Influence of lipid membranes on huntingtin exon 1 fibrillation

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Abstract

Huntington's disease (HD) is an inherited neurodegenerative disorder caused by a CAG repeat expansion within the first exon of the huntingtin (HTT) gene. Pathological extensions above a critical threshold of 37 CAG lead to an expression of a mutant huntingtin (Htt) protein comprising an expanded polyglutamine (polyQ) domain. The aberrant splicing and proteolytic cleavage of mutant Htt proteins result in highly toxic Htt fragments spanning exon 1 (Httex1), which are sufficient to reproduce most of HD's pathology. Recent studies support that biological membranes promote Httex1 misfolding and aggregation. This project aims to establish an approach for studying the amyloid fiber formation of Httex1 and to evaluate the impact of anionic lipid membranes. The aggregation of Httex1-37Q and Httex1-43Q variants were here evaluated by ThT fluorescence assays. Initially, a strategy for production of tag-free Httex1-37Q was developed using a solubilizing SUMO-fusion approach. However, ThT fluorescence assays revealed that tag-free Httex1-37Q was already aggregated before starting the assay. Instead, the mutant Httex1-43Q variant was purified as a fusion construct and the SUMO-tag was only removed during the ThT assay. Remarkably, tag-cleavage is almost instantly, avoiding interference on the assay. The fibrillation kinetics of Httex1-43Q show a typical sigmoid curve profile and that anionic lipid membranes potentiate its fibrillation, in accordance with current literature data. Our approach provides an excellent platform for studying Httex1 amyloid fibril formation in solution and in the presence of lipid membranes, allowing to investigate different variants, concentrations, and lipid compositions.

Introduction

Huntington's Disease (HD) is a progressive inherited neurodegenerative disorder caused by a CAG repeat expansion within the first exon (Httex1) of the huntingtin gene (*HTT*) [1]. HD shows physical symptoms, such as movement disturbances [2], and psychological symptoms such as loss of cognitive functions [3]–[5]. No effective cure is available for HD although treatments are being researched [6]. Patients rely mostly on available medication and supporting care to maintain their life quality.

Most of the pathological effects of HD can be attributed to Httex1, and individuals who express more than 37 glutamine residues in the polyQ domain are guaranteed to develop the disease [1]. Large extensions in the polyQ cause the protein to be unstable and form amyloid-like fibers that affecting cellular stability [7]. Httex1 has a global charge of 0 and consists of a polyQ domain flanked by a N-terminal 17 amino acid segment (Nt17) and a C-terminal proline-rich domain (PRD) [8], [9]. The PRD sequence does reduces the aggregation kinetics and aggregate stability [10]. The Nt17 domain seems to act as a catalyst to the aggregation process [11]. A study was able to show the effect of Nt17 is dominant over the aggregation-suppressive effect the PRD [12].

Amyloid fibers, present in HD, are formed by β-strands that assemble into fibrils following a nucleated polymerization growth model [13]. This model is graphically represented by a sigmoid curve that shows a lag phase (nucleation), an exponential growth (elongation), and finally a plateau [14]. The presence of amyloid fibers is commonly detected by the fluorescent probe thioflavin-T (ThT) that works as a molecular rotor [15]. ThT specifically binds to the β-sheet structure present in amyloid fibers and emits fluorescence [16]. However, ThT gives no information regarding amyloid fibers' morphology, that data is obtained by cryogenic electron microscopy (Cryo-EM) and atomic force microscopy (AFM) [17], [18].

Expression of amyloid-like proteins, and in this case Httex1, is usually done using recombinant *E. coli* cells [19]. Httex1 specifically has been fused to solubilization tags. Many of these tagging methods alter the structure of Httex1 post-cleavage, by cleaving at unspecific locations or by adding extra amino acids [20]. Small ubiquitin-like modifier (SUMO) tag is cleaved by highly efficient ubiquitin-like-specific protease 1 (ULP1), which recognizes the three dimensional structure of the SUMO domain [21], [22].

Lipid membranes are an important part of every cell, isolating the interior of the cell from the outside. It also incorporates several organelles [23], [24]. Studying the lipid-Httex1 interaction is fundamental work and has potential implications for disease understanding [25]. The Nt17 domain specifically has been shown to strongly interact with lipid membranes and influence the aggregation process, as the domain can insert itself into the membranes [26] [27]. Because of this, it has a negative role in membrane stability, affecting the bilayer's fluidity by increasing its rigidity [28].

The overall project aims to characterize the role of the lipidic membranes in the Httex1 aggregation and fibrillation pathway. First, we developed an approach for generating recombinantly expressed tag-free Httex1-37Q using a SUMO fusion strategy at the N-terminal and performed simple aggregation tests using ThT fluorescence assays [21]. Then His_6-SUMO-Httex1-43Q was expressed and purified as a fusion protein, to be used in ThT fluorescence assays both in the presence and absence of lipid bilayer structures.

Materials and Methods

Expression tests for the His_6-Htt-37Q protein

BL21(DE3) One Shot® chemically competent *E. coli* were transformed with the pET-SUMO-Httex1-37Q plasmid by heat shock, spread on a LB-Agar plate with Km and incubated overnight (O/N) at 37 º C. A 25 mL LB-Km O/N inoculum of a single colony was then performed (37 ºC, 200 rpm). Then the culture was renewed with 15 mL of the O/N culture and 100 mL of fresh LB/Km medium then incubated for 4 hours. The large-scale expression of His_6-SUMO-Httex1-37Q was performed in 1L LB/Km at 16 ºC, 180 rpm, and 0.6 mM of IPTG. The cells were harvested by centrifugation at 8000 rpm, 10 min, and 4 ºC and the pellet was resuspended in 60 mL of buffer A1, supplemented with 0.1 mM PMSF and 1 mini-tablet of protease inhibitors. The resuspended cells were stored at -80 ºC until purification.

Purification of tag-free Httex1-37Q protein

Cells were first sonicated on ice using the Branson Sonifier 250 with 9 cycles of 15 pulses and 5 minutes of rest between each cycle. The cell lysate was centrifuged at 17 600 *x g* at 4 ºC for 10 minutes and then for another 1 hour. The supernatant was then filtered through a 0.45 µm syringe filter (low protein binding). The His_6-SUMO-Httex1-37Q fusion protein was initially purified by an immobilized metal affinity chromatography (IMAC) in ÄKTA Purifier System (GE Healthcare) using a 5 mL HisTrap FF column. The fractions containing the His_6-SUMO-Httex1-37Q fusion protein were pooled and buffer was exchanged via dialysis O/N at 4ºC. To cleave the His_6-SUMO tag, the fusion protein was incubated with His_6-ULP1 and 1mM of DTT for about 3 hours on a rotator at 4 ºC. A second IMAC was performed to remove His_6-SUMO tag and the His_6-ULP1. A 1 mL HisTrap FF column was used and 500 µL fractions of the flow-through were collected. The fractions containing pure Httex1-37Q were flash-frozen in liquid nitrogen and aliquoted (100 µL) for storage at -80 ºC.

2.4. Production of the His_6-SUMO-Httex1-43Q fusion protein

The His_6-SUMO-Httex1-43Q fusion protein was recombinantly expressed, purified and stored as described for Httex1-37Q variant, with the following adjustments. For this pathogenic variant, the His_6-SUMO tag was not removed during the purification procedure due to its high aggregation propensity. After the IMAC, fractions containing the fusion protein were pooled and concentrated in SEC buffer with 5 mM DTT using an Amicon Ultra-15 10 kDa cutoff. The second IMAC was not performed. The His_6-SUMO-HTTex1-43Q fusion protein was further purified by multiple SECs.

2.7 ThT fluorescence assays to detect amyloid fibril formation

Large unilamellar vesicles (LUVs) composed of 25:75 POPC:POPS were prepared. LUVs were obtained by extrusion as described in the literature [29]. The fluorescence experiments were conducted in a

PolarStar Optima microplate reader (BMG Labtech) using a 96-well microplates (Gernier) non-binding protein. Briefly, samples were measured for 16 hours at 25 ºC and with 250 cycles. Aggregation kinetics was monitored by measuring the fluorescence intensity (excitation 440-10 nm, emission 480-10 nm). Before each assay, protein samples previously stored at -80 ºC were centrifuged at 12 000 *x g* for 2 minutes at 4ºC to remove large aggregates. Purified tag-free Httex1-37Q (5, 10, 12.5, 20, 30, 50 µM) was incubated with 50 µM ThT. In solution ThT was incubated with 5, 10 and 15 µM of Httex1-43Q fusion protein, ULP1 and 1mM DTT. To evaluate the impact of anionic lipid membranes in Httex1-43Q aggregation kinetics, 5 and 10 µM of fusion protein was incubated with ThT, 25 µM, 50 µM, 100 µM and 200 µM of LUVs.

Results

Expression and Purification Tests of Httex1-37Q

The expression His_6-SUMO-Httex1-37Q fusion protein had not been tested before, so that is what our lab began to do successfully. Having obtained the protein, it was submitted to an optimized purification procedure shown in Figure 1.

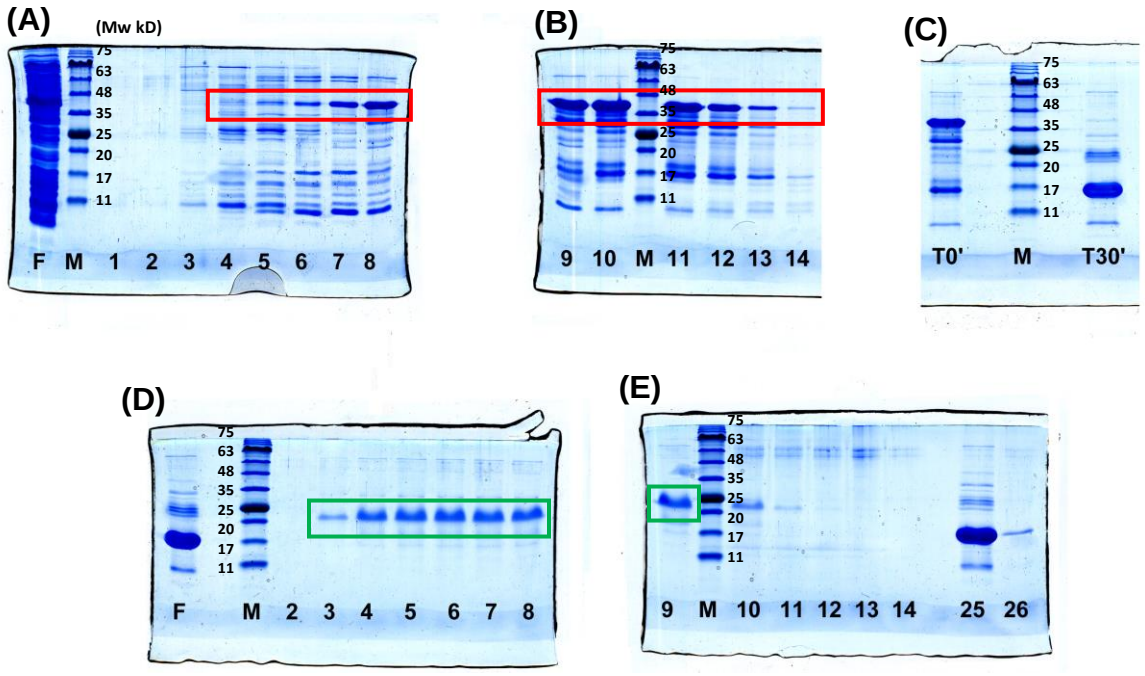

Figure 1: SDS-PAGE gels obtained in the optimized purification strategy for the Httex1-37Q protein. **(A)** and **(B)** First IMAC, where F – feed and 1 –14 represent the collected fractions, the red boxes highlight the location of the His_6-SUMO-Httex1-37Q. **(C)** Cleavage of the His_6-SUMO tag from the fusion protein after adding ULP1 with samples taken at each 30 minutes. **(D)** and **(E)** Second IMAC for purification of tag-free Httex1-37Q from His_6-SUMO and the

protease. F – feed; 2 – 14 and 25-26 represent the collected fractions. In all SDS-Page gels, M indicates the markers (NZYColour Protein Marker II).

Overall, the purification process uses IMAC procedures, showing satisfying results. In the first IMAC (Figure 1-A, B) the fusion protein can be seen near the 35 kDa region. While there are many bands above and below the one of interest, a vast part of the sample was purified. Then, the SUMO tag was cleaved by ULP1, generating tag-free Httex1-37Q. The results are shown in Figure 1-C, with the tag-free Httex1-37Q protein being near 25 kDa and the SUMO tag near 17 kDa. Finally a second IMAC was performed to isolate the tag-free protein, and the results can be seen in Figure 1-D,E. The tag-free Httex1-37Q fragment obtained was relatively pure – some faint bands can be seen above and below the 25 kDa region but most of the sample contains the protein of interest. It is important to note that the protein migrates at higher molecular weights due to its intrinsically disordered characteristics and abundance of proline and glutamine residues in its sequence. The protein concentration was determined and fractions of 100 µL were stored at -80 ºC until usage for the ThT fluorescence aggregation assay.

ThT fluorescence assays of tag-free Httex1-37Q

The tag-free Httex1-37Q variant was specifically chosen to evaluate the formation of amyloid fibrils by ThT fluorescence assays because it is located at the threshold for pathological aggregation in HD.

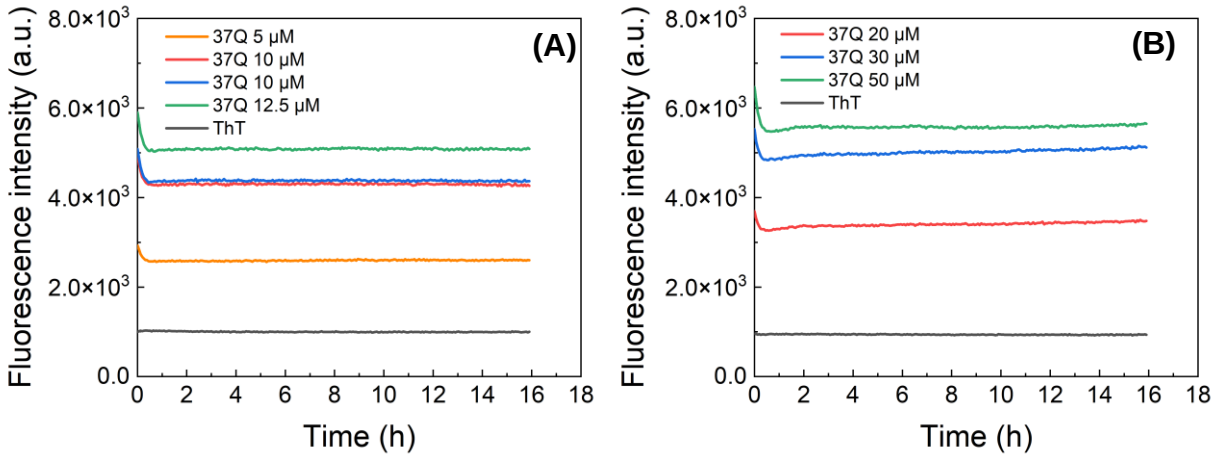

Figure 2: ThT fluorescence assays for monitoring the amyloid fibril formation of tag-free Httex1-37Q. **(A)** ThT fluorescence signal recorded upon incubation with distinct concentrations of Httex1-37Q: 5 µM (orange curve), 10 µM (red and blue curves), and 12.5 µM (green curve). **(B)** Higher Httex1-37Q concentrations were evaluated: 20 (red curve), 30 (blue curve), and 50 µM (green curve). The grey curves in both figures represent the control (only ThT in buffer F).

ThT fluorescence assays were initially performed with 5, 10 and 12.5 µM of tag-free Httex1-37Q in solution (Figure 2). For our control (ThT alone in buffer) was constant over the entire duration of the experiment (~16 hours). When ThT was incubated with the tag-free Httex1-37Q, ThT intensity was always constant within our experiments and simultaneously above the control condition. Moreover, the ThT fluorescence intensity increased with the protein concentration in the assay. The average fluorescence intensity for 5 µM of protein was 2597, for 10 µM was 4383 (and 4304), and for 12.5 µM was 5092 (the highest). The data in both graphs reveal that the tag-free protein was already aggregated before starting the assay and that further aggregation did not occur. If further aggregation had occurred, we would have seen an increase in ThT fluorescence intensity. Three more time points (29, 52 and 196 hours after starting the assay) were measured. For all conditions, ThT fluorescence intensities did not change, showing that fibrillation already attained is maximum at the beginning of the assay.

We further investigated high tag-free Httex1-37Q concentrations (Figure 2). The same trend is observed: (20 µM) has the lowest average value of fluorescence intensity 3401 and the highest concentration of protein explored (50 µM) shows the highest average value of fluorescence intensity 5584. For 30 µM concentration was obtained an average fluorescence intensity of 5015.

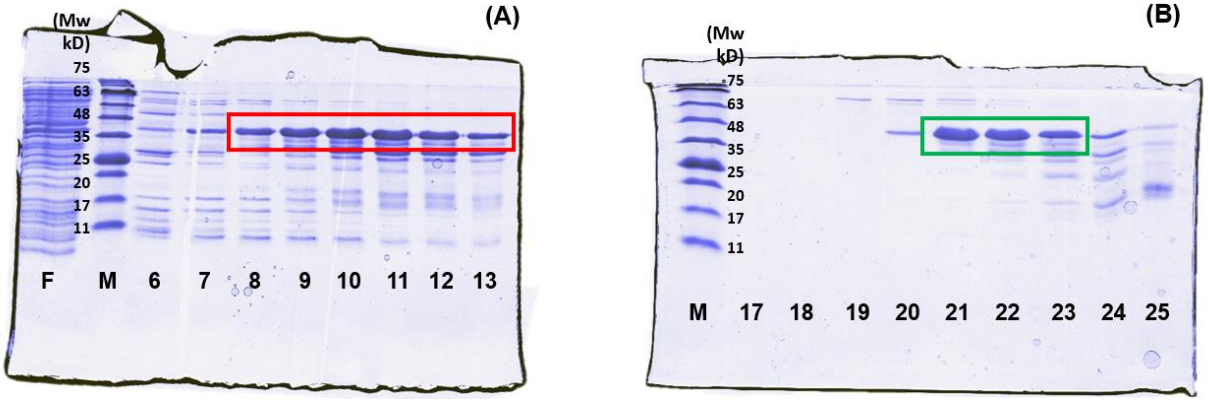

Figure 3: SDS-PAGE gels obtained for the purification of His6-SUMO-Httex1-43Q fusion protein. **(A)** IMAC; F – feed and 6-13 represent the collected fractions during the gradient with Buffer B2. The red box highlights the location of fusion protein and the fractions 8-13 were selected for pooling and concentration. **(B)** Fractions 17-25 collected during the SEC. The red box highlights the fractions that were then analyzed regarding their protein concentration and stored at -80 ºC for further experiments. M represents the markers (NZYColour Protein Marker II).

Considering the kinetic results using tag-free Httex1-37Q, we decided to try and purify the variant Httex1-43Q as a fusion protein, so the His_6-SUMO tag was not cleaved. This variant was chosen specifically because its polyQ-length is considered pathological and causes HD. However, it is also more challenging to purify because it contains more glutamine residues. The results are shown in figure 3 and are overall positive. The IMAC ran as expected and the SEC procedure yielded relatively pure protein.

Before advancing to ThT fluorescence assay studies using fusion protein Httex1-43Q and lipids, tag-free Httex1-43Q was purified and tested. The results are similar to the ones obtained in Figure 2, solidifying the idea that tag-free protein aggregates readily after purification and monitoring its fibrillation without a solubilizing tag is a big challenge.

Fibrillation of His_6-SUMO-Httex1-43Q with lipid vesicles

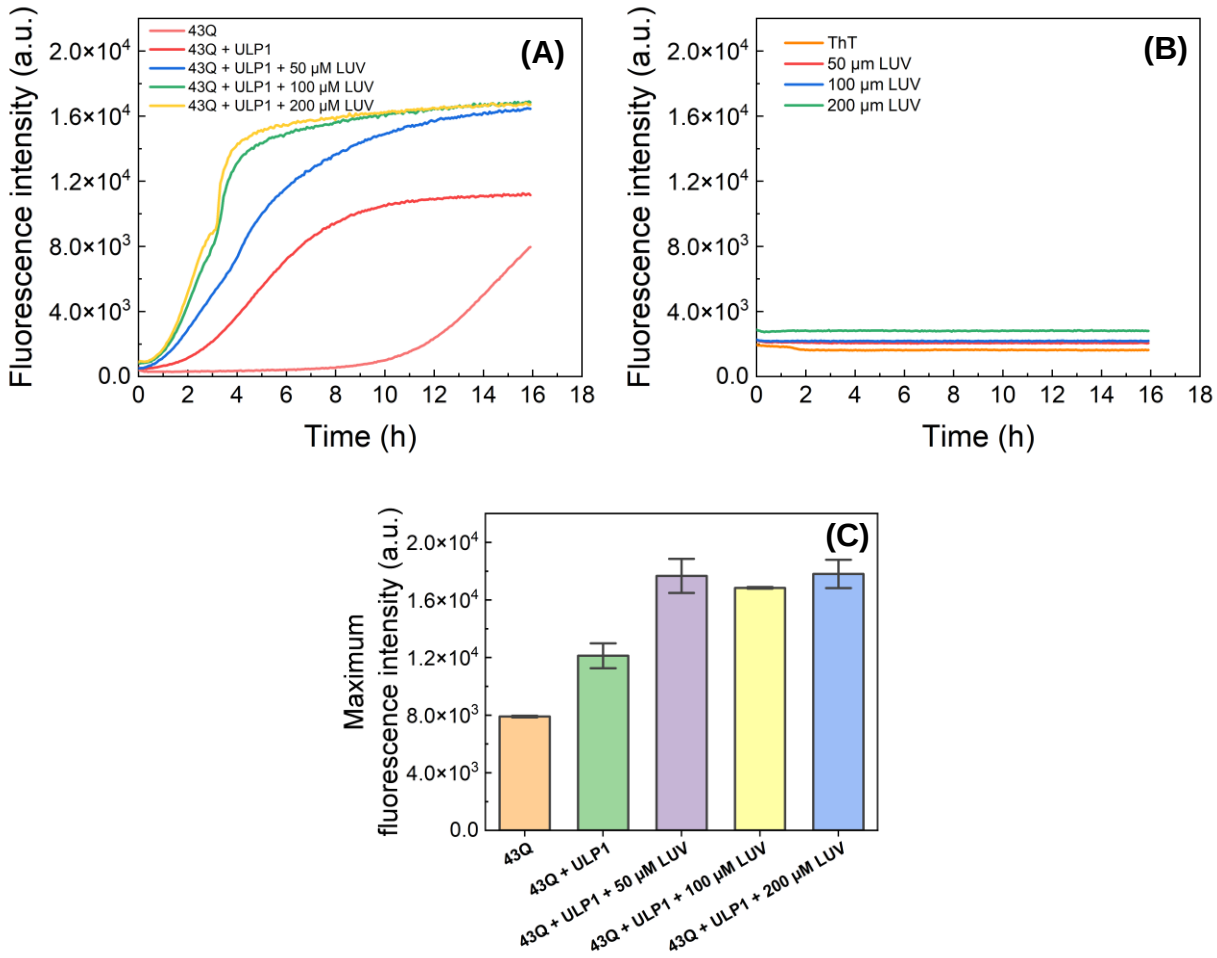

Figure 4: ThT fluorescence assays for 10 μM Httex1-43Q with anionic lipid vesicles. **(A)** ThT fluorescence intensities obtained with 10 μM of His_6-SUMO-Httex1-43Q fusion protein without ULP1 (without tag-cleavage); or with ULP1 (for tag-cleavage) in solution and in the presence of 50 μM, 100 μM or 200 μM LUVs 25:75 POPC:POPS. The data were corrected by subtracting the corresponding averaged control intensity. **(B)** Control conditions for the ThT fluorescence assays. The controls consist of ThT in buffer (ThT), and ThT with 50 μM, 100 μM or 200 μM of LUVs. **(C)** Bar graph illustrating the maximum fluorescence intensity at the end of the ThT assay for the experimental conditions represented in (A).

The main goal was to evaluate the impact of anionic lipid membranes in Httex-43Q fibrillation. ThT fluorescence assays were carried out with 10 μM of His_6-SUMO-Httex1-43Q fusion protein without ULP1; and also with ULP1 in solution, and then with different LUV concentrations (50 μM, 100 μM, and 200 μM). The results of the assay are shown in Figure 4. We note that our control experiments show similar trends to the previous fluorescence assays. ThT fluorescence in buffer is constant over time and with LUVs it slightly increased (but is not significant when comparing with samples with protein). Aggregation of the fusion protein (without ULP1) still occurred but with a slower kinetics; the fluorescence intensities remined constant in the first 8 hours and then increased, but a plateau was not reached in our experiment. This indicates that it is likely that even with solubilizing tags, the Httex1-43Q fragment is prone to fibril formation. When the fusion protein's tag was cleaved from the protein (by addition of ULP1), the lag phase was much shorter and after approximately 1 hour the fluorescence starts increasing in an exponential manner and a plateau was reached at a value of 11 000 after 14 hours of experiment. This reveals that the removal of the solubilizing tag has a significant effect on the Httex1-43Q fibrillation as expected.

The graphs illustrate the significant effect of lipid membranes on the aggregation of Httex1-43Q. The aggregation kinetics of Httex1-43Q in the presence of lipid membranes were faster than in solution (Figure 4-A). They also show a typical sigmoid curve profile associated with amyloid fibril formation. For 100 μM and 200 μM lipid, ThT fluorescence reached a plateau phase at 4 hours, and for 50 μM of lipid only seems to approach a plateau at 12 hours. In addition, our data supports that the extension of Httex1-43Q fibrillation was higher in the presence of anionic lipid membranes than in solution, since the ThT fluorescence intensities at the plateau were higher for samples with lipid (Figure 4-C). Considering that all samples contain the same protein concentration and only the lipid concentration was altered, we can conclude that anionic lipids have a high impact in Httex1-43Q aggregation.

Discussion

HD is a fatal and inherited neurodegenerative disease caused by the pathological expansion of the polyQ tract (above 37Q) within exon-1 of Htt protein [18]. Several evidence supports that biological membranes promote Httex1 aggregation and simultaneously these aggregates also cause membrane disruption/permeabilization, resulting in organelle dysfunction. In addition, the physical and chemical properties of lipids heavily influence its membrane interaction and mechanism of aggregation [54], [59].

The detailed description of the molecular mechanism of Httex1 aggregation is crucial for understanding its pathological contribution for HD and may lead to the development of new strategies for HD treatment. Early studies relied on the use of synthetic polyQ peptides (with extra lysine residues) for solubilization. However, recent studies support that both flanking polyQ regions, Nt17 and PDR, modulate Httex1 aggregation. Therefore, it is crucial to perform Httex1 aggregation assays in the context of the full-length

fragment. To achieve a better experimental design and more representative results, several labs have developed ThT fluorescence assays with Httex1 fusion constructs (for solubilization) and performing only the tag-cleavage during the experiment.

Not only that, but as seen by the results obtained in this project, the expression and purification procedures of Httex1 are extremely challenging due to its hydrophobic, intrinsically disordered, and aggregation-prone nature [65]. Therefore, several strategies have been developed for the recombinant expression in *E.coli*. In this thesis, we used a SUMO-fusion strategy that generates native tag-free Httex1- without extra amino acid residues after cleavage with ULP1 protease. However, there are many other tags that each may need different proteases and conditions to function properly. Because of this, the comparison of Httex1 aggregation kinetics comparison between labs is very challenging.

The plasmid containing the His_6-SUMO-Httex1-43Q construct was previously cloned in the lab and the expression and purification was optimized in this thesis with the best results being shown in Figure 1. The optimized process in this thesis removed the need for concentration in Amicons by replacing it with a dialysis step for buffer exchange and by employing a second IMAC procedure with a 1 mL HisTrap FF column to avoid concentration in Amicons and adsorption of protein to the membrane. As described in literature, the native structure of Httex1 fragments and their physicochemical properties make them intrinsically disordered and highly unstable [32], [66] and are highly prone to aggregate. The SUMO tag is a great solubilization solution, and it has been shown during the purification process that its respective protease ULP1 is extremely effective and fast at cleaving the tag from the Httex1 fragment. Not only that, but it is widely accepted that SUMO tag does not interact significantly with ThT, which allows for a much cleaner experimental design because most of the fluorescence registered can be attributed to ThT indeed binding to β-sheets present in amyloid fibers that are formed during the assay.

Many more studies both on brain cell membranes and mutant huntingtin will be needed to join these two areas and design new, innovative projects. Of course, cellular conditions affect the process of amyloid fibril formation. Knowing that cells have several compartments encapsulated by lipid membranes, studying the interaction between the pathological Httex1 fragment and artificial lipid membranes will give insight into the pathological mechanisms of HD [55]. However, the results obtained from the experiments done with purified protein and lipid vesicles show that anionic lipids promoted a faster aggregation rate for Httex1 fibrils.

Conclusion

Amyloidogenic intrinsically disordered proteins are exceptionally susceptible to degradation and aggregation, and thus extremely difficult to purify and work with. Previous research relied on the use of synthetic polyQs to study the effects of aggregation, but recently the importance of the flanking polyQ

domains of Httex1 has been highlighted. To produce stable protein that is more resistant to aggregation, solubilization tags are added, nevertheless some of the tags used cleavage may introduce new amino acids to the sequence. In the case of Httex1 and its variants used in this project, His_6-SUMO-tag was added then cleaved with ULP1. This method works very well because this procedure does not introduce new amino acids into the sequence, is highly efficient and does not take a long amount of time.

Furthermore, the thesis also aimed at investigating the effect of anionic lipids on Httex1 aggregation and fibrillation process. Using 10 μM of fusion protein Httex1-43Q, the obtained results support the great influence of lipids by potentiating aggregation.

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