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Factors Influencing Huntingtin Aggregation at Surfaces: Implications for Huntington's Disease

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**Factors Influencing Huntingtin Aggregation at Surfaces: Implications for
Huntington's Disease**

Sharon E. Groover

Dissertation submitted to the
Eberly College of Arts and Science
at West Virginia University
Morgantown, WV 26505

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Chemistry

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Factors Influencing Huntingtin Aggregation at Surfaces: Implications for Huntington's Disease

Abstract

Sharon E. Groover

Huntington's Disease (HD) is a genetic, neurodegenerative disease characterized by an abnormal polyglutamine (polyQ) expansion in the first exon of the huntingtin protein (htt). The polyQ domain facilitates aggregation and initiates the formation of a diverse collection of aggregate species, including fibrils, oligomers and annular aggregates. The first 17 amino acids of htt (Nt17) directly flank the polyQ domain and is a key factor in htt's association to membranous structures. In addition to Nt17 being an amphipathic α -helix, it also promotes aggregation through self-association and contains numerous posttranslational modifications (PTMs) that can modulate toxicity and subcellular localization. For in depth understanding of these mechanisms, particularly in the presence of lipid membrane surfaces, the PTM phosphorylation and macromolecular crowders found in subcellular environments were explored. Through the application of phosphomimetic mutations of htt to a variety of lipid systems, lipid-specific impacts of electrostatic interactions involved in htt/lipid interactions were elucidated. Cytosolic conditions mimicked through the addition of macromolecular crowders and htt were evaluated at both solid/liquid and membrane/liquid interfaces, with each crowder having a distinct effect on htt aggregation. The results presented here aid in the understanding of the multi-faceted nature of htt aggregation in the presence of cellular and subcellular surfaces

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List of Symbols and Abbreviations

α -syn	α -synuclein
A β	β -amyloid peptide
ACS	American Chemical Society
AD	Alzheimer's Disease
AH	Amphipathic α -helix
AFM	Atomic Force Microscopy
AKT	Protein Kinase B
BSL-1	Biological Safety Level-1
CR	Colorimetric Response
DOPG	1,2-dioleoyl-sn-glycero-3-phosphoglycerol
DRPLA	Dentatorubral-Pallidoluysian Atrophy
E. Coli	Escherichia Coli
EDTA	Ethylenediaminetetraacetic acid
EPR	Electron Paramagnetic Resonance
ER	Endoplasmic Reticulum
ERAD	Endoplasmic-Reticulum-Associated Protein Degradation
Exon1	First exon of the huntingtin protein
GST	Glutathione-S-Transferase
HCl	Hydrochloric Acid
HD	Huntington's Disease
HFIP	Hexafluoroisopropanol
Htt	Huntingtin Protein
Htt-Exon1	Huntingtin exon1
Htt-Exon1(46Q)	Huntingtin exon1 with 46 glutamine repeats
IDRs	Intrinsically Disordered Regions
IPTG	Isopropyl- β -D-thiogalactoside
LC	Liquid Chromatography
LLPS	Liquid/liquid Phase Separation
LUV	Large Unilamellar Vesicles
MLOs	Membraneless Organelles

NaOH	Sodium Hydroxide
Nt17	The First 17 N-Terminal Amino Acids of Htt
PEG	Polyethylene Glycol
PD	Parkinson's Disease
PDA	Polydiacetylene
PIPs	Phosphatidylinositol Phosphates
PolyQ	Polyglutamine
PolyP	Polyproline
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-(phospho-rac-(1-glycerol))
POPS	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine
PTMs	Post Translational Modifications
Q	One letter abbreviation for the amino acid glutamine
RMS	Root Mean Squared
SBMA	Spinal-bulbar Muscular Atrophy
SCA-1, SCA-2, SCA-3, SCA-6, SCA-7, SCA-12, SCA-17	Spinocerebellar Ataxia diseases
SEM	Standard Error of the Mean
SGs	Stress Granules
ssNMR	Solid-State Nuclear Magnetic Resonance
STEM	Science, Technology, Engineering and Mathematics
TBLE	Total Brain Lipid Extract
ThT	Thioflavin T
TRDs	Trinucleotide Repeat Disorders

1. Introduction: Role of the Structure and Functions of the Huntingtin Protein Related to Huntington's Disease

1.1 General Protein Misfolding Diseases

A hallmark of several neurodegenerative disorders is the systematic buildup of micron sized proteinaceous material in tissues and cellular compartments. These diseases include Parkinson's Disease (PD), Alzheimer's Disease (AD) and Huntington's Disease (HD). Clinical symptoms include impaired cognitive function and motor control and pathological changes comprise of synaptic abnormalities and neuronal cell loss.¹⁻³ In each of these diseases, a hallmark is the conformational change of specific proteins inducing aggregation and accumulation of amyloids within the brain.⁴⁻⁶ These insoluble amyloids are identified through their cross- β sheet rich structures and fibril morphologies.^{7,8} These general amyloid disorders are also classified as protein misfolding diseases, in reference to the misfolding of the normal, functional protein (Fig. 1).

Figure 1.1: Amyloid structure in HD. *Diseased neurons within HD patients' brains contain amyloid plaques which are comprised of a variety of structures stemming from the disordered protein, leading eventually to amyloid fibrils. These plaques are localized to the nucleus of the neuron. Beginning with the disordered protein, when misfolded, β -sheet structures (as shown by grey arrows) start to form. The misfolded proteins reach a critical nucleus and form an amyloid intermediate that eventually forms the β -sheet rich amyloid fibrils. Created in part with BioRender.com*

The driving force behind protein folding is the tendency of hydrophobic residues to sequester together within the protein interior due to a phenomenon known as the hydrophobic effect.⁹ Functional proteins are newly synthesized from linear chains of amino acids and conform to a well-defined three dimensional compact structure of minimal energy, consistent with the concept of a folding energy landscape funnel (Fig. 2).¹⁰ In this compact, native conformation, the hydrophilic residues are located on the protein surface and exposed to water. The pathway along the energy landscape leading to the native conformation contains local energy minima in which partially folded intermediates can become trapped.^{11–13} These intermediates risk misfolding and following alternative folding pathways leading to structures differing from the native conformation. In amyloidogenic proteins, these alternative intermediates lead to β -sheet rich monomeric or multimeric structures such as oligomers that can further promote the formation of fibrils, or amorphous aggregates.¹⁴ These altered conformations associated with amyloid result in affected biological properties and a toxic gain of function.^{15,16}

Figure 1.2: Protein folding funnel energy landscape. Unfolded proteins generally undergo a variety of conformations to reach the most compact and stable native state (green pathway). If the unfolded protein proceeds through an alternate pathway other than the native folding pathway by becoming trapped in energy minima, misfolded intermediates are formed (purple pathway). For amyloid proteins, these misfolding intermediates are β -sheet rich and continue to form amyloid aggregates of lower energy, with increasing β -sheet content. Created in part with BioRender.com

1.2 Huntington's Disease and the Role of the Huntingtin Protein

Huntington's Disease (HD) is a fatal, autosomal dominant neurodegenerative disease that results in dystonia, chorea, and loss of cognitive abilities.^{17,18} This disorder results from a mutation in the HTT gene found on the short arm of chromosome 4, which codes for the huntingtin protein (htt). The mutation is a CAG trinucleotide expansion, which codes for the amino acid glutamine (Q). Therefore, the expansion in the translated huntingtin (htt) protein is referred to as the polyglutamine (polyQ) domain, and this domain is located near the N-terminus of htt.¹⁹ HD is one of ten identified CAG-repeat or polyQ disorders. In these diseases, including HD, there is a high correlation between age of onset, polyQ length, and aggregation of proteins containing expanded

polyQ.^{20,21} While the polyQ domain is normally ~17-20 residues long in htt, a polyQ repeat length of ~35 is the critical threshold associated with HD, and disease severity increases as the polyQ length expands (Fig. 3).^{19,22–25}

Figure 1.3: Huntingtin protein, exon1 and polyQ thresholds. An abnormal trinucleotide (CAG) repeat length within the IT15 gene is the root cause of htt's pathogenicity. The first exon at the N-terminus, known as exon1, contains three distinct domains, Nt17 (green), polyQ (purple) and a proline rich domain (polyP) (grey). As the polyQ length increases, age of onset decreases, with the intermediate region indicating that disease will occur later in life (triangle).

The exact mass of htt is dependent upon the size of the polyQ domain, however, the full length of htt is comprised of 3144 amino acids, 67 exons and is ~350 kDa.¹⁹ The polyQ domain follows is located in the first exon of htt and is preceded by 17 amino acids commonly referred to as Nt17. Beyond Nt17, the polyQ domain is flanked in exon1 on the C-terminal side by a proline-rich domain that contains two polyproline (polyP) stretches. The flanking domains have varying roles. Nt17 initiates lipid binding and aggregation,^{26–30} and the polyP domain modulates htt-lipid interaction²⁶ and htt nucleation.³¹ Evidence suggests that N-terminal fragments comparable to exon1 are directly involved in HD,^{32–37} thus many studies have been completed with biologically relevant N-terminal htt fragments. Htt-exon1 is responsible for increased aggregation

and accrual of proteinaceous material in the striatum in HD patients,³⁸ and it is a stable fragment that is continuously produced in the brain through proteolysis of full-length htt.³² N-terminal fragments comparable to htt-exon1 are detected in knock-in mouse models expressing full length htt³², resulting in similar progressive neurological phenotypes and age-dependent accumulation.^{33,38,39} Evidence suggest the use of htt-exon1 is an ideal model for physiologically relevant, in vitro biophysical studies of oligomers and fibrils formed by purified htt-exon1, as htt-exon1 is responsible for increased aggregation and accrual of proteinaceous material in the striatum in HD patients.³⁸

1.3 Mechanisms and Toxicity of Aggregation

Similar to other neurodegenerative diseases, fibrils in HD form via a nucleation dependent polymerization growth pathway. Though the details of aggregation fluctuate for each protein, proteins containing expanded polyQ tracts tend to follow the basic polyQ-mediated aggregation schemes.¹⁴ Mutant htt assembles into a collection of various aggregates associated with amyloid diseases, such as fibrils, oligomers, annular structures, inclusion bodies and amorphous aggregates.^{20,40–42}

A rate limiting^{43,44} nucleation phase, known as the lag phase, is associated with the alteration of the native structure of the protein to non-native conformations.⁴⁵ A mono- or multimeric nucleus formed in the lag phase through several inter- and intramolecular forces causes the rapid elongation of β -sheet rich fibrillar structures.^{44,46,47} There are two dominant aggregation pathways of generic polyQ aggregation: a) direct nucleation from monomer to fibril⁴⁸ and b) the reordering of soluble oligomeric structures into β -sheet rich fibrillar structures.⁴² These two

mechanisms are not mutually exclusive; however, conditions that promote oligomerization as the dominant or “on pathway” aggregation pathway accelerate the process.¹⁴

Figure 1.4: Schematic model of htt aggregation pathways. Htt aggregation follows three main aggregation pathways, which begin with a misfolded, non-native conformation of a monomer (green). On pathway mechanisms eventually lead to the formation of β -sheet rich fibrils, by direct transition of misfolded monomer to fibril (bottom), or through a nucleation mechanism resulting in oligomeric species (second from bottom, circle). Off-pathway mechanisms include the formation of oligomers, amorphous and annular aggregates (top and second from top, pink). These pathways are not mutually exclusive, and together the various aggregates can form large inclusions (bottom right) seen in HD patients' brains. Reprinted from Adegboyiro, et al. *Proteins Containing Expanded Polyglutamine Tracts and Neurodegenerative Disease*. Copyright 2017 American Chemical Society.

The aggregation mechanisms can result in an assortment of aggregates co-existing, but there is limited knowledge of the exact toxicity associated with each specific aggregate species. This heterogeneity dictates the need for determining the exact aggregation mechanism, defining structural elements related with the toxic forms of htt aggregates and highlights the intricacy of mutant htt toxicity. Debate continues

regarding the focal cause of toxicity associated with htt aggregates. Inclusion body formation, while the hallmark of HD, may signify a protective cellular response to eliminate diffuse htt aggregates, which have been reported to be the acutely toxic.⁴⁷ The monomeric form of htt-exon1 encompasses intrinsically disordered domains that interact intramolecularly to encourage toxic oligomerization of htt.^{49–51} Soluble oligomers can vary in stability and size, ranging from small dimers and tetramers to larger octamers and dodecamers. Htt has been known to form a variety of oligomers, as observed by AFM (atomic force microscopy), which can initiate htt fibril formation *in vitro*.^{34,44} Oligomers morphologically and biochemically indistinguishable from those observed *in vitro* are detectable in transgenic cells expressing htt, HD mouse models, and patients' brains.^{50,52}

Particular attention has been fixated on how flanking sequences on either side of the polyQ domain promote certain aggregation mechanisms. Addition of polyP residues to the C-terminus forces a PPII-like structure into the polyQ domain, which competes against the aggregation-prone β -sheet.⁵³ Nt17 stimulates aggregation by encouraging formation of helical oligomers.^{26,53} The oligomer structure brings polyQ domains into closer proximity and lowers the energy barrier to nucleate β -sheet formation.⁵³ This key role of Nt17 in the upstream stages of htt aggregation suggests that it is a viable therapeutic target.

1.4 Structure and Functions of Nt17

Understanding the structural dynamics of Nt17 would provide a deeper understanding to the complexity of htt aggregation. Nt17 in ionic buffer is intrinsically disordered but demonstrates the ability to undergo conformational transitions leading to

an amphipathic α -helix (AH) when interacting with binding partners.^{20,30} The structure of an AH is favorable for establishing a collection of helix bundles in aqueous environment, driven by maximizing the interaction of the hydrophobic faces of adjacent helices.^{29,54} The formation of these interacting helices has been anticipated to play a role in the preliminary stages of htt aggregation. Through ordering into helical bundles, Nt17 promotes the stability of aggregates and an oligomeric intermediate aggregation pathway, leading to an increased rate of aggregation.^{55–57} Oligomers are a potentially critical toxic species; therefore, the promotion of oligomerization by Nt17 could have potentially important outcomes in htt-related toxicity. Intermolecular interactions leading to the self-association of Nt17 enables the formation of these helical oligomers, leading to β -sheet rich fibrils associated with the polyQ core; however, Nt17 maintains its AH structure in the fibril.²⁶

Peptides containing polyQ but lack the N-terminal flanking sequence tend not to form oligomeric intermediates, thus proceeding directly to fibrillar aggregates.^{26,30} However, pure polyQ-peptide aggregating on a surface have been discovered to form oligomers.^{33,54} Free Nt17, which lacks the polyQ domain, has been demonstrated to inhibit formation of Nt17Q₃₇P₁₀KK peptides in vitro.^{26,30} The mechanism appears to be identical to the mechanism by which nucleation occurs, by increasing the distance between adjacent polyQ tracts and the energy for polyQ fibrillization to occur.⁵⁸ Steric hinderances can also interfere with the ability of Nt17 to promote oligomerization. In cases where a Myc-tag preceded Nt17 in full length exon1, the formation of oligomers in vitro was reduced without changing the rate of fibril formation when equated to similar proteins that lacked the Myc-tag.²⁰ Increased structural complexation using small

molecules, antibody-antigen, or molecular chaperones that interacts directly with this domain are also practical means of inhibiting N-terminal aggregation.^{3,59–62}

Membrane binding AHs is a common motif in proteins and peptides, with Nt17 demonstrating these functions.²⁸ AH lipid binding begins with separation of the hydrophilic and hydrophobic sides of the AH structure. Hydrophilic residues are solvent exposed and accessible for multiple binding partners, while hydrophobic residues are buried in the acyl chain of the lipid membrane, leading to an AH structure parallel with the surface.^{28,63,64} This binding approach is driven by long-range electrostatic interactions between the peptide and lipid headgroups coupled with conformational rearrangements of the peptide that allow the hydrophobic residues to remain closer to the membrane core.⁶⁴

Supplementary AH abilities of Nt-17 function in the preliminary insertion of polyQ peptides into a lipid membrane structure, which can increase the local concentration of aggregation-prone polyQ region. This interaction strengthens the reactions between polyQ domains through a 2-dimensional diffusion on the membrane surface.^{54,65,66} AHs also have several practical features, including detection of defects induced by curvature, which allows preferential sensing and binding of highly curved membranes.⁶⁷ Since AHs weakly bind membranes, their interactions can be effortlessly maintained. For example, Nt17 can isolate truncated htt-exon1 peptides to regions of curvature on supported lipid bilayers.⁶⁸ Detailed understanding of Nt17 lipid binding mechanisms can elucidate the methods of htt aggregation at lipid surfaces.

1.5 Post Translational Modifications within Nt17

Within the Nt17 helix, there are several sites that undergo post-translational modifications (PTMs), with some sites associated with multiple PTMs.^{69–71} These PTMs include acetylation,^{72,73} oxidation,⁷⁴ SUMOylation,^{69,75,76} ubiquitination,⁷⁷ and phosphorylation.^{43,51,78–81} Other less known, but observed, PTMs include palmitoylation⁸² and transglutamination⁸³. Collectively, these PTMs of Nt17 have significant effects on htt function and disease progression. Factors of htt function including translocation,^{84–86} membrane association and binding,^{76,81,87} toxicity,^{29,69,70,75,78} and aggregation^{76,78,81,87} can be traced back to specific amino acids within Nt17.

Figure 1.5: View of Nt17 helix with PTMs. The view down the top of the barrel of the Nt17 α -helix shows hydrophobicity of each residue (as indicated by legend) and all reported PTMs. The center line represents the membrane of cells, in which all residues below the line are known to bury within the membrane due to their hydrophobic nature. Adapted in part with permission from Arndt, J.R. et al. *The Emerging Role of the First 17 Amino Acids of Huntingtin in Huntington's Disease*. Copyright 2015 Biomolecular Concepts.

The localization of lysine residues on the edge of the hydrophilic portion of the AH (K6 and K15) suggests a potential roles in stabilizing early htt oligomers.⁸⁸ Three lysine residues are found within Nt17, (K6, K9, K15) and all are available for acetylation,

SUMOylation, and ubiquitination. Acetylation of these residues decreases the severity of htt lipid binding, resulting membrane disruption, and promotes the formation of large globular aggregates instead of fibrils.⁸⁷ Proteomic mapping via mass spectrometry confirmed K9 is significantly acetylated in mammalian cell lysates.⁷²

SUMOylation of these lysine residues modify htt-induced pathogenesis for both cell and animal models^{69,75} and alter aggregation and the ability of htt to bind membranes.^{69,76} At K6 and K9, SUMOylation stabilized htt-exon1 fragments, effectively retarding aggregation and toxicity in *Drosophila* models.⁶⁹ SUMOylation by Rhes, a small G protein, specially binds mutant htt over wild type by acting as a SUMO E3 ligase, increasing toxicity and soluble levels of htt.⁸⁹ The natural ubiquitination process tags proteins for degradation and is known to compete for the same lysine residues as SUMOylation, and both reduce the toxicity of mutant htt.⁹⁰ However, the presence of ubiquitin in htt inclusions demonstrate that this degradation mechanism becomes inefficient in HD.⁹¹ Since SUMOylation and ubiquitination compete for the same lysines, it can be implied that one method could be used to control the other. For example, serine 13 (S13) and serine 16 (S16) phosphomimics in htt were found to regulate SUMO-1 modifications in cells and in vivo.^{43,80} These same phosphomimics also enhance K6 htt SUMOylation, which could modulate K9 acetylation.⁷⁵

There are three phosphorylation sites within the Nt17 of htt-exon1, S13, S16 and threonine 3 (T3), with even more sites in full-length htt. Extensive studies suggest that htt phosphorylation is associated with reduced levels of mutant htt toxicity, aggregation, and lipid binding.^{43,71,78,80,81,92,93} Phosphorylation at S13 or S16 reduce helical content.^{81,92} while T3 phosphorylation stabilizes helical content, yet phosphorylation at all

three sites reduced aggregation.^{94,95} A combination of serine phosphorylation mimics established that serine to aspartate mutations greatly reduced the stability of the fibril structure and formation of htt aggregates.^{43,58} Htt with bona fide phosphorylation at S13 and S16 aggregated similarly to htt-constructs containing phosphomimetic mutations at these same sites.^{43,80,92} Therefore, it is demonstrated that serine phosphomimetic mutations are effective mimics for probing the impact of htt phosphorylation on aggregation.

1.6 Significance of Lipid Interactions with Huntingtin Protein

In amyloid-based neurodegenerative diseases like HD, membrane perturbation and permeabilization is a commonly proposed toxic mechanism.^{26,96} Cellular membranes are predominately comprised of lipids, which are comprised of a hydrophilic head and hydrophobic tail. Lipids readily self-assemble to form bilayers in aqueous solution. Htt itself is highly associated with a variety of membranes within cells, and localization to these membranes is a prominent feature in many normal functions attributed to htt.^{55,87,91} Several membrane related functions in which the lipid-binding properties of Nt17 may play a role, include cholesterol and energy homeostasis,⁹⁷ cellular adhesion,⁹⁸ motility,^{99,100} and molecular scaffolding.¹⁰¹ Htt functions in the transport of lipid vesicles (including endocytic, synaptic or lysosomal), especially along microtubules.^{102–104}

Figure 1.6: Mechanism of membrane binding interactions of htt. A key function of the AH of Nt17 is the ability to sense membranes. Nt17 is crucial for the mechanism of lipid membrane association of htt in that it forms two faces, hydrophobic and hydrophilic, in the presence of binding partners. These allow for self-association in early stages of aggregation, or the ability to preferentially bind to lipid membranes.

Nt17 also facilitates the trafficking of htt-exon1 to membranes associated with membranous organelles including ER, mitochondria, Golgi and autophagic vacuoles.^{105,106} Membrane association is also important for htt localization to subcellular components, such as the nucleus and plasma membrane.¹⁰¹ The ability of the AH structural qualities of Nt17 to engage membrane curvature sensing demonstrates a mechanistic role in these cellular functions.²⁹ Specifically, htt trafficking between the nucleus and cytoplasm occurs through Nt17-regulated reversible association with several membranous organelles.¹⁰⁵

With regard to toxicity, htt aggregates alter membrane structure and stability.^{45,48,105,107–109} Large unilamellar vesicles (LUV) that interact with htt-exon1 fragments enhance fibril formation *in vitro*.⁹⁶ Mitochondrial and vesicular trafficking are impaired in htt knock-out mice,^{110,111} Htt interacts with acidic phospholipids¹⁰¹ which could be instrumental in nucleating aggregation. Cumulative evidence suggests that a fundamental component of HD pathology is the consequence of toxic loss-of function,

theoretically due to the disturbance of interactions between membranes and htt.^{14,17,112,113} Htt knockout mice display amplified apoptosis and premature embryonic lethality,^{17,112} and mice that are heterozygous for the htt knockout show significant neuronal degeneration,^{17,18} signifying that htt is a necessary protein for normal function. Membranes may represent direct targets of toxic htt aggregates. Htt-exon1 disturbs the structural integrity of lipid bilayers in a polyQ-dependent manner.^{114,115} Many aggregate species are localized to damaged membranes within cells,^{14,96,116,117} such as fibrils distorting ER morphology and dynamics in HD,¹¹⁷ and membranous structures are incorporated into htt inclusions.¹¹⁶ Htt-exon1 oligomers bind to and disrupt membranes consisting of brain lipids, and these interactions were correlated to membrane leakiness and neuronal toxicity.⁹⁶

Altering the lipid composition of membranes plays an important role in htt aggregation.⁶⁵ Lipid properties, including size, charge and degrees of saturation result in a broad array of physicochemical membrane features. Since physicochemical properties of a membrane system are governed by membrane composition,¹¹⁸ this explains why adjustments to membrane composition impact the interaction between htt and lipid surfaces. For example, the size of the lipid headgroup is key for developing the favored curvature of a bilayer system,¹¹⁹ while the size and saturation of the lipid tail impact features such as packing and fluidity.¹²⁰ Amplified membrane fluidity results in a greater tendency for the occurrence of membrane defects,¹²¹ which ties into the defect detecting properties of the Nt17 domain.²⁷ Structural analysis by solid state NMR demonstrates that Nt17 undergoes a structural transition from random-coil in solution to an α -helical structure upon binding lipid bilayers.^{28,101} In addition, htt monomers disrupt

the physical packing of POPC bilayers in a way that potentially favors intermolecular Nt17 association.⁶³ PC/PS vesicles catalyze htt aggregation by promoting specific orientation within Nt17 on the membrane and increasing the local htt concentration on the surface.⁶⁵ This effect is dependent on polyQ length and peptide to lipid ratios.¹²² Comparatively, the presence of total brain lipid extract (TBLE) diminishes fibrillization of htt.^{66,123} This supports the notion that lipid composition is a factor in the effect of a membrane on htt aggregation.

Nt17 plays an essential role in the ability of htt to bind membranes. While synthetic polyQ peptides lacking Nt17 did not appreciably interact with model lipid membranes, lipid membranes stabilized discrete oligomers of a Nt17Q₃₅ peptide, and a Nt17Q₃₅P₁₀ peptide formed a variety of htt aggregates that readily damaged bilayers.²⁶ Computational studies further back the vital role of Nt17 in htt/lipid interactions and deliver evidence that the presence of both Nt17 and polyP flanking regions enhance the interaction of htt with bilayers.¹²⁴ The supportive effect of Nt17 and the polyP domain in disrupting membranes may be principally significant considering interactions between these two domains in a cellular environment are altered when the polyQ domain is expanded beyond the threshold associated with HD.¹²⁵ Induced mechanical changes in the membrane due to the buildup of monomeric htt and prefibrillar aggregates engaging directly on the surface disrupt bilayer integrity and could serve a role in htt-related toxic mechanisms.^{115,126} As the previously discussed PTMs are known to be involved in htt translocating to specific organelles^{105,127} and should be considered in regulating the interaction of htt with lipid membranes mediated by Nt17, they likely also impact the aggregation of htt in the presence of lipid membranes.

1.7 Dissertation Rationale

Due to the importance of Nt17 in the interactions with membranous organelles and regulating aggregation, the roles that these interactions potentially play in HD pathology are crucial, yet unknown. Surfaces, particularly lipid surfaces associated with membranous organelles, have influences related with their interfaces. Such influences include electrostatic interactions, and the electrostatics between surfaces and htt have a huge role.

In Chapter 2, we explored combinations of changing the membrane electrostatics and changing the Nt17 electrostatics by mimicking a known post translational modification (PTM). Certain PTMs within Nt17 are known to modify HD pathology, and due to Nt17's inclination to interact with lipid membranes, the influence of these mutations were explored with various model lipid membranes. Specifically, phosphorylation at T3, S13, and/or S16 improves phenotype and delays fibrilization in HD models. The impact of introducing phosphomimetic mutations (T3D, S13D and S16D) into htt-exon1 changed htt's interaction with and aggregation in the presence of lipids, reliant on the lipid system. Lipid systems included total brain lipid extract (TBLE), POPC and POPG, and were chosen to mimic a complex neutral lipid system, a neutral zwitterionic model lipid system, and a negatively charged lipid system.

Given that aggregation happens in a much more complicated environment, specifically in the highly crowded cytosol, the effects of macromolecular crowders present in the cytosol were examined with htt. In Chapter 3, changes were explored in the interfaces of membranes through the addition of various macromolecular crowders Dextran, Ficoll and PEG, with htt. This poses the question, if we crowd the liquid

surface, i.e. the liquid portion of the solid/liquid interface, how does that impact the interactions at surfaces? The differences of crowding interactions with htt in a solid/liquid interface, represented by in situ experiments on mica, were compared to crowding a gel-like/liquid interface represented by in situ experiments on TBLE bilayers.

In addition, we adapted many of these assays and experiments used in the laboratory into new, upper level, undergraduate biochemistry laboratory experiments for students presented in an appendix. In short, over a six-week time span, students are immersed in a graduate level setting and perform an experiment with minimal guidance from prep work to completion. Students are asked to present their findings in a conference setting, with the final report written in the ACS journal *Biochemistry* format. Moving away from the traditional “cookbook” format of undergraduate laboratories, this experiment was well received as an addition to the undergraduate biochemistry curriculum.

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2. Phosphomimetic Mutations Impact Huntingtin Aggregation in the Presence of a Variety of Lipid Systems

ABSTRACT

Huntington's Disease (HD) is a neurodegenerative disorder caused by the abnormal expansion of a polyglutamine (polyQ) tract in the first exon of the htt protein (htt). PolyQ expansion triggers the aggregation of htt into a variety of structures, including oligomers and fibrils. This aggregation is impacted by the first 17 N-terminal amino acids (Nt17) of htt that directly precedes the polyQ domain. Beyond impacting aggregation, Nt17 associates with lipid membranes by forming an amphipathic α -helix. Post-translational modifications within Nt17 are known to modify HD pathology, and in particular, phosphorylation at T3, S13, and/or S16 retard fibrilization and ameliorates phenotype in HD models. Due to Nt17's propensity to interact with lipid membranes, the impact of introducing phosphomimetic mutations (T3D, S13D and S16D) into htt-exon1 on aggregation in the presence of a variety of model lipid membranes (total brain lipid extract, POPC, and POPG) was investigated. Phosphomimetic mutations altered htt's interaction with and aggregation in the presence of lipids; however, this was dependent on the lipid system.

2.1 Introduction

A prominent feature of many neurodegenerative diseases, e.g. Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), is the deposition of proteinaceous material in the form of plaques and inclusions within tissues and cellular compartments. These depositions are predominately comprised of amyloid

that are defined by a fibrillar morphology and a cross β -sheet structure. In particular, HD is an autosomal dominant genetic disorder caused by the expansion of a polyglutamine (polyQ) region in the first exon of the huntingtin protein (htt) above a critical threshold (~35 repeat glutamine residues),¹ and this expansion promotes htt's aggregation into fibrils.² Htt aggregation is a multifaceted process involving, beyond fibrils, a variety of other aggregate species, including oligomers, annular structures, and amorphous aggregates.^{2–4} Due to the heterogeneous nature of htt aggregation, debate continues about the role different aggregate species play in toxicity. In this regard, htt oligomers,^{5–7} fibrils,^{8,9} and inclusions¹⁰ have all been directly implicated as toxic entities.

Within the cellular environment, htt associates with a variety of membranous surfaces, e.g. mitochondria, endoplasmic reticulum (ER), the nuclear envelope, tubulovesicles, endosomes, lysosomes, and synaptic vesicles.^{11–16} These membranes, predominately comprised of lipids, provide subcellular environments that influence htt aggregation.¹⁷ In mouse models of HD, lipids accumulate in htt aggregates and inclusions,^{18–20} and htt aggregates disrupt lipid membranes^{21–24} and organelle integrity.^{10,15,16,25} Htt's interaction with membranous surfaces not only influences aggregation but may also be involved directly in toxic pathways. Several model lipid membranes influence htt aggregation *in vitro* in a variety of ways.^{22,23,26–31} POPC/POPS lipid vesicles accelerate fibril formation by promoting a distinct aggregation pathway;³² however total brain lipid extract suppresses fibrilization.^{21,33}

The first 17 N-terminal amino acids of htt (Nt17) that directly precede the polyQ region is implicated in both htt aggregation and interaction with lipid membranes.^{21,34} Nt17 is predominately disordered in solution but has a propensity to adopt an

amphipathic α -helical structure upon interaction with a binding partner. Nt17 domains of several htt proteins can associate to form α -helical rich oligomers.^{35,36} These oligomers enhance fibril formation by bringing together polyQ domains of different htt proteins.^{35,36} Beyond this role in promoting aggregation, the ability to form an amphipathic α -helix facilitate the binding of htt-exon1 to lipid membranes. A variety of post-translational modifications (PTMs) occur within Nt17, with implication for htt aggregation and membrane interactions. These PTMs include phosphorylation,^{37–40} oxidation,^{41,42} acetylation,^{30,43} SUMOylation,^{32–35} and ubiquitination.^{32–34} PTMs impact htt function, translocation, and toxicity.^{37,44–46} In particular, Nt17 contains three phosphorylatable residues: threonine 3 (T3), serine 13 (S13), and serine 16 (S16). Phosphorylation of S13 and S16 reduces accumulation of htt and pathogenesis in animal models, modifies htt subcellular localization, reduces aggregate stability, and inhibits fibril formation.^{37,39,47–51} Imitating threonine 3 (T3) phosphorylation enriches polyQ-mediated toxicity in *Drosophila* models of HD but increases the amount of insoluble htt-exon1 species.^{37,51} More recent literature on htt-exon1 peptides demonstrate that inducing phosphorylation^{50–53} or adding a negative charge^{51,54} through aspartic acid single point mutations to the T3 residue leads to a decrease in fibril elongation.^{51,55} Inhibition of fibrilization via phosphorylation also appears related to modifications in the nucleation process related to the formation of α -helical rich oligomers facilitated by Nt17.⁴⁷

Here, the aim was to determine how phosphorylation at T3, S13, and S16 influence the ability of htt-exon1 to directly interact with lipid membranes and how these interactions influence htt aggregation. To achieve this, aggregation of htt-exon1 proteins containing phosphomimetic mutations (T3D, S13D, or S16D) in the presence and

absence of lipid membranes was investigated by various biophysical techniques. Lipid systems investigated were TBLE, POPC, and POPG. Phosphorylation altered htt's interaction with and aggregation in the presence of lipids, but this was heavily influenced by the specific lipid systems exposed to htt.

2.2 Materials and Methods

2.2.1 Preparation of Phosphomimetic Constructs. Extracted DNA from a glutathione S-transferase (GST)-htt-exon1(46Q) fusion protein was inserted at cleaved sites BamH1 and Sal1 vectors within a pGEX-5X-1 plasmid. This region was replaced with three separate constructs, in which an aspartic acid residue replaced the three-phosphorylation post translational modification sites within the Nt17 domain (T3D, S13D and S16D). The construct plasmids were transformed into One Shot thermocompetent STBL3 *E. Coli* cells and made into glycerol stocks stored at -80°C for future purification in the same manner as the unmodified wild-type (WT).

2.2.2 Expression, Purification of (GST)-htt-exon1 Fusion Protein. (GST)-htt-exon1(46Q) fusion proteins (WT, T3D, S13D and S16D) were purified as described previously.⁵⁶ In short, GST-htt-exon1 was expressed with isopropyl B-D-thiogalactoside for 4 hours at 30°C in *E. Coli*. Cells were lysed with lysozyme (0.5 mg/mL) and sonication, and fusion proteins were purified using a 5 mL GST affinity column and liquid chromatography (LPLC, Bio Rad). Relevant protein bands were observed with gel electrophoresis technique and Coomassie blue staining to assess purity and appropriate fractions. Protein concentration was determined using Coomassie Bradford reagent. Before each experiment, pre-existing aggregates were removed through high speed centrifugation at 20,000g for 30 minutes at 4°C. Initiation of aggregation through

cleavage of the GST tag is performed by the addition of Factor Xa protease (New England Bio Labs). All experiments were performed in buffer A (50mM Tris-HCl, pH 7, 150mM NaCl).

2.2.3 Lipid Preparation. Total brain lipid extract (TBLE), 1-Palmitoyl-2-oleoyl-glycero-3-phosphatidylcholine (POPC), and 1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol (POPG) were purchased as lyophilized powders (Avanti Polar Lipids). Lipids were dissolved in chloroform and aliquoted for further use. Residual traces of solvent were evaporated overnight in high vacuum. Resulting lipid films were hydrated in buffer A at 60°C with vortex mixing until all lipid was suspended in solution. Vesicle formation was promoted by 10 freeze/thaw cycles using liquid N₂ followed by bath sonication at room temperature for 1 hour.

2.2.4 Thioflavin T (ThT) Aggregation Assay. Htt-exon1(46Q) constructs were diluted to a final concentration of 20 µM in the presence of 125 µM ThT (Sigma-Aldrich, St. Louis, MO) and the desired lipid vesicles if needed. In experiments with vesicles, a 10:1 lipid:protein ratio was used, resulting in a lipid concentration of 200 µM. Assays were performed in black Costar 96-well plates with clear flat bottoms (100 µL per well), and ThT fluorescence was monitored with a microplate reader (SpectraMax M2). Experiments were run at 37°C with readings every 5 minutes for 18 hours at 440 nm excitation and 484 nm emission. For an individual experiment, all conditions were run in triplicate, and a minimum of three independent experiments were performed.

2.2.5 Polydiacetylene (PDA) Vesicle Binding Assay. 10,12-tricosadiynoic acid (GFS Chemicals, Columbus, OH) and lipid system of choice were dissolved at a 2:3 molar ratio in a solution of 1:4 ethanol/chloroform. Solution was evaporated off under a gentle

stream of N₂, and the film was dissolved with buffer A at 70°C. The solution was sonicated for 10-minute intervals at 100 W using a sonic dismembrator (FisherSci) until solution was sufficiently opaque and stored at 4°C overnight to ensure self-assembly of the mixture into vesicles. Polymerization of the PDA was initiated by irradiation at 254 nm for 10 minutes at room temperature with stirring, resulting in a blue, opaque solution. The colorimetric response was monitored by UV-Vis spectroscopy to quantify protein binding to vesicles with a SpectraMax M2 microplate reader (Molecular Devices). The assay was performed in a 96-well plate format (100 µL total volume per well), and each condition was performed in triplicate per experiment. A minimum of three unique experiments were performed. Each plate contained negative controls (neat buffer A) and a positive control (final concentration of 100 µM NaOH). The NaOH control was used to standardize PDA results across experiments.⁵⁷ The percent colorimetric response (% CR) was determined every 5 min for 18 hours at 37°C by measuring the blue absorbance (640 nm, A_{blue}) and the red absorbance (500 nm, A_{red}) using the following equation:

$$(Eqn. 2.1) \quad \% CR = \left[\frac{PB_0 - PB_1}{PB_0} \right] * 100$$

PB is defined as A_{blue}/(A_{blue}+A_{red}). PB₀ is determined from the negative control, and PB₁ is obtained from each experimental condition.^{58,59}

2.2.6 Atomic Force Microscopy (AFM). For *ex situ* AFM experiments, htt-exon1(46Q) (20 µM) was incubated in the absence or presence of lipid vesicles (10:1 lipid to protein ratio) at 37°C and 1400 rpm in an orbital thermomixer. At designated time points, 2 µL aliquots of each sample were deposited onto freshly cleaved mica (Ted Pella Inc.,

Redding, CA) for 1 minute, washed with 200 μ L of 18 M Ω ultrapure water, and dried under a gentle stream of filtered air. Samples were imaged using a Nanoscope V Multi-Mode scanning probe microscope (VEECO) equipped with a closed loop vertical engage J-scanner. Silicon-oxide cantilevers with a nominal spring constant of 40 N/m and a resonance frequency of $\sim$ 300 kHz were used. Scan rate was set at 1.99 Hz with cantilever drive frequencies at 10% below resonance. For *in situ* AFM experiments a tapping mode fluid cell with an O ring was equipped with a cantilever a nominal spring constant of 0.1 N/m. To prepare the TBLE bilayer, lyophilized lipid film was reconstituted in 25 mM Tris buffer at 1 mg/mL. The resulting TBLE solution underwent ten freeze-thaw cycles followed by 10 min of bath sonication. The resulting TBLE vesicles were directly injected into the fluid cell (30 μ L), and the formation of a supported lipid bilayer was monitored via continuous AFM imaging. Once a continuous 40 μ m x 40 μ m patch of supported lipid bilayer formed, htt constructs that had been incubated on ice with factor Xa for 2 h was injected into the fluid cell to a final concentration of 10 μ M. The lipid bilayer exposed to htt was continuously imaged. Resulting images from both *ex situ* and *in situ* were then analyzed using the MATLAB image processing toolboxes (MathWorks) as described.^{60,61}

2.3 Results

2.3.1 Phosphomimetic mutations in Nt17 alter htt aggregation. All experiments presented here used GST-htt-exon1 fusion proteins with 46 repeat glutamines (htt-exon1(46Q)). Single amino acids within Nt17 (T2, S13, or S16) were mutated to aspartic acid to mimic phosphorylation. For convenience, the control htt-exon1(46Q) protein without mutations will be referred to as htt^{NT}, and the htt-exon1(46Q) protein with

phosphomimetic mutations will be referred to as htt^{T3D}, htt^{S13D}, and htt^{S16D} respectively. For all GST-htt fusion proteins, cleavage of the GST tag with Factor Xa initiates aggregation. While phosphomimetic mutations in Nt17 are known to slow fibrillization,^{39,47} the ability of the specific experimental system used here to recapitulate this feature needed to be verified. Fibril formation of each htt protein was monitored with a ThT assay (20 µM concentration, Fig. 1), and all three mutations reduced aggregation over a 9 h period. Htt^{T3D} impeded fibril formation to the greatest extent (a 75% reduction compared to htt^{NT} in the maximum ThT signal observed), followed by htt^{S16D} (61 % reduction). Of the three mutations, S13D had the mildest impact on fibril formation, reducing the maximum ThT signal by only 26%.

Figure 2.1: The impact of phosphomimetic mutations on htt aggregation. (A) Aggregation of htt^{NT}, htt^{T3D}, htt^{S13D}, and htt^{S16D} tracked by a ThT assay. (B) Quantification of the maximum ThT fluorescent signal observed. Error bars represent SEM. * corresponds to $p < 0.01$ and ** corresponds to $p < 0.05$ when compared with htt^{NT}. (C) Representative ex situ AFM images of aggregates formed at various time points from 20 μ M incubations of htt^{NT}, htt^{T3D}, htt^{S13D}, and htt^{S16D}. (D) Histograms of the average height along the contour of fibrils of htt^{NT}, htt^{T3D}, htt^{S13D}, and htt^{S16D} measured from AFM images. (E) Morphological correlation plots (height vs diameter) of individual oligomers observed in AFM images at 3 and 8 h for all four htt constructs. In addition, normalized height (top) and diameter (right) histograms are also presented.

To further characterize the impact of phosphomimetic mutations on htt aggregation, AFM analysis was performed (Fig. 1C). Htt^{NT}, htt^{T3D}, htt^{S13D}, and htt^{S16D} were incubated (20 μ M, 37°C), sampled at 1, 3, 5, and 8 h, and imaged by AFM. For htt^{NT}, a few short fibrils were observed after 1 h of incubation, and these fibrils grew in number and length with time. After 8 h, an extensive number of htt^{NT} fibrils were observed. With each phosphomimetic mutation, fewer fibrils were observed compared with htt^{NT}, and consistent with the ThT assay, htt^{S13D} had the smallest impact on fibrillization. The length of fibrils observed from htt constructs containing phosphomimetic mutations tended to be shorter compared to htt^{NT} due to the reduced rate of fibril formation. However, previous ssNMR studies demonstrates that phosphomimetic mutations in Nt17 do not significantly alter the underlying htt fibril structure.⁴⁷ That is, the polyQ core was unchanged even though the rate of formation and aggregate stability is altered. To assess if fibrils formed from the four htt constructs used here may have similar underlying structure, the average height along the contour of all fibrils observed in AFM images was determined and used to construct histograms (Fig. 1D). This measurement was chosen as even though the length of fibrils may be different, if the underlying structure is similar the thickness (measured as height along the contour) would be the same, and differences in the average height along the contour of amyloid fibrils are associated with polymorphic fibril structures associated with other amyloid proteins.⁶² Fibrils of all four constructs had similar average heights (mode of ~7 nm). The average height along fibril contours observed here is consistent with the phosphomimetic mutations not altering the underlying fibril core structure.

Beyond fibrils, htt aggregates into a heterogeneous mixture of oligomers. To determine if phosphomimetic mutations in Nt17 altered oligomer morphology, the height and diameter of oligomers was measured after 3 and 8 h of incubation using automated scripts in MATLAB. For this analysis, oligomers were defined as any feature more than 0.8 nm in height with an aspect ratio (longest distance across to shortest distance across) <3.0, indicating a globular structure. Correlation plots of diameter vs height, as well as histograms for each dimension, were constructed (Fig. 1E). Htt^{NT} formed a heterogeneous mixture of oligomers, which is particularly pronounced in the height histograms. While htt^{NT} oligomers were diverse in morphology, the mode height was 5-7 nm at 3 h with a slight shift to larger oligomers at 8 h. For example, there was a sizeable population of htt^{NT} oligomers larger than 10 nm in height after 8 h. Oligomers formed from phosphomimetic htt constructs displayed less heterogeneity. In general, oligomers formed from htt containing phosphomimetic mutations tended to be smaller, which may be consistent with previous reports that Ser-to-Asp mutations in htt reduce its ability to form α -helix rich oligomers.⁴⁷ Htt^{T3D} formed oligomers with a mode height of 2.5-3.5 nm at 3 h. There was an abrupt shift to larger oligomer species (mode height of 6-8 nm); however, only a few htt^{T3D} oligomers grew beyond 10 nm in height. Both htt^{S13D} and htt^{S16D} formed smaller oligomers than htt^{NT} at 3 h (mode heights of 2.5-4 nm and 2.5-6 nm respectively). While htt^{S13D} displayed a small shift in oligomer height at 8 h (mode height of 4-6 nm), the predominant morphological changes associated with htt^{S13D} and htt^{S16D} at 8 h was a broadening of oligomers that manifested as a slight shift (development of a tail) in the diameter histograms. Collectively, these observations suggest that phosphomimetic mutations impact htt aggregation at the oligomeric stage.

As α -helical rich oligomers mediated by Nt17 are implicated in mechanisms to nucleate fibrillization,^{35,36} reduction in fibril formation associated with phosphorylation may be due to differences in efficiency of formation and types of oligomers formed.

2.3.2 Phosphomimetics of htt alter aggregation in the presence of lipids. Having established an experimental system, investigations aimed at elucidating the role of phosphorylation on htt/lipid interactions were performed. For these studies, three lipid systems were chosen as lipid composition can heavily influence amyloid formation. The first lipid system was total brain lipid extract (TBLE). TBLE has been shown to alter htt aggregation by suppressing fibril formation^{21,33} and consists of a physiologically relevant mixture of lipid components. In addition, two simpler lipid systems were investigated (POPC and POPG). With the phosphomimetic constructs neutralizing the net positive charge of Nt17, these two simplified systems were chosen because they have the same tails but differently charged head groups, as POPC is zwitterionic and POPG is anionic.

To assess the impact of the three lipid systems on fibrillization of the four htt constructs, ThT assays were performed in the presence of lipid vesicles (Fig. 2). For these assays, the lipid to htt molar ratio was 20:1, and all vesicles were in the range of 140-180 nm in diameter as assessed by dynamic light scattering. Consistent with previous reports,^{21,33} TBLE reduced htt^{NT} fibrillization relative to the absence of lipid (compare the maximum ThT signals in Figure 2A to Figure 1B). This reduction in fibrillization associated with the presence of TBLE vesicles was also apparent for the three phosphomimetic htt constructs. Relative to htt^{NT} in the presence of TBLE, the phosphomimetic mutations further reduced fibrillization with the same pattern as observed in the absence of lipids. That is, htt^{T3D} produced the smallest ThT signal (77%

reduction compared to htt^{NT} in the presence of TBLE), followed by htt^{S16D} (54% reduction), and htt^{S13D} (29% reduction).

Figure 2.2. Phosphomimetic mutations influence htt aggregation in the presence of lipid vesicles. ThT assays and quantification of the maximum ThT fluorescent signal observed for htt^{NT}, htt^{T3D}, htt^{S13D}, and htt^{S16D}. Error bars represent SEM in the presence of vesicles comprised of (A) TBLE, (B) POPC, and (C) POPG. * corresponds to $p < 0.01$ and ** corresponds to $p < 0.05$ when compared with htt^{NT}.

With the addition of POPC or POPG vesicles, htt^{NT} fibrillization was enhanced compared with aggregation reactions in the absence of lipids (compare Figure 2B-C with Figure 1B), with POPG having a significantly larger effect. The fibrillization of the phosphomimetic htt constructs was also enhanced in the presence of POPC or POPG, and the extent of fibrillization continued to follow the same pattern. That is, htt^{T3D} aggregated the least, followed by htt^{S16D} and htt^{S13D} respectively. However, the relative inhibition of fibrillization with respect to htt^{NT} aggregation in the presence of POPC or POPG was reduced. With POPC, there was no significant difference between the ThT

signal produced by htt^{NT} and htt^{S13D}. With POPG, the ThT signal was not significantly different between htt^{NT} and htt^{S16D}. Furthermore, there was a significant increase in ThT signal in htt^{S13D} compared to htt^{NT} in the presence of POPG.

To further establish the impact of the three lipid systems on the aggregation of the htt constructs, AFM analysis was performed. As before, incubation experiments were performed with fresh preparations (20 μ M) of htt^{NT}, htt^{T3D}, htt^{S13D}, and htt^{S16D}, but this time they were incubated with vesicles of TBLE, POPC, or POPG (20:1 lipid to peptide ratio). Background images of these lipid systems deposited on mica without htt are shown in Figure 3. These incubations were sampled at 1, 3, 5, and 8 h.

Figure 2.3. Representative AFM images of backgrounds associated with deposited solutions of lipid vesicles. Clean mica and mica exposed to TBLE, POPC, or POPG vesicles are compared. The green line in each image corresponds to the height profile shown directly below each image.

Representative AFM images of aliquots removed from these solutions after 8 h of incubation are shown in Figure 4. Representative AFM images of the other three time points for incubations with TBLE, POPC, or POPG vesicles are shown as Figures 5, 6,

and 7 respectively. A variety of aggregates (oligomers and fibrils) are observed on mica under all conditions. The average height along the contour of fibrils was measured to determine if the presence of the lipids altered fibril structure (Fig. 4B). Despite different rates of formation, there were not any significant differences in fibril structures across the four htt constructs within a single lipid system. However, the fibril structure appeared altered by POPG. With TBLE and POPC, the average height along the contour of the fibril was ~6-7 nm, which is consistent with fibrils formed in the absence of lipids. With POPG, the average height along the contour ranged from 3-5 nm, suggesting that the structure of fibrils formed in the presence of POPG may vary from fibrils formed in the absence of lipid.

Figure 2.4. Phosphomimetic htt constructs form a variety of aggregates in the presence of lipid vesicles. (A) Representative *ex situ* AFM images of aggregates formed after 8 h of incubation (for other time points see figures S2-S4) from 20 μ M incubations of *htt^{NT}*, *htt^{T3D}*,

htt^{S13D}, and *htt*^{S16D} in the presence of vesicles comprised of TBLE, POPC, or POPG. (B) Histograms of the average height along the contour of fibrils of *htt*^{NT}, *htt*^{T3D}, *htt*^{S13D}, and *htt*^{S16D} formed in the presence of vesicles comprised of TBLE, POPC, or POPG measured from AFM images.

Figure 2.5. Phosphomimetic construct aggregates observed with ex situ AFM in the presence of TBLE. Representative ex situ AFM images of aggregates formed after 1, 3, and 5 h of incubation from 20 μ M incubations of *htt*^{NT}, *htt*^{T3D}, *htt*^{S13D}, and *htt*^{S16D} in the presence of TBLE.

Figure 2.6. Phosphomimetic construct aggregates observed with ex situ AFM in the presence of POPC. Representative ex situ AFM images of aggregates formed after 1, 3, and 5 h of incubation from 20 μ M incubations of htt^{NT} , htt^{T3D} , htt^{S13D} , and htt^{S16D} in the presence of POPC.

Figure 2.7. Phosphomimetic construct aggregates observed with ex situ AFM in the presence of POPG. Representative ex situ AFM images of aggregates formed after 1, 3, and 5 h of incubation from 20 μ M incubations of htt^{NT} , htt^{T3D} , htt^{S13D} , and htt^{S16D} in the presence of POPG.

Using the same criteria as before, the height and diameters of oligomers observed at 3 and 8 h of incubation were determined and used to build correlation plots and histograms (Fig. 8). The presence of all three lipid systems reduced the heterogeneity of htt^{NT} oligomers at a given time point. With TBLE, htt^{NT} oligomers were less heterogeneous than in the absence of lipids, displaying mode heights of 5-7.5 nm at 3 and 8 h. Phosphomimetic mutations promoted smaller oligomers than htt^{NT} in the presence of TBLE, with mode heights of 2-3.5 nm, 2-3 nm, and 2-6 nm for htt^{T3D} , htt^{S13D} , and htt^{S16D} respectively. After 8 h, the height distribution of oligomers formed from all phosphomimetic mutations shifted to larger size with increased heterogeneity. After 3 h

of incubation, POPC vesicles promoted small oligomers with all four htt constructs (mode heights of 2.5-4 nm, 2-3.5 nm, 2-3 nm, and 2-3 nm for htt^{NT}, htt^{T3D}, htt^{S13D}, and htt^{S16D} respectively). While oligomers formed from htt^{NT} and htt^{T3D} in the presence of POPC remained relatively small, oligomer formed from htt^{S13D} and htt^{S16D} were significantly larger at 8 h (mode heights of ~6-8 nm for both). POPG initially promoted smaller oligomers with all four htt constructs (mode heights in the 2-4 nm range at 3 h); however, a population of larger oligomers appeared with POPG after 8 h for all four constructs, resulting in a more heterogeneous population. This shift was the most pronounced with htt^{S16D}.

Figure 2.8. Lipids promote unique htt and phosphomimetic htt oligomer morphologies. Morphological correlation plots (height vs diameter) of individual oligomers observed in AFM images at 3 and 8 h for all four htt constructs in the presence of vesicles comprised of (A) TBLE, (B) POPC, and (C) POPG. In addition, normalized height (top) and diameter (right) histograms are also presented.

2.3.3 Phosphomimetics of htt impact binding in a lipid specific manner. As the presence of lipid vesicles altered htt aggregation (with and without phosphomimetic mutations) in a lipid specific manner, a series of colorimetric membrane binding assays using lipid/polydiacetylene (PDA) vesicles were performed to determine the impact of phosphomimetic mutations on membrane binding. These vesicles were made with either TBLE, POPC, or POPG as the lipid component. Lipid/PDA vesicles have varied colorimetric responses (CRs) when exposed to proteins or small molecules depending on the extent of interaction.^{58,59} The CR in the lipid/PDA vesicles is related to transitions of the PDA polymer backbone structure, which is affected by changes in tension associated with exogenous molecules interacting with and/or inserting into the vesicle.⁵⁹ By measurement of the absorbance of both the blue (640 nm) and red (500 nm) wavelengths of lipid/PDA vesicles upon exposure to htt-exon1(46Q), the percent CR was obtained, which directly corresponds to the protein–lipid interaction. In addition, these assays were normalized to an NaOH control, allowing for comparison across lipid systems.⁵⁷

Phosphomimetic mutations significantly enhanced the interaction of htt with TBLE as shown in Figure 9, with htt^{T3D} and htt^{S13D} having the strongest interaction with the membrane (~2x the maximum % CR compared to htt^{NT}, $p < 0.01$). With TBLE, the enhanced interaction of htt^{S16D} compared to htt^{NT} was less pronounced and not statistically significant. In contrast, phosphomimetic mutations delayed the interaction between htt and POPC for the first 3.5 h. The $\alpha\beta\alpha$ displayed by PDA/POPC vesicles exposed to htt^{NT} reached 9-10% within 1 to 2 h at which time the %CR leveled off to a relative steady state (max %CR of 13.1%). For all three phosphomimetic htt constructs

it took ~7-9 h to reach a similar %CR with POPC. After the 9 h experiments, only htt^{S16D} displayed a significantly smaller maximum %CR (10.7%, $p < 0.05$) compared with htt^{NT}. Within the time frame of these experiments htt^{NT} and htt^{S16D} invoked a larger %CR with POPC vesicles compared with TBLE. Both htt^{T3D} and htt^{S13D} more strongly interacted with TBLE compared to POPC. As all three phosphomimetic htt constructs had similar interactions with POPC, these cross-system differences may be due to the complexity of lipid composition of TBLE. The interaction of htt with POPG was the weakest of the three lipid systems, and there did not appear to be any significant differences associated with htt^{NT} and the phosphomimetic htt constructs.

Figure 2.9. Phosphomimetic mutations in htt alter its ability to bind a variety of lipid vesicles. Normalized PDA/lipid binding assays and the maximum relative %CR associated with exposure of PDA vesicles with the lipid component comprised of (A) TBLE, (B) POPC, or (C) POPG exposed to the four htt constructs. Error bars represent SEM. * corresponds to $p < 0.01$ and ** corresponds to $p < 0.05$ when compared with htt^{NT}.

2.3.4 Phosphomimetics of htt influence disruption of lipid bilayers. While the three lipid systems impacted htt aggregation as demonstrated by ThT and *ex situ* AFM assays, both techniques monitor bulk aggregation within an incubation. That is, they do not distinguish between htt aggregation occurring on a membrane surface or in bulk solution. In addition, there did not appear to be a direct correlation between the extent of htt/lipid interaction as measured by PDA and fibrillization associated with the phosphomimetic htt constructs. As htt aggregation occurs via a unique mechanism on POPC/POPS lipid surfaces,³² it was hypothesized that phosphomimetic mutations may be impacting aggregation at membrane surfaces in a distinct manner that is masked by the bulk aggregation assay already employed. As a result, *in situ* AFM was employed to directly observe aggregation of the four htt constructs directly on lipid membranes.

For these assays it is important to obtain stable, supported lipid membranes that are free of nanoscale defects. Unfortunately, such supported membranes were not reproducibly produced for the pure POPC or POPG systems. Supported TBLE bilayers were easily produced and were systematically exposed to the four htt constructs (final htt concentration of 10 μ M in the fluid cell). Upon injection of htt, TBLE bilayers were continuously imaged to directly observe changes in morphology and the development of htt aggregates (Figure 10). Such supported bilayers of TBLE have been extensively used to investigate their interaction with amyloid-forming proteins via *in situ* AFM.^{62–66} Across trials, the total concentration of lipid in the AFM fluid cell was maintained; however, observations were restrained to a 30 $\times$ 30 μ m patch of supported bilayer that was verified to be defect-free (at the level of resolution of the AFM) prior to exposure to any htt constructs. Prior to exposure to htt, TBLE bilayers had a smooth morphology

(RMS roughness ~ 0.2 - 0.4 nm) and can be continuously imaged by AFM for at least 24 h without inducing any morphological changes.

Figure 2.10. Phosphomimetic mutations alter htt aggregation on the surface of a TBLE bilayer. (A) In situ AFM images of the same region of supported TBLE bilayers exposed to 10 μ M htt^{NT}, htt^{T3D}, htt^{S13D}, or htt^{S16D} as a function of time. Black arrows indicate the same region in consecutive images. Quantification of (B) the RMS roughness of regions of altered morphology and (C) the % area disrupted of supported TBLE bilayers exposed to phosphomimetic htt constructs as a function of time. Error bars represent SEM.

Supported TBLE bilayers were systematically exposed to htt^{NT}, htt^{T3D}, htt^{S13D}, or htt^{S16D} at a final htt concentration within the fluid cell of 10 μ M. Protein aggregation on the bilayer was directly monitored by continuous *in situ* AFM imaging for 3 h (Fig. 10). To quantify the extent of morphological changes on TBLE bilayers associated with exposure to htt constructs, the RMS roughness of the altered areas on the bilayer was determined and the % area of the bilayer displaying altered morphology (Fig. 10B-C).

Upon initial exposure of the TBLE bilayer to htt^{NT}, discrete patches displaying a roughened morphology developed on the TBLE bilayer. These patches often contained features reminiscent of htt oligomers; however, fibrillar morphology was rarely observed. With time, the area of the bilayer altered by exposure to htt^{NT} increased ($16\pm4\%$ after 0.5 h of exposure to $36\pm7\%$ after 3 h). The roughness associated with these altered regions doubled within the same time frame (1.4 ± 0.6 nm at 0.5 h to 2.8 ± 1.0 nm after 3 h). In comparison, htt^{T3D} deposited more aggressively on the TBLE bilayer, significantly altering its morphology. Initially, htt^{T3D} appeared to form patches that resembled densely packed small fibrils. However, with time these patches became granular in appearance (RMS roughness of 6.5 ± 1.3 nm by 3 h) and dominated the image, covering $65\pm2\%$ of the surface. Htt^{S13D} initially appeared as discrete fibrillar aggregates on TBLE bilayers. Htt^{S13D} did not develop the granular morphology observed with htt^{T3D}; rather, large regions of altered morphology protruding above the unperturbed bilayer regions developed. These regions were relatively smooth in appearance, but because they protruded so far above the unperturbed bilayer, had the largest observed RMS roughness (8.8 ± 0.8 nm) after 3 h of exposure to htt^{S13D} and covered $61\pm1\%$ of the surface. Exposure to htt^{S16D} also resulted in regions of altered morphology on the

bilayer surface, but these regions often had fibril-like features extending from their periphery. The RMS roughness (5.9 ± 1.3 after 3 h) associated with these regions was larger than htt^{NT}, but not as big as the roughness observed for the other phosphomimetic constructs. After 3 h of exposure, $54 \pm 1\%$ of the bilayer surface was altered by httS16D.

The relative trend of phosphomimetic mutations increasing the area of TBLE bilayers displaying altered morphology is roughly similar to the PDA assay; however, the extent of the increased interaction appears much larger with the PDA assay. This is likely due to the more pronounced morphological changes (as quantified by RMS roughness measurements) producing larger mechanical strain on PDA vesicles, amplifying the signal. A peculiar observation is that phosphomimetic mutations appeared to promote fibrillar htt aggregates on the TBLE surface; however, the ThT assays suggested that these mutations reduced fibrillization in the presence of TBLE vesicles. This may be due to differences in what is being monitored between the two assays. ThT measures aggregation in the entirety of the incubation (solution and membrane surface); however, in situ AFM only detects changes that appear on the membrane surface. As such, this suggests that there may be distinct aggregation pathways occurring simultaneously, one in bulk solution and one at the membrane interface. Such a notion is supported by EPR studies.³²

2.4 Discussion

PolyQ expansion drives htt aggregation and toxicity, but flanking sequences adjacent to the polyQ domain heavily influence these processes.^{67–69} In particular, Nt¹⁷ impacts htt localization, aggregation, and degradation in cells,^{17,45,48,70} and

phosphorylation within Nt17 further regulates these phenomena.^{37–39,71} Based on its ability to form an amphipathic α -helix, a key function of Nt17 is interacting and binding lipid membranes.^{32,70} Here, we investigated the impact of phosphomimetic mutations within the Nt17 on htt interaction and aggregation in the presence of a variety of model lipid membranes. In general, phosphomimetic mutations at T3, S13, or S16 slowed fibril formation in the presence of lipids; however, this was highly dependent on the specific lipid system used. As aberrant htt/lipid interactions and subcellular membrane damage are observed in HD, understanding how PTMs like phosphorylation impact htt/lipid interactions could reveal new therapeutic targets and strategies.

While the current study demonstrates that altering the charge of Nt17 via phosphomimetic mutations heavily influences htt aggregation in the presence and absence of lipids, there are several caveats to these studies that need to be addressed. Phosphomimetic mutations have been used to study the impact of phosphorylation on htt aggregation, and the consensus is that these mutations reduce fibrillization and fibril stability.^{37,39,47,50,51,54,72} However, such mutations are imperfect models of phosphorylation.⁷³ Recent studies have explored the impact of bona fide phosphorylation within Nt17 on htt aggregation.^{74,75} Actual phosphorylation at S13 and/or S16 impeded fibrillization and disrupted the α -helix formation in Nt17; S13D and S16D mutations did not strongly inhibit α -helix formation.⁷⁴ This inhibited α -helix formation strongly reduced the interaction with lipid membranes. Phosphorylation at T3 stabilizes α -helix formation.⁵⁴ An additional caveat here is that only single phosphomimetic mutants were investigated. There is crosstalk between different PTM sites within Nt17 with implications for aggregation. Combinations of phosphorylation at

T3, S13, and S16 have profound impacts on Nt17 structure and htt aggregation.⁷⁴ In addition, there is crosstalk associated between T3 and K6, with acetylation at K6 reversing the inhibitory impact on htt aggregation associated with phosphorylation at T3.⁵⁴ Despite these caveats, there is still value in using phosphomimetic mutations to understand how Nt17 phosphorylation regulates htt aggregation and membrane activity as introduction of phosphomimetic mutations (S13D/S16D) in the context of the full-length mutant htt improved phenotype in an HD mouse model.³⁹ This suggests that, while phosphomimetic mutations may not provide exact models of the impact of phosphorylation of htt in HD, these mutations do modify phenotype and biophysical characterization can provide insights into potential toxic mechanisms. As such, understanding the biophysical impact of phosphomimetic mutations on htt aggregation and membrane activity may provide mechanistic detail concerning htt related toxicity.

The differences in aggregation associated with the different lipid systems underscores the complexity of htt/lipid interactions. Experiments and simulations indicate that the structure and orientation of Nt17 on membranes is an amphipathic α -helix that sequesters nonpolar residues toward the membrane hydrophobic core.^{76,77} Electrostatic interactions between Nt17 and lipids influences the affinity of Nt17 for phospholipid bilayers,^{78,79} and a phosphomimetic double mutation (S13D and S16D) reduced the binding affinity of htt for POPC/POPS mixed lipid systems, requiring 15 times the lipid content to reach saturation and suggesting an introduced electrostatic repulsion.²⁶ Similar to what was observed here for aggregation in the presence of POPC or TBLE vesicles, the double mutation (S13D and S16D) suppresses htt aggregation with POPC/POPS vesicles.²⁶ While we generally observed a large decrease in the

membrane binding of htt with phosphomimetic mutations with TBLE based on PDA assays, changes in membrane binding associated with phosphomimetic mutations observed here with POPC and POPG vesicles were subtle and often not significant. Beyond the use of different lipid systems between these studies, a potential explanation is that the single mutations used here only neutralized the net charge of Nt17 while the double mutation resulted in a net negative charge. Here, POPG enhanced aggregation even when the net charge of Nt17 was neutralized with little impact on htt membrane affinity. Taking into account that Nt17 contains numerous residues (T3, K6, K9, S13, K15, and S16) that can undergo charge altering PTMs³⁴ and that crosstalk between these residues influence their modification and aggregation,^{54,74} modifying charge via combinations of PTMs in Nt17 can serve a potential role for targeting specific membranes of differing phospholipid composition. In this regard, phosphorylation and acetylation modifies the localization of huntingtin throughout cells.^{17,38,39,70,80,81}

The direct observation, via *in situ* AFM, that the introduction of phosphomimetic mutations in htt-exon1 altered the morphology of aggregates on supported TBLE bilayers further demonstrates the complex role interfaces play in these processes. While determining all the factors influencing aggregation *in vivo* remains difficult, a key component of the cellular environment is the presence of interfaces associated with membranes. Solid/liquid interfaces profoundly impact the aggregation process (both kinetically and morphologically) of numerous amyloid-forming proteins.⁸² For example, β -amyloid peptide ($A\beta$) displays distinct aggregation pathways on chemically diverse surfaces. On the anionic surface of mica, $A\beta$ forms small, highly mobile oligomers that coalesce into prefibrillar aggregates.⁸³ In contrast, epitaxial effects associated with

exposure to graphitic surfaces promotes the formation of extended nanoribbons with distinct orientations on the surface.⁸³ The introduction of point mutations clustered around the central hydrophobic core of A β (E22G Arctic mutation, E22K Italian mutation, D23N Iowa mutation, and A21G Flemish mutation) impact the rate of A β aggregation in bulk solution, but do not alter the morphology of aggregates once formed. However, these mutations have a pronounced impact on aggregate morphology when aggregation was directly observed on either mica⁸⁴ or supported TBLE bilayers,⁶⁴ demonstrating that a change in a single amino acid strongly influences protein/surface interactions. Regarding physiological relevance, cellular and subcellular membranes provide important quasi-solid/liquid interfaces within cells. The ability of the lipid membranes to influence htt aggregation has been well established, with different lipid systems often having a distinct impact.^{22,32,33,85} Like the afore mentioned point mutations in A β , phosphomimetic mutations in htt-exon1 promoted distinct aggregate morphology on TBLE bilayers. This adds another layer of complexity when unraveling the heterogeneous mixture of aggregates forming in the cellular environment and assigning toxic function to specific aggregate forms. Htt/lipid interactions and factors modifying these interactions can have a pronounced impact therapeutic strategy aimed at modifying the aggregation process. In this regard, the presence of lipid membranes abolished the ability of the small molecule curcumin from inhibiting htt fibrillization.⁸⁵

Understanding the interplay between htt and membranous surfaces is critical for understanding a variety of associated toxic mechanisms. A number of biophysical functions are associated with lipid binding amphipathic α -helices⁸⁶ such as Nt17. These include detecting and binding highly curved lipid membranes,^{87,88} and Nt17

demonstrates this propensity.^{26,31} Amphipathic α -helices bind membranes weakly and are regulated by a variety of PTMs. Phosphorylation in Nt17 appears to perform such a regulatory role in cellular trafficking of htt,^{37,39,71,74} suggesting that phosphorylation plays a role in the selectivity of htt binding to a variety of subcellular membranes of varying lipid composition. The impact of phosphorylation to stabilize or destabilize α -helix formation is one contributing factor in altering htt's affinity for membranes,⁷⁴ but selectivity likely works in conjunction with membrane composition. Lipid composition determines the physicochemical properties of membranes that influence amyloid/membrane interactions.⁸² Beyond electrostatic considerations, the aforementioned propensity of Nt17 for curved membranes suggest that selectivity of htt-exon1 for different membranes may be related to lateral membrane pressure and transient packing defects, both of which are readily influenced by curvature.⁸⁹ The introduction of phosphomimetic mutations, especially T3 and S13, enhanced the affinity of htt for TBLE, yet TBLE strongly inhibited fibril formation while stabilizing oligomer size. The complexity of TBLE, which is comprised of a complex, physiologically relevant ratio of zwitterionic and negatively charged lipids (9.6% phosphatidylcholine, 16.7% phosphatidylethanolamine, 1.6% phosphatidylinositol, 10.6% phosphatidylserine, 2.8% phosphatidic acid, etc.), provides a varied surface that provides an array of transient defects to interact with Nt17. TBLE also contains ~20-25% cholesterol, which significantly alters htt aggregation on membranes.²² In this regard, brain extracts promote α -helical structure within Nt17.²⁷ The affinity of htt for POPC and POPG was less impacted by phosphorylation, suggesting that tuning of membrane affinity of htt-exon1 by phosphorylation is more sensitive to complex lipid membranes.

2.5 References

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3. Macromolecular Crowding Alters Huntingtin Aggregation at Interfaces

ABSTRACT

Huntington's disease (HD) is a fatal neurodegenerative disease caused by an extended polyglutamine (polyQ) domain within the first exon of the huntingtin protein (htt). PolyQ expansion directly invokes the formation of a heterogeneous mixture of toxic htt aggregates, including fibrils and oligomers. While htt is a cytosolic protein, it also associates with numerous organelle surfaces within the cell, leading to altered organelle morphology and dysfunction. Here, the impact of macromolecular crowding on htt aggregation in bulk solution and at solid/liquid or membrane/liquid interfaces was investigated.

Dextran, ficoll, and polyethylene glycol (PEG) were used as crowding agents. In bulk solution, crowding enhanced the heterogeneity of non-fibrillar aggregate species formed in a crowder dependent manner. Crowding agents interfered with the deposition and formation of htt fibrils on mica. Crowding in the aqueous phase enhanced deposition of htt aggregates onto supported total brain lipid extract (TBLE) supported bilayers, leading to distinct morphological alteration of the bilayer surface. Collectively, these observations point to the complexity of htt aggregation at interfaces and that crowding in the aqueous phase profoundly influences this process.

3.1 Introduction

Deposition of aggregated, proteinaceous material as plaques and inclusions is a hallmark of several neurodegenerative diseases, i.e. Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD). These deposits are

predominately comprised of amyloid, characterized by a fibrillar morphology and cross β -sheet structure. Of these diseases, HD is caused by a mutation in the huntingtin gene that encodes for an abnormal expansion (greater than ~35 repeats) of a polyglutamine (polyQ) tract in the first exon of the huntingtin protein (htt).¹ PolyQ expansion correlates with htt aggregation, which is a multi-pathway, complex process involving a variety of aggregate species including oligomers, amorphous aggregates, fibrils, and annular structures.²⁻⁴ Due to the variety of aggregates possible, htt aggregation results in heterogeneous mixtures of aggregates, making it difficult to elucidate relationships between structure and toxic gain of function. Fibrils,^{5,6} oligomers,⁷⁻⁹ and inclusions¹⁰ are all implicated as potential toxic entities. Due to this, it is imperative to understand how exogenous factors within the cytosolic environment influence aggregation.

While aggregation is a heterogeneous process, the complexity of the intracellular environment can have a pronounced impact on htt aggregation. The cytosolic environment of cells is extremely crowded with proteins and other large macromolecules. Depending on location and cell type, macromolecular concentrations within cells can reach up to 400 mg/mL and up to 40% of the available volume is occupied by macromolecules,¹¹⁻¹⁴ and such crowded environments influence protein behavior.¹⁵ For example, macromolecular crowding agents sterically reduce configurational entropy in proteins via an excluded volume mechanism.¹⁶ In addition, proteins nonspecifically interact with crowding agents via van der Waals forces, hydrophilic/hydrophobic effects, and electrostatics.¹⁷ These interactions further influence protein conformation. As such, the physicochemical properties of the crowder molecules profoundly influence proteins in numerous ways with implications for amyloid

formation.¹⁸ Amyloid formation in a crowded environment can differ by orders of magnitude from that observed in bulk solution.^{16,19} The impact of crowders on different amyloid-forming proteins varies greatly depending on factors such as crowder concentration, and the extent of interaction between the crowding agent and the aggregating protein. For example, α -synuclein aggregation is accelerated by numerous crowders, i.e. polyethylene glycol (PEG), dextran, ficoll, lysozyme, and bovine serum albumin; however, larger macromolecular crowders promoted aggregation faster than smaller crowders.²⁰ On the other hand, crowders can stabilize multimeric protein states, suppressing nucleation of fibrillization.²¹ In terms of chemical interactions, the extent of saccharide based crowders interaction with A β determined whether crowding promoted or inhibited fibril formation.²²

Beyond crowded environments, the presence of liquid/surface interfaces alter amyloid formation.²³ Solid surfaces,^{24,25} including mica, graphite, gold, and Teflon, induce conformational changes in proteins, which alter aggregation of a variety of amyloid forming proteins.^{26–30} Surfaces of particular physiological relevance are lipid membranes, which also exert influence on protein aggregation.²³ In this regard, htt is highly associated with membranous surfaces within cells, and these interactions further modify aggregation. For example, htt and a variety of its aggregated forms localize to the ER, mitochondria, nuclear envelope, tubulovesicles, endosomes, lysosomes, and synaptic vesicles.^{31–35} PolyQ expansion enhances htt/phospholipid interactions.^{36–38} Htt aggregation disrupts lipid membranes, leading to organelle dysfunction.^{30,39–41} Brain lipids and membranous structures accumulate in htt aggregates,^{39,42–44} suggesting a role of lipids in the aggregation process. Indeed, the impact of a variety of membranes,

ranging from total brain lipid extracts (TBLE) to pure lipid systems, alter the rate of aggregation and the resulting aggregate species observed.^{30,45–48} Htt-exon1 fibril formation is enhanced in the presence of POPC/POPS^{45,49,50} mixed vesicles and pure POPC, POPG, or POPS vesicles.⁵¹ On the other hand, brain lipid extracts reduce fibrillization,^{30,38,52} and the addition of exogenous lipid components, i.e. cholesterol, sphingomyelin, and GM1,^{47,48} to these extracts further influence aggregation.

Here, the impact of macromolecular crowding on htt aggregation was investigated. Three different crowders (Dextran, Ficoll, and polyethylene glycol (PEG)) were used. In particular, the impact of macromolecular crowding in the aqueous phase on htt aggregation and solid/liquid or membrane/liquid interfaces was determined. Continuous imaging using in situ atomic force microscopy (AFM), allowing for the tracking of individual aggregates on surfaces, monitored aggregation at these interfaces. Despite the different macromolecular crowders being considered chemically inert,^{53,54} each exerted differing influence on htt aggregation in bulk solution with Ficoll promoting a unique aggregate morphology. While ThT assays suggested that fibrils still formed, crowders interfered with fibril deposition onto mica. When monitoring aggregation occurring directly on mica, a variety of oligomers and fibrils were observed, but the extent of surface aggregation was impeded by crowding in the aqueous phase, with PEG completely blocking the observation of fibrils. When supported total brain lipid extract (TBLE) bilayers were exposed to htt, crowding in the aqueous phase by all three macromolecules enhanced htt deposition on the bilayer, resulting in increased membrane morphological disruption. Here, PEG was associated with the most aggressive extent of membrane damage.

3.2 Materials and Methods

3.2.1 Expression, Purification of (GST)-htt-exon1 Fusion Protein. A (GST)-htt-exon1(46Q) fusion protein was purified as described previously.⁵⁵ In short, GST-htt-exon1 was expressed in *E. Coli*. Resulting cells were lysed with lysozyme (0.5 mg/mL) and probe sonication. The target fusion protein was purified from these lysates using a GST affinity column and liquid chromatography (BioLogic LPLC, Bio Rad). The purity of the target protein in relevant Fractions was assessed by SDS-PAGE. Protein concentration was determined by a Bradford assay. Prior to each experiment, pre-existing aggregates were removed via high speed centrifugation (20,000 ×g at 4°C for 30 minutes). Factor Xa protease (New England Bio Labs) was used to cleave GST and initiate aggregation. All experiments were performed in Tris buffer (50mM Tris-HCl, pH 7, 150mM NaCl).

3.2.2 Macromolecular Crowder Preparation. Stock solutions (800 mg/mL) of Ficoll 400, Dextran 20,000, and Polyethylene Glycol 20,000 (PEG) of macromolecular crowders were made in Tris buffer heated to 60°C, resulting in viscous solutions. These solutions were incubated in an orbital thermomixer at 60°C and 1400 rpm, followed by bath sonication at 60°C for 1h. These stock solutions were diluted to reach the final desired crowder concentrations in various experiments.

3.2.3 Peptide Preparation. A custom, synthetic peptide based on htt-exon1, Nt17Q₃₅P₁₀KK (AnaSpec) was prepared based on a variation of previously described protocols.⁵⁶ Briefly, peptide was dissolved in a 1:1 mixture of hexafluoroisopropanol (Acros Organics) and trifluoroacetic acid (Acros Organics) for 12 h without agitation. Excess solvent was evaporated off using a vacufuge. The resulting films were dissolved

in DMSO at a concentration of 2000 μM . These DMSO stock solutions were diluted into Tris buffer (20 μM , 1% DMSO), and this solution was injected directly into the AFM fluid cell (final concentration of 10 μM , 0.5% DMSO).

3.2.4 Lipid Vesicle Preparation. Total brain lipid extract (TBLE; Avanti Polar Lipids) was hydrated in Tris buffer with vortex mixing at 60°C until all lipid was suspended in solution. Vesicle formation was promoted by 10 freeze/thaw cycles using liquid nitrogen, followed by bath sonication for 1 hour at room temperature. The vesicle size distribution was analyzed by dynamic light scattering (NanoBrook Omni particle size analyzer, Brookhaven).

3.2.5 Thioflavin T (ThT) Aggregation Assay. Htt-exon1(46Q) was diluted to a final concentration of 10 μM in the presence of 125 μM ThT (Sigma-Aldrich, St. Louis, MO). Assays were performed in a 96-well plate format (100 μL per well) using a microplate reader (Molecular Devices, SpectraMax M2). All assays were run at 37°C, and readings were taken every 5 min with 440 nm excitation and 484 nm emission. All conditions were run in triplicate with a minimum of three independent experiments performed. In ThT assays with lipids, a 10:1 lipid:protein ratio was used.

3.2.6 Polydiacetylene (PDA) Vesicle Binding Assay. 10,12-tricosadiynoic acid (GFS Chemicals, Columbus, OH) and TBLE was dissolved at a 2:3 molar ratio in a solution of 1:4 ethanol/chloroform. Excess solution was evaporated off under a stream of N_2 , and the resulting film was resuspended in Tris buffer at 70°C. Probe sonication (10-minute intervals at 100 W) using a sonic dismembrator (FisherSci) was performed to achieve an opaque solution. This solution was stored at 4°C overnight to allow self-assembly into vesicles. Polymerization of the PDA was initiated by irradiation at 254 nm for 10

minutes at room temperature with stirring, resulting in a blue color. The assay was performed in a 96-well plate format using a microplate reader (Molecular Devices, SpectraMax M2). The percent colorimetric response (% CR) was determined every 5 min by measuring the blue absorbance (640 nm, A_{blue}) and the red absorbance (500 nm, A_{red}) using the following equation.

$$(Eqn. 3.1) \quad \% CR = \left[\frac{PB_0 - PB_1}{PB_0} \right] * 100$$

PB is defined as $A_{\text{blue}}/(A_{\text{blue}}+A_{\text{red}})$.^{57,58} PB_0 was determined from the negative control of exposure of the vesicles to neat buffer, and PB_1 was obtained is obtained from each experimental condition. A positive control of exposure to 100 μM NaOH was performed and used to standardize the PDA assays across independent trials.⁵⁹ All conditions were run in triplicate with a minimum of three independent experiments performed.

3.2.7 Atomic Force Microscopy (AFM). For *ex situ* AFM experiments, htt-exon1(46Q) (10 μM) was incubated in the presence and absence of macromolecular crowders at 37°C and 1400 rpm in an orbital thermomixer. At designated time points, 2 μL aliquots of each sample were deposited onto freshly cleaved mica (Ted Pella Inc., Redding, CA) for 1 minute, washed with 200 μL of 18 M Ω ultrapure water, and dried under a gentle stream of filtered air. For the fibril deposition experiment, htt-exon1(46Q) (20 μM) was incubated at 37°C and 1400 rpm in an orbital thermomixer for 24 h to pre-form fibrils. An aliquot of this sample was diluted into neat buffer (control) or macromolecular crowders

to achieve a final concentration of 10 μM of htt and 100 mg/mL of each crowder. These samples were deposited on mica as described above. All samples were imaged using a Nanoscope V Multi-Mode scanning probe microscope (VEECO) equipped with a closed loop vertical engage J-scanner and silicon-oxide cantilevers with a resonance frequency of ~ 300 kHz and a nominal spring constant of 40 N/m. For *in situ* AFM experiments, a tapping mode fluid cell equipped with an O ring and cantilever with a nominal spring constant of 0.1 N/m were used. For experiments of peptide aggregation on mica, samples of peptide (10 μM) in neat buffer or with macromolecular crowders (100mg/mL) were mixed and injected directly into the fluid cell. Aggregation on mica was monitored by continuous imaging. For experiments on lipid membranes, prepared vesicles were injected into the fluid cell and allowed to fuse onto the surface to form a supported bilayer. Once a continuous 40 μm x 40 μm patch of supported lipid bilayer formed, htt-exon1(46Q) that had been incubated on ice with factor Xa for 1 h was injected with and without macromolecular crowders into the fluid cell for a final concentration of 10 μM of htt and 25 mg/mL of crowders. Aggregation on the bilayer was monitored by continuous imaging. For *in situ* AFM experiments on mica or supported bilayers, scan size was periodically increased to ensure that the imaging process was not influencing aggregation. All AFM images, from both *ex situ* and *in situ* experiments, were analyzed using MATLAB equipped with the image processing toolbox (MathWorks) as described.^{60,61}

3.3 Results

3.3.1 Crowding alters htt aggregation in bulk solution. To investigate the impact of macromolecular crowders on htt aggregation in bulk solution, a GST-htt-exon1 fusion

protein with 46 repeat glutamines (htt-exon1(46Q)) was used as a model system. Cleavage of the GST tag with Factor Xa initiates aggregation. Htt-exon1(46Q) (10 μ M) was incubated in the presence of Dextran (20,000 da), Ficoll (400 da), or PEG (20,000 da), and fibril formation was monitored with a ThT assay (Figure 1). A concentration gradient (50, 100, and 200 mg/mL) was investigated for each crowder. For most conditions, there were not statistically significant changes in fibril formation compared to htt-exon1(46Q) incubated in the absence of crowders; although, there were some trends associated with each crowder. With dextran, ThT signal was typically larger than control, but this was only significantly different with 200 mg/mL dextran when comparing the maximum ThT signal ($p < 0.05$). Ficoll also appeared to increase fibril formation with increasing crowder concentration; however, the 200 mg/mL ficoll condition did not quite reach statistical significance. Unlike in the presence of the other two crowders, htt aggregation appeared slightly reduced in the presence of PEG, but this reduction did not reach statistical significance under any condition.

Figure 3.1: The impact of macromolecular crowders on htt-exon1(46Q) fibril formation as assessed by a ThT assay. Macromolecular crowders used were (A) dextran, (B) ficoll, and (C) PEG, and each was investigated at 50 mg/mL, 100 mg/mL, and 200 mg/mL. Htt-exon1(46Q) concentration was 20 μ M. Buffer control represents experiments without any crowding agent. Error bars represent standard error of the mean (SEM) and are provided every 30 min.

While the three crowders did not have a pronounced impact of fibril formation as measured by a ThT assay, this assay does not provide information concerning fibril

morphology or other non-fibrillar aggregates such as oligomers. To determine if crowding impacted fibril morphology and oligomer formation, htt-exon1(46Q) incubations with macromolecular crowders and sampled after 8 h for AFM analysis. Htt-exon1(46Q) incubations in the absence of crowders were monitored as a control. Representative AFM images of these incubation with 100 mg/mL of each crowder are shown in Figure 2A, with background images of each crowder (no htt-exon1) deposited on mica are shown in Figure 3. Representative AFM images of htt-exon1(46Q) with other concentrations (50 mg/mL and 200 mg/mL) of the crowders are presented in Figure 4. Based on the ThT assays, we expected to see significant populations of htt fibrils under all conditions; however, fibrils were only observed in the control incubation (Figure 2A). As a result, a control experiment was designed to determine if the crowders interfered with the deposition of fibrils onto mica (Figure 2B). 20 μ M htt-exon1(46Q) solutions were seeded with pre-formed fibrils and incubated to obtain extensive fibril populations. These pre-aggregated htt-exon1(46Q) samples were diluted to obtain a final protein concentration of 10 μ M with either neat buffer containing no crowding agents or with macromolecular crowders at a final concentration of 100 mg/mL. After 5 min of mixing, these solutions were sampled and deposited on mica for AFM analysis of fibril coverage. Comparison of the number of fibrils per unit area for each sample demonstrated that all three crowders prevent fibrils from depositing on mica to a significant extent (Figure 2C). PEG had the largest impact on the fibril density on mica, reducing it by 92%, followed by ficoll (73% reduction) and dextran (49% reduction). This analysis suggests that the absence of fibrils observed from incubations of htt-exon1(46Q) with each crowder may be due to interference with the deposition process.

Despite the crowders interfering with fibril deposition, the different macromolecular crowders promoted distinct morphologies of non-fibrillar aggregates that adhered to mica (Figure 2A). To quantify this altered morphology, the height and diameter of each non-fibrillar aggregate was measured using automated scripts in MATLAB^{60,61} and used to construct height vs diameter correlation plots (Figure 2D). To verify that features observed in the AFM images derived from htt-exon1(46Q) were unique to the addition of protein, control crowder background images (Figure 4) were analyzed and also plotted in the correlation plots for comparison. Htt-exon1(46Q) in neat buffer formed a heterogeneous mixture of globular oligomers with heights ranging predominately from 1-8 nm and diameters ranging from ~30-80 nm. While the dextran background contained numerous features (heights ~1-5 nm, diameters ~30-60 nm) that overlapped with the size of the smaller htt-exon1 oligomers, a unique set of significantly larger globules were observed in incubations of htt-exon1(46Q) incubated with dextran at 100 mg/mL. These large globules displayed tighter correlation between their heights and diameters with a significant population growing beyond the range (height > 10 nm, diameters > 80 nm) of features observed in the htt-exon1 control. These larger aggregates associated with htt-exon1 aggregation in the presence of dextran were present in the 50 mg/mL sample but were not observed at dextran concentration of 200 mg/mL.

Analysis of htt oligomers formed in the presence of ficoll is complicated due to the extensive features appearing in the ficoll blank control that had significant overlap with features observed when htt-exon1(46Q) was incubated with ficoll at 100 mg/mL. However, there was a clearer shift of features distinct from the background at 50 mg/mL

and 200 mg/mL ficoll experiments. Nevertheless, the distribution of potential htt-exon1(46Q) oligomers in the correlation plots associated with the presence of ficoll are distinct to those observed in the htt-exon1(46Q) control. With htt-exon1(46Q), very few features appeared on the mica surface in the presence of PEG, and few features appeared as background from control PEG experiments. However, observed oligomers of htt-exon1(46Q) formed in the presence of PEG at 100 mg/mL displayed a “fried egg” like morphology. That is, a flat feature around the periphery of each aggregated surrounded a distinct globular structure in the middle. While the measured height of these features were similar to oligomers observed in control htt-exon1(46Q) incubations, their diameters were significantly larger (60-120 nm) due to the peripheral portion of the aggregate. While ThT did not detect any difference associated with fibril formation and elongation, AFM images suggest that each crowder altered the morphology of aggregate intermediates to varying extent. This observation is limited, however, to the htt-exon1(46Q) aggregates that readily deposited on mica.

Figure 3.2: Ex situ AFM analysis of *htt*-exon1(46Q) aggregates formed in the presence of 100 mg/ML macromolecular crowders. *Htt*-exon1(46Q) was incubated at 20 μM with dextran, ficoll, or PEG. These incubations were sampled for AFM imaging after 8 h of incubation, and (A) representative AFM images are presented. (B) Preformed *htt*-exon1(46Q) fibrils were mixed with 100 mg/mL macromolecular crowders, deposited on mica, and imaged by AFM to determine if the crowders interfered with the deposition of fibrils. (C) Quantification of the fibrils observed per unit area on mica from the fibril deposition experiment in (B). Error bars represent standard error of the mean (SEM), and * indicated $p < 0.01$. (D) Correlation plots of height vs diameter of non-fibrillar aggregates observed in (A). For each correlation plot, features observed in the corresponding blank (crowder only) control are provided for comparison.

Figure 3.3: Representative AFM images of mica exposed to (A) neat buffer or 100mg/mL of either (B) dextran, (C) ficoll, or (D) PEG. Height profiles, as indicated by the green lines, are provided below each image.

Figure 3.4: Ex situ AFM analysis of htt-exon1(46Q) aggregates formed in the presence of 50 or 100 mg/ML macromolecular crowders. Htt-exon1(46Q) was incubated at 20 μ M with dextran, ficoll, or PEG. These incubations were sampled for AFM imaging after 8 h of incubation, and (A) representative AFM images are presented. (B) Correlation plots of height vs diameter of non-fibrillar aggregates observed in (A). For each correlation plot, features observed in the corresponding blank (crowder only) control are provided for comparison.

3.3.2 Htt aggregation at a solid/liquid interface is modified by macromolecular

crowders. As all three crowders interfered with fibril deposition onto mica and were

associated with unique oligomeric species appearing on mica, it is possible that a

crowded liquid phase alters the interaction of htt-exon1 with mica. As a result, we next

aimed to elucidate if macromolecular crowding in the aqueous phase influenced

aggregation occurring at a mica surface. Due to complications associated with the GST

tag, a synthetic htt peptide that mimics htt-exon1 was used. This peptide was comprised of the first N-terminal 17 amino acids (Nt17) of htt that precede the polyQ domain, a polyQ domain of 35 repeats, a polyP stretch of 10 residues, and two lysine residues at the C-terminus to aid in solubility. Due to this, the peptide is referred to as Nt17-Q₃₅-P₁₀-KK. The real-time aggregation of Nt17-Q₃₅-P₁₀-KK on mica was tracked by continuous *in situ* AFM imaging (Figures 5 and 6). Solutions of Nt17-Q₃₅-P₁₀-KK were prepared in neat buffer or with crowders and injected directly into the AFM fluid cell to a final protein concentration of 10 μ M and crowder concentration of 100 mg/mL.

Figure 3.5: Tracking of individual Nt17-Q₃₅-P₁₀-KK aggregates in mica as a function of time with no crowders in the aqueous phase. (A) A series of *in situ* AFM images of the same regions on mica. Black circles indicate aggregates in consecutive images that were initially classified as oligomers that remain stable or eventually transition into fibrils. Green circles identify oligomers in consecutive images that eventually desorb from the mica surface. The blue box indicates in consecutive images two fibrils that grow together. (B) Analysis of the time that 128 distinct oligomers remained stable on mica.

Figure 3.6: Comparison of Nt17-Q₃₅-P₁₀-KK aggregation on mica with different macromolecular crowders in the aqueous phase. (A) Time-sequence, representative *in situ* AFM images of Nt17-Q₃₅-P₁₀-KK (10 μM) aggregating on mica with buffer, dextran, ficoll, or PEG in the aqueous phase. The macromolecular crowder concentration was 100 mg/mL. As the height of features varied, the blue boxes represent areas of the image that correspond to the color scale with blue font. (B) Quantification of the % surface area covered by Nt17-Q₃₅-P₁₀-KK aggregates as a function of time. Error bars represent standard deviation.

Nt17-Q₃₅-P₁₀-KK in neat buffer (no crowders) formed a variety of oligomers and putative fibrils on mica within the timeframe of the first image (~8-10 minutes after injection, Figure 5 and 6A). The mica area occupied by these Nt17-Q₃₅-P₁₀-KK aggregates steadily increased with time, and the predominate aggregate species on the surface were fibrillar in morphology within 17-20 minutes. These fibrillar species grew in length from both ends as more peptide was incorporated into the aggregate. Branching was often observed, and fibrils growing into each other frequently occurred (Figure 5A). Several of these fibrils had clear oligomer precursors, but the majority of them did not. That is, they already had a fibrillar morphology upon their initial appearance. There were

several observed mechanisms for oligomer disappearance. Some oligomers transitioned into fibrils, some desorbed from the mica surface, and others were incorporated into a pre-existing fibril that extended toward the oligomer. The relative stability of individual oligomers varied greatly. The fate of 128 individual oligomers were tracked from image to image to determine how long each remained in an oligomer conformation on the mica surface (Figure 5B). Just under half of these oligomers disappeared within ~10 minutes (or by the next image). However, ~10% of identified oligomers persisted for over an hour on mica. The total amount of aggregation on mica was quantified by determining the percent surface area occupied by aggregates as a function of time (Figure 5B). Based on this analysis, total aggregation of Nt17-Q₃₅-P₁₀-KK in neat buffer steadily grew, covering $39.2 \pm 2.9\%$ of the mica surface after ~200 min.

With the addition of macromolecular crowders in the aqueous phase, aggregation of Nt17-Q₃₅-P₁₀-KK on mica was significantly reduced (Figure 6A). With dextran and ficoll, a smaller number of aggregates (both oligomers and putative fibrils) appeared within 8-10 min compared to control. With time, fewer additional aggregates appeared on mica. The aggregates that did appear were mostly fibrillar and their morphology was indistinguishable to control. These fibrils elongated and branched with time. However, the extent of aggregation on the surface was significantly diminished, as only $2.6 \pm 0.5\%$ and $5.6 \pm 3.7\%$ of the mica surface was occupied by aggregates after ~200 min (Figure 6B). With PEG, fibrils were not observed on mica, and only a few oligomers were present (occupying less than 0.5% of the total surface for over 200 min; Figure 6B). The observed oligomers in the presence of PEG were typically smaller than

oligomers observed under all other conditions; however, very few of these small oligomers were observed across all trials with PEG, making comparisons difficult.

3.3.3 Htt aggregation on lipid bilayers is modified by macromolecular crowders.

As crowders altered htt peptide aggregation at a solid/liquid interface and lipid membranes can also impact amyloid formation, the ability of crowders in the aqueous phase to impact htt-exon1(46Q) on lipid bilayers was investigated. The model lipid system chosen for these studies was total brain lipid extract (TBLE) because it contains a physiologically relevant mixture of lipid components. To determine if the macromolecular crowders altered the ability of htt-exon1(46Q) to bind lipid membranes, TBLE/PDA vesicle lipid binding assay was performed (Figure 7). This is a colorimetric assay in which vesicles comprised of a mixture of phospholipids and polymerized PDA exhibit a rapid color change from blue to red due to mechanical stress induced by protein binding. By monitoring the %CR peptide–membrane interactions and interfacial membrane processes can be quantified. First, each macromolecular crowder (at concentrations of 50, 100, and 200 mg/mL) was tested to see if they invoked a CR in the TBLE/PDA vesicles in the absence of htt (Figure 8). The crowders did not invoke a CR in the absence of protein. Next, TBLE/PDA vesicles were exposed to htt-exon1(46Q) at 10 μ M in the presence of each crowder at the various concentrations, and the %CR was measured over 9 h (Figure 7). Relative to control (htt-exon1(46Q) in the absence of crowders), all three crowders typically enhanced htt-exon1(46Q) interaction with TBLE vesicles. For dextran, the largest enhancement was observed at 50 mg/mL crowder concentration. At higher concentrations of dextran, htt-exon1(46Q) lipid binding was enhanced relative to control to a lesser extent. A similar trend was

observed with Ficoll, as the lower concentrations of ficoll induced larger %CRs. Although, 200 mg/mL ficoll resulted in an initial jump in the %CR that was followed by a slight reduced %CR compared to control. PEG, at all concentrations, invoked an initial large response that dropped off after ~1-2 h. After this time frame, the %CR either did not increase or tracked closely with control. A caveat is that the combination of PEG and htt-exon1(46Q) often caused the TBLE/PDA vesicles to fall out of solution, suggesting that the large initial response was destabilizing the suspension and causing the smaller measured %CR. Nevertheless, the assay suggests that all three crowders enhanced the ability of htt-exon1(46Q) to bind vesicles.

Figure 3.7. The impact of macromolecular crowders on htt-exon1(46Q) interaction with TBLE vesicles as assessed by a PDA assay. Macromolecular crowders used were (A) dextran, (B) ficoll, and (C) PEG, and each was investigated at 50 mg/mL, 100 mg/mL, and 200 mg/mL. Htt-exon1(46Q) concentration was 20 μ M. Buffer control represents experiments without any crowding agent. Error bars represent standard error of the mean (SEM) and are provided every 30 min.

Figure 3.8. Control TBLE/PDA lipid binding assays assessing if macromolecular crowders invoke a colorimetric response. Macromolecular crowders used were (A) dextran, (B) ficoll, and (C) PEG, and each was investigated at 50 mg/mL, 100 mg/mL, and 200 mg/mL.

As the macromolecular crowders altered the ability of htt-exon1(46Q) to interact with lipid vesicles, we next used a ThT assay to assess if these crowders altered fibril formation in the presence of TBLE vesicles (Figure 9). To further assess the impact of the crowders on fibrillization of htt, for these assays, the lipid to htt molar ratio was 10:1, and the vesicles were ~140-180 nm in diameter as assessed by dynamic light scattering. The impact of the three crowders on fibril formation in the presence of TBLE vesicles as assessed by ThT followed a similar trend as that observed in the absence of TBLE vesicles. That is, dextran and ficoll appeared to increase fibrillization; PEG appeared to slightly inhibit fibrillization. However, with the addition of both vesicles and crowders, the noise in these assays was increased, and these differences did not reach statistical significance. However, the ThT assay comprehensively measures fibril formation of the entire sample and cannot distinguish between fibrils formed in bulk solution or at the vesicle surface. It also does not provide information regarding non-fibrillar aggregates.

Figure 3.9. The impact of macromolecular crowders on htt-exon1(46Q) fibril formation in the presence of TBLE vesicles as assessed by a ThT assay. Macromolecular crowders used were (A) dextran, (B) ficoll, and (C) PEG, and each was investigated at 50 mg/mL, 100 mg/mL, and 200 mg/mL. Htt-exon1(46Q) concentration was 20 μ M. TBLE vesicles were added at a lipid to peptide ratio of 10:1. Buffer control represents experiments without any crowding agent. Error bars represent standard error of the mean (SEM) and are provided every 30 min.

To more directly investigate htt-exon1(46Q) aggregation on TBLE bilayers, *in situ* AFM was employed to observe aggregation occurring on a supported TBLE bilayer with a crowded aqueous phase (Figure 10). For these experiments, it is important that the supported bilayer is stable under continuous imaging. Therefore, control experiments were performed to verify that bilayers were stable when crowders were injected into the fluid cell. Supported bilayers were first formed on mica in neat buffer, and then macromolecular crowders were injected into the fluid cell. Based on these control experiments, it was determined that the supported bilayers were reliably imaged with up to 25 mg/mL of crowder in the fluid cell for several hours. Larger concentrations of crowders often resulted in membrane damage. As a result, these experiments were performed with a 25 mg/mL final concentration of crowding macromolecules. Only continuous TBLE bilayers (40 $\times$ 40 μ m in size), as assessed by AFM imaging in neat buffer, were exposed to htt-exon1(46Q), and observations were limited to these verified continuous bilayers. A freshly prepared TBLE bilayer had an RMS roughness of 0.26 ± 0.06 nm prior to being exposed to htt-exon1(46Q) (Figure 10B). Htt-exon1(46Q)

was injected to a final concentration of 10 μM in the fluid cell. For experiments with crowders, htt-exon1(46Q) and the crowder were mixed together and injected into the fluid cell at the same time.

Figure 3.10. Comparison of htt-exon1(46Q) aggregation on supported TBLE bilayers with different macromolecular crowders in the aqueous phase. (A) Time-sequence, representative in situ AFM images of htt-exon1(46Q) (10 μM) aggregating on supported TBLE bilayers with buffer, dextran, ficoll, or PEG in the aqueous phase. The macromolecular crowder concentration was 25 mg/mL. As the height of features varied, the blue boxes represent areas of the image that correspond to the color scale with blue font. (B) Quantification of the RMS roughness of unperturbed (solid bars) and disrupted (striped bars) regions of the bilayer. The RMS roughness of a bilayer prior to exposure to htt-exon1(46Q) is provided by the purple bar. Error bars represent standard deviation. (C) Height and diameter histograms of htt-exon1(46Q) aggregates as a function of time. (D) Quantification of the % area of the bilayer disrupted by htt-exon1(46Q) as a function of time. Error bars represent standard deviation.

When TBLE was exposed to htt-exon1(46Q) in the absence of crowders, numerous oligomers appeared on the surface within 10 min, and often small defects in the bilayer were observed (Figure 10A). With time, regions of the bilayer displayed

disrupted morphology characterized by increased surface roughness and a highly granular appearance. Most of the bilayer maintained a smoother appearance. Often, these roughened areas developed around previously observed oligomers. These disrupted regions grew in area and became rougher with time. To quantify this roughening effect, regions of the bilayer displaying increased roughness were identified and the RMS roughness of these regions was determined (Figure 10B). The RMS roughness of the unaltered regions of the bilayer was also determined. The roughness of the smooth regions was comparable to freshly prepared TBLE bilayers. However, the RMS roughness of the disrupted portion of the membrane was 0.57 ± 0.13 nm after ~9 min, and this increased to 1.22 ± 0.33 nm after ~140 min. The dimensions of the oligomers observed were also measured, and these oligomers swelled in size (predominately in the lateral dimension) with time (Figure 10C). To quantify the extent of the interaction of htt-exon1(46Q) with the supported bilayers, the percentage of the surface that displayed any altered morphology was determined as a function of time via AFM image analysis (Figure 10D). For this analysis, the area occupied with aggregates was considered as a disrupted region. After ~130-145 min, the extent of the bilayer disrupted leveled off at about 13-15% of the total bilayer area.

The interaction and aggregation of htt-exon1(46Q) with the supported TBLE bilayers was altered by the addition of macromolecular crowders in a crowder dependent manner. When the aqueous phase was crowded with dextran, oligomers appeared on the surface within the first 10 min of exposure to htt-exon1(46Q) in a similar fashion to the control experiment without crowders, and these oligomers increased in size with time (Figure 10A). Similar to control, regions of increased

roughness with a granular appearance also developed on the bilayer, except these regions occupied more of the surface area with time (Figure 10C), and the RMS roughness of these regions became larger after ~100 min (Figure 10B). With Ficoll, htt-exon1(46Q) aggregation was quite different (Figure 10A). Large globular features appeared on the bilayer surface within the first 10 minutes. Rather than being associated with regions of a disrupted granular morphology, these large aggregates swelled in size, reaching heights as large as 20 nm above the bilayer surface and diameters on the order of 300-500 nm (Figure 10C). As these oligomers swelled, they occupied an increasing area of the bilayer. Additionally, holes spanning the entire bilayer appeared near these features within ~100 min, and these holes grew in size with time.

With PEG, regions of altered morphology formed on the bilayer within the first 10 min, but unlike the control, these regions were not granular in appearance (Figure 10A). Rather, they displayed a smoother, raised morphology. The bilayer between these regions was also slightly rougher (0.33 ± 0.04 nm) than a typical TBLE bilayer (Figure 10B). By ~30 min with PEG, a considerably large population of small oligomers appear on the bilayer, and the regions of altered morphology grow. By ~60 min, large aggregates start to appear on the surface. Holes exposing the mica substrate developed around these large aggregates as they grew in size. With time, these holes covered a larger area (microns in size). Meanwhile, the rest of the bilayer became rougher, with greater than 95% of the surface displaying some type of altered morphology within ~115 min (Figure 10D).

3.4 Discussion

While toxicity has been attributed to numerous aggregate forms of htt, survival analysis of neurons expressing htt-exon1 correlated diffusely distributed htt within the cell to cell death. However, this diffuse htt can be comprised of various types of aggregate species. For example, a significant population of htt oligomers are detectable in mouse and cellular models of HD that occupy this diffuse fraction.^{62,63} However, htt fibrils are also present in the diffuse fraction, as detected by super-resolution microscopy.⁶⁴ Here, using simple macromolecules, we demonstrated that a crowded environment modifies the heterogeneous distribution of htt-exon1 aggregates formed *in vitro*. While the crowded environment within the cytosol is much more complicated, these results suggest that crowding effects likely exert a strong influence on the composition of the diffuse htt fraction linked to toxicity. In addition, potential toxic mechanisms attributed to htt aggregation involves damaging membranes associated with a variety of organelles, including the ER, mitochondria, and nuclear envelope.^{10,35,40,65} Here, we demonstrate that crowding in the aqueous phase alters the interaction with htt at solid/liquid and solid/membrane interfaces. In particular, the interactions with lipid membranes was enhanced in by crowding in the aqueous phase, and this was highly dependent on the chosen crowder. This suggests that the crowded environment of the cytosol can play an important role in htt/membrane interactions that are associated with toxicity.

There are several caveats in comparing the experiments on bilayers and mica. First, they used different models for htt aggregation, a synthetic N-terminal fragment of htt shorter than exon1 on mica and a full htt-exon1 protein on bilayers. Second, the crowder concentrations that were feasible to use varied, 100 mg/mL with mica and 25

mg/mL with bilayers. However, there are some interesting difference worth noting. On mica, the extent of aggregates observed was altered, but with dextran and ficoll, the morphology of aggregates, especially fibrils, appearing on mica was identical to those observed in control. With PEG, only small oligomers were observed and not in great enough quantity to make meaningful comparisons. However, on TBLE bilayers, the different crowders promoted unique aggregate morphologies. The morphology of aggregates of polyQ containing peptides formed in the presence of mica is predominately influenced by polyQ length, not flanking sequences.^{3,30} On the other hand, a variety of membrane properties, lipid composition, and flanking sequences within htt-exon1 influence the interaction of htt with bilayers.^{30,47,51,66} This dichotomy of aggregation behavior between the two surfaces with different macromolecular crowders suggests more than a simple mechanism associated with protein diffusion via crowded environments. Indeed, the lack of meaningful crowder concentration dependent difference in fibril elongation as measured by ThT assays (both with and without lipids) supports that htt diffusion is likely not significantly inhibited, as impeded diffusion is known to slow amyloid elongation.^{67,68}

All of the macromolecular crowders accelerated accumulation of htt-exon1(46Q) on TBLE bilayers within the first 1-2 h of exposure as measured the PDA assay and *in situ* AFM. This may be due to exclusion of htt-exon1(46Q) (and possibly membrane active aggregate species) from the solvent to the membrane interface. Localization and condensation of soluble proteins at lipid membrane interface is known to be sensitive to macromolecular crowding due to excluded volume effects.^{69,70} This excluded volume mechanism likely plays a larger role with dextran, as the htt-exon1(46Q) aggregates

and bilayer morphological changes resembled that observed in the absence of crowders. Beyond a simple excluded volume explanation, there appeared to be crowder specific interactions associated with ficoll and PEG with htt-exon1(46Q) as each promoted unique aggregate morphologies and changes in the bilayer. This may be due to local concentration effects that promote protein/protein and/or protein/lipid interactions at the membrane/liquid interface. Their enhanced local concentration promotes protein:lipid and protein:protein interactions, oligomerization and/or aggregation, while soluble proteins and crowders that do not associate with the membrane are largely excluded from the interface.⁷¹ In this regard, changing macromolecular crowders enhance accumulation of RBC-modifying enzymes on cell surfaces up to 440-fold.¹⁴

Considering the unique aggregate morphologies observed on TBLE with the addition of ficoll and PEG, a question arises as to if these aggregates formed in solution before binding the membrane or if they developed from smaller species once bound to the membrane. The imaging frequency of the continuous *in situ* AFM imaging was not fast enough to settle this question; however, it offers some hints toward answering this question. With ficoll, the large aggregate appeared within the timeframe to take the first image after injection of htt-exon1(46Q). These aggregates could have formed in solution, as the *ex situ* AFM analysis revealed similar sized features forming in the presence of ficoll, with large portions of these aggregates being unique from the ficoll background at 50 and 200 mg/mL. This suggests that it is possible for these large features to have formed in solution prior to binding the surface. Once on the surface, these aggregates steadily grew in size and were associated with the development of

holes in the bilayer, suggesting they may accumulate membrane lipids as well. While some morphological features on bilayers were associated with relatively large aggregates in the presence of PEG, most of the morphological changes were associated with smaller feature or ubiquitously spread across the bilayer, suggesting that a large portion of the htt-exon1(46Q) interacting with the bilayer was not extensively aggregated. All of these experiments were pre-formed with the same lipid system. The observed differences in aggregation as a function of crowder suggest that htt-exon1(46Q) localization to membrane interfaces is modified by the extent and type of crowding in the aqueous phase above the membrane surface.

3.5 References

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4. Future Work and Concluding Remarks

The work presented in this dissertation was aimed at elucidating interactions of htt with surfaces, based on an electrostatic and lipid interface perspectives. To begin, an experimental system was established using mutations in Nt17 to htt phosphorylation. Once this system was validated, investigations were aimed at understanding the impact of phosphorylation on htt/lipid interactions with a variety of lipid systems. Understanding biophysical impact of phosphomimetic mutations on htt aggregation and lipid membrane activity may provide mechanistic detail regarding htt membrane association linked to toxicity. This was followed by a study aimed at elucidating how a crowded liquid phase influences htt aggregation at interfaces. A variety of macromolecular crowders were used, and each had a profound impact on aggregation at a mica/liquid interfaces and a membrane/liquid interface. The major goal of these studies individually was to understand fundamental aspects of htt aggregation, including charge and membrane interactions, in the presence of cellular and subcellular surfaces. The ultimate overall goal was to identify and expand upon potential targets for preventing mutant huntingtin aggregation; hopefully leading to more available, effective therapeutic and preventative treatments for HD. To further the work completed in this dissertation in context, implications for future studies are discussed.

4.1 Impacts of PIP Lipids and Htt on Membranes

Htt is associated with a variety of distinct membranes within neurons, each comprised of a distinct mix of lipid components. While htt can damage these different membranes, different aggregate species have been associated with specific organelles.

For example, oligomers have been implicated in mitochondrial membrane dysfunction;^{1,2} fibrils deform and damage the ER;³ and inclusion disrupt the nuclear envelope.⁴ Thus, it is important to understand how different lipid species influence htt/membrane interactions and aggregation. Nt17 forms a membrane targeting AH, which facilitates htt-exon1's direct association with a variety of cellular and subcellular membranes via direct contact with phospholipids within the membrane.⁵⁻⁷ For example, mutant htt has increased binding affinity to numerous phospholipids,^{6,7} and mutant htt fragments can directly disrupt phospholipid bilayer integrity.⁷ Htt has displayed a high affinity for a small class of lipids called phosphatidylinositol phosphates (PIPs). Of the seven known PIPs, the low abundance (0.1% of total lipids) multivalent PIPs, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂ and PI(3,4,5)P₂, associate more strongly with htt.⁷⁻⁹ Due to the positive charge associated with the lysine residues in Nt17 which are integral to lipid binding;¹⁰ and the negative charge of PIPs, it is possible that lysines within Nt17 underscore the high affinity of htt for PIP enriched membranes.

Charged phospholipids, such as PIPs, interact with ions via Coulombic forces. Divalent cations and Li⁺ with POPC can change mechanics and shift membranes to a more gel-like state.¹¹ It is then expected that cation induced clustering of PIP with Ca⁺² should rigidify the membrane by diminishing lateral diffusion, reduce leakage, and lessen the damage when htt is bound to the membrane. It is feasible to expect that changing the ionic strength could have an effect on how the membrane interacts with the htt protein. PIP₂ forms clusters *in vitro*.^{12,13} Most models with PIP clustering use higher concentrations of divalent ions and PIP lipids greater than what is naturally present in the plasma membrane.^{12,14,15} To date, the effects of PIP clustering on htt

binding and aggregation are unknown. Calcium clearly plays a vital role in both PIP lipids and htt, but little evidence has been presented at physiological concentrations.

This clustering would have additional consequences in downstream applications of htt, for example, PIP enriched membranes play a key regulatory role in AKT-mediated phosphorylation of htt.⁷⁻⁹ While the interaction of primary PIPs, such as PI(4,5)P₂, with htt has been analyzed in the literature¹⁶, the structural changes resulting from interaction of secondary multivalent PIPs PI(3,4)P₂ and PI(3,4,5)P₃ with htt has not been explored in depth. Most studies focus toward animal models and immune or cytotoxicity evidence that can be related back to the kinases and pathways,^{17,18} with little information on the actual binding event that initiates these processes. A model for htt recruitment to the plasma membrane by PIPs during growth factor signaling shows that the type III kinase uses PI(4)P₂ and PI(4,5)P₂ as substrates to create PI(3,4)P₂ and PI(3,4,5)P₃ at the membrane surface.¹⁶ Experiments with lipid overlay indicate that htt binds multivalent PIPs which is consistent with an electrostatic mode of association.⁸ PIPs provide a high affinity binding site on the inner cytosolic membrane surface for proteins like AKT and htt.⁹ Htt is a substrate for AKT and phosphorylation of htt by AKT is crucial for neuroprotective effects but is altered by mutant htt.¹⁹ Due to PIPs clustering ability with Ca⁺², it appears that mutant htt and AKT could accumulate along with GSKβ to activate cell death pathways with mutant htt.⁷

Figure 4.1: Proposed interaction of Nt17 association with PIP and PIP/Ca⁺² vesicles. The binding of Nt17 to PIP/Ca⁺² clusters on a lipid membrane (left) can expect to have a stronger response than the binding of Nt17 to PIP enhanced lipid membranes (right). This is due to increased electrostatic interactions, however both proposed interactions lead to downstream therapeutic pathways focused on reduced toxicity.

Administration of PIPs or altering PIP kinases as a therapeutic target in animal models have included direct application to the brain, altering embryonic or similar developing cells in animal models¹⁶ or non-physiological conditions,²⁰ which is unrealistic for future treatment of HD. Recent efforts have shown that increasing the levels of PIP decreases the amount of protein aggregation associated with several neurodegenerative diseases,¹⁶ but little is known about the mechanism. Similar to the approach used in Chapter 2, mechanisms underscoring the high affinity of htt for PIP and the potential role of lysine residues can be determined. For example, lysine residues in Nt17 are often acetylated, altering their charge and modifying htt-exon1's ability to associate with lipid membranes. Acetylation mimicking mutations in Nt17 can be employed to investigate their role in binding with membranes enriched with physiologically relevant amounts of PIP. By targeting these lipids, Nt17, and optimizing physiologically similar conditions to study these underlying concepts, clear mechanisms

will be associated with htt aggregation that can potentially lead to the rationale design of strategies to target htt/PIP binding events for therapeutic purposes. Additional significance is providing extended fundamental knowledge of the membrane mediated htt aggregation.

4.2 Exploration of PTM Crosstalk, Charged and Complex Lipid Systems, and Organelles with Htt

As mentioned in Chapter 2, certain PTMs are crucial modulators of htt aggregation and toxicity.^{21,22} The high number of overall PTMs within Nt17 implies that these modifications are essential to the functions of htt, in health, and disease.²³ In particular, Nt17 contains numerous residues for two charge altering PTMs, acetylation and phosphorylation.²⁴ The juxtaposition of the phosphorylation and acetylation PTMs induces crosstalk between the residues, establishing a molecular switch for modifying the dynamics of htt aggregation and function.²³ Clarifying the regulatory molecular mechanisms of these PTMs and the consequences of such modifications, is crucial for elucidating the basis of many htt functions within HD. For example, the acetylation of K6 residues in htt reverse aggregation inhibitory effects of T3 phosphorylation, due to the close proximity and stabilizing role of K6 in Nt17 helix formation.²⁵ However, more detailed studies are necessary to determine the various mechanisms in which PTMs crosstalk, and their downstream effects on htt aggregation and lipid binding *in vivo*.

Figure 4.2: Comparison of phosphorylation and acetylation PTM sites on Nt17. Overhead view of the barrel of the Nt17 α -helix, showing hydrophobicity of each residue (as indicated by legend) and all reported PTMs. The phosphorylation PTM sites (green) of T3, S13 and S16 are in close proximity to the acetylation PTM sites (purple) of K9 and K6. The center line is representative of the membrane of cells, and K6, K15 and S13 are all noticeably closer to the membrane. This suggests that selectively altering these PTMs together should have even stronger effects on lipid binding. Adapted in part with permission from Arndt, J.R. et al. *The Emerging Role of the First 17 Amino Acids of Huntingtin in Huntington's Disease*. Copyright 2015 Biomolecular Concepts.

There are also implications that membrane affinity influenced by phosphorylated htt is sensitized to complex, defect riddled membranes. In further expansion of the ideas outlined above, increased modification of electrostatic interactions using double and triple site phosphomimics and increasing cross talk via combinations of acetylation and phosphorylation PTMs mimics in Nt17, both serve as potential outlets for targeting specific cellular and subcellular membranes of differing composition. Additionally, phosphorylation and acetylation in tandem modify the localization of huntingtin throughout cells.^{26–31} This suggests that including the study of complex membranes found on cellular organelles, such as mitochondria and endoplasmic reticulum (ER), would be suitable future directions to the expansion of this study.

Another hallmark of HD is altered mitochondrial morphology, with various irregularities in dissimilar cell types.³² Modified mitochondrial structures correspond to mitochondrial dysfunction in HD cells, established by numerous factors including decreased electron transport chain activity.^{32,33} In particular, neurons are distinguished by amplified mitochondrial fragmentation.^{34–37} Additionally, ER stress progresses in many neurodegenerative diseases including HD,^{32,38,39} and it is a key influence in the deterioration of cells.^{34,40–42} As misfolded proteins intermingle and aggregate in the cell, toxic species arise that cause ER stress and disrupt normal cellular function.³⁴ With a cytosolic protein such as htt, intrusion of oligomers with ER-associated degradation (ERAD) mechanisms has been elucidated in HD to be an influential process of initiating ER stress.^{43,44} Fundamental concepts elucidated in Chapter 2 could be further applied to see if increasing electrostatic interactions through PTMs leads to protective effects in downstream aggregation in the presence of these complex organelles; or determine if there is increased selective affinity of various PTMs within Nt17 for these membranes.

Other charged, pure lipid systems such as the anionic POPS or DOPG lipids, or mixed model lipid systems could also be studied to fine tune the effects of particular lipids found within the complex membranes of cells. Insertion depths of PTM residues on various membranes could be monitored in an attempt to shed light on particular binding mechanisms that occur, to further elucidate pre-existing findings associated with PTMs, including crosstalk between different PTMs. PTMs likely play a prominent role in htt's normal function and modify mutant htt toxicity. Understanding the complex relation between PTMs, lipid association, aggregation, and toxicity would hopefully lead to novel therapeutic strategies targeting Nt17.

4.3 Liquid/liquid Phase Separations, Stress Granules, and Htt Aggregation

Mechanisms *In Vivo*.

In Chapter 3, macromolecular crowding impacted the formation of htt aggregate species and aggregation at interfaces. In particular, large htt granules were observed under some crowding conditions that morphologically were similar to stress granules (SGs) found in cellular environments. Traditionally, isolation of molecules into organelles is attained by encompassing the organelles with lipid membranes.⁴⁵ Micro-niches within the cell that demonstrate liquid-like qualities, including SGs, nucleoli, germ granules and paraspeckles, have been collectively labelled as biomolecular condensates⁴⁶ or membraneless organelles (MLOs).^{47–49} MLOs are intracellular assemblies that are key to numerous fundamental mechanisms within in cell, including protein transport.^{50,51} Association of MLOs occurs through a process termed liquid-liquid phase separation (LLPS), a metastable de-mixing process of proteins facilitated by temporary multivalent interactions.^{52–56} Several diverse interactions influence LLPS that in turn, promotes formation of explicit MLOs with varying functions, as demonstrated by SGs and countless others.^{46,49,50} Solutions of macromolecules that endure LLPS condense into a dense phase that is similar to liquid droplets, coalescing with a dilute phase.⁵⁷ The fundamental driving force of this process being the switch of macromolecule/water interactions for energetically favorable water/water interactions and macromolecule/macromolecule interactions.⁵⁷ The propensity of a solution to engage in LLPS depends on numerous factors, including concentration and identities of the macromolecules involved, temperature, salt concentration, co-solutes, and volume exclusion.^{47,50,57}

The cytosol in particular contains high levels of disordered proteins that are not entirely neutral.⁵⁸ The formation of macroscopic puncta (or granules/bodies) in the cell from these proteins adds an additional level of complexity.⁴⁷ The cytoplasm and nucleoplasm of a multitude of cells are interspersed with condensates, that are disconnected from the adjacent fluid, which are dynamically established and liquefied over time.^{47,58,59} SGs are particularly relevant to neurodegenerative diseases, as functions associated with SG response have mutations associated with motor neuron diseases.⁴⁵ Therapeutic tactics intended for hindering SG accretion have shown to reduce disease advancement in animal models of neurodegenerative diseases.^{45,60,61}

Figure 4.3: Location of stress granules (SGs). While found in numerous cellular environments, SGs can be found in close proximity to the nucleus of motor neurons. This is significant as *htt* also accumulates in and around the nuclei of neurons, leading to the assumption that SGs interact with and possibly affect *htt* aggregation. Created in part with BioRender.com

Additionally, there is significant overlap among proteins that undergo LLPs and those that aggregate in neurodegenerative diseases.^{50,62–64} Proteins containing intrinsically disordered regions (IDRs) stimulate LLPS yet come at the cost of being highly aggregation prone, suggesting that this overlap might be connected in disease pathogenesis.^{50,62–65} *In vitro* models of purified proteins and synthetic crowding agents

have been explored to determine the effect of crowding on MLOs,⁴⁷ though research is lacking in this area with htt. *In vitro* studies are essential to providing insight to the functions and qualities of MLOs on biomolecular processes,^{46,49,52–54,65–73} though the effect of macromolecular crowding on LLPS is rarely discussed in detail despite them being a common addition in these studies.⁴⁷ Thus, macromolecular crowding influences phase separation in mixtures that would not otherwise demonstrate crowder-mediated LLPS in living cells.⁴⁷

Figure 4.4: Lipid/Lipid phase separation (LLPS) of htt. Normal htt (blue, top left and right) in cellular conditions can undergo a reversible, transitional process in which aggregates (pink, top right) begin to form, but not in enough severity to form fibrillar structures. This dense collection of proteins and macromolecules (represented by green, orange and purple dots) then begin to condense into a liquid-like phase within the surrounding fluid. From here, in cases of expanded polyQ, the liquid phase can progress into a solid-like phase. Alternatively, this normal htt can undergo a similar process, where aggregates form and condense into solid-like structures such as fibrils (red, bottom). In this alternative pathway, the presence of expanded polyQ regions within htt can promote a direct transition to these solid-like structures. Created in part with BioRender.com

Current understanding in the formation of MLOs such as SGs within cells, raises another possible aggregation mechanism for htt, as the aggregation mechanism of htt *in vivo* is still unclear.⁷⁴ Htt-exon1-fluorescent fusion proteins frequently accumulate into micron-sized aggregates, orders of magnitude larger than the deposits frequently analyzed *in vitro*.⁷⁴ Ultrastructural studies allude that these aggregates contain granular and/or fibrillar substructures.^{75–77} Solidification of protein condensates such as SGs has also been associated to other neurodegenerative diseases such as PD and AD, in which they lose their liquid-like properties and begin forming amyloid fibrils.^{78,79} Investigation of the electrostatic interactions which influence multiple factors involved in the thermodynamic mixing of LLPS, could prove insightful to determining the threshold at which some MLOs become too dense and begin to form condensates that affect normal cellular function. In addition to defects, Nt17 has a distinct relationship with the curvature of surfaces.⁸⁰ The presence of multiple complex lipid membranes and the surfaces of MLOs indicates exploring details about the fluidity, composition and curvature of these surface components could prove useful in elucidating *in vivo* mechanisms of htt aggregation. Together, these concepts provide motivation to determine how macromolecular crowding induces LLPS in htt and if that in turn, furthers the severity of aggregation.

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Appendix: Huntingtin Protein Purification and Experimentation with Macromolecular Crowders: An Innovative Undergraduate Biochemistry Experiment

ABSTRACT

Traditional undergraduate biochemical laboratory curricula are confronted with multitudinous challenges in improving student learning outcomes due to limitations with time, increased student enrollment, and high-cost laboratory resources. However, with the advent of cost-effective instrumentation and incorporation of creative approaches to circumvent inflexible undergraduate schedules, it is now possible to overcome these challenges. Thus, we have designed an interdisciplinary, medically relevant laboratory experiment intended to provide undergraduate students with enhanced pedagogical training through guided- and independent-based inquiry. This experiment seeks to mediate limitations common to traditional biochemical experimentation using high-level instrumentation in tandem with graduate-level student engagement.

A1. Introduction

Science, technology, engineering, and mathematic (STEM) educational programs have seen an increase in majors, including subdivisions such as biochemistry, bioinorganic chemistry, bioorganic chemistry, biophysical chemistry, agriculture, and food science.^{1–3} Rigorous coursework and laboratories, along with undergraduate research experiences, are essential for STEM majors; however, not all students can participate in undergraduate research. Thus, students do not obtain the crucial training or engaged learning needed for upper-level educational programs or STEM careers. The need for undergraduate laboratory experiments that provide relevant graduate-level experiences is becoming increasingly necessary. Available

biochemistry laboratories have seen very little graduate-level experimentation because laboratory design tends to follow an expository style format where students acquire very limited and superficial knowledge through passive completion of a “cookbook” protocol.⁴ This is due in part because most undergraduate scientific laboratory experiments have limitations with both equipment and time.^{4–8}

Typical biochemistry laboratory courses consist of a two-semester model where the first semester focuses on acquiring basic biochemical skills, including proper pipetting techniques, preparation of solutions and buffers, and familiarization with basic instrumentation such as electrophoresis, colorimetric ultraviolet-visible spectroscopy and size exclusion chromatography. The second semester centers on higher-level biochemical techniques for the analysis of biological species and processes, including cell growth and protein expression. Emphasis is applied on providing knowledge of biochemical techniques from a practical perspective and developing critical thinking skills to prepare graduates. This model, although established and convenient, is problematic in that the time constraints of most undergraduate biochemistry laboratories allow for unoriginal applications that do not facilitate intellectual growth through critical thinking skills development that is provided via research-based experiences. Thus, these educational labs lack the veracities associated with higher level biochemical employment or education. The idea of straying from a traditional learning format by changing experimentation to a more research-based approach has been well received.^{4,9–11}

Cost effective availability of biological instrumentation has made it possible to add a more advanced component to biochemical laboratory experiments. Refrigerated

centrifuges, microplate reader spectrophotometers, digital incubators, and specialized chromatography columns are now available and could provide valuable experiences when transitioning to graduate level education or industry. For example, protein expression and purification in undergraduate laboratories has been successful with diverse recombinant proteins in *Escherichia Coli* (*E. Coli*). Opportunities for experimental modifications exist within the protein expression and purification processes⁹⁻¹² and have been demonstrated with versatility in chromatography.¹¹ Despite these modifications, without a change in the style of learning, students will not be able to reap the benefits of a graduate level experience.

We have designed an undergraduate biochemistry laboratory experiment involving the huntingtin protein (htt) that incorporates guided and inquiry-based experimentation with emphasis on instrumentation and graduate-level participation. This experiment models other independent graduate level work being conducted but has been adapted to accommodate the inflexible undergraduate timeline. The experiment focuses on immersion into the graduate school environment coupled with the use of advanced instrumentation, which will provide excellent undergraduate learning opportunities that can be expanded and are markedly useful when moving forward in a scientific career. Further, interest amongst the students will persist and will encourage independent research on the topic by using a popular and medically relevant protein, in contrast to traditional enzymatic based biochemistry experiments.^{9,12-14}

A2. Background

The primary genetic cause of several neurodegenerative diseases is the expansion of trinucleotide repeats to a greater number than what occurs in the naturally

occurring stable gene.^{15–17} Trinucleotide repeat disorders (TRDs) result from an abnormal expansion of repetitive codon sequences within a gene and primarily affect the nervous system. A subclassification of TRDs consist of a group of 10 disorders in which the codon CAG, which encodes for the amino acid glutamine (Q), is repeated and results in an expanded polyglutamine (polyQ) tract within the translated protein. This subset of TRDs is commonly referred to as CAG repeat disorders or polyQ diseases. The 10 identified CAG repeat disorders include Huntington's Disease (HD),¹⁸ dentatorubal-pallidoluysian atrophy (DRPLA or Haw River Syndrome),¹⁹ the spinocerebellar ataxias, type 1 (SCA-1), type 2 (SCA-2), type 3 (SCA-3 or Machado-Joseph disease), type 6 (SCA-6), type 7 (SCA-7), type 12 (SCA-12), type 17 (SCA-17)^{20–26} and spinobulbar muscular atrophy (SBMA or Kennedy's disease).²⁷

Generally, expansion of polyQ beyond a critical threshold is required for disease. Onset of symptoms and age are strongly correlated to polyQ length. In HD, polyQ repeat lengths of approximately 35 in htt are considered the critical threshold, with increased severity as the repeat length increases.^{18,28–31} A key hallmark of the aforementioned expanded polyQ diseases is the accumulation of abnormal proteins into proteinaceous deposits. These deposits are predominately comprised of aggregate species termed amyloid that are defined by a fibrillar morphology and a cross β -sheet structure. In particular, polyQ expansion promotes htt's aggregation into fibrils.³² However, multiple aggregation pathways exist, involving a variety of other aggregate species, including oligomers, annular structures, and amorphous aggregates.^{15,32,33} Within the literature, htt oligomers^{34–36} and fibrils^{37,38} have been classified as toxic entities.

The six-week experiment outlined demonstrates the *E. Coli* based expression and purification of a GST-htt-exon1 fusion protein with a disease length polyQ domain. Cleaving the GST induces aggregation that can be tracked kinetically by a fluorescence-based aggregation assay. Students used this assay to determine the impact of variety of factors on htt aggregation. Specifically, to test if mimicking the crowded environment of the cytosol with macromolecular crowders impacted htt aggregation. Emphasis was placed on facilitation of advanced critical thinking when designing the independent experiment, graduate level presentations, and manuscript writing. Collaboration and feedback was provided throughout the weekly experiments, including personalized feedback during preliminary oral presentations.

A3. Overview of Experiment

In this six-week experiment, students completed the weekly laboratory work in a large group, 10-15 students per laboratory session, with a total of 22-30 students completing the experiment each semester. Smaller sub-groups were generated and rotated based on weekly tasks (Table A1), which allowed for participation in all the various components of the experimental procedure.

Similar to graduate level education or STEM careers, students completed both oral and written presentations of their scientific findings. Students were asked to focus on providing a professional presentation that could be delivered at a scientific conference. They were also expected to provide weekly written records along with a final manuscript that replicated the publication style of a peer-reviewed scientific journal; student manuscripts were in the style of the American Chemical Society (ACS) *Biochemistry* journal.

Week 1: Several inoculations of *E. coli* with the vector to express a GST-htt-exon1 fusion protein were generated to demonstrate various stages of protein expression. The starting times of these inoculations were offset to allow students to participate in the entire process, allowing the entire process to fit within a typical three-hour laboratory period.

Students alternate between stations to gain experience with each step of *E. coli* growth and protein expression, including the preparation of growth media and autoclave sterilization techniques. Students set up an overnight inoculation of a strain of *E. Coli* containing the plasmid for the GST-htt fusion protein, prepare fresh media with ampicillin, and continue through the protocol with a previously prepared overnight culture. Protein expression is induced using isopropyl- β -D-thiogalactoside (IPTG) and cell harvesting is demonstrated; however, protein expression and cell harvesting of the offset inoculation was completed outside of the lab period.

Week 2: Alternating stations, students prepare buffers needed for purification, suspend cell pellets, and perform protein extraction by lysing cells with lysozyme and sonication. Using small-scale glutathione (GST) affinity centrifugation columns, each student participates in the purification process. Additionally, assays to measure protein concentration and dialysis techniques are demonstrated.

Week 3: Use of a microplate reader spectrophotometer and plate organization is discussed to prepare students for the following week's experiment (Week 4). Students then participate in an excel workshop that focuses on scientific data analysis, which students will complete independently during Week 4, and include in both their oral presentation and manuscript.

Week 4: Students complete the required aggregation assays without significant guidance from the instructor; minimal assistance is key to ensure students are collaborating and developing metacognition skills. Although the impact of macromolecular crowding on htt aggregation was examined here, there are several experimental adaptations available.

Week 5/6: Students combine hypotheses, testing procedures, observations, results and conclusions into a scientific manuscript that replicates the publication style of a biochemical scientific journal. Students also presented their findings as if at a scientific conference, allowing early opportunities for students to receive positive feedback on how to present scientific information in various media forms when preparing for scientific careers.

Table A1: Devised Solutions to Traditional Experimental Setbacks

Weekly Breakdown	Traditional Setback	Solution
<i>Week 1: Protein Expression in E. coli</i>	Instrument availability, time management	<i>E. coli</i> are staggered at various stages of growth for protein expression. Students are distributed into groups to gain experience with all aspects of these processes.
<i>Week 2: Protein Purification</i>	Instrument availability, time management	Small scale purification methods enable many students to actively participate in protein purification.
<i>Week 3: Excel Workshop</i>	Limited knowledge of Excel, specifically with advanced data analysis	The Excel workshop focuses on scientific data analysis, and its presentation in both oral and manuscript forms. Students utilize mock data with open discussions encouraged.
<i>Week 4: Kinetic Experiment</i>	Lack of independent laboratory investigations that require advanced scientific design and assessment	Students design an experiment testing various conditions based on previously learned techniques, specifically with macromolecular crowders. Minimal supervision is provided as students are responsible for experimental setup and execution.
<i>Weeks 5 & 6: Professional Oral Presentations and Scientific Reporting</i>	Limited professional, data centered public speaking opportunities, significant deficiencies in scientific writing	Students drafted and presented their analyzed data over a two-week period. Emphasis was placed on providing presentations that could be delivered at professional scientific conferences. Weekly notebooks were combined into a scientific manuscript that replicates the publication style of a scientific journal.

A4. Hazards

Bradford reagent, hydrochloric acid (HCl), and sodium hydroxide (NaOH) can cause burns and are corrosive. Sodium phosphates can irritate the nose and throat when inhaled and could result in respiratory difficulties; sodium phosphates can also pass through the skin and irritate the eyes. Glycerol can be harmful if swallowed. Ampicillin may cause allergic reactions. Trizma base, isopropyl- β -D-thiogalactoside (IPTG), ethylenediaminetetraacetic acid (EDTA), yeast and tryptone may cause skin and eye irritation and may be harmful if swallowed or inhaled. Thioflavin T is corrosive and can cause burns, serious eye irritation and targets respiratory

system. Huntington protein and all related waste is classified as Biological Safety Level -1 (BSL-1) and should be treated with a 10% bleach solution and autoclaved prior to disposal.

A5. Results and Discussion

This experiment can be utilized in a biochemistry or biochemistry-related laboratory course where students have been introduced to protein and kinetic topics in previous coursework. The experiment requires four three-hour laboratory sessions with two additional three-hour sessions for oral presentations as outlined above.

Typical student results obtained during the initial three laboratory sessions (Weeks 1-3) included an average absorbance value between 0.500-0.700 at 600 nm for the optical density of the larger batch broth cultures. Following purification during Week 2 of experimentation, students generally obtained approximately 10-12 mL of 0.2-0.4 mg/mL of htt. If the concentration of the protein fractions alone was greater than 20 μ M, neat buffer was used to dilute the sample until the concentration was below 20 μ M. This was to ensure minimal htt loss due to premature aggregation during dialysis and provide a sufficient quantity of diluted htt to generate mock data. During Week 3, students completed an excel data analysis using mock data. In the final week of experimentation (Week 4), students determined the concentration of htt after dialysis with typical concentrations ranging from 2-10 μ M depending on the success of the purification. Students also developed a plate layout for the three macromolecular crowders used, Dextran, Ficoll, and polyethylene glycol (PEG). A final concentration of each macromolecular crowder at either 100 mg/mL or 50 mg/mL in 100 μ L (the volume in each well) was achieved while still accounting for the final target concentration of htt (10

μM). Crowder volumes for these target concentrations were determined from a dilution scheme by the students using previously prepared stocks.

A Thioflavin T (ThT) assay was used to assess fibril formation of htt incubated with the three macromolecular crowders. Students were assigned final concentrations of either 100 mg/ml or 50 mg/ml of the macromolecular crowders with the assay being completed in triplicate. Figure A1 demonstrates student obtained ThT assay data of htt in the presence and absence of crowders. Students assessed trends in fibril formation by the location of each signal in relation to htt alone. A signal higher than the htt control (no crowders) indicated enhancement of fibril formation. Polyethylene glycol (PEG) at both concentrations produced a diminished signal relative to control, indicating inhibition of fibril formation.

Figure A1: Representation of student obtained data during experiment. Student data with 10 μM htt in the presence and absence of Ficoll, Dextran and PEG at (A) 50 mg/mL and (C) 100 mg/mL. Quantification of the maximum ThT fluorescent signal observed for Ficoll, Dextran and PEG at (B) 50 mg/mL and (D) 100 mg/mL with respect to htt alone is present. Error bars represent SEM with * corresponding to $p < 0.01$ and ** corresponds to $p < 0.05$.

In addition to traditional weekly laboratory notebooks, students were assessed by the oral presentations and final manuscripts. For the laboratory notebooks, sections such as the introduction to the experiment, experimental objectives, materials and methods, results and discussion, conclusion and laboratory questions were requested

each week. The laboratory questions as well as the experiment objectives section in the written pre-laboratory assignment were key pieces for evaluating basic knowledge of the pedagogical goals prior to completing the weekly experiments. Post-laboratory results, discussion, and conclusion sections judged the level the students understood the weekly experimental goals, identifying what information would be relevant to continue for the following week's task, and demonstrated if students were thinking critically about what to include for their manuscript. Students scored exceptionally well for the laboratory notebooks, with an average grade of 91%. High scores are indicative that the pedagogical goals of each week were effectively communicated and achieved.

The oral presentations following the completion of week #4 were used as a measure of assessment for the combination of the student's overall understanding of the experiment, professional speaking, and communication skills. Prior to the presentations, students were encouraged to follow guidelines with regards to the background information, experimental procedures, results, and conclusions. It was stressed that focus should be placed on the results, what they mean and why they were significant to the broader scientific community. Current literature and proper identification of the overall goal were also critically evaluated. Proper referencing, slide aesthetics and knowledge during the question sessions from peers and instructors were also provided as guidelines and judged to help create an immersive, conference-style setting. Again, students scored well for the oral presentations, with an average grade of 84%. For most of the students, this style of evaluation and experience was their first opportunity within the program, adding a much-needed component for an upper level undergraduate course.

Upon completion of the oral presentations, students submit a manuscript of their results written in *Biochemistry* format. A template for the manuscript is provided, and students are expected to use the information from their laboratory notebooks and feedback from their presentations. Students were presented with a list of appropriate sections, suggestions of what to include in each section, and were encouraged to read literature in similar areas of study and format. Deeper understanding of the ThT results, htt aggregation and fibril structure, and relation of the experiment to real world situations were expected to be elucidated. Determining if results were significant through statistics and areas for improvement were also emphasized strongly. The average grade for the manuscripts was 77%, again suggestive that students were retaining the knowledge learned and effectively using critical thinking skills to structure the manuscript. This was the first experience with writing a publication-type document for students within the major, so a learning curve was to be expected.

Overall, student evaluations revealed that 79% of students enjoyed the experiment. Students responses also indicated increased satisfaction with the hands-on nature of the experiment, use of multiple lab spaces, newly gained knowledge of the preparation required for larger-scale experimentation, and group collaboration. Students also indicated the significant usefulness of learning upper-level techniques and instrumentation, including spin GST-affinity based column chromatography, and LC GST-affinity based column chromatography, large scale recombinant protein expression and cell lysis. Specific student comments encompassing the entirety of the experiment included “we were able to take part in all the steps without using multiple lab days”, “the excel lab taught functions that were useful for data analysis and helpful for future

classes”, “that we actually learned how to generate complicated graphs we see in papers” and “we finally had a coursework lab that incorporates techniques used in real graduate level labs. It gave the lab group a sense of autonomy and responsibility in dividing and conquering work.”

Conversation and positive encouragement were fostered throughout each week of the experiment as students encounter common experimental issues or struggle to envision the experimental goals. This is especially critical for students who have had no exposure to undergraduate research or have been conditioned for traditional “cookbook” protocols that are expected to work with little error. Additionally, early communication between student and instructor helps to bridge the guided laboratory structure to the desired independent inquiry-based format. Through these activities’ students gained important knowledge in data analysis and the use of sophisticated scientific instrumentation, acquired insight into upper-level biochemical experimentation, and have a better understanding of real-world issues currently faced in biochemistry and biochemistry related fields.

A6. Summary

When compared to traditional biochemical experiments involving protein expression and purification, this experiment allowed for an atypical, interesting, and impactful protein to impart some new life into a curriculum that is steadily becoming stale and less relevant for students seeking STEM careers. Additionally, these modified protein expression, purification, and experimental protocols successfully demonstrated their innovative use by circumventing the time-constraints typically plaguing an undergraduate laboratory course along with allowing for cost effective use of equipment

and materials. Experimental focus on improving communication and critical thinking skills are both useful and necessary for career paths in STEM. Students also have a better understanding of a biologically relevant protein and its role in HD, along with how the efficacy of potential treatments are evaluated in disease prevention.

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A8. Brief Overview of Additional Information for Instructors

The material following this manuscript is a collection of the key components of the Supporting Information document. Grading rubrics, questionnaires, student manuals, equipment lists, CAS registries and protocols were not included for conciseness. Sections included in the lab summary and instructor notes contain important details to aid in the execution of the new lab, including adaptations and troubleshooting tips collected in several semesters of teaching this new laboratory exercise here at West Virginia University for biochemistry undergraduate students.

A8.1 Lab Summary for Instructors

Huntington disease (HD) is a fatal neurodegenerative disorder characterized by psychiatric symptoms, cognitive degeneration and motor instabilities.¹ A mutation in the huntingtin gene caused by a CAG trinucleotide repeat results in a polyglutamine (polyQ) expansion in the translated huntingtin protein (htt). This polyQ expansion spurs htt aggregation into fibrils and ultimately into intracellular proteinaceous inclusions.^{2, 3} Disease onset is directly correlated with polyQ length where 35-39 repeats may result in disease, 40-60 repeats result in adult onset, and repeat lengths greater than 60 are observed in juvenile disease onset.² Further, there is increasing evidence that htt aggregation may be associated with disease progression and severity, suggesting a larger need for research exploring aggregate formation.^{2, 3}

Novel strategies that directly target the HD gene and prevent production of the toxic mutant htt are being investigated. One current strategy involves an antisense oligonucleotides (ASO)-based RNase activation system via spinal injection to HD patients.⁴ While successful, HD progresses slowly, and this treatment becomes expensive if prolonged. Additionally, protein misfolding and abnormal htt can interfere with several cellular processes, including cell trafficking.^{1,2} This presents several challenges not only for obtaining cost-effective treatments but in understanding the complete mechanism of the disease progression.⁵ Investigation into physiologically relevant or less invasive therapeutic drug targets exploring htt folding remains a necessary and interesting topic of investigation.

As an undergraduate student, the independent use of advanced instrumentation is not always a possibility due to budgetary limitations and the restrictions caused by a scheduled laboratory period, which greatly limits individualistic exploration and critical

thinking. These limitations can be modified if the student participates in undergraduate research; however, this is not always a possibility for all students. Thus, we have developed a laboratory experiment where it is now possible to expand protein growth and purification methods to additional proteins that have otherwise been underutilized in an undergraduate biochemical setting through modifications made in the laboratory timeline. Specifically, students will complete experimental preparative methods (*E. Coli* growth, protein purification, etc.) that mirror advanced protocols completed in biochemical research through guided-inquiry and then complete independent experimentation with the htt protein quantifying amyloid formation and prevention using macromolecular crowders. These technical experiences are relevant in a broader, real world context. For example, high throughput assays in plate format are a common method of screening for potential therapeutics, which relies on many of the design principles and data analysis employed in this laboratory experiment.

Time and curriculum level

Students should perform the experiment in an advanced laboratory course after they have been introduced to basic biochemistry techniques and lab practices; skills and topics relating to protein expression and purification, kinetics, instrumentation, and critical thinking will be utilized. The laboratory experiment combines aspects of biochemistry and biophysical chemistry, and therefore can be utilized for several topics of discussion in biochemistry or physical chemistry courses. Completion of this laboratory experiment should occur during lectures that include topics of protein expression, protein structure/folding, spectroscopic analytical procedures, and kinetics. In total, this experiment requires six, three-hour laboratory sessions. Students work in

larger groups during experimentation for four, three-hour sessions, and two, three-hour laboratory sessions for oral and written scientific presentations with emphasis on developing advanced written and oral communication skills required for upper-level education and STEM careers.

Procedures, techniques, and concepts presented

During this experiment, students will utilize advanced laboratory techniques to determine the effects of macromolecular crowders on htt aggregation kinetics. After preparing solutions, such as lysogeny broth (LB) and buffers, students express htt-exon1 containing an aggregation prone polyQ domain as a GST-fusion protein in *Escherichia Coli* (*E. coli*). Once optimum density of *E. coli* is confirmed in the large batch inoculations through optical density measurements, protein expression is induced via isopropyl- β -D-thiogalactoside (IPTG). The cells are then washed and harvested as pellets via centrifugation. For purification, these pellets are re-suspended in buffer and lysed by treatment with lysozyme followed by probe sonication. Cellular debris is pelleted and discarded. The cell lysate, which contains the fusion protein, is diluted with buffer and loaded onto a prepared glutathione-S-transferase (GST)-based affinity spin column for purification via centrifugation. Upon completion, protein concentration is preliminarily determined by a Bradford assay, using a standard curve generated with bovine serum albumin (BSA) standards. The purified protein is dialyzed for 48 hours at 4°C, with two dialysis buffer exchanges. The concentration of the dialyzed protein is determined using the Bradford assay; however, the purified protein usually needs to be concentrated via centrifugal concentrators prior to use in aggregation assays.

Students will also prepare thioflavin T (ThT) stocks and several macromolecular crowder solutions for testing; they will determine the amount of protein per well that is needed for analysis in addition to calculating the appropriate concentrations of thioflavin T and crowders. Students then plan and prepare their samples in triplicate using a 96-well plate and complete kinetic testing via ThT fluorescence using a microplate reader. Once completed, students analyze and present their finding to their peers.

Performing this laboratory experiment provides students with an opportunity to utilize important upper level laboratory techniques such as protein expression and purification, affinity-based column chromatography, and fluorescence-based assays. These experiments enable the student to conceptualize how a protein such as htt functions and is analyzed. Students also recognize how simple spectroscopic techniques can be efficiently used to monitor the progress of purification and determine an unknown concentration of a protein. Students can compare htt aggregation in the presence of macromolecular crowders, providing first-hand experimental knowledge of the potential impact of the intracellular crowded environment on htt. This laboratory lies at the interface between biology and physical chemistry, an important interdisciplinary field to which many students are not exposed, particularly in a laboratory setting. Additionally, students become familiar with useful data analysis and presentation techniques that will be advantageous in advanced STEM educational programs and careers.

Usefulness of experiment

This laboratory will be useful for illustrating principles of biochemistry and biophysical chemistry, including protein expression, purification, and aggregation.

Protein purification and aggregation is monitored through analytical techniques, including spectroscopic and fluorescence-based assays. The hands-on experience that this laboratory experiment provides conceptualization of how these simple techniques can be applied to understanding relevant, biochemical phenomena, e.g. toxic protein aggregation. Additionally, students gain experience using a microplate reader to kinetically monitor aggregation and investigate how molecularly crowded environments that mimic the cytosol can influence this process. Further, using a disease-relevant protein such as htt provides real world relevance and a greater appreciation of amyloid diseases. Students gain a greater understanding of the complete, fundamental process of protein expression and purification followed by application of the obtained protein in an assay that mimics exploratory laboratory research that one would encounter in industry or in academic research labs.

Summary of student evaluations

Student evaluations were completed following each week of experimentation with 50 student participants spanning two semesters. From the results of the evaluations, most students did not have extensive experience working in a research laboratory nor did most complete anything like the weekly experimentation in previous coursework or research experiences.

Common responses to both protein expression (Week #1) and protein purification (Week #2) experimentations included “the experiment included components never done before in other graded labs like stock preparation” and “this lab was good to learn about lab prep work and what actually goes into prepping for experiments as a whole”.

Throughout the protein purification process (Week # 2), students valued the use of

“multiple higher-level instruments” and techniques for completing the experiment and “learning multiple things in a lab period that were touched upon in lecture or in the past coursework”. Students also reported enjoying group work, which “allowed open communication and collaboration” as the experiment was being completed. One comment indicated that it was interesting to learn a biochemical approach to column chromatography when students are usually only exposed to a version of it in organic chemistry.

The Excel workshop (Week #3) reported an average of 76% of students who valued the learning experience, and an average of 95% of students suggested it should be continued in future semesters. Students enjoyed the “scientific approach to excel”, including the ways to “create data plots that resemble those seen in literature” and appreciated tips on how to simplify data analysis.

Week #4 of experimentation, which consisted of independent experimental design and implementation, showed students overall enjoying the increased hands-on working opportunities; students also valued the heightened anticipation of completing their designed experiment and seeing how the individual weekly laboratories were applied to that final design. Student data indicated an average of 82% of students enjoying the six-week long experiment overall as well as an average of approximately 80% of students enjoying each week individually.

A8.2 Instructor Notes

Pre-laboratory assignment

It is recommended that students complete Experiment # 0 prior to the first three-hour laboratory session. Completing this experiment is optional; however, it is suggested for students to practice making biological solutions that are not conventionally generated in a typical undergraduate laboratory course. Further, completing this experiment will reduce laboratory time, ensuring completion of the htt laboratory experiment, and provide students with an obvious example of how preparation is essential in biochemical research.

Alternative experiments

This laboratory experiment is advantageous in that it can be readily adapted to investigate other relevant intracellular components. For example, using the ThT assay, students could explore the use of other physiologically relevant macromolecular crowder molecules or small molecules, such as epigallocatechin gallate (EGCG), curcumin and rizuole. Potential therapeutic drug screenings could also be completed where students could compare and differentiate their findings on different drug targets. Another simplistic laboratory assay, such as polydiacetylene assay (PDA), could be used to study the effects of crowders, small molecules or potential drugs on htt binding to lipids. This assay may possibly be expanded further by exploring different lipid systems or incorporating lipids into the ThT assay. Depending on instrumentation and skill level of students, variation of instrumentation in the final experiment can occur. Circular dichroism (CD), atomic force microscopy (AFM), solid state nuclear magnetic resonance (ssNMR), and scanning electron microscope (SEM) are all examples that can be used to modify the experiment. For example, AFM can be used to study the morphologies of various aggregates produced and can complement kinetic assays

using similar experimental conditions. Other techniques including immunohistology experiments (Western blots, slot and dot blots) can also be used to identify different protein aggregates.

Laboratory preparation

Students can work in large groups with individual students taking turns completing tasks at various stages throughout each week of experimentation. Students should be provided with appropriate equipment and chemicals.

Solution preparation

Solution preparation and some procedural steps will need to be completed outside of the scheduled laboratory period by the instructor or teaching assistant. Several salt, bleach and buffer solutions will need to be prepared as described. During Week #1, *E. coli*. large batch inoculations from the overnight culture should be started and close to the appropriate optical density (OD) prior to the start of the first laboratory session. Depending on the polyQ repeat length used, the incubation period will need to be started 24 hours in advance with the expression step occurring between two and four hours prior to the start of the lab session. In Week #2, the protein should be dialyzed for 48 hours with aliquots of dialysis buffer being replaced twice. Note: The protein prepared by students will be useable for up to 10 days, however, ideal use would be within 5-7 days.

Multiple htt samples can be combined to generate mock data for use in Week #3 experimentation. Additionally, a large quantity of protein should be purified three days prior to Week #4 laboratory session to ensure freshness and prevent seeding. If

preferred, a larger sample of protein could be purified using a liquid chromatography (LC) system or multiple purifications could be completed and combined using the spin column purification method as described in Week #2.

Instrumental instructions

Prior to starting each three-hour laboratory session, instructors should turn on the refrigerated centrifuge and plate reader one to two hours prior to the experiment to ensure appropriate temperature equilibration. UV-Vis spectrophotometer, sonicator, and bath sonicator can all be turned on during the experiment as they do not have lengthy start up times. Additional details are outlined in the experimental procedures each week.

Tips for success

Notes are provided to students to assist in proper experimental completion; however, some additional areas where difficulties may occur should be announced to students. Starting with the first week, encourage students to think of how the instrumentation and weekly experiment applies to their overall experimental objectives. Participation by each student should be monitored during the laboratory sessions, as students may not be self-motivated, interested in teamwork, or have no experience in gradate-related research labs. Encouragement and deeper conceptual inquiries with the instructor are encouraged, as these interactions are more representative of a research setting and helps to promote the desired self-guided approach; opportunities to discuss the written laboratory reports and oral presentations prior to submission and in-class presentations are also suggested.

Troubleshooting

Special care should be taken with some experimental protocols. For example, the probe sonicator has a very delicate tip that is easily damaged. Students should be encouraged to handle the probe sonicator gently and monitor the protein consistency for any necessary adjustments in probe wattage. Additionally, if the concentration results in a lower yield than expected, procedural modifications can be made by reducing the number of wells to two instead of three or reducing the protein concentration per well. This ensures useful data will be achieved and demonstrates the unpredictability of scientific research.

Adapting This Experiment

Htt is useful at demonstrating additional biochemistry topics, such as protein structure and aggregation mechanisms, depending on instrumentation and desired difficulty level. If htt is not available, the protein could easily be substituted with β -amyloid or α -synuclein peptides. These peptides can be ordered commercially but would not require purification, which would eliminate cell growth and purification from the experimental procedure. However, this change could allow for the introduction of other experimental methods and instrumentation involved in peptide preparation, including lyophilizers and vacuum pumps.

Students could prepare the stock solutions required for the experiment, including 1 M Na_2PO_4 , 1 M NaH_2PO_4 , 5 M NaCl, 1 M Tris (pH 7) and a 10% bleach solution; this would accompany in-lab preparation of purification solutions such as dialysis buffer, buffer A, buffer B and aggregation buffer.

Students should be cognizant of experimental procedures completed outside of the scheduled laboratory period, such as dialysis or expression steps. If available, students can purify larger quantities of the protein using a low-pressure liquid chromatography (LC) system, which would provide protein for multiple experimental comparisons on one microfluorescent plate. Comparisons of similar or different targets could be completed with students generating a scientific case for why their target should be explored further.

Calculations could be removed or added to modify the difficulty level during each week of experimentation. Additionally, a more in-depth literature search of htt along with a chosen assay could be conducted.

A8.3 References

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