Determining the in vitro saturation concentration using fluorescence microscopy

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Abstract

The saturation concentration (Csat) is a key parameter of proteins undergoing liquid-like phase separation. It is defined as the concentration above which a protein demixes from solution and forms biomolecular condensates. Here, we present a protocol for determining the Csat of a fluorescently labelled protein *in vitro*. Determining the Csat at various control parameters, such as salinity or pH allows deriving multidimensional phase diagrams.

Introduction

The formation of intracellular biomolecular condensates is emerging as a principle to describe the organization of the eukaryotic inside (Banani 2017). Condensates form by the process of liquid-liquid phase separation. In its simplest form, a two-component system, a buffer (which may already be complex) and a polymer compound, such as a protein, can demix from one another to form a polymer rich-phase which coexists with a polymer-poor phase (Hyman, 2014). We are at the beginning of understanding the underlying principle, but the diversity and complexity of the phenomenon occurring in biology is overwhelming and in these rather early days we are often left with more questions than answers.

Condensates may be present throughout the cell cycle but may also form or disassemble during developmental or environmental changes. While the functional aspects of some condensates are emerging, examples may be the nucleolus (Feric, 2016) or centrosomes (Woodruff, 2017), many more condensates are being identified and described, yet often their function remains unclear. Likewise, the characteristics and molecular mechanisms underlying the assembly process remain a quest of intense research.

The ability to identify and isolate key components of cellular condensates enables the discovery of underlying molecular interactions that regulate the formation and function of condensates inside of cells. A key challenge has been to express and purify those components. This is, because many of these proteins exhibit rich phase behavior and are aggregation prone. Understanding that the interactions within condensates appear to be of electrostatic nature facilitated the isolation process. Using buffer with increased salinity successfully suppressed the aggregation and phase separation during the purification process, often yielding pure and stable protein samples (REF alberti 2018)

The assembly of condensates is a highly concentration dependent process. For a self-interacting system, such as a protein prone to phase separation, it will persist in an unassembled, maybe monomeric state at small protein concentrations. The limited stability of proteins renders them unstable and increasing the concentration increase the likelihood for a protein to self-interact. The system abruptly demixes to form a rich-phase. The concentration at which the protein forms condensates is defined as the saturation concentration (Csat). In other words, at concentrations below Csat the monomers (or small species) persist in solution, but at concentrations above Csat, excess molecules interact to forms a phase forms (like salt crystals precipitate from a supersaturated salt solution). More complex system exists. E.g. many proteins interact with ligands, such as DNA or RNA and thus the probability to undergo phase separation is determined by the concentration-dependent interaction of the protein and the interaction partner.

To enable the discovery of the structure-function relationship of condensates, it is of critical importance to understand Csat as function of control parameters, such as pH, temperature, salinity and ligands. The control parameters determine the strength of intra- and intermolecular interactions and thus route the protein (or protein-ligand complex) into distinct material states, such as liquid-like condensates, hydrogels or even aggregates and crystals. With these tools at hand, one provides a mechanistic understanding of the molecular interactions and enables the description of the structure-function relationship.

Here, we set out to determine the Csat of a model protein (DDX4-YFP, Nott (2015)) as a function of various control parameters, including pH and salinity, which

sets the base for subsequent characterization of the molecular interactions and condensates material properties.

Reagents, Materials and Equipment

Buffers

- 40 mM PIPES, pH 7.6-6.0
- 20 mM Tris, pH 8.0-7.5
- NaCl 5M in H2O
- ddH2O
- Note: Fresh buffers should be prepared and filtered.

Pipettes and pipette tips

- 1000 µL
- 100 µL
- 10 µL
- 2 µL
- Note: some condensates are very fragile. Shear forces induced by pipetting can result in dissolution or aggregation of the condensates.

Sample chamber

- 384 microtiter well plate. For short term, a plastic cover is suggested to reduce evaporation. For intermediate incubation, the sample may also be covered with mineral oil.
- Alternatively, samples can be mounted onto microscopy glass-slides, with spacing between the glass and cover slide.

Protein

- Here we use DDX4-YFP as a model system.
- Stock concentration: 65 µM
- Buffer: 20 mM Tris, pH 8.0, 500 mM NaCl

Vails

- 0.1-1.5 ml plastic vials for liquid handling of buffers and protein.
- Low/Non-binding recommended

(optional, if necessary)

- Centrifuge for sample rotation (removal of nucleation seeds or aggregates)
- Nanofiltration devices (removal of nucleation seeds or aggregates)

Microscope and objectives

- widefield or confocal microscope
- Imaging of condensates can be carried out with 20x air, 60x water or 100x oil emulsion objectives and should be chosen according to condensate size and volume fraction phase separated.

Procedure

Prepare protein

1. Thaw 15 µL protein per condition rapidly, holding the samples in your hands.
2. Combine all vials to a single one.
3. Spin the sample at max. speed for 2 min.
4. Prepare 20 µL of a 1:10 dilution of the stock protein solution using the storage buffer (50 mM Tris, pH 8.0, 500 mM NaCl) as dilution buffer. This 1:10 dilution is used for setting up small protein concentrations.

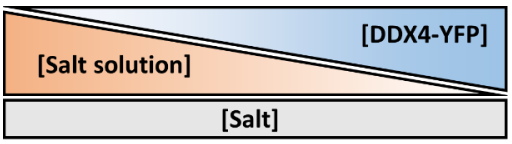
Prepare buffer solutions

1. Prepare 500 µl 2 x buffer for each condition

Salt compensating solution

1. Because the protein is stored in a solution high salinity, a large amount of salt it carried over from the stock solution into the final reaction. Consequently, pipetting different amounts of protein, yields different concentrations of salt carried over. This must by compensated.
2. Prepare 1 ml salt compensating solution, 500 mM NaCl in H2O
3. 900 µL ddH2O + 100 µL 5 M NaCl

Setting up the well plate



1. Add the buffer to the respective wells
2. Add the water to the respective wells
3. Add the salt to the respective wells
4. Mix the well plate by shaking gently
5. Add the protein last. Important: Do not use your pipette for mixing the solution. Condensates are fragile and shear forces may

dissociate the condensates and induce aggregation.

6. Cover the well plate with a plastic cover
7. Incubate the well plate for 15-30 min
8. Optional: you may mix the solution by placing the well plate on a well-plate shaker, not exceeding 800 rpm
9. Image the well plate

Data analysis

Manual data analysis

- Open microscope images and score the absence or presence of biomolecular condensates 0 or 1.
- Form the mean average of the scoring per conditions.
- Plot the mean average score as a function of the protein concentration to derive a saturation concentration curve.
- Form a linear extrapolation of the baseline before phase separation and a linear extrapolation of linear part of saturation curve. Determine Csat from the intersection of the two linear extrapolations.

Computer aided data analysis

- Use a fiji base script to
  - segment the condensates in each field of view per condition
  - measure the mean fluorescence intensity and area of condensates
  - determine the mean average background intensity and area
  - calculate the fraction phase separated from the total condensates intensity normalized to the total fluorescence

$$Fraction = \frac{\sum I_{condensate}}{(\sum I_{Condensate} + \sum I_{background})}$$

- Use R / Rstudio to statistically analyze and plot the fraction phase separated
- Fit the data to the a generic sigmoid function using non-least square fitting:

$$fraction = \frac{(f_{min} - f_{max})}{1 + e^{\frac{x - x_0}{dx}}} + f_{max}$$

where f_{min} and f_{max} described the minimum and maximum value of the observed phase separated fraction, respectively, x_0 denominates the value at which half of the fraction is saturated and dx the slope at half saturation.

- Derive the maximum slope in the turning point of the sigmoid

$$m_{dx} = \frac{f_{max} - f_{min}}{4 * dx}$$

- To approximate Csat use the following equation can be used

$$Csat \sim \frac{f_{min}}{m_{dx}} - \frac{f_{min} + f_{max}}{2 * m_{dx}} + x_0$$

Figures

Figure 1: Schematic representation of the domain structure of the 2xNT-DDX4-YFP model construct. NT: N-terminal domain (green), note: NT was duplicated. this construct exhibits a reduced Csat compared to 1xNT, YFP: Yellow Fluorescence Protein (yellow), C-terminal extension depicted in blue.

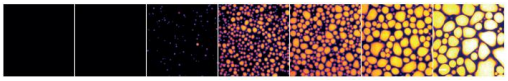


Figure 2: Representative fluorescence images of recombinantly expressed and purified DDX4-YFP at various protein concentrations in 20 mM TRIS, 125 mM NaCl, pH 8.0. Images were taken on the bottom of a multi well dish. The average size of and the area covered by condensates increases as the function of protein concentration.

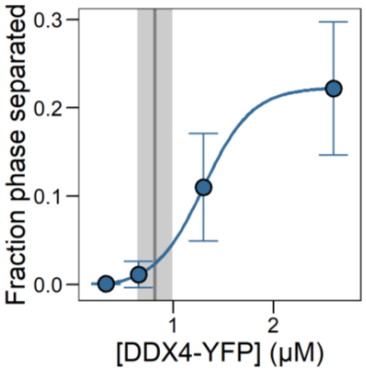


Figure 3: Analysis of the Fraction phase separated (blue) as a function of DDX4-YFP concentration. Trendline fitted to the datapoints is depicted in blue. Csat for robust phase separation is depicted in grey. Note: The experiment conditions differ from those shown in Figure 1, resulting in a different Csat.

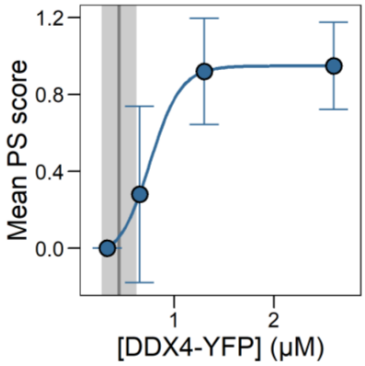


Figure 4: Analysis of the phase separation behavior of DDX4-YFP as a function of protein concentration. In this analysis, the average of 36 fields of views was analyzed and scored for the presence and absence of condensates. The mean per condition was calculated to derive Csat. This analysis predicts the nucleation concentration. Trendline fitted to the datapoints is depicted in blue. Csat for early phase separation is depicted in grey.

Discussion and Conclusions

DDX4-YFP is a multidomain protein that consists of three distinct parts, a disordered N-terminal region (NT) followed by the folded YFP and a short, disordered C-terminal extension (Figure 1). In this course we used a variant of DDX4, in which the N-terminal region was duplicated (2xNT-DDX4-YFP). The N-terminal domain of DDX4 self-interacts and provides the multivalent interactions to drive phase separation (Figure 2 and Nott (2015)). According to the previous characterization, the N-terminal domain determines the Csat and duplication of this region lowers the Csat even further, demonstrating that the interactions among N-terminal regions plays an important part in regulating the solubility of the protein and the phase separation process. Varying the concentration of DDX4-YFP allows determining and quantifying the assembly transition (Figure 2-4). The assembly transition depends on the interaction strength among DDX4 molecules. These interactions are subject to control parameters, such as pH and salinity. We derive saturation concentration from various conditions, combine them in a multidimensional phase diagram to establish an understanding of the influence and interdependence of the control parameters.

References

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Appendix - Examples of pipetting schemes

[DDX4YFP]	65,0	µM					
Storage buffer	20 mM Tris, pH 8.0, 500						
eactions Volume	20,0	µL					
Final [NaCl]	100	mM NaCl					
pH	8	pH					
Buffer	50	mM Tris / PIPES					

[DDX4-YFP] (µM)	Add buffer (µl)	Add H2O (µl)	salt compensating buffer volume (µl)	Volume DDX4-YFP Stock solution (µl)	Volume DDX4-YFP 1/10 Dilution (µl)	[NaCl] (mM) carry over	Total (µl)
0,0	**10,0**	**6,0**	**4,0**	0,0	**0,0**	0,0	20,0
0,1	**10,0**	**6,0**	**3,7**	0,0	**0,3**	7,7	20,0
0,2	**10,0**	**6,0**	**3,4**	0,0	**0,6**	15,4	20,0
0,3	**10,0**	**6,0**	**3,1**	0,0	**0,9**	23,1	20,0
0,4	**10,0**	**6,0**	**2,8**	0,0	**1,2**	30,8	20,0
0,5	**10,0**	**6,0**	**2,5**	0,0	**1,5**	38,5	20,0
0,8	**10,0**	**6,0**	**1,7**	0,0	**2,3**	57,7	20,0
1,0	**10,0**	**6,0**	**0,9**	0,0	**3,1**	76,9	20,0
2,0	**10,0**	**6,0**	**3,4**	**0,6**	0,0	15,4	20,0
3,0	**10,0**	**6,0**	**3,1**	**0,9**	0,0	23,1	20,0
4,0	**10,0**	**6,0**	**2,8**	**1,2**	0,0	30,8	20,0
5,0	**10,0**	**6,0**	**2,5**	**1,5**	0,0	38,5	20,0
7,5	**10,0**	**6,0**	**1,7**	**2,3**	0,0	57,7	20,0
10,0	**10,0**	**6,0**	**0,9**	**3,1**	0,0	76,9	20,0

Table 1: Pipetting scheme for a protein concentration series in 25 mM, pH 8, 100 mM NaCl

[DDX4YFP]	65,0	µM					
Storage buffer	20 mM Tris, pH 8.0, 500						
eactions Volume	20,0	µL					
Final [NaCl]	200	mM NaCl					
pH	8	pH					
Buffer	50	mM Tris / PIPES					

[DDX4-YFP] (µM)	Add buffer (µl)	Add H2O (µl)	salt compensating buffer volume (µl)	Volume DDX4-YFP Stock solution (µl)	Volume DDX4-YFP 1/10 Dilution (µl)	[NaCl] (mM) carry over	Total (µl)
0,0	**10,0**	**2,0**	**8,0**	0,0	**0,0**	0,0	20,0
0,1	**10,0**	**2,0**	**7,7**	0,0	**0,3**	7,7	20,0
0,2	**10,0**	**2,0**	**7,4**	0,0	**0,6**	15,4	20,0
0,3	**10,0**	**2,0**	**7,1**	0,0	**0,9**	23,1	20,0
0,4	**10,0**	**2,0**	**6,8**	0,0	**1,2**	30,8	20,0
0,5	**10,0**	**2,0**	**6,5**	0,0	**1,5**	38,5	20,0
0,8	**10,0**	**2,0**	**5,7**	0,0	**2,3**	57,7	20,0
1,0	**10,0**	**2,0**	**4,9**	0,0	**3,1**	76,9	20,0
2,0	**10,0**	**2,0**	**7,4**	**0,6**	0,0	15,4	20,0
3,0	**10,0**	**2,0**	**7,1**	**0,9**	0,0	23,1	20,0
4,0	**10,0**	**2,0**	**6,8**	**1,2**	0,0	30,8	20,0
5,0	**10,0**	**2,0**	**6,5**	**1,5**	0,0	38,5	20,0
7,5	**10,0**	**2,0**	**5,7**	**2,3**	0,0	57,7	20,0
10,0	**10,0**	**2,0**	**4,9**	**3,1**	0,0	76,9	20,0

Table 2: Pipetting scheme for a protein concentration series in 25 mM, pH 8, 200 mM NaCl