple layers of cells with the top layer consisting primarily of dead cells. As seen in figure 6, even though 7 wells were still covered in cells after the staining, the morphology of the cells was abnormal. There are no clear individual cells, nor are the nuclei clearly visible. Furthermore, the green signal from the cells had a low intensity with <100 counts; earlier measurements showed intensities up to 400 counts. This means that the green fluorescent light is most likely caused by autofluorescence Figure 6 also shows no substantial difference between the cells within the coated and uncoated wells.

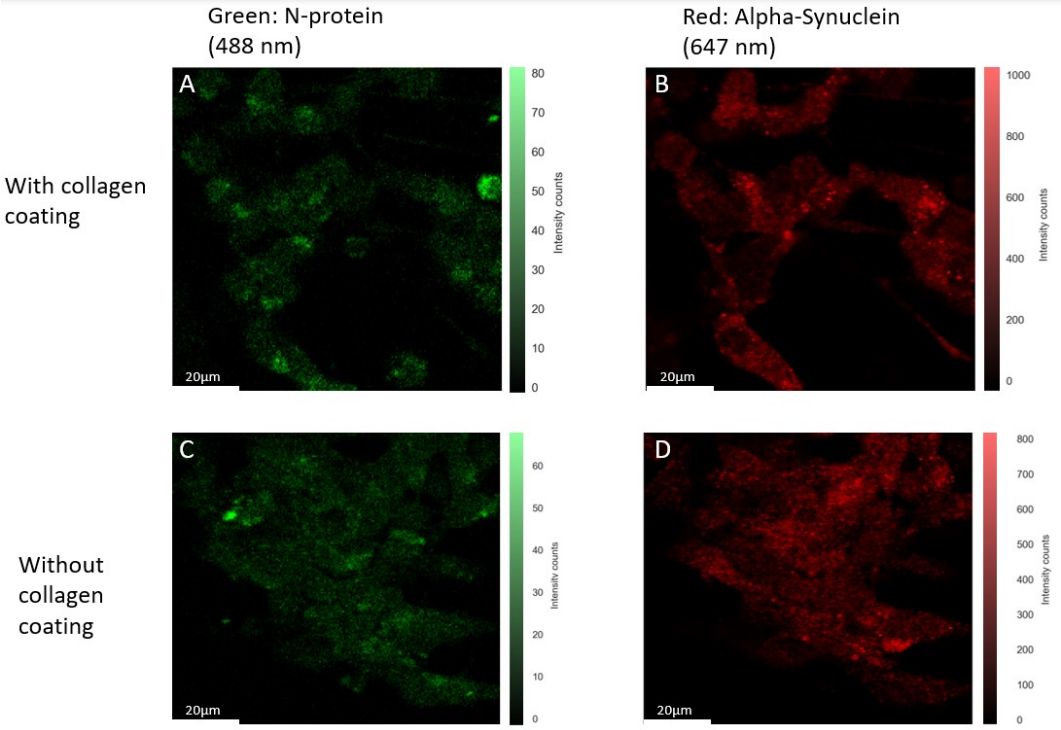

Figure 6: *Differentiated SH-SY5Y cells after ICC staining with rabbit α-synuclein C-Tail Antibody and Alexa Fluor™ 647. A) was also stained with SARS-CoV-2 Nucleocapsid Protein Mouse mAb and Alexa Fluor™ 488. A and B) The cell culture was done in wells coated with collagen. C and D) The cell culture in wells without coating. Extra measures were taken to ensure minimum cell loss during the staining process.*

During the staining, the wells were checked after each step. This made it clear that the cells detached from the well during the first two wash steps with PBS. Most of the cells were already removed from the wells before the actual staining process. This means that the staining process was not the main cause that led to the low cell count in the wells. It was suspected that the confluency of 100% could cause the cells to detach. To solve the problem of the high confluency, the cells were replated in a ratio of 1:6 48 hours before the staining.

Once again, not all wells had sufficient cells. On the 8-well plate with collagen coating only the wells with differentiated SH-SY5Y cells were covered in cells. On the 8-well plate without coating all wells had a sufficient cell count. This indicates that the coating and differentiation of the SH-SY5Y do have an effect on the attachment of the cells.

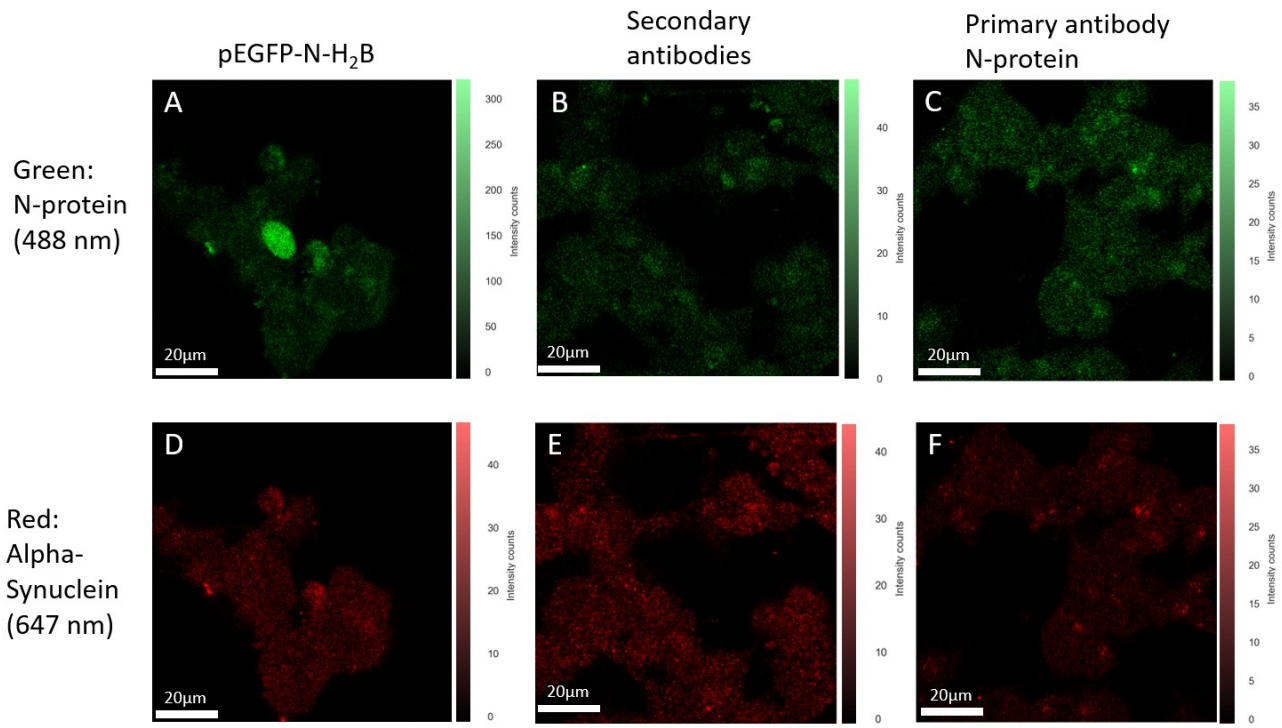

Figure 7: *The autofluorecence and apecfic binding of the Antibodies. A and D) There are no antibodies present and the cell has undergone transfection with pEGFP-N-H_2B. B and E) show the nonspecific binding of the secondary antibodies rabbit Alexa Fluor™ 647 and mouse Alexa Fluor™ 488. C and F) The control for the specific binding of the primary SARS-CoV-2 Nucleocapsid Protein Mouse mAb antibody.*

With sufficient cells in the wells, it is now possible to look at the autofluorescence and the nonspecific binding of the antibodies. Previously, no signal was seen in the controls however, it was unclear if this was because there were no cells present or if the autofluorescence was close to zero. Now with wells covered in cells figure 7 shows that there is autofluorescence, but the intensity is significantly lower than the intensity of specific bound antibodies. In figure 7 D and F there are no anitbodies for α-Synuclein present. This means that the signal seen in these images is the autofluorescence of the cells at 647 nm. In figure 7 E there are secondary antibodies for α-Synuclein present, but the intensity is the same as the images without antibodies. This means that the nonspecific binding of the secondary antibodies for α-Synuclein is almost zero.

Figure 7 A-C show the intensity around 488 nm. In figure 7 B there are secondary antibodies for the N-protein. Even though there is no N-protein in the cells there is an intensity of 40 counts. This is most likely not the secondary antibodies binding non-specifically, but autofluorescence as the intensity is the same for α-Synulcien without antibodies present. Since in figure 5 there were only a dozen cells and yet multiple of them had a strong signal at 488 nm, it was speculated that the primary SARS-CoV-2 N-Protein antibody would bind non-specifically. Yet, figure 7 C has the same intensity as B indicating that the primary antibody does not bind non-specifically. Furthermore, in figure 7 A the cells have undergone transfection with pEGFP-N-H_2B. pEGFP-N-H_2B causes the histone H2B to become green fluorescence which makes the nucleus green in the staining. Figure 7 A has an intensity of 400 counts which is significantly higher than the autofluorescence seen in the other images. This means that the signal from the α-Synculein and N-protein can be distinguished from the autofluorescence by looking at the intensity.

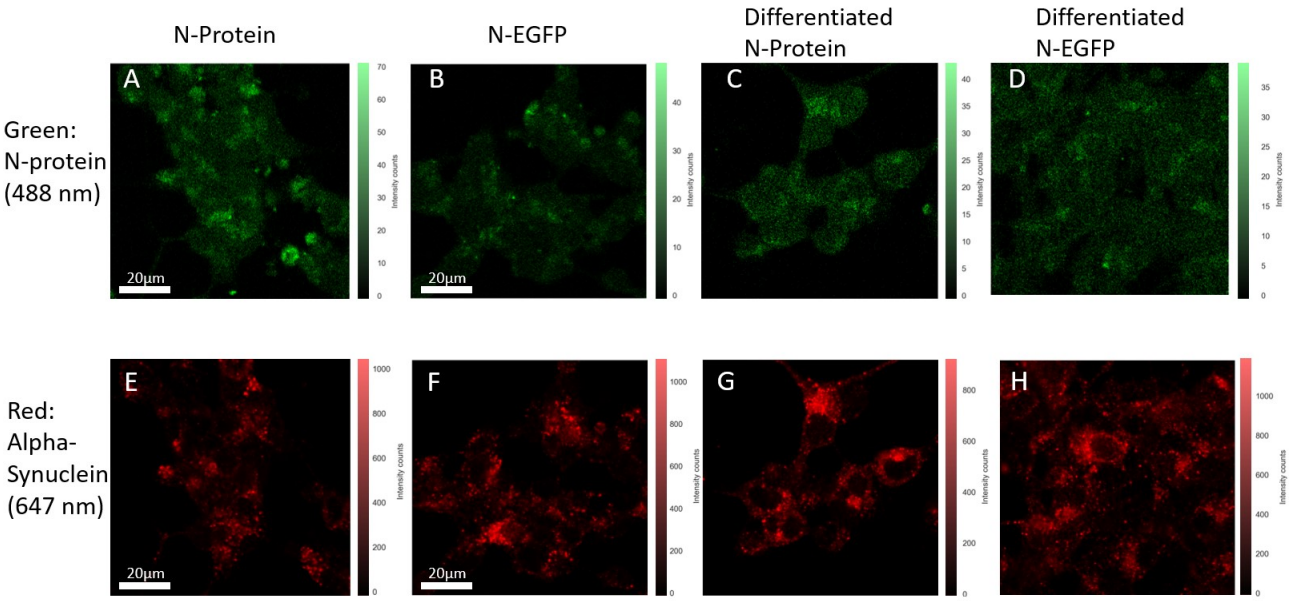

Figure 8: *SH-SY5Y cells and differentiated SH-SY5Y cells after ICC staining with rabbit α-synuclein C-Tail Antibody and Alexa Fluor™ 647. A and C were also stained with SARS-CoV-2 Nucleocapsid Protein Mouse mAb and Alexa Fluor™ 488.*

Figure 8 E-H shows the α-Synuclein in both SH-SY5Y cells and differentiated SH-SY5Y cells. There is no clear difference in intensity between SH-SY5Y cells and the differentiated cells. Furthermore, figure 8 A-D all have an intensity lower than 100 counts. This means that the fluorescence seen in these images is autofluorescence and not the presence of the N-protein. This shows that the transfection efficiency of the N-protein and N-EGFP protein was low since there were no cells found with high intensity at 488 nm.

4 Discussion

4.1 Microscale Thermophoresis

To analyze the binding between the SARS-Cov-2 N-protein and α-Synuclein a Microscale Termpohoresis (MST) measurement was done. Figure 1 shows that there is a binding curve so the N-protein does bind to α-Synuclein. Multiple data points with lower concentrations had to be removed. Because of this, the lower plateau can not be determined from the data points. The unreliability of the points with a low concentration is most likely caused by inaccuracy during the preparation of the samples. Due to the low concentration a small deviation from the needed concentration, due to a pipetting error for example, causes a relatively bigger error than with higher concentrations.

The reliability of the Kd curve can be determined with the Kd confidence. Table 1 shows that the graph of the measurement where 80% MST power is the most accurate since it has the lowest Kd Confidence. If the MST power increases, the fluorescence decreases faster and thermophoretic movement increases resulting in a higher resolution of the binding curve. [20] This explains why the 80% measurement shows a binding curve whereas the 20% and 40% show a more linear correlation.

The Kd found with 80% MST power is approximately 0.5 µM, this is a low Kd value, which means that there is a strong binding affinity between the N-protein and α-Synuclein. Furthermore, the Kd value of 0.5 µM is in line with the value found in literature, which is an EC50 of 0.3 µM. [13] The EC50 is the half-maximal effective concentration. In this context, the EC50 is the concentration when the curve reaches half the value of the upper plateau. Even though the EC50 and the Kd are different quantities, in this context, they should be in the same order of magnitude. This is the case with a Kd of 0.5 µM and an EC50 of 0.3 µM.

For future experiments, it would be better to do the MST measurements in triplicate. If there is a measurement where multiple data points cannot be used, another triplicate could be used. Furthermore, with multiple measurements, an average and margin of error could be established for the Kd.

4.2 Thioflavin T aggregation assay

To confirm that the SARS-Cov-2 N-protein does enhance the aggregation of α-Synuclein a Thioflavin T aggregation assay. When no N-protein was present the lagtime was >450 hours. When the N-protein was added this reduced to <20 hours which is a decrease of 95%. This is a significant decrease so it is clear that the N-protein has an effect on the aggregation of α-synuclein. However, it was not determined whether or not the N-protein itself causes an increase in ThT fluorescence. While the N-protein does contain beta-strands, it does not contain enough beta-sheets to activate ThT. [21] The research of Semerdzhiev et al. further supports this since they did include a control with only the N-protein and it showed no reaction with the ThT. [13]

To simulate a test tube experiment closer to the in vitro situation of α-Synuclein aggregation in SH-SY5Y cells, cell lysate was added to the buffer. The highest concentration used was 73%. This was chosen since this completely replaces the MilliQ used in the buffer without cell lysate. It would have been better if the concentration was closer to 100%, but this was not possible without changing the concentrations of the vital components of the buffer. If this was done the measurements with cell lysate could no longer be compared with previous measurements seen in figure 2 and table 2.

Table 3 shows that cell lysate increases the lagtime of α-Synuclein aggregation in the presence of the N-protein. This was expected since the cell lysate contains the cell organelles, proteins and nucleic acids that are present in SH-SY5Y cells. This causes steric hindrance which reduces α-Synucleins accessibility. It is therefore harder for the N-protein to bind to α-Synuclein and this increases the lagtime. The lagtime in table 3 is still lower than the lagtime without N-protein recorded in table 2. This indicates that the effect of the N-protein is not nullified by the cell lysate.

Furthermore, it is expected that increasing the concentration of cell lysate would increase the steric hindrance which would cause the lagtime to increase. However, table 3 shows no clear correlation between the concentration of cell lysate and the lagtime.

The aggregation of α-Synuclein is a stochastic process that is also sensitive to the conditions in which the aggregation takes place. This causes high variability in the lagtime and poor reproducibility. [16] Table 2 shows a lagtime of 486 hours and a standard deviation of 36 hours. The lagtime of α-Synuclein is compared to literature longer than average. However, the lagtime in the literature differs greatly as values between 17 and 250 hours can be found. [13, 16, 22, 23]

The lagtime of α-Synuclein and the N-protein was measured twice. One has a lagtime of 17.5 hours and can be seen in table 2 and the other indicates a lagtime of 4 hours as shown in table 3. The lagtime of 4 hours is closer to the lagtime found in literature. [13] The deviation is most likely caused by the fact that a different batch of N-protein was used for these experiments. However, both lagtimes are $<$20 hours so the difference is not significant.

During the duration of this research, N-protein labelled with GFP was unavailable. To still be able to look at the effect of GFP on the aggregation of α-Synuclein in the presence of the N-protein, GFP was added to the aggregation mixture. Adding GFP separately instead of bound to the N-protein causes some new variables. The control with only GFP added to α-Synuclein shows no aggregation within the measured 1100 hours. The control with only α-Synulein, on the other hand, has a lagtime of about 485 hours. This implies that GFP inhabits the aggregation of α-Synuclein. GFP is a large protein with a size of 27 kDA. [24] In comparison, the α-Synuclein is only about half that size with 14 kDA. [25] The size of the GFP protein could cause steric hindrance, causing less α-Synuclein proteins available for aggregation.

However, there is only a neglectable difference in lagtime between α-Synuclein in the presence of the N-protein with and without GFP. This shows that the steric hindrance caused by the GFP has no measurable effect on the aggregation between the N-protein and α-Synuclein.

4.3 ICC Staining

The ICC staining was done to see the effect of the N-protein on α-Synuclein in SH-SY5Y cells. However, due to the lack of cells in multiple staining attempts, the focus turned to increasing the attachment of the cells.

The first wells to be stained can be seen in figure 4. At the time, the most likely cause of the low cell count was the fact that the wells were kept dry after the staining. However, the next measurement where the wells were kept covered in PBS had the same low cells count. At this point, measures were taken to increase the cell count. Yet, the cells were not observed with the EVOS microscope between steps. If this was done, it would have become clear that most cells detached in the first wash steps with PBS. This would have saved time as transfection and differentiating cells can take up to two weeks.

To find the cause of the high detachment of cells a process of elimination was used. This method was used as most known solutions to cell detachment are focused on the detachment during the culture of the cells. This was not the problem as all the wells had a high confluency of attached cells before the staining. With the process of elimination, it was determined that the low cell count was not caused by the low working volume. Nor by the stock of formaldehyde solution or the time the PFA was on the cells. The power of the vacuum aspiration system was also not the cause of the low cell count. Lastly, it was speculated that the high confluency of the could be the cause of the detachment of the cell. Yet, even when the cells were replated 48 hours before the staining only 12 of the 16 wells had a sufficient number of cells.

Only two variables seem to have an effect on the increased number of cells in the last staining. The wells containing differentiated cells had in both the staining of figure 6 and 8 more cells than the undifferentiated cells. Furthermore, the 8-well without collagen coating had more undifferentiated cells in the staining of figure 8 than the 8-well with collagen coating. This is unusual since the collagen coating should support cell attachment. Therefore, it would be more logical if the wells with coating had more cells. It is possible that there was a problem with the collagen coating of this batch of 8-well plates. This is further supported by the fact that during the culture of the cells, there was no noticeable difference between the wells with and without collagen coating.

Figure 5 has high-intensity spots of α-Synuclein. These high-intensity spots are caused by aggregated α-Synculein. [26] Figure 5 also shows the presence of the N-protein in the SH-SY5Y cells. The high intensity

of green fluorescence is mostly found in the cytosol of the cell. This is in accordance with the literature since the SARS-COV-2 N-protein binds directly with the mRNA in the cytosol as earlier studies have shown. [27]

Even though figure 5 shows both the presence of the N-protein and aggregated α-Synuclein, there is no definitive correlation between the presence of the N-protein and the aggregated α-Synuclein. Not all aggregated α-Synuclein are spots where the N-protein concentration is high. Moreover, the sample size is only a dozen cells with only a view where transfection had taken place. This sample size is too small to draw any conclusions about the correlation between the N-protein and the aggregated α-Synuclein.

Figure 8 E-H indicates that there is no difference in intensity of α-Synuclein between the differentiated and non-differentiated SH-SY5Y cells. According to literature, the differentiated SH-SY5Y cells should have more α-Synuclein. [19] It is unclear why this is not the case in this experiment.

Figure 8 shows that the transfection efficiency is close to zero in the measured cells. This could be increased by further reducing the confluency. Another method to increase the efficiency is the use of a stable cell line. If the transfection is successful in only a few cells, these will be selected by adding the specific antibiotic belonging to the antibiotic resistance of the transfected genetic material. These cells could then be selected with fluorescence cell sorting and cultured into a cell population with a 100% transfection efficiency. The stable cell line could also be used to investigate the toxicity of the N-protein with a viability assay. Unfortunately, there was not enough time to produce a stable cell line.

5 Conclusion

This study showed, with an MST measurement, that there is a strong binding affinity between α-Synuclein and SARS-Cov-2 nucleocapsid protein. A ThT aggregation assay also proved that the N-protein significantly reduces the lagtime of the α-Synuclein aggregation and that this effect is still present when cell lysate is added to the buffer. No conclusion could be drawn on the effect of the N-protein on the aggregation α-Synuclein in SH-SY5Y cells due to the detachment of cells during the staining process. It is unclear what exactly caused the detachment even though numerous factors have been ruled out as the cause. It is advised to use a stable cell line to further investigate the effect of the N-protein in SH-SY5Y cells. The results of the study indicate that the SARS-Cov-2 N-protein has an effect on the aggregation of α-Synuclein. It is therefore still plausible that there is a link between SARS-CoV-2 infection and post-infectious parkinsonism.

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