Highly Potent Peptide Therapeutics To Prevent Protein Aggregation In Huntington's Disease

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Safety statement

No unexpected or unusually high safety hazards were encountered.

Contents

1. Expression and Purification of Htt Constructs

The plasmids (Figure S1) harboring the cloned constructs were transformed in *E. coli* BL21 (DE3) for protein expression.

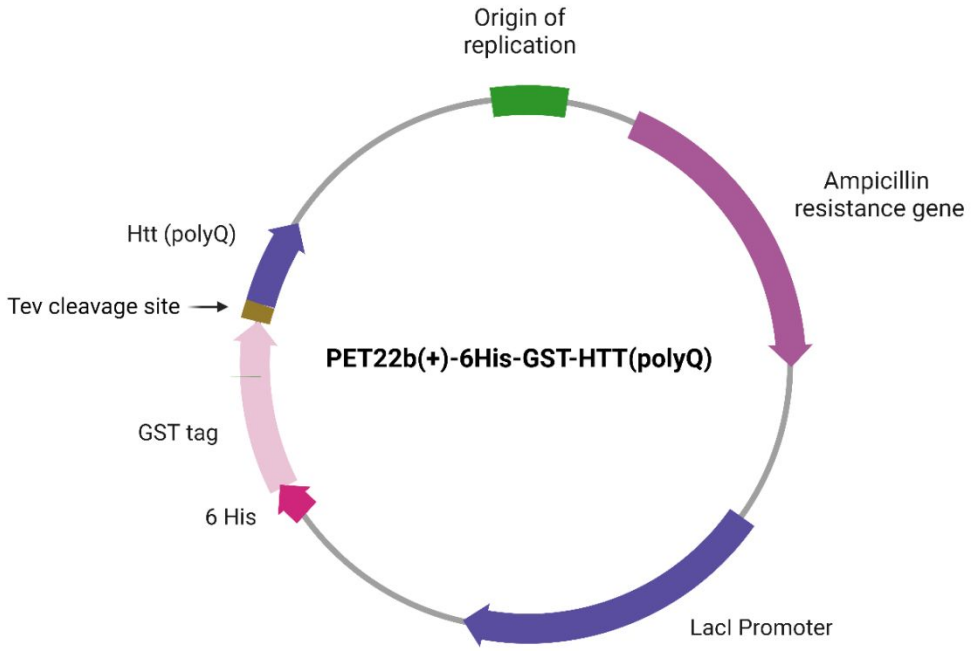

Figure S1. Plasmid map of PET22b(+) vector with GST-Htt construct.

Glycerol cell stocks containing the constructed plasmids were grown at 37 °C, 200 rpm in the shaking incubator overnight. Overnight culture of GST-Htt(Q103) was diluted in fresh LB with a 1:100 ratio and grown in a 37 °C shaking incubator until the optical density OD_{600} reached 0.4 and was induced by the addition of 1mM of IPTG. The cells were then incubated at 37 °C, 200 rpm for 4 hours. Fusion proteins, GST-Htt-(Q46), and GST-Htt-(Q25), were expressed using ZYM5052 autoinduction media.

GST tag was fused to stabilize and prevent the polymerization of Htt proteins, as described previously [1]. Both wildtype GST-Htt-(Q25) and mutant GST-Htt-(Q46) and GST-Htt-(Q103)

fusion proteins were cloned in pET22b (+) vector under the T7 promoter. A TEV protease cleavage site was cloned between the 6His-GST and N terminal of Htt Exon1 proteins. The plasmids harboring the cloned constructs were transformed in *E. coli* BL21 (DE3) for protein expression. The details of the cloning, expression and purification of all the GST-Htt constructs used in this study is given in the Supporting Information (Figures S1−S3 and Table S1-S2).

The overnight cultures grown in LB were diluted in autoinduction media in a 1:100 ratio and were incubated at 37 ^{o} C, 200 rpm for 20-24 hours. The cell pellet was resuspended in imidazole binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4). Cells were lysed by three rounds of Freeze-thaw and then subjected to sonication at 30% Amp, with 10 seconds pulse on and 10 seconds pulse off for 5 minutes at 4 ^{o}C. Cell homogenates were then centrifuged at 13000 xg for 1 hour, 4 ^{o}C. The supernatant was filtered using 0.45 μm filters. Fast protein liquid chromatography (FPLC) was used to purify his tagged proteins from the whole cell lysate. The proteins were purified using a Ni-NTA column in AKTA start and were eluted in imidazole elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). The purified proteins were then desalted by AKTA start and Hi-Trap desalting column (5 mL).

Figure S2 shows insoluble highmolecular-weight aggregates stuck in stacking gel in lane 6 and lane 9. Lane 6 and lane 9, both show protein in the well. These are fibrils of Htt Q103 and Htt Q46 respectively. Western blot was performed with anti-polyQ antibody. Figure S3 shows western blot result for GST-Htt(25Q), GST-Htt(46Q) and GST-Htt(103Q).

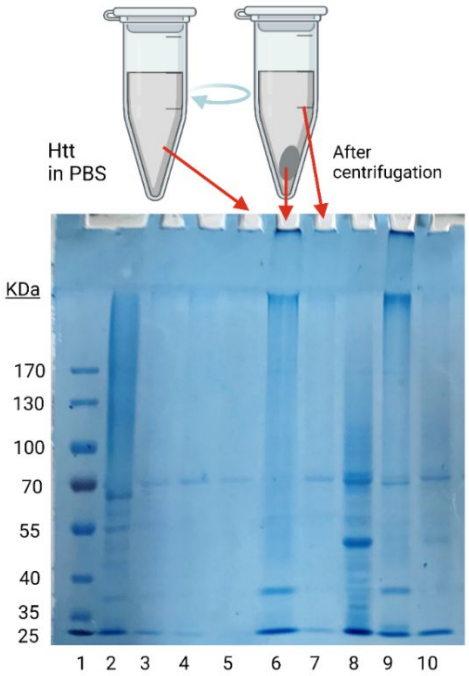

Figure S2. SDS-PAGE gel. Lane1-10: 1:Protein marker, 2: GST-Htt (Q103), 3:GST-Htt (Q103) with tev protease, 5: Htt (Q103) in PBS. 6: 5 days old Htt (103Q) pellet. 7: 5 days old Htt (103Q) supernatant, 8: GST-Htt (Q46), 9: 5 days old Htt (46Q) pellet. 10: 5 days old Htt (46Q) supernatant.

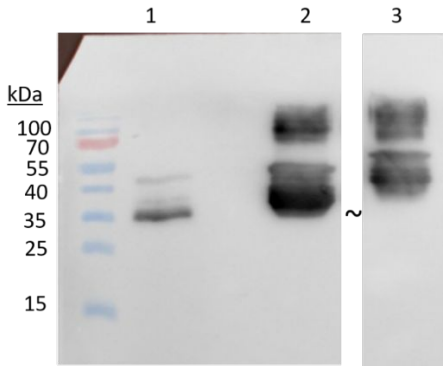

Figure S3. Western blot analysis with polyQ antibody for GST-Htt(Q25) (lane 1), GST-Htt(Q46) (lane) and GST-Htt(Q103)

2. Congo Red Assay to Visualize Aggregation

Purified Htt-(Q103) in PBS was incubated at room temperature for five days. After five days, 4 µL of 50X Congo red dye was added to 200 µL purified protein and was centrifuged at 11000 xg for 10 minutes. Congo-Red dye has a high affinity towards beta-sheet enriched Htt fibrils and is a commonly used histological dye for detecting amyloid fibrils [2]. A red pellet was observed after centrifugation, showing the presence of fibrils in in Htt(Q103) (Figure S4).

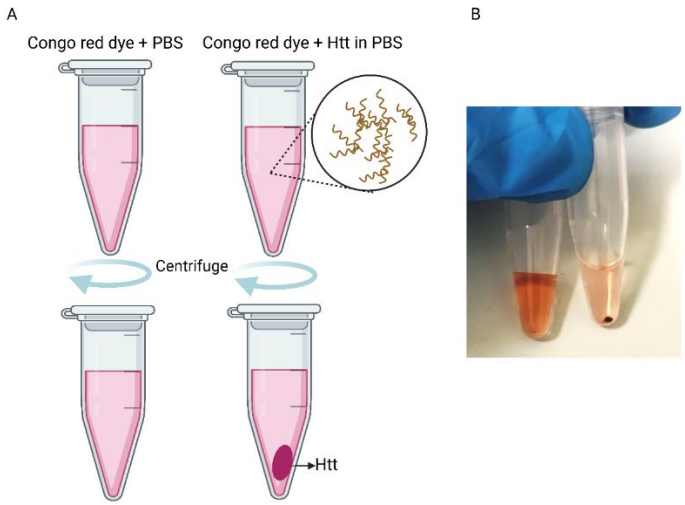

Figure S4: A) Schematic representation of Congo Red staining for Htt(Q103) fibrils. B) Congo Red staining for Htt (Q103) fibrils.

3. ThT Assay to Visualize Aggregation in Real-time

The peptides used in this study were produced by solid-state synthesis and were purchased from Peptiteam. For initiating aggregation, TEV protease was added to 7 µM of GST-Htt

protein. 7 μM of each peptide was then added to the peptide-treated groups. The reaction was completed to 200 μL in each well by tev reaction buffer, and 0.5 μL of 25 μM ThT was added. The 96-microwell plate was then sealed with adhesive tape to prevent evaporation. The ThT fluorescence intensity of each sample was recorded by spectraMax M5 Microplate Reader spectrophotometer (Molecular Devices) with 438/495 nm excitation/emission filters at cutoff at 475 nm after every 10 minutes for 16-20 hours. Each reading was preceded by 5 s for orbital agitation. All readings were set up as triplicates. The ThT data were normalized as described before [3], and percentage fluorescence was plotted against time (Figure S5).

4. Aggregation Kinetics

Freshly purified Htt proteins were centrifuged at 18000 x g for 20 minutes to remove pre-existing aggregates [4]. The TEV cleavage reaction was set up to initiate aggregation using 10 μM GST-Htt(Q103). 500 μL volumes of reactions were incubated at room temperature. 2 hours after the administration of TEV protease, 10 μM peptide was introduced at time zero. After 2,4,8,10,12 and 24 hours, 5 μL of samples were aliquoted from both peptide-treated and untreated Htt(Q103) for AFM deposition. On SiO_2 wafers, a 5 μL sample was deposited in order to get topography pictures in air. The wafer was cleaned with ultrapure water for two minutes

and then dried with argon. In order to examine the impact of inhibitory peptides on the aggregation kinetics of GST-Htt(Q46) and GST-Htt(Q25), the purified proteins were diluted to a final concentration of 30 μM in 1X TEV reaction buffer (pH 8.0) and TEV Protease was added to remove 6xHis-GST and commence the aggregation. 500 μL volumes of reactions were incubated at room temperature. 2 hours after the administration of TEV protease, equimolar peptide was introduced at time zero. After 4,8, and 24 hours, 5 μL of samples were aliquoted from the peptide-treated and untreated Htt(Q25) and Htt(Q46) experimental groups. The sample deposition for AFM was carried out in the same manner.

5. AFM Imaging

AFM images of AFM surface topography were captured with an Oxford Instruments MFP-3D Origin in tapping mode, which employs air topography. Budget AFM probes with a 300 kHz resonance frequency and 40 N/m spring constant Sensors were used for measuring purposes. Samples were scanned between 1.2Hz and 2.2Hz, depending on the size of the scan area. AFM measurements were conducted in ambient conditions at room temperature. Matlab FiberApp was used to quantify each data set's fibril length. Matlab AFM pictures were evaluated and adjusted

for scan artifacts using the Gwyddion v2.60 application. The two-way ANOVA test compared the fibril length of peptide-treated Htt proteins and untreated Htt proteins.

Table S1. List of primers used to clone the Htt constructs in pET22b (+) AmpR Vector

Primers	Sequence 5'-3'
pCEO-69	GCAGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTCATCAAGGTCGGTGCAGAGGCTC
pCEO-70	GTTTGGTGGTGGCGACCATCCTCCAAAAGGATCCGAAAACCTGTATTTTCAGGGCactagtATGGCGACCCTGG

Table S2 : GST-Htt DNA sequences

Htt constructs	Sequence
GST-Htt(Q25)	ATGAACACCATTCATCACCATCACCATCACAACACTAGTATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGGAATATCTTGAAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAATGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTTATTATATTGATGGTGATGTTAAATTAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGCACAACATGTTGGGTGGTTGTCCAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGATACGGTGTTTCGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTCGAAGATCGTTTATGTCATAAAACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATGTTGTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGCGTTCCCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATAAGTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTCCAAAAGGATCCGAAAACCTGTATTTTCAGGGCa

	ctagtATGGCGACCCTGGAAAAGCTGATGAAGGCCTTCGAGTCCCTCAAAAGCTTCC AACAGCAGCAACAGCAACAACAGCAGCAACAGCAACAACAGCAGCAACAGCAA CAACAGCAGCAACAGCAACAACCGCCACCACCTCCCCCTCCACCCCCACCTCCTC AACTTCCTCAACCTCCTCCACAGGCACAGCCTCTGCTGCCTCAGCCACAACCTCCT CCACCTCCACCTCCACCTCCTCCAGGCCCAGCTGTGGCTGAGGAGCCTCTGCACCG ACCT
GST- Htt(Q46)	ATGAACACCATTCATCACCATCACCATCACAACACTAGTATGTCCCCTATACTAGG TTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGGAATATCTTG AAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAATGGCGAA ACAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTTATTATATTGATGGT GATGTTAAATTAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGCACA ACATGTTGGGTGGTTGTCCAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGC GGTTTTGGATATTAGATACGGTGTTTCGAGAATTGCATATAGTAAAGACTTTGAA ACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTCGAAG ATCGTTTATGTCATAAAACATATTTAAATGGTGATCATGTAACCCATCCTGACTTC ATGTTGTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGC GTTCCCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATA AGTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCAC GTTTGGTGGTGGCGACCATCCTCCAAAAGGATCCGAAAACCTGTATTTTCAGGGCa ctagtATGGCGACCCTGGAAAAGCTGATGAAGGCCTTCGAGTCCCTCAAAAGCTTCC AACAGCAGCAACAGCAACAACAGCAGCAACAGCAACAACAGCAGCAACAGCAA CAACAGCAGCAACAGCAGCAACAGCAACAACAGCAGCAACAGCAACAACAGCA GCAACAGCAACAACAGCAGCAACAGCAACAACCGCCACCACCTCCCCCTCCACCC CCACCTCCTCAACTTCCTCAACCTCCTCCACAGGCACAGCCTCTGCTGCCTCAGCC ACAACCTCCTCCACCTCCACCTCCACCTCCTCCAGGCCCAGCTGTGGCTGAGGAGC CTCTGCACCGACCT
GST- Htt(Q103)	ATGAACACCATTCATCACCATCACCATCACAACACTAGTATGTCCCCTATACTAGG TTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTTCTTTTGGAATATCTTG AAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAATGGCGAA ACAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTTATTATATTGATGGT GATGTTAAATTAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGCACA ACATGTTGGGTGGTTGTCCAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGC GGTTTTGGATATTAGATACGGTGTTTCGAGAATTGCATATAGTAAAGACTTTGAA

	ACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTCGAAG ATCGTTTATGTCATAAAACATATTTAAATGGTGATCATGTAACCCATCCTGACTTC ATGTTGTATGACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGC GTTCCCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATA AGTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCCAC GTTTGGTGGTGGCGACCATCCTCCAAAAGGATCCGAAAACCTGTATTTTCAGGGCa ctagtATGAAGGCCTTCGAGTCCCTCAAAAGCTTCCAACAGCAGCAACAGCAACAAC AGCAGCAACAGCAACAACAGCAGCAACAGCAACAACAGCAGCAACAGCAACAA CAGCAGCAACAGCAACAACAGCAGCAACAGCAACAACAGCAGCAACAGCAACA ACAGCAGCAACAGCAACAACAGCAGCAACAGCAACAACAGCAGCAACAGCAAC AACAGCAGCAACAGCAACAACAGCAGCAACAGCAACAACAGCAGCAACAGCAA CAACAGCAGCAACAGCAACAACAGCAGCAACAGCAACAACAGCAGCAACAGCA ACAACCGCCACCACCTCCCCCTCCACCCCCACCTCCTCAACTTCCTCAACCTCCTC CACAGGCACAGCCTCTGCTGCCTCAGCCACAACCTCCTCCACCTCCACCTCCACCT CCTCCAGGCCCAGCTGTGGCTGAGGAG

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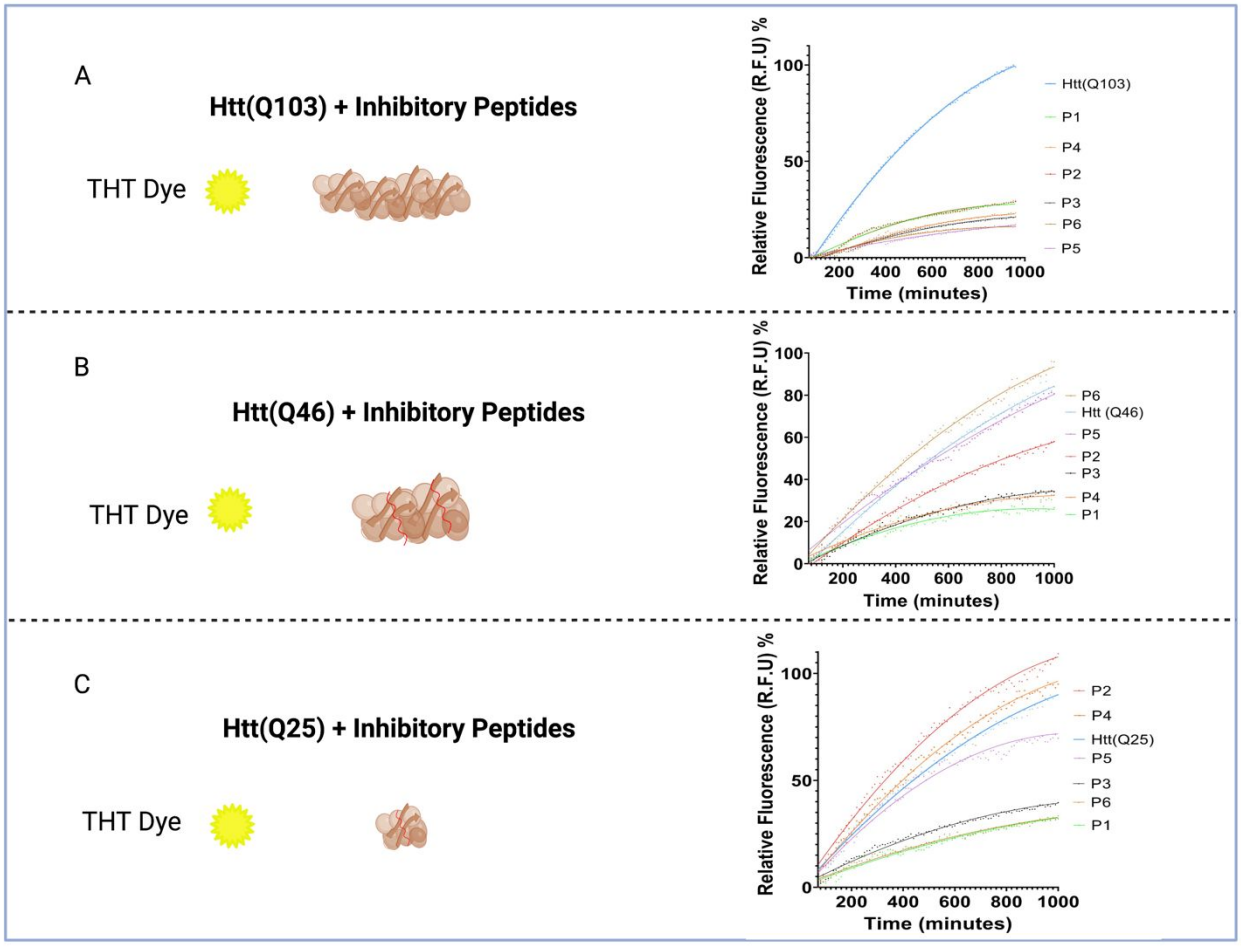

Figure S5. ThT assay for evaluating the effect of peptides on the fibril kinetics of Htt proteins.

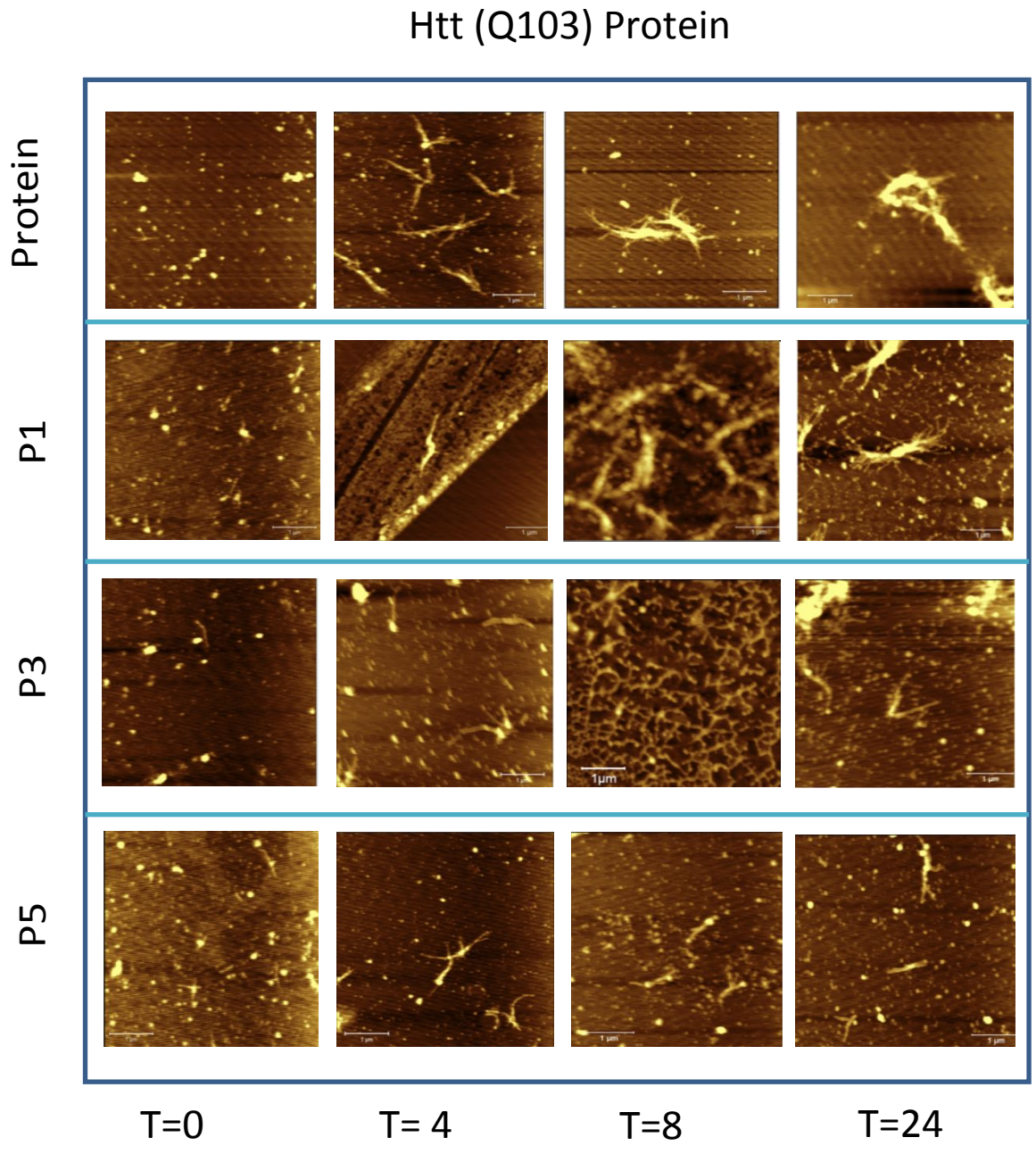
Htt (Q103) Protein
Protein
P1
P3
P5
1µm
T=0
T= 4
T=8
T=24

Figure S6. Aggregation kinetics of HttQ103, both in the absence and presence of inhibitory peptides. The AFM images show samples obtained at 0 hour, 4 hours, 8 hours, and 24 hours. The first row of Images corresponds to the fibril growth kinetic of the Htt Q103 protein. The second row of images corresponds to the fibril growth kinetics of HttQ103 in the presence of inhibitory peptide 1 (HHGANSLLGLVQS). The third row shows fibril growth kinetics of HttQ103 in the presence of inhibitory peptide 3 (HGLHSMHNKLLQT) and the last row shows fibril growth kinetics of HttQ103 in the presence of inhibitory peptide 5 (WMFPSLKLLDYH). Scale bar is 1 μm.

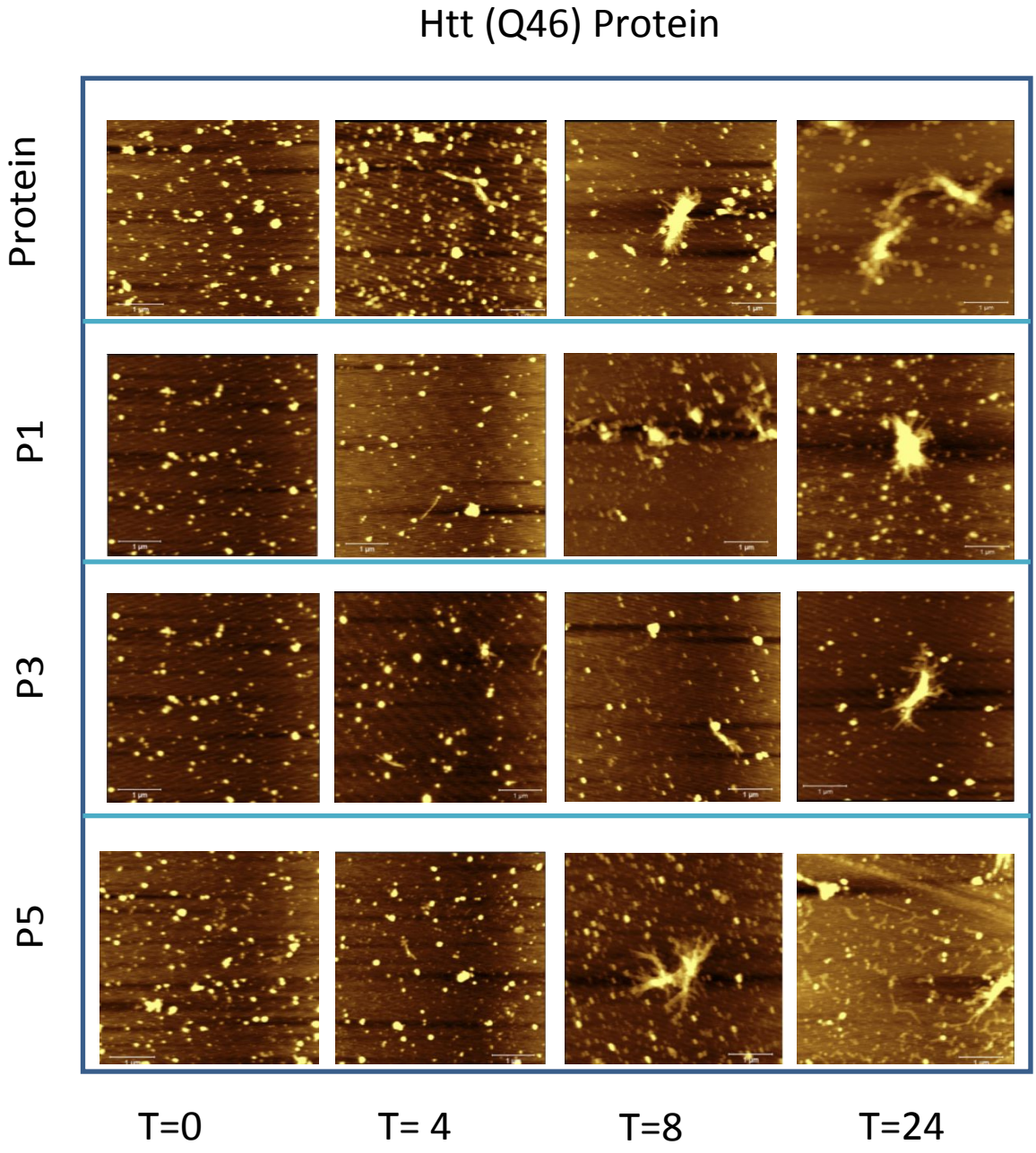

Figure S7. Shows the aggregation kinetics of Htt(Q46), both in the absence and presence of inhibitory peptides. The AFM images show samples obtained at 0 hour, 4 hours, 8 hours, and 24 hours. The first row of Images corresponds to the fibril growth kinetic of the Htt Q46 protein. The second row of images corresponds to the fibril growth kinetics of HttQ46 in the presence of inhibitory peptide 1 (HHGANSLGLVQS). The third row shows fibril growth kinetics of HttQ46 in the presence of inhibitory peptide 3 (HGLHSMHNKLQT) and the last row shows fibril growth kinetics of HttQ46 in the presence of inhibitory peptide 5 (WMFPSLKLLDYH). Scale bar is 1 μm.

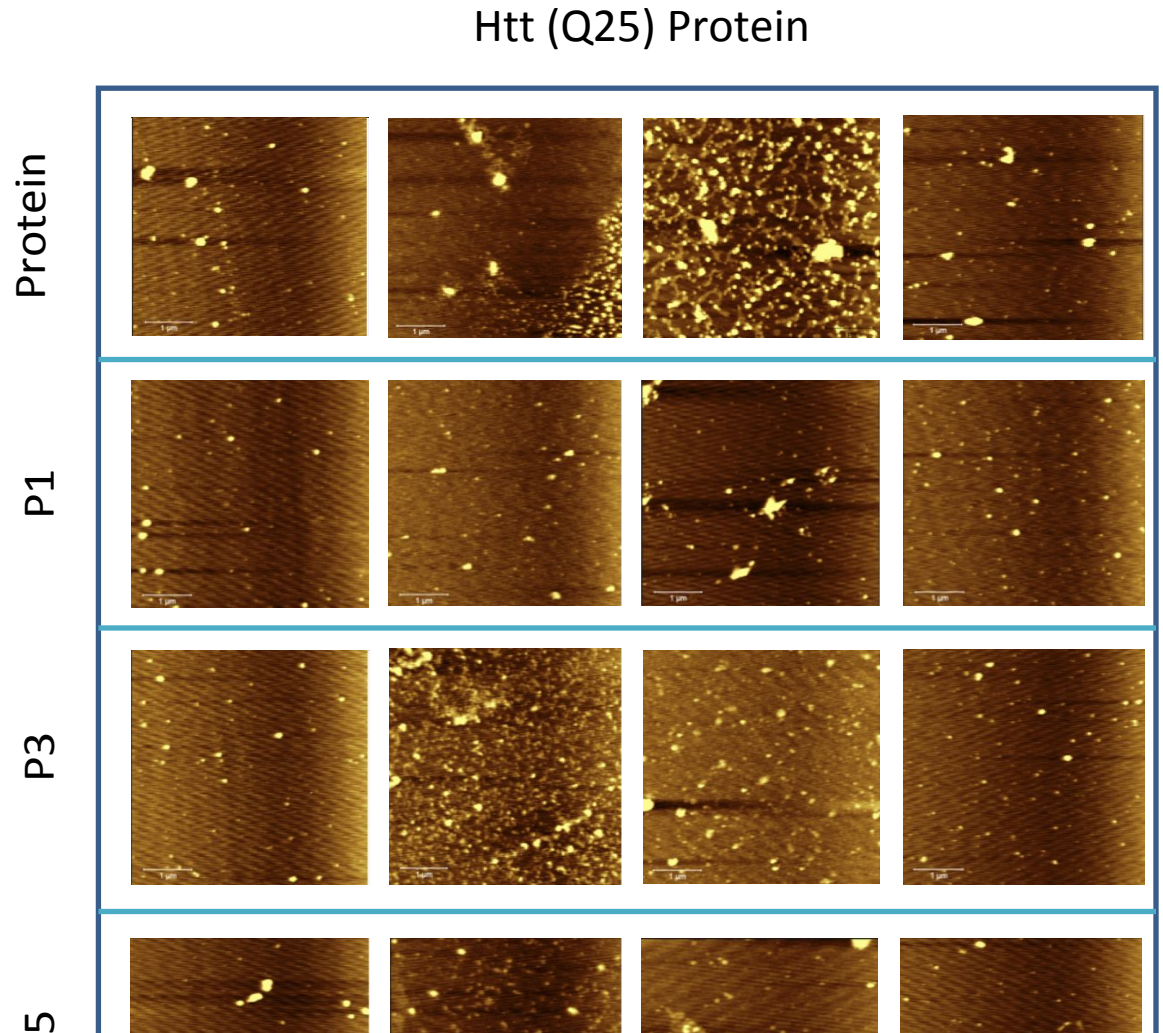

Figure S8. Shows the aggregation kinetics of Htt(Q25), both in the absence and presence of inhibitory peptides. The AFM images show samples obtained at 0 hour, 4 hours, 8 hours, and 24 hours. The first row of Images corresponds to the fibril growth kinetic of the Htt(Q25) protein. The second row of images corresponds to the fibril growth kinetics of HttQ25 in the presence of inhibitory peptide 1 (HHGANSLGLVQS). The third row shows fibril growth kinetics of HttQ25 in the presence of inhibitory peptide 3 (HGLHSMHNKLQT) and the last row shows fibril growth kinetics of HttQ25 in the presence of inhibitory peptide 5 (WMFPSLKLLDYH). Scale bar is 1 μm.

6. Peptide Selection

The peptides used in this study were selected by a biopanning process. Utilizing yeast surface display procedure offers the opportunity to express numerous peptides and proteins with high stability. As a eukaryotic expression system it provides advantages to display a wide array of proteins, particularly mammalian proteins.[5] Displaying proteins like pathogenic amyloids on the surface of yeast cells will result in less toxic effects compared to their presence in the cytosol[6].

The peptides used in this study were screened and selected against the monomeric units of Htt proteins, both wild-type (Htt(Q25)) and mutant Htt fragments (Htt(Q46) and Htt(Q103)). Yeast cells expressing Htt proteins on their surfaces were obtained after various cloning steps [7]. Each of the monomeric Htt proteins was individually expressed on the surface of yeast cells. These yeast cells were then mixed with a phage library carrying peptides on their surfaces, and peptides that bind to Htt monomers were selected.

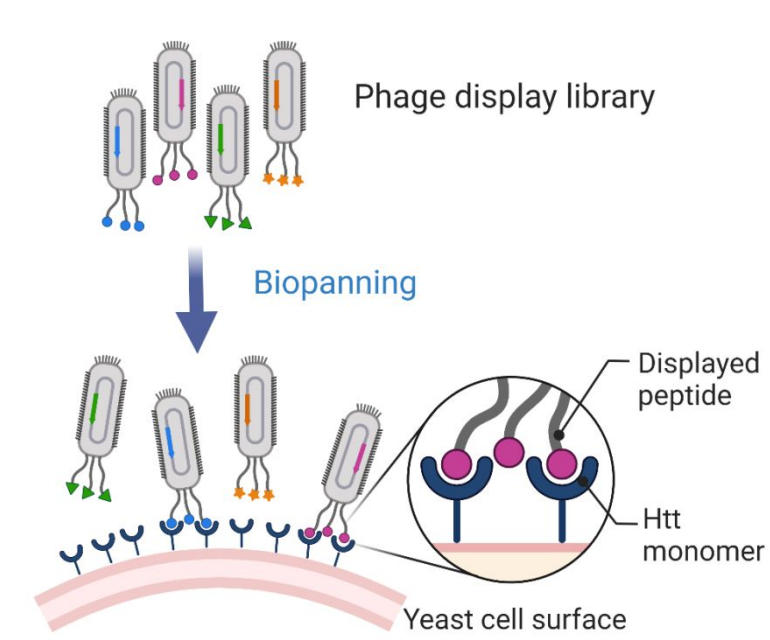

Figure S9: Schematic representation biopanning procedure for selection of Htt monomers (Created with BioRender.com)

7. Circular Dichroism (CD) spectroscopy

Circular dichroism (CD) data were measured from 195 nm to 250 nm with Jasco J-815 CD spectropolarimeter. The CD data was analyzed to determine the secondary structural elements employing the online tool BestSel [8]. It has been observed that the increased percentage of beta-sheet secondary structure observed in the produced Htt monomer proteins correlated with the increasing number of glutamine residues (Figure S10, S11).

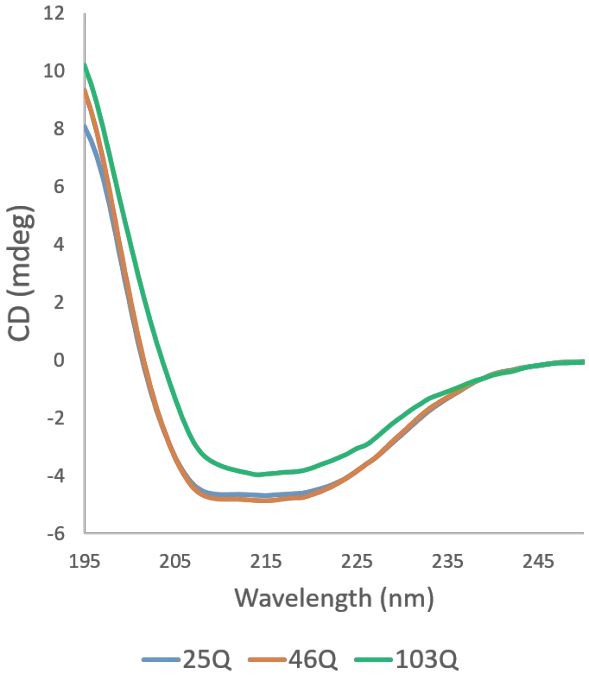

Figure S10: CD spectra of GST-Htt25Q, GST-Htt46Q and GST-Htt103Q

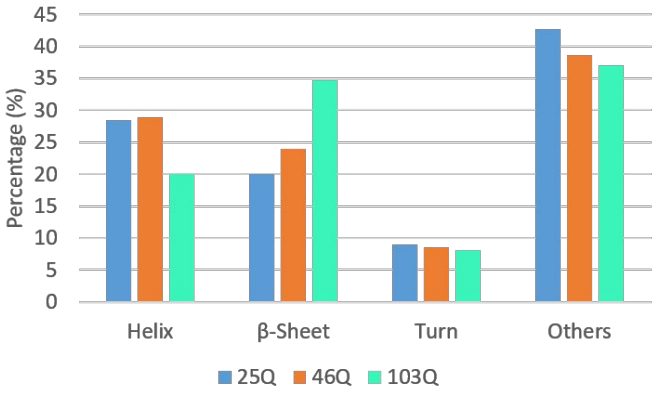

Figure S11: Secondary structure elements predictions of GST-Htt25Q, GST-Htt46Q and GST-Htt103Q obtained from CD data.

8. Cytotoxicity Assay

The cytotoxic effects of Htt monomers and peptide application were evaluated by selecting peptide 5 among the peptides determined as a result of aggregation kinetics and Tht assay data. HT22 mouse hippocampal neuronal cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.5 g/mL glucose, 10% fetal bovine serum (FBS), 1X penicillin/streptomycin, 2 mM L-glutamine as final concentrations. The growth conditions were achieved by incubating cells at 37 ºC, 5% CO_2, humidified fixed incubator. For checking viability of HT22 cells with the existence of Htt proteins and peptide 5, 50000 cells/ mL were used, and 100 µL cell suspension was aliquoted into 96-well plates to use 5000 cells/well. After 24 hours of incubation with described growth condition for attachment of the cells to the plate surface, first, 2µL of 100 µM monomeric 25Q-Htt, 46Q-Htt, and 103Q-Htt samples were added to the cells separately to obtain 2 µM protein final concentration. Immediately after protein addition, peptide solution was placed in wells. All conditions were done in triplicates. After mixing proteins and the peptide with cells, incubation was done at 37 ºC, 5% CO_2, humidified fixed incubator for 24 hours. At the end of the incubation, MTT assay was started. For this assay, 10 µL of 5 mg/mL MTT stock solution was added to each well and incubated for 4 hours at growth conditions. Subsequent to incubation period, the media was discarded and 100 µL of DMSO was added to solubilize the formazan crystals. The solubilization was achieved by shaking the plate at room temperature for 10 minutes at dark. The samples were measured for absorbance values at 570 nm (Figure S12). The effect of peptide application aimed at preventing increased aggregation and toxicity in cells due to the expanded polyglutamine has been observed. When the peptide was applied alone, it resulted in an approximate 20% decrease in viability; however, when compared to its application with htt monomers with an increased polyglutamine length, it was observed that they had relatively lower toxicity.

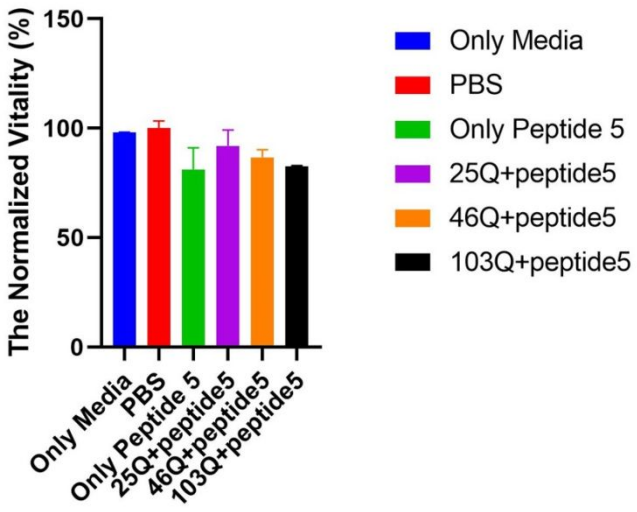

Figure S12: Effect of Htt proteins and peptide mixes to cell viability.

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