Classification of Higher Order Assemblies of FUS and Their Role in Amyotrophic Lateral Sclerosis

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Abstract

Mutations in the nuclear RNA-binding protein Fused in Sarcoma (FUS) gene are responsible for 5% of inherited ALS and 1% of spontaneously acquired ALS. FUS proteins contain an intrinsically disordered low complexity (LC) domain. This domain allows the protein to reversibly self-assemble, undergoing phase transition into condensates. This FUS assembly occurs in an RNA-dependent manner, showing little RNA specificity. These assemblies can then bind the CTD of RNA polymerase II and affect transcription. Mutations within the LC domain of FUS can lead to altered assembly formation. It can also cause protein mislocalization to the cytoplasm. We performed experiments to understand more about both mutagenic and wild type FUS, as there is still much that is unknown. We created mutant primers to establish a library of recombinant FUS plasmids. Each of these primers targeted a tyrosine residue in the LC domain. We also used dynamic light scattering (DLS) to investigate the size and structure of monomeric full length, wild-type FUS. Finally, we performed DLS assays on the FUS to induce assembly formation. These assays allowed for an investigation of both FUS-FUS and FUS-RNA interactions in vitro.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that kills motor neurons which are responsible for voluntary muscular functions such as chewing, talking, and breathing. Symptoms of ALS don't typically show until an average age of 55 and most people survive only 3-5 years after diagnosis. There is currently no cure or reversal treatment for the disease. Mutations in the nuclear RNA-binding protein Fused in Sarcoma (FUS) gene are responsible for 5% of inherited ALS and 1% of spontaneously acquired ALS. FUS proteins contain an intrinsically disordered low complexity (LC) domain. This domain is an SYQG-rich region which is largely disordered, which allows it to exist in many different conformational states. It also allows the protein to reversibly self-assemble, undergoing phase transition into condensates[1,4,5,8,10]. This FUS assembly occurs in an RNA-dependent manner, showing little RNA specificity[3,9,12]. These assemblies can then bind the CTD of RNA polymerase II and affect transcription[6,9]. Mutations within the LC domain of FUS can lead to altered assembly formation[4,8]. It can also cause protein mislocalization to the cytoplasm[2,7,11]. We performed experiments to understand more about both mutagenic and wild type FUS, as there is still much that is unknown. We created mutant primers to establish a library of recombinant FUS plasmids. Each of these primers targeted a tyrosine residue in the LC domain. We also used dynamic light scattering (DLS) to investigate the size and structure of monomeric full length, wild-type FUS. Finally, we performed DLS assays on the FUS to induce assembly formation. These assays allowed for an investigation of both FUS-FUS and FUS-RNA interactions in vitro. It is important to investigate the behavior of wild type and mutagenic FUS monomer and assemblies, so we can find the link between FUS and neurodegeneration in patients with ALS.

Methods and Materials

Mutagenesis Primer design

The mCherry FUS plasmid sequence was downloaded and all the tyrosine residue sequences in the low complexity (LC) domain were marked. From those, tyrosine codons that were flanked by 11-13 bases on either side, had a GC content from 40-60%, and had a melting temperature below 65°C as indicated by OligoAnalyzer were chosen. A single base in each codon was changed so that each would code for a serine instead of a tyrosine. Primers were then ordered from Eton Bioscience.

Site-Directed Mutagenesis

Site-directed mutagenesis was performed using Q5 Site-Directed Mutagenesis Kit (E0554). First, 12.5 µL Q5 Hot Start High-Fidelity 2X Master Mix, 1.25 µL of 10 µM forward primer (segment of sequences shown in Figure 1), 1.25 µL of 10 µM reverse primer (complement of sequences shown in Figure 1), 1 µL of mCherry FUS template DNA, and 9.0 µL of nuclease-free water were mixed in a thin-walled PCR tubes and transferred into the thermocycler. The thermocycler conditions were set to an initial denaturation at 98°C for 30 seconds, then 25 cycles of 10 seconds at 98°C, 10 seconds at 60°C, and 3.5 mins at 72°C, followed by a final extension for 2 minutes at 72°C. 1 µL of each sample was then mixed with and incubated at room temperature with 5 µL 2X KLD Reaction Buffer, 1 µL 10X KLD Enzyme Mix, and 3 µL nuclease-free water. Samples were then transformed using bacterial transformation in NEB 5-alpha Competent E. coli cells.

Bacterial transformation in E. coli

NEB 5-alpha Competent E. coli cells were thawed on ice. They were then gently mixed and 50 µL of cells were pipetted into a transformation tube on ice. 1-3 µL of 1 pg-100 ng of mCherry FUS plasmid DNA was added to the cell mixture. The tube was then carefully flicked 4-5 times to mix the cells and DNA. The mixture was then placed on ice for 30 minutes. It was then heat shocked at 42°C for 30 seconds. It was then placed on ice for 5 minutes. Then 950 µL of room temperature LB broth was added to the mixture. The mixture was incubated at 37°C for 60 minutes with 250 rpm shaking. 250 µL of culture was then spread on an agar + amp plate and incubated overnight at 37°C.

Protein expression using IPTG

A single colony from the transformation described above was picked with a sterile pipette and put into a culture tube with 3 mL LB broth + amp. The tube was then incubated overnight at 37°C and 250 rpm. The starter culture was then added to a larger baffle flask with either 250 mL, 1 L, or 1.5 L LB and incubated at 37°C and 250 rpm until at an O.D. between 0.6 and 0.8 (measured by UV-vis). 1mM IPTG was then added to the large culture and it was incubated overnight at 17°C and 250 rpm. The large culture was then spun down for 15 minutes at 4°C and 4000 g and the supernatant was removed, and the pellet was flash frozen with liquid nitrogen and stored at -80°C.

Protein purification

Frozen pellet from induction was taken and added to 10-20x pellet volume of lysis buffer (1% NP40, 1.5mM b-mercaptoethanol, and FUS Buffer (1M urea, 1M KCl, 50mM Tris pH 8.0, 10mM imidazole, 5% glycerol)). The following steps were performed on ice as much as possible. The pellet + lysis buffer was then stirred to homogeneity and the cells were lysed using a French press at 1000 psi. The lysate was centrifuged at 16000 g at 4°C for 20 minutes. 2-5 mL of nickel beads were equilibrated to FUS Buffer by being suspended in the buffer, mixed, and centrifuged at 600 g for 2 minutes. The supernatant was then removed. Repeated 3 times. The supernatant from the centrifuged lysate was then added to the equilibrated beads and incubated at 4°C for 1-2 hours with gentle rocking (beads appear white when bound to FUS). The lysate supernatant + beads were then centrifuged at 600 g at 4°C for 2 minutes and the new supernatant was removed and discarded. The beads were then gently washed with FUS Buffer 4 times with the same technique used to equilibrate the beads. 2 mL of elution buffer (10 mL PBS or FUS Buffer, 250 mM imidazole, 1.5 mM b-mercaptoethanol, urea ranging from 0-4 M, and adjust pH 7.4) was added to the nickel beads and allowed to sit until the beads turned blue again. The protein was then eluted from a 15 mL econo-pac chromatography column. The eluted protein was then immediately flash frozen and stored at -80°C or centrifuged for 30 minutes at 12,000 g at 4°C with a 0.1-micron Ultrafree centrifugal filter tube before performing DLS.

SDS-PAGE

Samples were taken at each step in the purification process. 25 µL of each sample was mixed with 10 µL of 4X sample buffer. Each sample was then pipetted up and down to mix and was heated at 95°C for 3-5 minutes. 20 µL of each sample was then added to an individual well of a 7.5% SDS-PAGE gel surrounded by 1X running buffer. The gel was then run at 150 V for 40-45 minutes. The gel was then stained with Coomassie blue, washed, and imaged using a ChemiDoc.

Dynamic light scattering
Dynamic light scattering was performed using a Wyatt DynaPro NanoStar. The fixed parameters were modified to only show radii from 1-300 nm or all radii greater than 1 nm. The instrument parameters were modified to have an acquisition time of 10 seconds and a total of 10 acquisitions. The temperature was kept at 20°C for all experiments. The solvent parameters were modified based upon solvent being used. The sample parameters were modified so the rg model was "Globular Proteins" and "Random Coil." The data analysis model used was "coils" and radius (x-axis) was plotted against % mass (y-axis). Resolution was set to "optimal resolution." One at a time, samples were inserted into cuvettes (Eppendorf UVette Cuvets) and then places in the machine. Arrow on cuvette pointed toward the back of the machine and was on the right side of the cuvette. When the cuvette was in the machine and all parameters were set, the machine was connected to the computer program Dynamics (Version 6.12.0.3). The acquisitions were collected and automatically averaged and turned into a measurement by Dynamics. Each sample was recorded as a different measurement. Data from the measurements was exported into an excel document for further analysis.

Results

Site-directed mutagenesis to investigate role of tyrosine residues in FUS LC domain
The tyrosine residues of the low complexity (LC) domain of FUS are important for pi-pi stacking which allows FUS proteins to interact with one another and form assemblies. Ten mutant primers were made to perform quick-change mutagenesis. Each primer contained one tyrosine to serine point mutation. These primers were going to be used to create mutagenic FUS plasmids containing one, two, or three tyrosine to serine mutation. This would allow for a library of mutants to locate residues critical for FUS protein function. Six of the ten primers were successful in implementing the correct point mutation into the plasmid sequence ***(Figure 1)***. Of those six, five contained additional point mutations or insertions that were not supposed to be in the sequence. This was likely due to the structure of the LC domain of FUS. Most of the domain is a very SYQG region and it is likely that primers dimerized or otherwise had difficulty binding to the correct sequence.

Developing a physiologically relevant buffer for elution and storage of FUS
Usually, FUS is eluted in FUS, which has much higher salt concentrations than a normal cellular environment. PBS is a buffer with salt concentration that is much closer to physiological salt concentrations. To make experimental conditions more physiologically relevant, FUS was eluted in PBS instead of FUS Buffer. Dynamic light scattering was used to measure percent mass and hydrodynamic radii of molecules in the solution (***Figure 2a)***. With this data, it was seen that monomeric FUS was present when initially eluted with PBS. This was slightly surprising as FUS was expected to instantaneously form assemblies. For the FUS eluted in FUS Buffer, the data is unimodal with a maximum at 4.2 nm and the peak lies within 3.2 nm and 13.3 nm ***(Table 1)***. For the FUS eluted in PBS, there is also a peak at 4.2 nm and the peak lies within 3.2 nm and 10.0 nm. Whether FUS was eluted in FUS Buffer or in PBS, there was a peak at 4.2 nm, and each sample had a similar range of species.

Stability of FUS protein in urea and effect of urea on protein structure
FUS was eluted in PBS with urea for two reasons. The first reason was to investigate urea's effect on the stability of the monomer in long term storage. We hypothesized that higher concentrations of urea would be good for storing the protein because FUS Buffer has high salt concentration and can maintain

the protein as a monomer. The second goal was to learn more about the structure of the full length FUS protein, and whether it is present in a coiled or elongated state. Since urea is a denaturant, we hypothesized that if the FUS protein exists in a largely coiled state, increasing urea will cause the protein to elongate. This should lead to an shift right in the average hydrodynamic radius of the protein as indicated by dynamic light scattering. On the other hand, if the FUS is already in an elongated state, we wouldn't expect to see much of a change in the hydrodynamic radius with increasing urea concentrations. To do this, different samples of FUS were eluted in buffers of PBS mixed with urea ranging from 0.25M to 4.0M and were then analyzed with dynamic light scattering from 1-300 nm (***Figure 3)***. The samples were then each aliquoted into two tubes and flash frozen in liquid nitrogen and stored at -80°C for one week. After one week, one of the two aliquots of each urea concentration was thawed on ice and again analyzed with dynamic light scattering from 1-300 nm. After two weeks, the second of the two aliquots of each urea concentration was thawed on ice and analyzed with dynamic light scattering from 1-300 nm.

For FUS eluted in PBS with 0.25M urea, the initial elution shows a maximum at 5.4 nm and the peak lies within 3.0 nm and 9.5 nm ***(Table 2)***. After one week frozen, there is a maximum at 5.3 nm and the peak lies within 4.0 nm and 9.4 nm. After two weeks frozen, there are no peaks in the 1-300 nm range.

For FUS eluted in PBS with 0.5M urea, the initial elution shows a maximum at 5.4 nm and the peak lies within 3.0 nm and 9.5 nm. After one week frozen, there is a maximum at 7.0 nm and the peak lies within 5.3 nm and 9.4 nm. After two weeks frozen, there are no peaks in the 1-300 nm range.

For FUS eluted in PBS with 1.0M urea, the initial elution shows a maximum at 5.4 nm and the peak lies within 3.0 nm and 9.5 nm. After one week frozen, there is a maximum at 7.0 nm and the peak lies within 5.3 nm and 12.5 nm. After two weeks frozen, there is a maximum at 7.0 nm and there is one peak to the right at 9.4 nm.

For FUS eluted in PBS with 4.0M urea, the initial elution shows a maximum at 5.4 nm and the peak lies within 3.0 nm and 12.7 nm. After one week frozen, there is a maximum at 9.4 nm and the peak lies within 7.0 nm and 16.6 nm. After two weeks frozen, there is a maximum at 7.0 nm and the peak lies within 5.3 nm and 16.6 nm.

The 1.0M and 4.0M urea solutions retained monomeric FUS for the duration of the two weeks, while the 0.25M and 0.5M urea solutions did not. This is consistent with urea stabilizing the protein. Additionally, as the protein was stored in higher concentrations of urea and for longer periods, a shift right in the average hydrodynamic radius can be seen. This gives evidence for the hypothesis of FUS being an elongated protein.

The effect of RNA and temperature on condensate formation

Within the nucleus of the cell, there exist both FUS-RNA interactions and FUS-FUS interactions. FUS proteins can assemble with one another in the absence of RNA. FUS proteins can also seed onto RNA, leading to assembly. FUS was eluted in PBS and 0.4 μM of a short strand of RNA was added. Dynamic light scattering was then used to visualize the full range of radii present in solution ***(Figure 4)***. Samples were incubated at three different temperatures (4°C, room temperature, and 37°C) to see how temperature would affect protein assembly. Previously published literature stored FUS at 4°C to

induce condensate formation, so that temperature was used even though it lacks physiological relevance. The temperature 37°C is most physiologically relevant, but the protein is more prone to crashing out of solution at this temperature, so room temperature was also used to get closer to physiological temperature, but also to keep FUS from crashing out of the solution. The experiment was performed three times, although an extra step was included in trials two and three. First, FUS was eluted in PBS. The elution was then spun down at 12,000 g for 30 minutes. Ten cuvettes were then labeled. Cuvette one had 100 µL of PBS only. Three cuvettes had 100 µL of PBS + 0.4 µM RNA. Three cuvettes had 100 µL of the elution from the purification. Three cuvettes had 100 µL of the elution from the purification + 0.4 µM RNA. All these samples were gently pipetted up and down, parafilmed, and analyzed by dynamic light scattering for all radii. One cuvette from each sample type was incubated at 4°C for 24 hours. The second cuvette from each sample type was incubated at room temperature for 24 hours. The third cuvette from each sample type was incubated at 37°C for 24 hours. After incubation, all the samples were analyzed with dynamic light scattering for all radii. In trials two and three, all cuvettes were incubated for an additional 24 hours at room temperature. After incubation, they were then again analyzed by dynamic light scattering for all radii. Following are the DLS analyses for each of the three trials.

With elution buffer alone, there is a maximum at 22.5 and the peak extends from 16.9 nm to 53.0 nm ***(Figure 2a).*** With elution buffer alone, there is a maximum at 1.7 nm and the peak extends to 2.3 nm. There is a second maximum at 125.0 nm and the peak extends from 39.8 to 295.1 nm. There is a third maximum at 38259.2 nm and the peak extends from 21585.9 nm to 67811.4 nm ***(Figure 2b).*** With elution buffer alone, there is a maximum at 70.5 nm and the peak extends from 39.8 to 125.0 nm. There is a second maximum at 295.0 nm and the peak extends from 125.0 nm to 522.9 nm. There is a third maximum at 5160 nm and the peak extends from 3876.5 nm to 9147.3 nm. There is a fourth peak at 283588.0 nm and the peak extends from 120182.0 nm to 377546.0 nm ***(Figure 2c).***

There is sight variation in the different FUS + RNA before incubation samples, but all show a maximum at 5.3 nm and the peaks extend from about 3.0 nm to 39.9 nm ***(Figure 2a).*** There is slight variation in the different FUS + RNA samples before incubation, but all show a maximum at 5.3 or 4.0 and the peaks extend from 3.0 nm to 93.9 nm ***(Figure 2b).*** In the FUS + RNA samples before incubation, there is a maximum at 4.0 nm and the peak extends from 2.3 nm to 125.0 nm ***(Figure 2c).***

In the FUS + RNA sample incubated at 4°C, there is a maximum at 4.0 nm and the peak extends from 3.0 nm to 29.9 nm ***(Figure 2a).*** In the FUS + RNA sample incubated at 4°C, there is a maximum at 1.7 nm and the peak extends right to 2.3 nm. There is second maximum at 7.1 nm and the peak extends from 5.4 nm to 9.5 nm. There is a third maximum at 70.5 nm and the peak extends from 53.0 to 93.9 nm ***(Figure 2b).*** In the FUS + RNA sample incubated at 4°C, there is a maximum at 5.4 nm and the peak extends from 3.0 nm to 16.9 nm. There is a second maximum at 53.0 nm and the peak extends from 29.9 nm to 93.9 nm ***(Figure 2c).***

In the FUS + RNA sample incubated at RT, there is a maximum at 5.3 nm and the peak extends from 4.0 nm to 39.8 nm ***(Figure 2a).*** In the FUS + RNA sample incubated at RT, there is a maximum at 7.1 nm and the peak extends from 5.4 nm to 16.9 nm. There is a second maximum at 295.0 nm and the peak extends from 16.9 nm to 926.7 nm ***(Figure 2b).*** In the FUS + RNA sample incubated at RT, there is a maximum at 5.4 nm and the peak extends from 2.3 nm to 22.5 nm. There is a second maximum at 70.5 nm and the peak extends from 22.5 nm to 125.0 nm ***(Figure 2c).***

In the FUS + RNA sample incubated at 37°C, there is a maximum at 5.3 nm and the peak extends left to 4.0 nm. There is second maximum at 29.9 nm and the peak extends right to 53.0 nm. There is third maximum at 696.3 nm and the peak extends from 523.0 nm to 927.0 nm. There is a fourth maximum at 38261.3 nm and the peak extends from 21587 nm to 50938.1 nm ***(Figure 2a).*** In the FUS + RNA sample incubated at 37°C, there is a maximum at 5.4 nm and the peak extends left to 4.0 nm. There is a second maximum at 39.8 nm and the peak extends from 29.9 to 53.0 nm. There is a third maximum at 3876.4 nm and the peak extends from 2911.7 nm to 5160.7 nm. There is a fourth maximum at 159995.0 nm and the peak extends from 67804.5 nm to 3777535.0 nm ***(Figure 2b).*** In the FUS + RNA sample incubated at 37°C, there is a maximum at 3.0 nm and the peak extends left to 2.3 nm. There is a second maximum at 39.8 nm and the peak extends from 29.9 nm to 53.0 nm. There is a third maximum at 295.1 nm and the peak extends from 125.0 nm to 927.2 nm. There is a fourth maximum at 53.0 nm and the peak extends from 927.2 nm to 93.93 nm. There is a fifth maximum at 50949.1 nm and the peak extends from 21591.7 nm to 160055.0 nm ***(Figure 2c).***

In the FUS + RNA sample incubated at 4°C then at RT for another day, there is a maximum at 12.7 nm and the peak extends right to 16.9 nm. There is a second maximum at 125.0 nm and the peak extends from 53.0 nm to 295.0 nm. There is a third peak at 67808.3 nm and the peak extends from 50933.1 nm to 160004.0 nm ***(Figure 2b).*** In the FUS + RNA sample incubated at 4°C then at RT for another day, there is a maximum at 295.1 nm and the peak extends from 221.7 nm to 523.1 nm ***(Figure 2c).***

In the FUS + RNA sample incubated at RT then at RT for another day, there is a maximum at 12.7 nm and the peak extends from 9.5 nm to 29.9 nm. There is a second maximum at 93.9 nm and the peak extends from 29.9 nm to 2911.6 nm. There is a third maximum at 283574.0 nm and the peak extends from 90268.1 nm to 377528.0 nm ***(Figure 2b).*** In the FUS + RNA sample incubated at RT then at RT for another day, there is a maximum at 2.3 nm and the peak extends from 1.7 nm to 3.0 nm. There is a second maximum at 9.5 nm and the peak extends from 7.2 nm to 16.9 nm. There is a third maximum at 70.6 nm and the peak extends from 53.0 nm to 94.9 nm ***(Figure 2c).***

In the FUS + RNA sample incubated at 37°C then at RT for another day, there is a maximum at 295.0 nm and the peak extends from 166.5 nm to 523.1 nm. There is a second maximum at 6872.3 nm and the peak extends from 2912.4 nm to 38265.1 nm. There is a third maximum at 283651.0 nm and the peak extends from 38265.1 nm to 377630.0 nm ***(Figure 2b).*** In the FUS + RNA sample incubated at 37°C then at RT for another day, there is a maximum at 392.9 nm and the peak extends from 295.1 nm to 523.1 nm. There is a second peak at 377632.0 nm and it extends left to 283652.0 nm ***(Figure 2c).***

From the DLS data, species in solution appear to change based on temperature exposure. After exposure to temperatures warmer than 4°C, the species experience shifts to the right. This indicates an increase in size of the species present. From the data it is not possible to form a complete understanding of the difference between samples with FUS only (not shown here) and samples with FUS + RNA show similar data.

Discussion

With regards to the mutagenesis experiment, only six of the ten mutations successfully inserted, and only one didn't contain other point mutations or large insertions. A hypothesis for this is that it is due to the repetitive nature of the LC domain of FUS, as the primers likely bind to multiple locations in the sequence and this causes difficulty in controlling which sequences are amplified by PCR. Since it was so difficult to successfully mutate FUS by this method, we decided to move on past this experiment. For future experiments, this difficulty should be taken into consideration. Ordering premade mutagenic plasmids may be more viable.

Eluting FUS in PBS works just as well as eluting in FUS Buffer and is more biologically relevant. Monomeric FUS tends to crash out of solution when stored in just PBS for prolonged periods. But adding urea to the PBS can aid the storage of FUS as a monomer so that it can be eluted and stored for a prolonged period. The 1.0M and 4.0M urea concentrations worked for preserving the monomer, whereas the 0.25M and 0.5M concentrations did not, so using 1.0M urea seems to be a good solution for long term storage of FUS. For further experimentation, it would be good to try urea concentrations between 0.5M and 1.0M to find the lowest concentration that can be used. It would also be good to try storing the protein for longer, up to four weeks. Additional experiments were performed that involved ten freeze-thaws to the protein in succession and not much change was seen with DLS. Therefore, amount of time stored seems to be relevant as opposed to just looking at freeze-thaw cycles alone.

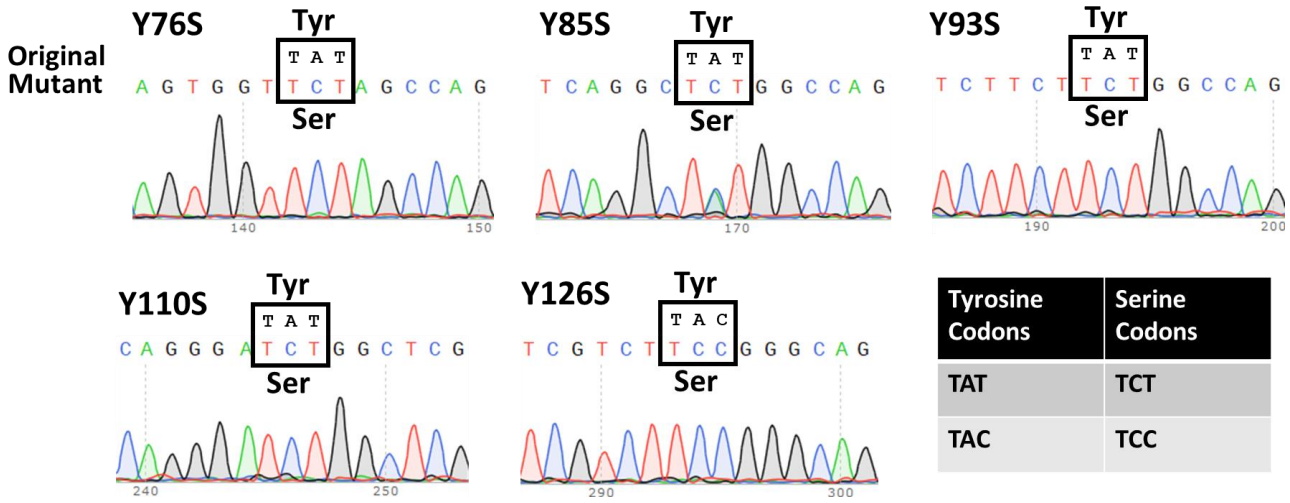
Furthermore, whether eluted in FUS or PBS, the hydrodynamic radius of monomeric FUS is consistently found to be about 4.2-5.3 nm. This gives us more information that can be used to model the assembled state of FUS and understand more about the composition and behavior of the assemblies. Knowing about the size of the monomer, the percent water content of polymers can be calculated with more certainty. Before, the size of FUS was overapproximated a bit and the percent water content of assemblies was calculated to be about 97.5%, but with the radii found here, the percent water content has been calculated to be about 95%.

There is a slight shift right in the monomeric peak of FUS as more urea is present. This means that the average hydrodynamic radius of FUS is slightly increasing in the presence of more urea. The width of the range of peaks also increases upon longer storage and with higher urea concentrations. A hypothesis is that FUS is a folded protein and urea, which is a denaturant when used at concentrations of about 8.0M, forces the molecule to elongate somewhat when present in concentrations below 8.0M.

When RNA was added to FUS and stored at 4°C, there was little shift in the monomeric peak. Previous literature has used 4°C conditions to induce condensate formation, but those were only performed on the FUS LC domain, whereas these experiments used full length FUS. When stored at room temperature, a larger peak begins to emerge, and eventually multiple distinct peaks can be seen to the right of the monomeric peak. When stored at 37°C, the protein was more prone to largely crash out of solution, but when the crashed-out part settled, distinct peaks like those seen in the room temperature sample could be seen. After being stored for 24 hours in one of the three temperatures, all samples were left at room temperature, and even the samples that previously had only a monomeric peak experienced a shift to the right. A hypothesis is that FUS forms condensates of distinct sizes instead of being present at a continuum of sizes. It remains to be seen why the room temperature induced formation more effectively than the 4°C treatment, but this is a more biologically relevant

temperature so mimics cellular conditions better. More experimentation and analysis must be performed to gain a more complete picture of the effect of RNA on FUS condensates. Dynamic light scattering appears to be a promising method of analysis for the formation of these FUS assemblies and more replicates of the experiments performed here are necessary. Extensions of this experiment should also be performed, including varying concentrations of FUS and RNA and addition of crowding reagents and other materials that further mimic cellular condition.

Tables and Figures



Tyrosine Codons	Serine Codons
TAT	TCT
TAC	TCC

Figure 1. DNA sequence chromatogram for six mutations made in the FUS low complexity (LC) domain. The plasmid used was mCherry FUS. Mutations are point mutations from tyrosine to serine and were made by site-directed mutagenesis and then amplified by PCR. These specific tyrosine residues were chosen because they were in the LC domain and a primer of adequate length and melting temperature could be made for them. Mutation to serine was made to remove the aromatic ring but to keep the hydroxyl side chain.

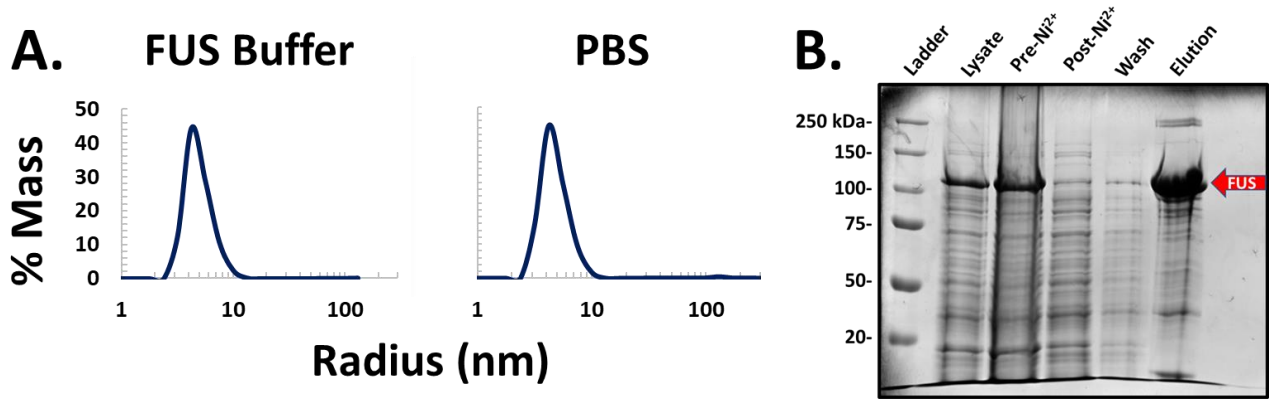


Figure 2. Dynamic light scattering data for FUS in FUS Buffer and PBS and SDS-PAGE gel. Protein was eluted using protocol explained in the methods section. **A.** In one trial, the protein was eluted using FUS Buffer and in the other trial, the protein was eluted using PBS. After the elution, samples were analyzed via dynamic light scattering. The graphs show the percent mass within the range from 1-300 nm. This was to show that both buffers can effectively elute monomeric FUS off the nickel beads. **B.** SDS-PAGE gel run on a 7.5% gel for samples collected from the different steps of the purification, with the last lane being the purified FUS sample at slightly above 100 kDa.

	FUS Buffer		PBS	
	Radius	% Mass	Radius	% Mass
Most abundant species	4.24	44.52	5.37	45.91
Smallest Species	3.19	12.79	3.18	15.72
Largest Species	13.32	0.20	9.99	1.85

Table 1. Dynamic light scattering data for FUS in FUS Buffer and PBS. Protein was eluted using protocol explained in the methods section. In one trial, the protein was eluted using FUS Buffer and in the other trial, the protein was eluted using PBS. After the elution, samples were analyzed via dynamic light scattering. The table shows the percent mass of the monomeric peak within the range from 1-300 nm. This was to show that both buffers can effectively elute monomeric FUS off the nickel beads.

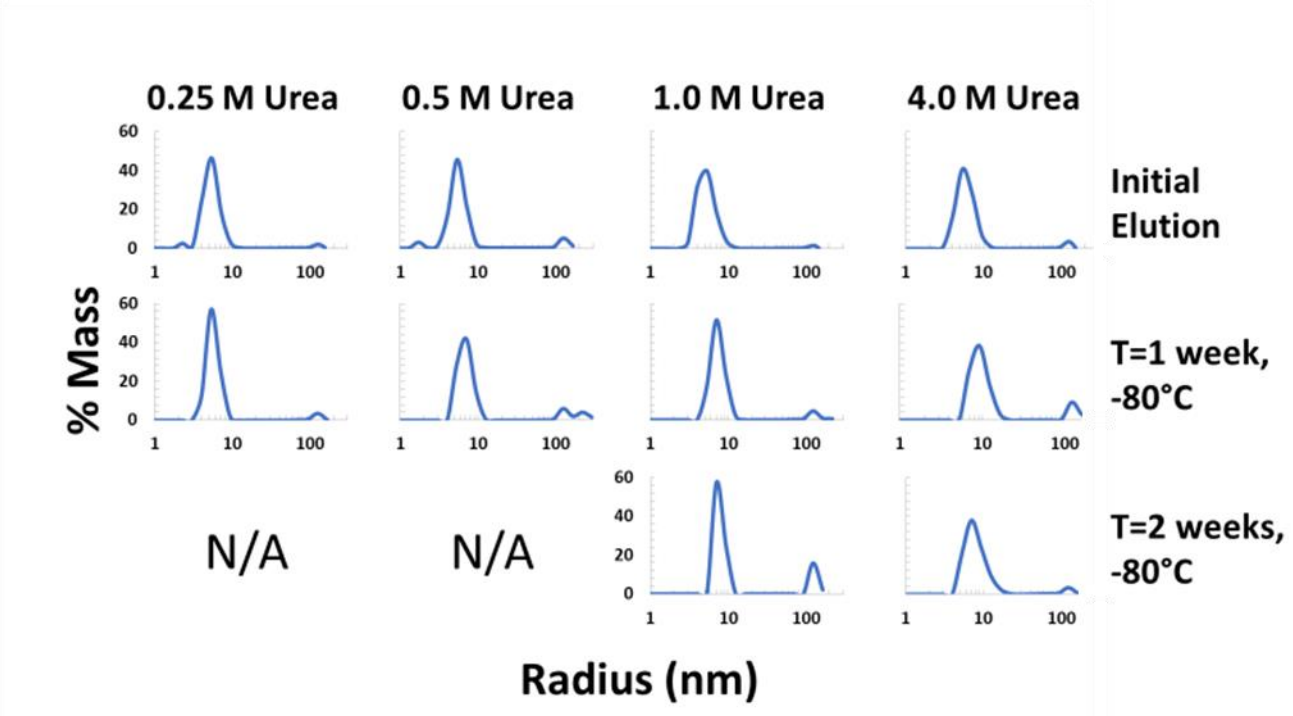


Figure 3. Dynamic light scattering data for FUS in PBS + different urea concentrations. Protein was eluted in PBS + 0.25M, 0.5M, 1.0M, or 4.0M urea. After elution, samples were analyzed by dynamic light scattering. The graphs show the percent mass of the species in the samples. The sizes were collected in the range from 1-300 nm. The samples were then aliquoted into two different tubes and were flash frozen with liquid nitrogen and stored at -80°C. The first aliquot of each concentration of urea was thawed after one week and analyzed by dynamic light scattering. The second aliquot of each concentration of urea was thawed after two weeks and analyzed by dynamic light scattering. After one week, each aliquot still showed monomeric peak in the 1-300 nm range. After two weeks, the PBS + 1.0M and 4.0M urea aliquots still showed monomeric peak in the 1-300 nm range, while the PBS + 0.25M and 0.5M urea aliquots had no peaks in the range.

	0.25M Urea Initial		0.5M Urea Initial		1.0M Urea Initial		4.0M Urea Initial	
	Radius	% Mass	Radius	% Mass	Radius	% Mass	Radius	% Mass
Most abundant species	5.37	46.42	5.37	45.91	5.37	39.89	5.37	40.70
Smallest Species	2.28	2.51	2.28	0.06	3.03	2.89	3.03	0.97
Largest Species	12.67	0.22	12.67	0.23	12.67	0.47	12.67	0.85
	0.25M Urea 1 Week		0.5M Urea 1 Week		1.0M Urea 1 Week		4.0M Urea 1 Week	
	Radius	% Mass	Radius	% Mass	Radius	% Mass	Radius	% Mass
Most abundant species	5.30	57.00	5.29	28.82	7.05	51.49	9.38	38.23
Smallest Species	3.98	12.55	7.05	41.77	5.29	17.40	7.05	26.00
Largest Species	9.39	1.40	12.49	0.56	12.49	1.91	16.63	3.04
	0.25M Urea 2 Weeks		0.5M Urea 2 Weeks		1.0M Urea 2 Weeks		4.0M Urea 2 Weeks	
	Radius	% Mass	Radius	% Mass	Radius	% Mass	Radius	% Mass
Most abundant species	-	-	-	-	7.05	57.59	7.04	37.86
Smallest Species	-	-	-	-	7.05	57.59	5.291	20.81
Largest Species	-	-	-	-	9.38	24.35	22.13	0.34

Table 2. Dynamic light scattering data for FUS in PBS + different urea concentrations. Protein was eluted in PBS + 0.25M, 0.5M, 1.0M, or 4.0M urea. After elution, samples were analyzed by dynamic light scattering. The tables show the percent mass of the monomeric peaks within the range from 1-300 nm. The samples were aliquoted into two different tubes and were then flash frozen with liquid nitrogen and stored at -80°C. The first aliquot of each concentration of urea was thawed after one week and analyzed by dynamic light scattering. The second aliquot of each concentration of urea was thawed after two weeks and analyzed by dynamic light scattering. After one week, each aliquot still showed monomeric peak in the 1-300 nm range. After two weeks, the PBS + 1.0M and 4.0M urea aliquots still showed monomeric peak in the 1-300 nm range, while the PBS + 0.25M and 0.5M urea aliquots had no peaks in the range.

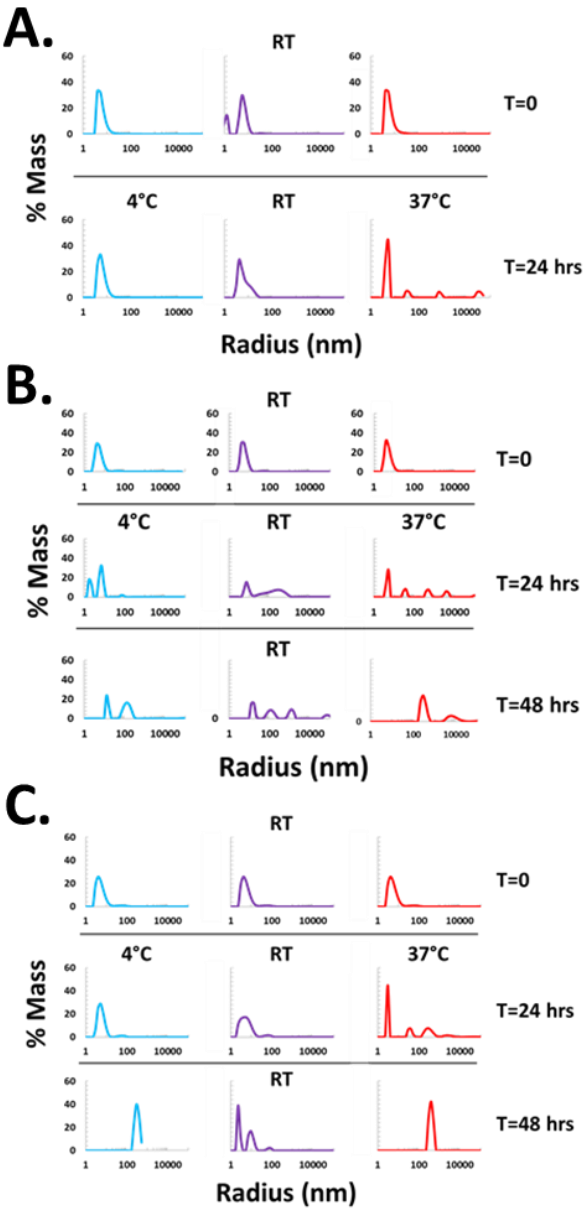


Figure 4. Three biological replicates of dynamic light scattering analysis of pure hMBP FUS with RNA. Protein was eluted in PBS and was spun down with a 0.1-micron filter. 100 µL was aliquoted into three different tubes and 0.4 µM RNA was added to each aliquot. Samples were analyzed via dynamic light scattering. The graphs show the percent mass within the range from 1 to 1-10^5 nm. Aliquot one from each replicate was left at 4°C for 24 hours, analyzed via DLS, returned to 4°C for 24 more hours, and analyzed again. This same procedure was followed for aliquot two from each replicate but was stored at room temperature. Same procedure followed for aliquot three of each replicate but stored at 37°C.

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