Introduction. Transactive response DNA-binding protein of 43 kDa (TDP-43) is a ubiquitous, well conserved protein belonging to the ribonuclear binding protein family.[1] It is involved in diverse, essential cellular functions including RNA transport and alternative splicing. TDP-43 is also the primary component of cytoplasmic aggregates that are the hallmark of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease with very limited treatments and an average survival-after-diagnosis of 2-3 years.[2] Some of these aggregates are amyloid—aggregates characterized by a fibrillar morphology and β-sheet-rich structure as well as a prion-like ability to propagate the amyloid structure.[3] Amyloid proteins are closely linked to
human disease, including many neurodegenerative diseases like Alzheimer's and Parkinson's. One role TDP-43 performs in the cytoplasm is protecting RNA during cellular stress by forming membraneless organelles known as stress granules (SGs).[4] SGs are concentrated droplets of RNA and RNA-binding proteins that are formed *via* liquid-liquid phase-separation (LLPS), the spontaneous de mixing of a solution into a metastable concentrated droplet phase and a dilute phase driven by transient, multivalent interactions. Phase-separated membraneless organelles have recently been discovered to play critical roles throughout the cell, but the complete composition and internal protein conformations of these droplets are not fully understood.[5] Long-lived SGs (the result of prolonged cellular stress) have also been shown to lose fluidity and become proteinaceous aggregates, suggesting a connection between protein aggregation and phase-separation.[4] A link between LLPS and amyloid aggregation is likely, as many amyloidogenic proteins can phase-separate without the need of other proteins or RNA.[6] In fact, it has been shown that LLPS droplets made from the C-terminal domain (CTD) of TDP-43 act as an intermediate for amyloid aggregation in some conditions (Figure 1).[7]

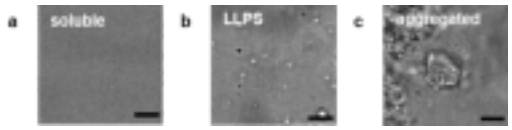


Figure 1. TDP-43 states. $TDP\text{-}43_{CTD}$ in (a) soluble (b) phase-separated, and (c) aggregated states as viewed by brightfield confocal microscopy. Scale bars are 10 μm.

The study of protein conformation inside any LLPS droplet has been very limited. Better understanding of the basic composition of droplets (i.e. water content) as well as the secondary structure of protein in LLPS droplets will help characterize how it may serve as an aggregation intermediate. Investigating whether either of these factors change with droplet age will add further to the story. For example, is droplet aging and accompanying loss of droplet fluidity due to changes in protein conformation, water exclusion, or another factor? Does secondary structure of constituent proteins change as droplets age, or do proteins remain disordered throughout droplet lifetime? Do aggregates formed *via* a phase-separation intermediate differ substantially from those formed in non-phase-separating conditions? Being able to address these questions will help 1) better understand LLPS and how it carries out its many cellular roles, and 2) identify possible drug targets if TDP-43 LLPS proves to be linked to pathological amyloid aggregation. This work aims to understand secondary structure of TDP-43 in phase-separated droplets and track changes that may occur in droplet hydration and protein conformation during the transition from LLPS to aggregate.

Methods and Experimental Design. I propose to utilize Raman micro-spectroscopy—a sensitive type of vibrational spectroscopy which utilizes Raman scattering—coupled to a microscope, which allows for spatial resolution (Figure 2a).[8] Water scatters strongly in the 3000–3600 cm^{-1} range, allowing for an approximation of hydration in droplets based on the intensity of the water bands versus a standard. The Raman fingerprint region (1000–2000 cm^{-1}) informs on the secondary structure of the protein (Figure 2b). Specifically, the formation of β-sheet-rich structures upon amyloid aggregation results in a characteristic

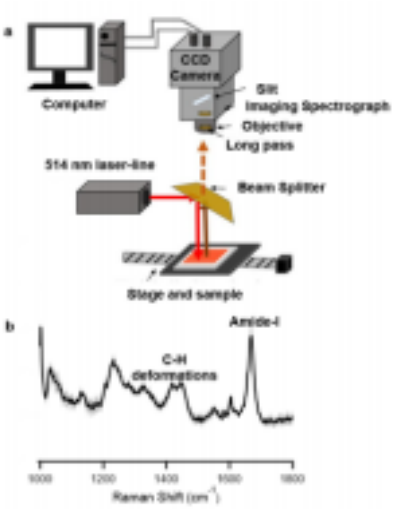
peak at ~1665 cm^{-1}.[9] Sidechain packing can be analyzed *via* C-H deformation modes at 1300-1500 cm^{-1}.[9] One of the strengths of Raman spectroscopy is that a single droplet can be observed over time with Raman spectra taken at multiple time points. Raman data from Murthy *et al.* of another phase-separated amyloid protein similar to TDP-43 suggests that protein within the droplets resembles soluble protein at early time points, but it is unknown if this structure is sustainable or if it naturally proceeds to a more amyloid-like secondary structure.[10] By following the lifetime of TDP-43 droplets, I can determine changes in hydration and protein structure as the droplets age and lose fluidity, filling the gaps left by Murthy *et al.* and expanding the work to a protein more relevant to ALS pathology.

Aim One: Collect Raman spectra inside of TDP-43 LLPS droplets

$TDP\text{-}43_{CTD}$ will be used to prototype the experiment because it reliably forms β-sheet-rich amyloid, and I have previously characterized its aggregation kinetics in a variety of conditions. $TDP\ 43_{CTD}$ can phase-separate on its own and has been reported to drive aggregation of the full-length protein.[11] After proof-of-concept, full-length TDP-43 will be expressed and purified. The protein will be placed in phase-separating solution conditions (high salt, neutral pH), and phase-separation will be confirmed *via* brightfield microscopy (Figure 1b). I will create a catalog of Raman data from each time point giving a detailed view of the structural changes during droplet solidification. I hypothesize that: 1) the conformation of TDP-43 will differ between its soluble, LLPS, and aggregated forms, 2) water content in droplets will decrease as droplets age and water becomes excluded from the stacked β-sheets formed by proteins in the amyloid conformation, and 3) as the phase-separated droplet ages, the secondary structure band around 1600-1700 cm^{-1} will narrow and sharpen, indicating a transition to β-sheet that mimics amyloid aggregates. Taken together, this data would demonstrate that LLPS can act as a mechanistic intermediate during TDP-43 amyloid formation.

Aim Two: Characterize Differences between Aggregates

Figure 2. Raman spectroscopy



(a) Diagram illustrating the set-up of a Raman microscope, adapted from [12] (b) Representative Raman spectra of $TDP\text{-}43_{CTD}$ fibrils with evident C-H deformations an amide I band reporting on β-sheet content.

TDP-43 can aggregate even when phase-separation is not present. I will use Raman spectroscopy to analyze the secondary structure of aggregates formed with and without the phase-separation. This will reveal any polymorphism in structure as Raman spectra are sensitive to not just secondary structure, but also side chain packing as seen in the C-H deformation bands. I will also use Raman to examine samples propagated from the brains of ALS patients (patient samples are used to 'seed' recombinant protein and, due to the prion-like nature of TDP-43, structure is preserved). By comparing the Raman spectrum of the *in vitro* phase-separated and non-phase-separated aggregates to the spectra of the patient-propagated samples, insight into whether disease-related aggregation stems from a phase-separated intermediate will be gained. **Resources and Suitability.** I have significant experience using Raman micro-spectroscopy in Dr. Jennifer Lee's lab at the NIH. Paired with my prior work with TDP-43 and strong record of independence and publication, this makes me uniquely well-suited to pursue this project. The work will be conducted utilizing

the Raman micro-spectrometer at the University of Wisconsin Centers for Nanoscale Technology.

Intellectual Merit. By characterizing the general hydration and composition of LLPS droplets, this project will provide foundational information on LLPS, a process of interest to fields ranging from polymer chemistry to cell biology. Additionally, if LLPS are shown to be intermediates in amyloid aggregation, this opens new doorways for drug development targeting the proteins in droplets.

Broader Impacts. This project illustrates the value of an interdisciplinary approach in studying human health and disease processes. Specifically, it demonstrates how physical chemistry methods—the most micro scale—can offer foundational, useful information on emergent processes in complex biological systems. I hope that demonstrating the utility of physical chemistry, which can often feel hopelessly far removed and theoretical for students, will pique the interest of the next generation of chemists as I work with them as a teaching assistant and research mentor.

References. [1] Y. Sun, *Biochemistry* **2017**. [2] M. Neumann, *Science* **2006**. [3] J. L. Robinson, *Acta Neuropathol* **2013**. [4] C. M. Dewey, *Brain Res* **2012**. [5] S. Boeynaems, *Trends Cell Biol* **2018**. [6] S. Elbaum-Garfinkle, *J Biol Chem* **2019**. [7] W. M. Babinchak, *J Biol Chem* **2019**. [8] R. R. Jones, *Nanoscale Res Lett* **2019**. [9] Z. Movasaghi, *Appl Spectrosc Rev* **2007**. [10] A. C. Murthy, *Nat Struct Mol Biol* **2019**. [11] A. E. Conicella, *Structure* **2016**. [12] S. Lohumi, *Appl Sc.* **2018**.