



Phase separation & interfacial phenomena of a model biomolecular condensate

Word count: 36 861

Jack Holland

St Peter's College
University of Oxford

Supervisors: Dr Timothy J. Nott & Prof Dirk G.A.L.
Aarts

A thesis submitted for the degree of
Doctor of Philosophy

Trinity Term 2022

Summary

The organization of intracellular space into distinct, membraneless, compartments is a ubiquitous feature of living organisms. It has recently become apparent that many of these biomolecular condensates may be formed by liquid-liquid phase separation. Representing (liquid-like) regions enriched in specific proteins and nucleic acid species, they partition biomolecules by providing a chemical environment distinct from the surrounding intracellular milieu. Regulation of their interfacial phenomena allows cells to localise them to specific regions of intracellular space, as well as control the kinetics of their formation and growth.

Through identifying and studying constituent proteins of biomolecular condensates, researchers have deciphered many of the sequence features necessary for phase separation. Of principal importance was the development of model biomolecular condensate systems *in vitro*. This has enabled rigorous physical modelling in a controlled environment, allowing investigations into the molecular specifics driving condensate formation through phase separation. In this thesis, we advance the study of biomolecular condensates, basing our research around the Ddx4^N model system focusing on condensate formation and interfacial phenomena.

Ddx4^N is the intrinsically disordered N-terminal tail of human ATP-dependent DEAD-box RNA helicase 4, shown to be necessary and sufficient for phase separation *in vitro* and in cells. Since its discovery as a condensate forming protein, its sequence features have been identified in numerous other condensate associated proteins, making Ddx4^N an ideal model system. First, we expand current knowledge concerning its phase separation. By investigating mutant sequences *in vitro*, the role of protein net charge is revealed, whilst also emphasising the effect of phenylalanine/tyrosine on cation- π interactions. Furthermore, we show the effect of net charge is reproducible in a cellular environment, using

in cell fluorescence images of exogenous Ddx4^N condensates.

Our attention is then drawn to the development of an experimental pipeline affording the precise characterisation of *in vitro* condensate interfacial phenomena through drop shape analysis. Confocal microscopy, the Young-Laplace equation and bespoke image analysis are combined to allow measurement of capillary lengths and contact angles of condensates resting on solid surfaces. Measuring condensate contact angles using our automated technique is found to be superior to a recently reported manual method.

We then focus on the fluid-fluid interfacial tension of condensates, presenting calculations based around the theory of van der Waals, implemented through the work of Scheutjens and Fler. Then we aimed to verify these calculations by measuring condensate capillary lengths through drop shape analysis, with a view to extracting their fluid-fluid interfacial tensions. However, we saw an unexpected increase of capillary length with condensate size. After close interrogation of the experimental technique and possible physical origins, we were unable to find an explanation for this phenomena. As such, the origin of our observed positive relationship between capillary length and condensate size remains an open question, requiring further study.

Finally, motivated by the observation that membrane-associated condensates are localised through specific interactions with membrane bound species, we decided to investigate the non-specific wetting of membrane surfaces by biomolecular condensates. Through measurement of contact angles on a supported lipid bilayer, we found condensates to be repelled by the membrane. Further measurements of condensate contact angles on hydrophilic and hydrophobic glass surfaces revealed a role for hydrophobic interactions, likely serving to repel condensates from necessarily hydrophilic headgroups of lipid bilayers. These data suggest repulsion between biomolecular condensates and membranes is responsible for the maintenance of endogenous condensates in the cytoplasm/nucleoplasm, away from intracellular membranes. Furthermore, we discover a positive line tension at the three phase contact line, with a deleterious impact on condensate wetting at the micrometer lengthscale.

Declaration

This thesis is submitted for the degree of Doctor of Philosophy at the University of Oxford. No part of this thesis has been accepted or is currently being submitted for any degree, diploma, certificate or other qualification in this University or elsewhere. This thesis is wholly my own work, except where indicated.

Common abbreviations

Biomolecules

Ddx4^N: N-terminal intrinsically disordered tail of the nuage-associated ATP-dependent DEAD-box RNA helicase.

Ddx4^{N*}: Mutant Ddx4^N carrying a sequence-wide cysteine to serine substitution.

DNA: Deoxyribose nucleic acid.

RNA: Ribose nucleic acid.

Physical constants

N_A: $6.0221415 \times 10^{23} \text{mol}^{-1}$ - Avogadro's number.

k_B: $1.380649 \times 10^{-23} \text{J K}^{-1}$ - Boltzmann's constant.

R: $8.3145621 \text{J K}^{-1} \text{mol}^{-1}$ - the gas constant, equal to $N_a k_B$.

g: 9.81m s^{-2} - acceleration due to gravitational interaction with Earth, close to its surface.

Contents

Summary	i
Declaration	iii
Common abbreviations	v
1 Introduction	1
1.1 Organisation of cellular biochemistry & membraneless compartments . . .	2
1.2 Model biomolecular condensates & proteins	5
1.3 Emergent properties of biomolecular condensates	9
1.3.1 Internal phenomena	9
1.3.2 Interfacial phenomena	10
1.4 Outstanding research questions	12
1.5 Thesis outline	13
2 Generation of Materials & Standard Experimental Techniques	15
2.1 Buffers & reagents	16
2.2 Spectrophotometry	18
2.3 Gel electrophoresis	19
2.4 Protein sequences	19
2.5 Molecular cloning	24

2.5.1	Generation of chemically competent cells	24
2.5.2	Transformation of <i>E. Coli</i>	25
2.5.3	Gene amplification	25
2.5.4	Restriction digest & ligation	26
2.6	Protein expression	27
2.6.1	Protocol	28
2.7	Protein purification	29
2.7.1	Protocol	30
2.8	Sample preparation	33
3	Tuning Ddx4^{N*} Phase Separation	35
3.1	Introduction	36
3.1.1	Design of constructs	37
3.2	Theoretical background	39
3.2.1	Mixing & the lattice model	40
3.2.2	Entropy of mixing	41
3.2.3	Internal & free energy of mixing	43
3.2.4	Phase separation	46
3.2.5	Phase diagrams	48
3.2.6	Applicability of Flory-Huggins theory to Ddx4 ^{N*} condensates	52
3.3	Methods	53
3.3.1	Microscopy & thermal stage setup	55
3.3.2	Data collection & image analysis	55
3.3.3	Fitting to Flory-Huggins theory	60
3.3.4	<i>In cell</i> partitioning measurements	61

3.4	Results & discussion	63
3.4.1	Phase diagram fits & interaction parameters	63
3.4.2	Release of water & counterions drives Ddx4 ^{N*} phase separation . . .	66
3.4.3	F→Y substitution enhances Ddx4 ^{N*} phase separation	69
3.4.4	Reduced net charge favours phase separation in a sequence context dependent manner	70
3.4.5	Modulation of intracellular electrostatic interactions	73
3.5	Conclusions & outlook	75
4	Implementation of Axisymmetric Drop Shape Analysis	79
4.1	Introduction	80
4.2	Theoretical background	81
4.2.1	Curved surfaces	81
4.2.2	The Young-Laplace equation for a sessile drop	83
4.3	Methods	85
4.3.1	Sample preparation	85
4.3.2	Confocal imaging	87
4.3.3	Image analysis	92
4.3.4	Droplet profile fitting	95
4.3.5	Manual measurement of contact angles using ImageJ	99
4.4	Results & discussion	100
4.4.1	Comparison with manual measurements of contact angle	100
4.5	Conclusions & outlook	102
5	Calculation & Measurement Of Fluid-Fluid Interfacial Tensions	105
5.1	Introduction	106

5.1.1	Methods to study fluid-fluid interfacial tension	109
5.2	Theoretical background	111
5.2.1	Mean field calculation of Interfacial Tensions	111
5.2.2	The capillary length & sessile drops	113
5.3	Methods	116
5.3.1	Sample preparation & imaging	116
5.4	Results & discussion	117
5.4.1	Scheutjens-Fleer calculations	117
5.4.2	Measurement of capillary length	119
5.5	Conclusions & outlook	129
6	Molecular Determinants of Condensate Wetting	131
6.1	Introduction	132
6.2	Theoretical background	134
6.2.1	Wetting & interfacial tension	134
6.2.2	Line tension	136
6.3	Methods	138
6.3.1	Supported lipid bilayer preparation by vesicle fusion	138
6.3.2	Sample preparation, imaging & analysis	141
6.4	Results & discussion	142
6.4.1	Ddx4 ^{N*} condensates exhibit a positive line tension	142
6.4.2	Ddx4 ^{N*} condensates are repelled by lipid bilayers	147
6.4.3	Hydrophobic determinants of condensate wetting	150
6.5	Conclusions & outlook	155
	Bibliography	159

Chapter 1

Introduction

ABSTRACT

This chapter is intended to give the reader an overview of biomolecular condensates, whilst motivating the research performed in this thesis. We begin with a broad introduction to membraneless intracellular compartments as biomolecular condensates, emphasising their ubiquity. Then we introduce the recent ‘phase separation’ hypothesis regarding their liquid-like nature and biogenesis. Then we focus on studies performed in model condensate systems, revealing the typical sequence features of proteins implicated in the formation of endogenous biomolecular condensates. Next we cover emergent phenomena exhibited by biomolecular condensates, illustrating these with key examples from the literature. Penultimately, we discuss the outstanding research questions forming the subject of this thesis, before concluding with an outline of the following chapters.

1.1 Organisation of cellular biochemistry & membraneless compartments

Life as we know it is fundamentally compartmentalised. On the most familiar scale, we humans are discrete entities with our own identities, personalities and genetic material, occupying ‘our own’ volume of the universe at any given time. However, broadly speaking, we and all other lifeforms on this planet, are composed of the same subset of atoms. What distinguishes us from crabs, daffodils, foxes and even each other, is the organisation of these atoms into a hierarchy of structures, from the angstrom scale of chemical bonds and micrometer scale of cells, to the centimetre and metre scale of shells, stems and skeletons. Generally, feedback between these scales allows organisms to perform chemical reactions to extract free energy from their environment, enabling their longevity and reproduction. Living cells are found to take a special place in this hierarchy, as they represent the smallest unit of life capable of this operation [1]. An understanding of how cells compartmentalise their constituent molecules is of fundamental interest to those fascinated by life.

At the end of the 19th century, Edmund Wilson delivered a lecture entitled "the structure of protoplasm" [2]. He observed specimens of protoplasmic (intracellular) material, obtained from starfish eggs, and saw the coalescence of spherical globules, leading him to hypothesize "the living protoplasm...is a liquid, or rather a mixture of liquids, ... in which are suspended drops ... of different chemical nature". Dr Wilson very likely bore witness to behaviour now deemed characteristic of intracellular membraneless compartments [3–5]. Distinct from their membrane-bound counterparts, membraneless compartments concentrate specific biomolecules, with their constituent components often in constant dynamic exchange with the surrounding protoplasm [6–12].

Representing a major organizing principle of cellular biochemistry, intracellular membraneless compartments are identifiable in all kingdoms of life [45, 46]. Their discovery arguably dates back even further than the observations of Wilson, nearly 200 years, to

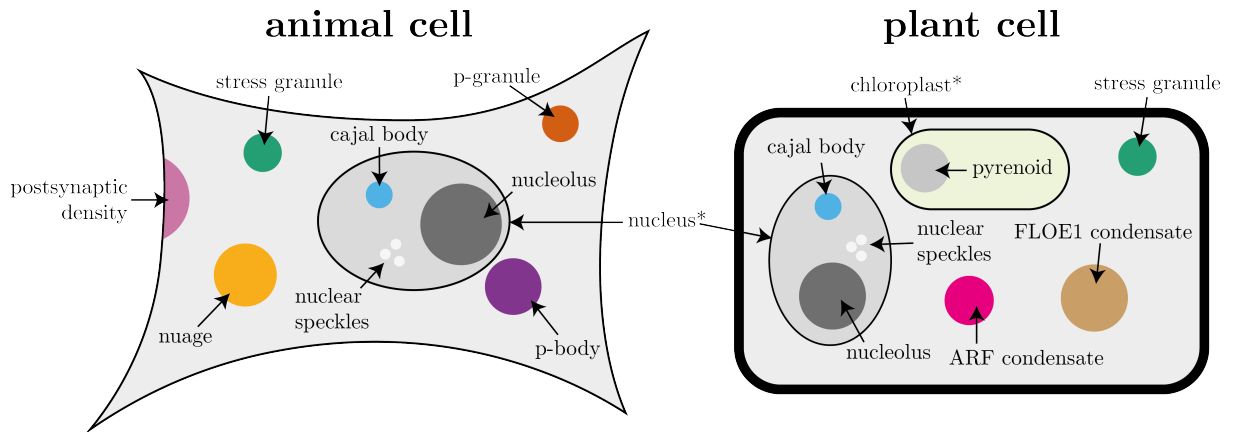


Figure 1.1: Highly simplified diagram of an animal and plant cell highlighting a selection of endogenous biomolecular condensates. *Nuclei and chloroplasts are membrane bound compartments.

Table 1.1: Functions of the endogenous biomolecular condensates illustrated in Figure 1.1.

condensate	function
P-body [13–15]	translational regulation, mRNA decay
P-granule [9, 16, 17]	translational regulation
nuage [18–21]	silencing of retrotransposons
stress granule [12, 14, 22, 23]	translational regulation
cajal body [24–26]	mRNA splicing, telomere maintenance
postsynaptic density [27–29]	neurotransmission
nucleolus [30–32]	ribosome biogenesis
nuclear speckles [33–37]	mRNA splicing
pyrenoid [38–40]	carbon fixation in algae
photobodies [41, 42]	storage of photoreceptors
ARF condensate [43]	auxin signalling
FLOE1 granule [44]	water sensing during seed germination

the observations of nucleoli by Wagner and Valentin [47, 48]. Figure 1.1 and Table 1.1 illustrates and details the function a selection of membraneless compartments found in plant and animal cells. Some membraneless compartments, e.g. nuclear speckles and cajal bodies - both involved in RNA processing [24–26, 33–37], are almost ubiquitous in plants and animals. Other compartments, such as the pyrenoid and postsynaptic density, serving as sites for carbon fixation in algae and neurotransmission respectively, are only found in more highly specialised cells [27–29, 38–40].

Despite the ubiquity of membraneless compartments, a physical understanding of their behaviour eluded researchers for well over a century, until the seminal work of

Brangwynne *et al* in 2009 [9] . By carefully monitoring the redistribution of P-granules in fertilised *Caenorhabditis elegans* oocytes during the first cell division, Brangwynne and co-workers found their formation and dissolution to be entirely consistent with a phase separation event [9]. Furthermore, they observed these membraneless compartments to coalesce, drip and exchange components rapidly with the surrounding intracellular milieu, phenomena reminiscent of a liquid droplet, with interfacial tension, in coexistence with the cytoplasm. They proposed liquid-liquid phase separation as a general mechanism, governing the organization of the protoplasm into membraneless compartments (echoing the view of Wilson).

Liquid-liquid phase separation describes the spontaneous condensation of disperse molecules from solution, to form a new, distinct, liquid phase. Since Brangwynne's discovery, the phase separation hypothesis has proven extremely fruitful, responsible for a surge of research on endogenous membraneless compartments *in vivo*, model phase-separating biomolecular systems *in vitro* and the incorporation of such compartments into synthetic biology systems [3, 4, 49–51]. The field of intracellular phase separation has since settled on the term "biomolecular condensate", coined by Banani *et al* in 2017, to define any liquid-like phase enriched in biomolecules [4].

Much of the current physical understanding of biomolecular condensates has been derived from *in vitro* studies of reconstituted "model" condensate systems. This thesis aims to enhance the molecular understanding of biomolecular condensates, choosing to study the phase separation and interfacial phenomena of an established model condensate. Immediately following is an overview of key results obtained from *in vitro* studies, with emphasis on the molecular detail of protein-protein interactions contributing to condensate integrity. Then we discuss emergent properties of biomolecular condensates, including their interfacial phenomena, to build a picture of functional condensates and give the reader an insight into recent advances. We then identify the outstanding research questions which form the subjects of this thesis. This chapter concludes with an outline of the remaining thesis.

1.2 Model biomolecular condensates & proteins

Proteins are a highly diverse set of biological macromolecules, implicated throughout biochemistry. They catalyse reactions, enact as structural elements, and along with ribose nucleic acids (RNAs), form a major constituent of endogenous biomolecular condensates. It is alterations to the chemical structures of proteins, facilitated by random mutations to deoxyribose nucleic acid (DNA), which enables Darwin's theory of natural selection [52].

Like RNA and DNA, proteins are polymeric, composed of a linear sequence of monomers. Unlike RNA and DNA, which are composed from a mixture of four (ribo)nucleotides, proteins are composed of 20 amino acids, linked together by amide bonds along the protein 'backbone'. Figure 1.2 (a) shows the chemical structure of these amino acids - also referred to as residues. They may be classified based on their functional groups (aromatic, aliphatic, sulfuric, basic, acidic, hydroxylic, amidic), or their typical interaction mode (hydrophobic, charged, polar), and defined by their full chemical name, three letter abbreviation or single letter code. Different sequences therefore impart different potential interactions. Principally, intramolecular interactions may cause the protein to fold into a well defined tertiary structure [53], or remain disordered, a continuum of conformations in solution, akin to random-flight polymers [54, 55].

How the sequences of proteins found inside endogenous biomolecular condensates contribute to their integrity began to unravel following the 2009 work of Brangwynne *et al* [9], when several researchers independently identified macromolecular multivalent intrinsically proteins capable of phase separation *in vitro* [56–60]. Although *in vitro* experiments may not capture the intrinsic complexity of intracellular environments, the control investigators have over the solution compositions and experimental variables have proven them to be a powerful method of investigation in protein science.

In 2015, the intrinsically disordered regions of P-granule protein Laf-1 [57], stress granule protein hnRNPA1 [58] and nuage protein Ddx4 [59], were shown to form liquid-

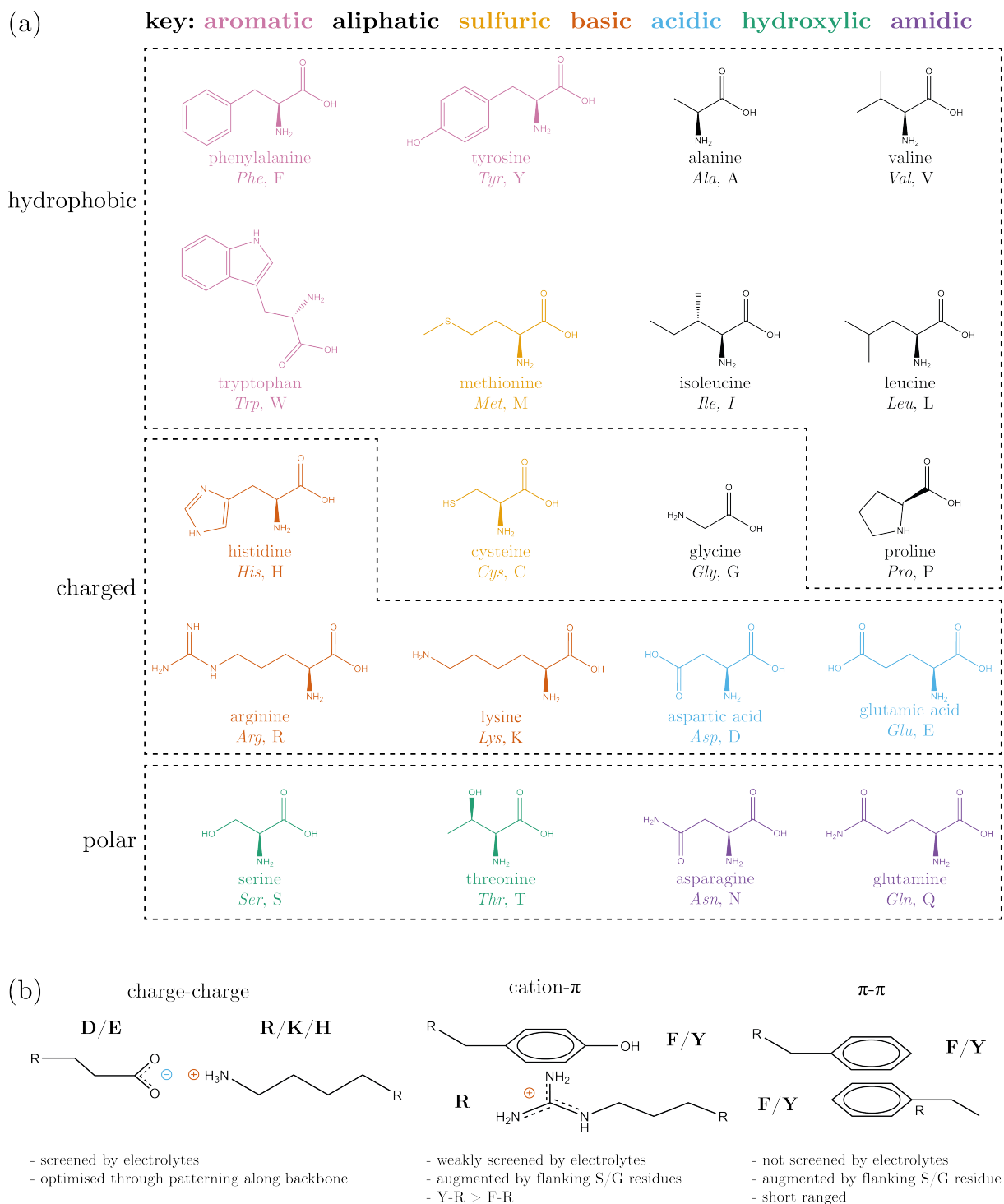


Figure 1.2: (a) The 20 genetically encoded common amino acids with their three letter and single letter codes. Colours categorise amino acids by their functional groups, dashed boxes categorise amino acids by their typical interaction types. Cysteine can form disulfide covalent bonds, glycine is the smallest amino acid, lacking any carbon side chain. (b) Interactions between amino acids shown to contribute to biomolecular condensates formed by phase separation of intrinsically disordered proteins.

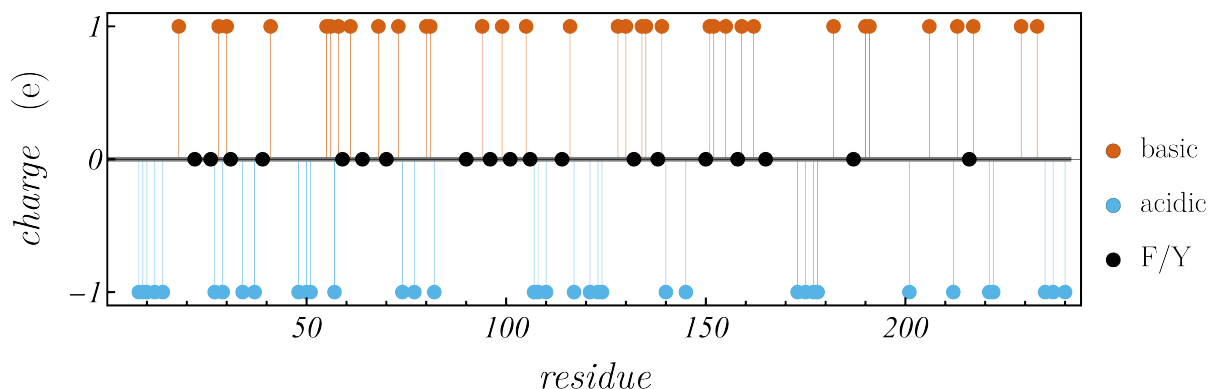


Figure 1.3: Distribution of (charged) acidic and basic residues and phenylalanine (F) and tyrosine (Y) residues along the 241 amino acid sequence of recombinantly expressed Ddx4^N.

like, model protenacious condensates, suspended in aqueous buffer, in a manner inhibited at increased salt concentrations. This effect was attributed to screening of electrostatic interactions between charged residues in a Debye-Hückel fashion [61], revealing the importance of charge-charge contacts in the phase separation of intrinsically disordered proteins (IDPs).

Nott *et al* further investigated the sequence context of acidic and basic residues in Ddx4^N and its contribution to their interactions. They scrambled the positions of the charged residues in the Ddx4^N, disrupting this patterning, and witnessed a marked decrease in its phase separation [59, 62]. The arrangement of charges along the Ddx4^N backbone, appearing as a polyampholyte (see Figure 1.3), seems to be encoded in a fashion which promotes its phase separation. The impact of charge patterning has subsequently been reproduced in studies of other phase separating IDPs, both physiological [63, 64] and synthetic [65–67].

In addition to charged amino acids, phase separating IDPs (including LAF-1, hn-RNPA1 and Ddx4^N) are often enriched in aromatic residues phenylalanine and tyrosine [58, 59, 65, 68]. Patterned along the sequence of Ddx4^N (see Figure 1.3), these residues are able to interact in a hydrophobic manner, releasing their solvating water molecules to form $\pi - \pi$ contacts [62, 69–71]. By investigating the effect of arginine methylation of Ddx4^N phase separation, Nott *et al* uncovered another, now archetypal,

interaction driving IDP phase separation [59, 70, 72, 73]. This methylation greatly hindered its phase separation, in a fashion attributed to direct cation- π interactions between arginine and phenylalanine/tyrosine residues, subsequently verified by nuclear magnetic resonance studies [62]. Similar to $\pi - \pi$ contacts, cation- π interactions are reliant on particular stacking configurations [74], and enhanced when interacting residues are flanked by small amino acids glycine and serine [59]. Mutational studies on model condensates formed by the intrinsically disordered Fused in Sarcoma (FUS) protein, have subsequently demonstrated the increased strength of Y-R cation- π contacts, compared to F-R cation- π contacts, refining the molecular grammar governing biomolecular condensate formation [70].

The major amino acid interactions driving the phase separation of IDPs identified thus far are summarised in Figure 1.2 (b). These interactions have since been implicated in not only *in vitro* studies of IDPs [75–77], but also *in cell* studies of endogenous and exogenous condensates [70, 72]. It is now apparent that whilst containing tens to hundreds of distinct chemical components, the behaviour of endogenous condensates is often dominated by small number of species [78–82], referred to as ‘scaffolds’ [83]. Aside from IDPs, scaffolds may also comprise RNAs. RNAs have been found to have a synergistic effect on phase separation with IDPs; in model condensates formed by LAF-1, Whi3 and hnRNPA1, RNA was seen to modulate both their phase separation and viscosity [57, 58, 60]. A common theme amongst scaffolds, is the need to be macromolecular, enabling the formation of a transient network of stabilising interactions [70, 84, 85]. The ability to dictate cytoplasmic/nucleoplasmic scaffold concentration, also alter their structures through posttranslational modifications, gives cells exquisite control over the regulation of biomolecular condensates. Of course, biomolecular condensates are more than just condensed regions of biomolecules, they are also packaged with a range of emergent properties, which we will now discuss.

1.3 Emergent properties of biomolecular condensates

Representing liquid-like regions of intracellular space enriched in specific biomolecules, biomolecular condensates give rise to a range of emergent properties, with physiological consequences. In Figure 1.4 we separate these into internal (chemical environment and material state) and interfacial phenomena (interfacial tension and wetting).

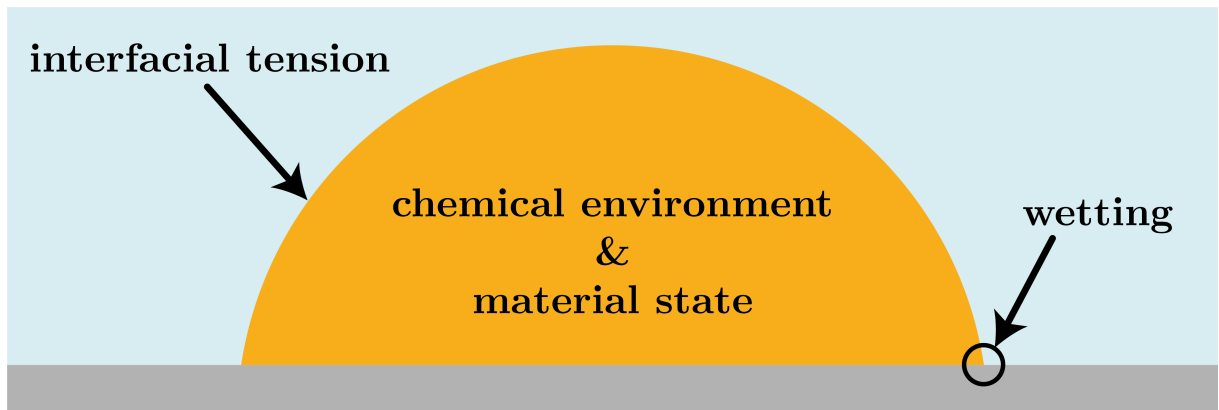


Figure 1.4: Emergent properties of biomolecular condensates. The internal composition of condensates is determinant of their chemical environment and material state. Their interfacial phenomena, (fluid-fluid) interfacial tension and wetting, are governed by interactions at their fluid-fluid interface and three phase contact regions respectively. Orange represents the condensed condensate phase, blue represents its coexisting fluid phase, gray represents a third phase, which may be liquid-like or solid.

1.3.1 Internal phenomena

Likely their most widely utilised feature, the unique chemical environment of endogenous condensates facilitates organization of the intracellular milieu through their partitioning of biomolecules [43, 86–88]. A clear example from biology is the sequestration of translation regulator protein mTORC1 in stress granules. This has the knock-on effect of greatly reducing protein synthesis in the cell, used as a method to combat stress [89, 90]. Partitioning of biomolecules is also demonstrated by *in vitro* phase separating IDP systems, further supporting their utility as model condensates. Ddx4^N condensates partition double stranded nucleic acids in a length dependent manner; absorbing shorter duplexes,

while excluding those longer than 16 base pairs. Furthermore, DNA duplexes inside these Ddx4^N condensates were found to be unwound, demonstrating passive biochemical activity in this highly simplified model system [91].

Whilst the high density of interacting proteins and nucleic acids provides a novel solvent environment, it also serves to restrict molecular motion, with many condensates exhibiting markedly increased viscosities relative to the surrounding aqueous environment [57, 62, 92–95]. So far we have described biomolecular condensates as ‘liquid-like’, this is because condensates may display a range of material states [96, 97]. Jawerth *et al* reported condensates formed by an IDP constituent of P-granules - PGL-3, exhibit gel-like viscoelasticity, behaving as liquids under low strain rates, but solids at high strain rates [98]. Condensates can also age, showing time-dependent material properties [99] and even undergoing deleterious liquid to solid transitions, as seen in endogenous FUS condensates [72, 100]. Reduced liquidity of condensates is not always a bad thing, and is used by keratoyalin granules, for example, to acquire mechanical stability during skin barrier formation [101].

1.3.2 Interfacial phenomena

Opposed to the internal phenomena of condensates, naturally governed by their internal composition, interfacial phenomena are dependent on the composition of the two (or more) phases in contact. A fundamental feature of condensed matter, interfaces are ubiquitous in nature, and are associated with a necessarily positive interfacial tension¹ [102]. The constantly rearranging molecules of liquid-like biomolecular condensates flow, and reorganize their interface to minimize interfacial energy, driving them towards spherical shapes [9, 18, 24, 30, 103]. This same principle drives condensate coalescence and subsequent relaxation into a larger sphere, lowering the amount of (unfavourable) interface present in the system [9, 76, 92, 95, 98, 104–107].

¹A negative interfacial tension would drive the infinite mixing of the contacting phases, eventually forming a single homogenous phase.

High interfacial tension drives rapid coalescence, whilst high condensate viscosity slows it down. It seems biology is aware of this; Caragine *et al* hypothesized the high viscosity (3000 Pas) and low interfacial tension ($1.5 \mu\text{N m}^{-1}$) of nucleoli are tuned to give slow coalescence, leaving the critically important nucleolar processes undisturbed [95]. Cells are also observed to modulate coalescence through the use of interfacial protein clusters acting as Pickering agents, stabilising P-granules with respect to coarsening [108]. Interfacial tension is expected to, and indeed does, impact biomolecular condensate formation through nucleation, with reduced interfacial tension corresponding to accelerated phase separation [105, 109, 110]. This is seen in cell nuclei, where seeding the nucleation of nucleoli on freshly transcribed ribosomal RNA affords spatiotemporal control over nucleolar formation [109], driven by specific protein-RNA interactions which lower interfacial energy.

Favourable interactions of liquids with surfaces can result in entirely localised phase separation, forming phases which would otherwise be unstable [111]. This ‘prewetting’ phenomena, has recently been attributed to the localised formation of Klf4 condensates on DNA [112]. Here, wetting refers to the tendency of a liquid to spread over another liquid or solid phase [102]. Wetting is shown to dictate the interior architecture of the nucleolus; appearing as a condensate-within-a-condensate, the outer liquid-like layer totally wets the inner layer [11]. Total wetting is also used to control the branching of microtubules, where TPX2 granules wet their surface, before transitioning into a beads-on-a-string formation and recruiting tubulin for microtubule growth [113, 114]. While many endogenous condensates are suspended in the cytoplasm/nucleoplasm, some are associated with intracellular membrane surfaces [115]. Localised to specific membrane regions through interactions with membrane proteins or the cortical cytoskeleton, these condensates exhibit partial wetting behaviour [27, 29, 115–125]. Interestingly, specific partial wetting of condensates on membranes has recently been implicated in membrane remodelling, leading to the formation of autophagosomes [124].

1.4 Outstanding research questions

While much progress has been made since Brangwynne’s phase separation hypothesis [9], there is still much to be understood about the physical nature of biomolecular condensates. Many phase separating intrinsically disordered proteins are enriched in charged amino acids, yet carry low net charge per residue [57–59, 70]. Furthermore, net charge is also known to influence protein aggregation [126, 127] and the conformational properties of IDPs [128]. At the onset of this thesis project, we identified protein net charge as an area of interest, representing an uninvestigated sequence feature, likely regulating the formation and properties of biomolecular condensates.

The degree to which the relationship between protein sequence and phase separation is understood has facilitated the design of de-novo phase separating proteins [65, 129], phase separation predictors [130–132] and accurate computational simulations [74, 133, 134]. The same cannot be said for the interfacial phenomena of biomolecular condensates, with many fewer investigations on their molecular origins. The 2018 work of Jawerth *et al* has arguably made the most progress, where they saw salt reduce the fluid-fluid interfacial tension of PGL-3 condensates, implicating electrostatic interactions. In 2021, Wang *et al* [135] cited the lack of user-friendly techniques to measure the fluid-fluid interfacial tension as the reason behind our reduced understanding. Aside from rough estimates [11, 57, 136], there have been no calculations of biomolecular condensate fluid-fluid interfacial tensions, despite the prevalence of such calculations in the soft matter community [137–141].

We also noticed many endogenous biomolecular condensates remain suspended in the protoplasm, away from intracellular membranes. Those that associate with the membrane, are localised by specific interactions with membrane proteins or the cortical cytoskeleton [27, 29, 115–125]. It is possible that protoplasmic condensates are maintained by active processes, or through association with the cytoskeleton, however, we suspect this is due to a lack of wetting at membrane surfaces, governed by the interfacial structure of

condensates.

1.5 Thesis outline

The overarching goal of this thesis is to advance the understanding of the relationship between protein sequence, phase separation and interfacial phenomena of biomolecular condensates. To do so, we base our work around the Ddx4^N model condensate system, previously used to reveal many of the sequence features now known to be widespread amongst intrinsically disordered proteins associated with endogenous condensates [59,62,91].

The *in vitro* work throughout uses a novel mutant Ddx4^N, wherein its four cysteine residues are substituted for serine. This Ddx4^{N*} construct is unable to form disulfide linkages, removing this confounding variable from our studies of condensate interfacial phenomena. The protein sequences and reagents used in this work, as well as standard experimental techniques, including protein expression and purification from bacterial cell culture, are given in **Chapter 2**.

In 2018, the beginning of this thesis project, augmentation of phase separation through F→Y substitutions was recently presented in FUS condensates [70]. We decided to look at the effect of F→Y substitutions on Ddx4^{N*} model condensates. **Chapter 3** comprises application of the Flory-Huggins thermodynamic framework to Ddx4^{N*} phase separation, allowing us to infer the molecular mechanism through which these sequence features tune condensate stability. We also investigate the effect of protein net charge on intracellular phase separation, combining *in vitro* and *in cell* experiments, revealing commonalities between *in vitro* model condensates and exogenous intracellular condensates.

It is common in the study of interfacial phenomena, to measure the shapes of sessile liquid drops in contact with solid surfaces [142,143]. Observation of the deformation of droplets under their own weight enables a measure of fluid-fluid interfacial tension, whilst

measuring the the contact angle between the fluid-fluid surface of drops and solid surfaces allows the characterisation of their wetting. This may be achieved, to high precision, through axisymmetric drop shape analysis (ADSA) [144–148]. In **Chapter 4**, through confocal microscopy and bespoke image analysis, we implement this technique to study microscopic *in vitro* biomolecular condensates. As confocal microscopes are widespread in cell biology and biophysics laboratories, we hope to inspire the use of this technique as a readily implementable method, expanding the existing experimental toolbox used to interrogate emergent condensate properties [149].

Chapter 5 concerns Ddx4^{N*} fluid-fluid interfacial tension. We introduce the mean-field Scheutjens-Fleer theory, an extension of Flory-Huggins theory, previously utilised in the calculation of the interfacial tension of phase separated polymer mixtures [150, 151], and apply it to our Ddx4^{N*} condensates. In conjunction, we present measurements of Ddx4^{N*} capillary length, enabled through ADSA, with a view to comparing these values with those predicted by the mean-field calculations of fluid-fluid interfacial tension.

The final chapter (**Chapter 6**) uses ADSA measurements of Ddx4^{N*} contact angles to investigate the molecular determinants of biomolecular condensate wetting phenomena. By measuring the dependency of contact angles on condensate size, we reveal the role of the structure at the three phase contact line in determining their wetting behaviour. Based on observations of the intracellular localisation of endogenous biomolecular condensates, we hypothesize the presence of a repulsive interaction between condensates and intracellular membranes. To test this hypothesis, we introduce a model membrane, composed of a supported lipid bilayer, into our *in vitro* system. By measuring Ddx4^{N*} contact angles on this surface, as well as on two more surfaces with differing hydrophobicity, we reveal the molecular nature of the condensate-membrane interactions.

Chapter 2

Generation of Materials & Standard Experimental Techniques

ABSTRACT

This thesis studies condensates formed by recombinant intrinsically disordered Ddx4 proteins. This chapter lists the buffers and reagents, as well as the sequences of the recombinant proteins, used throughout this thesis. The methodology allowing the generation of plasmids for subsequent protein expression is given. Then the protocol for the purification of Ddx4 proteins from bacterial cell culture for the in vitro work presented in subsequent chapters is detailed. Finally, the standard sample preparation technique for in vitro experiments is described.

2.1 Buffers & reagents

All buffers are aqueous, prepared using MilliQ ultrapure water with a resistivity of $18.2 \text{ M}\Omega \text{ cm}^{-1}$ at 25°C and maximum total organic content (TOC) of 5 ppm. Small particulates were removed by filtration through MF-Millipore™ $0.22 \mu\text{m}$ CE membranes for all buffers except lysis buffer, which was filtered using $0.45 \mu\text{m}$ Regenerated Cellulose filters. Unless stated otherwise, buffers were stored in autoclaved (121°C for 15 min) Duran® bottles, placed in cupboards at room temperature for short term storage, or at 4°C for long term storage.

10× phosphate pH 7.4: $5.24 \text{ g L}^{-1} \text{ NaPO}_4 \cdot \text{H}_2\text{O}$, $28.83 \text{ g L}^{-1} \text{ Na}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. pH measured using

Nickel-A: one part in ten 10× phosphate buffer providing 20 mM Na_2PO_4 , 500 mM NaCl, 20 mM imidazole. pH adjusted to range 7.4 - 8 using HCl or NaOH, verified by dropping a few μL onto Fisherbrand™ pH Indicator Paper Sticks.

Nickel-B: one part in ten 10× phosphate buffer providing 20 mM Na_2PO_4 , 500 mM NaCl, 500 mM imidazole. pH adjusted to range 7.4 - 8 using HCl or NaOH, verified by dropping a few μL onto Fisherbrand™ pH Indicator Paper Sticks.

Lysis buffer: one part in ten 10× phosphate buffer providing 20 mM Na_2PO_4 , 6 M GdmCl, 10 mM imidazole.

High imidazole buffer: 5 M imidazole, one part in ten 10× phosphate buffer providing 20 mM Na_2PO_4 , 500 mM NaCl. pH adjusted to 8.5 by addition of HCl, verified by dropping a few μL onto Fisherbrand™ pH Indicator Paper Sticks. Prepared in quantities of 5 mL and stored in 15 mL Greiner Bio-One™ CELLSTAR™ Test Tubes at -20°C .

1 M DTT: Prepared in quantities of 5 mL and stored in 15 mL Greiner Bio-One™ CELLSTAR™ Test Tubes at -20°C .

TAE buffer: To make 1 L use 4.84 g TRIS, 1.142 mL acetic acid, 0.372 g ethylenediaminetetraacetic acid (EDTA).

Ddx4^{N*} storage buffer: 20 mM Tris_(aq) (1.907 g L⁻¹ tris HCl, 0.945 g L⁻¹ Trizma base), 300 mM NaCl. pH verified using a Beckman Coulter PHI 350 Digital pH / Temperature / mV Meter as 8.0 at 22 °C.

Ddx4^N reducing storage buffer: 20 mM TRIS (1.907 g L⁻¹ TRIS HCl, 0.945 g L⁻¹ Trizma base), 300 mM NaCl, 5 mM TCEP. pH adjusted using to 8.0 at 22 °C by addition of NaOH, verified using a Beckman Coulter PHI 350 Digital pH / Temperature / mV Meter.

2× TRIS buffer: 3.814 g L⁻¹ TRIS HCl, 1.890 g L⁻¹ Trizma base. Gives a solution of 20 mM TRIS pH 8.0 at 22 °C when diluted 1:1 with MilliQ ultrapure water (verified using a Beckman Coulter PHI 350 Digital pH / Temperature / mV Meter).

100 mM CaCl₂ 15% (v/v) glycerol: 50 mL prepared and autoclaved at 121 °C for 15 min.

SUV buffer: 150 mM NaCl 2 mM CaCl₂.

Terrific Broth (glycerol free): Prepared in 1 L quantities by Oxford Biochemistry Dept. MGSL. 12 g tryptone, 24 g yeast extract, 9.4 g KH₂PO₄, 1.1 g K₂HPO₄, distilled water. Autoclaved at 121 °C for 15 min.

AIM solution I: 750 mM Na₂HPO₄, 750 mM KH₂PO₄, 1.5 M NH₄Cl, 150 mM Na₂SO₄.

AIM solution II: 0.75 (w/v)% D-glucose, 60 (w/v)% glycerol.

AIM solution III: 10 (w/v)% α-lactose monohydrate.

AIM solution IV: 1 M MgSO₄.

Auto induction media: Prepared in 1 L quantities composed of 905 mL Terrific Broth (glycerol free), 33 mL AIM solution I, 20 mL AIM solution II, 40 mL AIM solution III and

2 mL AIM IV.

50 mg mL⁻¹ kanamycin: Prepared using kanamycin sulfate from *Streptomyces*. Stored as 1 mL aliquots in 1.5 mL Eppendorf® tubes at -20°C .

LB high salt: Prepared by Oxford Biochemistry Dept. MGSL. 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl. Autoclaved at 121°C for 15 min.

Alexa-488: Stocks were prepared by resuspending Alexa Fluor™ (Thermo Fisher Scientific™) crystals in nuclease free water (Invitrogen™), syringe filtering through a 0.22 μm membrane and stored at -20°C as 20 μL aliquots in PCR tubes.

Cy3B: Stocks were prepared by resuspending Cy3B acid (AAT Bioquest) crystals in nuclease free water, syringe filtering through a 0.22 μm membrane and stored at -20°C as 20 μL aliquots in PCR tubes.

2.2 Spectrophotometry

Protein and nucleic acid concentrations were measured on a 1 μL using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific™) by recording the absorbance A at 260 nm and 280 nm respectively. The Beer-Lambert law [152],

$$A = \epsilon cl, \tag{2.1}$$

then gives the species concentration, c , as a function of path length l and the wavelength specific molar decadic extinction coefficient, ϵ . The representative concentration of a sample was determined as the mean of 5 replicates.

The concentrations of fluorescent dyes, Alexa-488 and Cy3B, were determined using a NanoDrop® ND-1000 Spectrophotometer, recording the absorbance at 495 nm and 558 nm. Extinction coefficients $73\,000\text{ cm}^{-1}\text{ mol}^{-1}$ and $120\,000\text{ cm}^{-1}\text{ mol}^{-1}$ were used with

Equation 2.1 to retrieve the species concentration.

The optical density (OD600) of bacterial cell culture was measured as absorbance at 600 nm, using an Ultrospec 2100 pro UV/Visible Spectrometer (Amersham Biosciences).

2.3 Gel electrophoresis

To assess the content of protein samples, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12 or 17-well precast Invitrogen™ NuPAGE™ 4-12%, Bis-Tris polyacrylamide gels. Samples were prepared at a ratio 3:1 with Invitrogen™ NuPAGE™ LDS Sample Buffer (4X), before boiling at 95 °C for 1 min. 10 µL of samples were then loaded onto gels, along with 8 µL PageRuler™ Unstained Protein Ladder, serving as a standard, before running the gel at 200 V for 35 min. Gels were stained overnight with InstantBlue® Coomassie.

The content of DNA samples were assessed using a 1% agarose gel, prepared by mixing 0.7 g agarose with 60 mL TAE buffer. This was then microwaved at 600 W until all agarose was dissolved. 5 µL of SYBYR Safe was then added to the agarose solution, which was then poured into a mold and allowed to set. 10 µL sample was then loaded to each lane, along with purple loading dye (New England Biolabs® inc.) at a 6× dilution. 10 µL standard was also loaded, prepared as 1 µL 1 kb ladder (New England Biolabs® inc.), 10 µL Nuclease free water (Invitrogen™). Agarose gels for DNA analysis were ran at 100 V for 40 min.

2.4 Protein sequences

The work in this thesis expands on previous work done by Nott *et al* on Ddx4^N [59,62,91], who's sequence corresponds to the first 236 residues of the consensus human sequence of

DEAD-box RNA helicase Ddx4, flanked by a cloning artefact GAMGS at its N-terminus. The primary sequence of this construct contains four cysteine residues, which promote multimerisation through the formation of covalent disulfide bonds. Often, this is alleviated *in vitro* through the addition of a reducing agent, such as DTT or TCEP, to the solution. However, reducing agents have a limited lifespan and are oxidised by atmospheric O₂. To remove any possibility of multimerisation during the course of the drop shape analysis experiments performed in Chapters 4 and 5, a cysteine-less mutant of Ddx4^N, referred to as Ddx4^{N*} throughout this thesis, was generated by substituting the four cysteine residues in the wild-type sequence with serine. This is a common substitution in protein science, owing to their similar chemical properties, with serine being slightly smaller, more polar and unable to crosslink. As such, the effect of the mutation on the Ddx4^N proteins' phase separation is expected to be mild.

The dimerisation propensity of Ddx4^{N*} was compared to Ddx4^N by defrosting two recombinantly expressed and purified aliquots, leaving them at room temperature, then taking samples at time intervals and freezing them with gel loading dye. The samples were analysed using SDS-PAGE, with the result shown in Figure 2.1. Ddx4^N shows multimerisation as soon as 10 min after removal from cryogenic storage, despite the protein being stored with 5 mM TCEP, a common reducing agent. There was no multimerisation visible, or obvious decay of Ddx4^{N*} observed up to one week after removal from storage.

The sequences of the five recombinantly expressed Ddx4^{N*} mutants studied in this work, along with Ddx4^N are shown in Figure 2.2. The assigned names refer to the length of wild-type sequence in the construct, noting that the true sequence length is the stated number plus five, due to the GAMGS cloning artefact. In Chapter 3 *in cell* experiments are presented, where mutants of Ddx4^N are expressed through transient transfection. These sequences contain length mutants of the N-terminal disordered tail of Ddx4^N fused to yellow fluorescent protein mCitrine, followed by the C-terminal disordered tail of Ddx4^N. Figure 2.3 gives these sequences, including mCitrine.

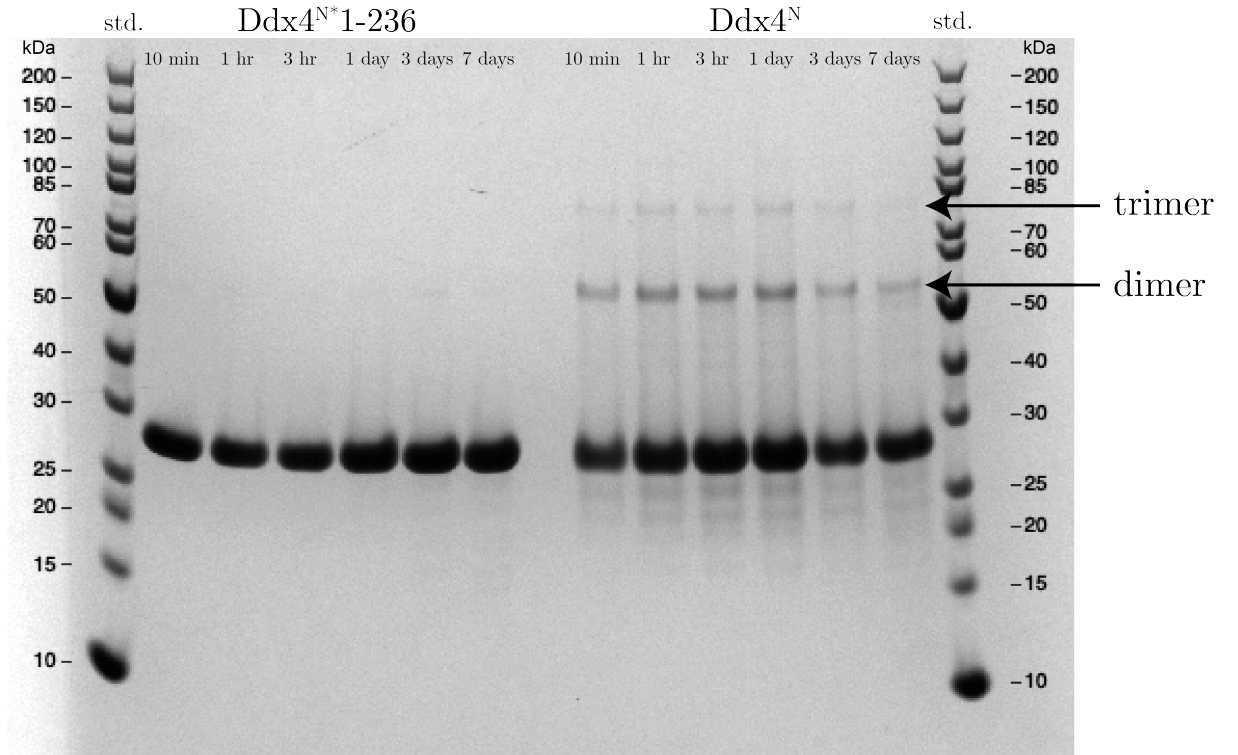


Figure 2.1: SDS-PAGE gel showing multimerisation of recombinantly expressed and purified Ddx4^{N} is removed by a full sequence cysteine to serine substitution.

Table 2.1: Properties of the recombinantly expressed protein sequences used in this work.

sequence	no. amino acids	Mr (kDa)	ϵ ($\text{M}^{-1} \text{cm}^{-1}$)
$\text{Ddx4}^{\text{N*}} 1-229$	234	25109.54	23950
$\text{Ddx4}^{\text{N*}} 1-231$	236	25309.74	23950
$\text{Ddx4}^{\text{N*}} 1-234$	239	25552.96	23950
$\text{Ddx4}^{\text{N*}} 1-236$	241	25769.15	23950
$\text{Ddx4}^{\text{N*}} 1-236 \text{ F} \rightarrow \text{Y}$	241	25993.14	44810
Ddx4^{N}	241	25833.39	23950

	1	10	20	30	40	50
Ddx4N	GAMGSMGDEDWEAEINPHMSSYVPIFEKDRYSGENGDNFNRTPASSSEMD					
Ddx4N*1-236	GAMGSMGDEDWEAEINPHMSSYVPIFEKDRYSGENGDNFNRTPASSSEMD					
Ddx4N*1-234	GAMGSMGDEDWEAEINPHMSSYVPIFEKDRYSGENGDNFNRTPASSSEMD					
Ddx4N*1-231	GAMGSMGDEDWEAEINPHMSSYVPIFEKDRYSGENGDNFNRTPASSSEMD					
Ddx4N*1-229	GAMGSMGDEDWEAEINPHMSSYVPIFEKDRYSGENGDNFNRTPASSSEMD					
Ddx4N*1-236FtoY	GAMGSMGDEDWEAEINPHMSSYVPIYEKDRYSGENGDNYNRTPASSSEMD					

	60	70	80	90	100
Ddx4N	DGP SRRDHEFMKSGFASGRNFGNRDAGECNKRDNTSTMGGFGVGKSFGNRG				
Ddx4N*1-236	DGP SRRDHEFMKSGFASGRNFGNRDAGESNKRDNNTSTMGGFGVGKSFGNRG				
Ddx4N*1-234	DGP SRRDHEFMKSGFASGRNFGNRDAGESNKRDNNTSTMGGFGVGKSFGNRG				
Ddx4N*1-231	DGP SRRDHEFMKSGFASGRNFGNRDAGESNKRDNNTSTMGGFGVGKSFGNRG				
Ddx4N*1-229	DGP SRRDHEFMKSGFASGRNFGNRDAGESNKRDNNTSTMGGFGVGKSFGNRG				
Ddx4N*1-236FtoY	DGP SRRDHYMKSGYASGRNYGNRDAGESNKRDNNTSTMGGYGVGKSYGNRG				

	110	120	130	140	150
Ddx4N	FSNSRFEDGDSSGFWRRESSNDCEDNPTNRNRF SKRGGYRDGNNSEASGPY				
Ddx4N*1-236	FSNSRFEDGDSSGFWRRESSNDS EDNPTNRNRF SKRGGYRDGNNSEASGPY				
Ddx4N*1-234	FSNSRFEDGDSSGFWRRESSNDS EDNPTNRNRF SKRGGYRDGNNSEASGPY				
Ddx4N*1-231	FSNSRFEDGDSSGFWRRESSNDS EDNPTNRNRF SKRGGYRDGNNSEASGPY				
Ddx4N*1-229	FSNSRFEDGDSSGFWRRESSNDS EDNPTNRNRF SKRGGYRDGNNSEASGPY				
Ddx4N*1-236FtoY	YSNSRYEDGDSSGYWRRESSNDS EDNPTNRNGY SKRGGYRDGNNSEASGPY				

	160	170	180	190	200
Ddx4N	RRGGRGSFRGCRGGFGLGSPNNDLDPDECMQRTGGLF GSRRPVLSGTGNG				
Ddx4N*1-236	RRGGRGSFRGSRGGFGLGSPNNDLDPDESMQRTGGLF GSRRPVLSGTGNG				
Ddx4N*1-234	RRGGRGSFRGSRGGFGLGSPNNDLDPDESMQRTGGLF GSRRPVLSGTGNG				
Ddx4N*1-231	RRGGRGSFRGSRGGFGLGSPNNDLDPDESMQRTGGLF GSRRPVLSGTGNG				
Ddx4N*1-229	RRGGRGSFRGSRGGFGLGSPNNDLDPDESMQRTGGLF GSRRPVLSGTGNG				
Ddx4N*1-236FtoY	RRGGRGSYRGSRGGYGLGSPNNDLDPDESMQRTGGLYGSRRPVLSGTGNG				

	210	220	230	240
Ddx4N	DTSQSRSGSGSERGGYKGLNEEVITGSGKNSWKSEAEGGES			
Ddx4N*1-236	DTSQSRSGSGSERGGYKGLNEEVITGSGKNSWKSEAEGGES			
Ddx4N*1-234	DTSQSRSGSGSERGGYKGLNEEVITGSGKNSWKSEAEGG..			
Ddx4N*1-231	DTSQSRSGSGSERGGYKGLNEEVITGSGKNSWKSEA.....			
Ddx4N*1-229	DTSQSRSGSGSERGGYKGLNEEVITGSGKNSWKS.....			
Ddx4N*1-236FtoY	DTSQSRSGSGSERGGYKGLNEEVITGSGKNSWKSEAEGGES			

```

1      10      20      30      40      50      60
Ddx4WT1-236mCitrineDdx4WT676-724 MGDEDWEAEINPHMSSYVPIFEKDRYSGENGDNFNRTPASSSEMDDGPSRRDHFMSGFA
Ddx4WT1-234mCitrineDdx4WT676-724 MGDEDWEAEINPHMSSYVPIFEKDRYSGENGDNFNRTPASSSEMDDGPSRRDHFMSGFA
Ddx4WT1-231mCitrineDdx4WT676-724 MGDEDWEAEINPHMSSYVPIFEKDRYSGENGDNFNRTPASSSEMDDGPSRRDHFMSGFA
Ddx4WT1-229mCitrineDdx4WT676-724 MGDEDWEAEINPHMSSYVPIFEKDRYSGENGDNFNRTPASSSEMDDGPSRRDHFMSGFA
mCitrine .....

70      80      90      100     110     120
Ddx4WT1-236mCitrineDdx4WT676-724 SGRNFGNRDAGECNKRDNSTMGGFVGKSFNGRGFSNSRFEDGSSGFWRESNDCEDN
Ddx4WT1-234mCitrineDdx4WT676-724 SGRNFGNRDAGECNKRDNSTMGGFVGKSFNGRGFSNSRFEDGSSGFWRESNDCEDN
Ddx4WT1-231mCitrineDdx4WT676-724 SGRNFGNRDAGECNKRDNSTMGGFVGKSFNGRGFSNSRFEDGSSGFWRESNDCEDN
Ddx4WT1-229mCitrineDdx4WT676-724 SGRNFGNRDAGECNKRDNSTMGGFVGKSFNGRGFSNSRFEDGSSGFWRESNDCEDN
mCitrine .....

130     140     150     160     170     180
Ddx4WT1-236mCitrineDdx4WT676-724 PTRNRGFSKRGGYRDGNNSEASGPYRRGGGSGFRGCRGGFGLGSPNNLDLPDECMQRTGG
Ddx4WT1-234mCitrineDdx4WT676-724 PTRNRGFSKRGGYRDGNNSEASGPYRRGGGSGFRGCRGGFGLGSPNNLDLPDECMQRTGG
Ddx4WT1-231mCitrineDdx4WT676-724 PTRNRGFSKRGGYRDGNNSEASGPYRRGGGSGFRGCRGGFGLGSPNNLDLPDECMQRTGG
Ddx4WT1-229mCitrineDdx4WT676-724 PTRNRGFSKRGGYRDGNNSEASGPYRRGGGSGFRGCRGGFGLGSPNNLDLPDECMQRTGG
mCitrine .....

190     200     210     220     230     240
Ddx4WT1-236mCitrineDdx4WT676-724 LFGSRRPVLSTGTNGDTSQSRSGSGSERGGYKGLNEEVITSGSKNSWKS...EAEGGES...SDMV
Ddx4WT1-234mCitrineDdx4WT676-724 LFGSRRPVLSTGTNGDTSQSRSGSGSERGGYKGLNEEVITSGSKNSWKS...EAEGG...SDMV
Ddx4WT1-231mCitrineDdx4WT676-724 LFGSRRPVLSTGTNGDTSQSRSGSGSERGGYKGLNEEVITSGSKNSWKS...EAE...SDMV
Ddx4WT1-229mCitrineDdx4WT676-724 LFGSRRPVLSTGTNGDTSQSRSGSGSERGGYKGLNEEVITSGSKNSWKS...E...SDMV
mCitrine .....

250     260     270     280     290     300
Ddx4WT1-236mCitrineDdx4WT676-724 SKGEELFTGVVPIILVELDGDVNGHKFSVSSEGEEDATYKLTTLKFICTTGKLPVPWPTLV
Ddx4WT1-234mCitrineDdx4WT676-724 SKGEELFTGVVPIILVELDGDVNGHKFSVSSEGEEDATYKLTTLKFICTTGKLPVPWPTLV
Ddx4WT1-231mCitrineDdx4WT676-724 SKGEELFTGVVPIILVELDGDVNGHKFSVSSEGEEDATYKLTTLKFICTTGKLPVPWPTLV
Ddx4WT1-229mCitrineDdx4WT676-724 SKGEELFTGVVPIILVELDGDVNGHKFSVSSEGEEDATYKLTTLKFICTTGKLPVPWPTLV
mCitrine .....

310     320     330     340     350     360
Ddx4WT1-236mCitrineDdx4WT676-724 TTFGYGLMCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEEDTLVN
Ddx4WT1-234mCitrineDdx4WT676-724 TTFGYGLMCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEEDTLVN
Ddx4WT1-231mCitrineDdx4WT676-724 TTFGYGLMCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEEDTLVN
Ddx4WT1-229mCitrineDdx4WT676-724 TTFGYGLMCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEEDTLVN
mCitrine .....

370     380     390     400     410     420
Ddx4WT1-236mCitrineDdx4WT676-724 RIELKGIDFKEDGNILGHKLEYNYNSHNHYIMADKQKNGIKVNFKIRHNIEDGSVQLADH
Ddx4WT1-234mCitrineDdx4WT676-724 RIELKGIDFKEDGNILGHKLEYNYNSHNHYIMADKQKNGIKVNFKIRHNIEDGSVQLADH
Ddx4WT1-231mCitrineDdx4WT676-724 RIELKGIDFKEDGNILGHKLEYNYNSHNHYIMADKQKNGIKVNFKIRHNIEDGSVQLADH
Ddx4WT1-229mCitrineDdx4WT676-724 RIELKGIDFKEDGNILGHKLEYNYNSHNHYIMADKQKNGIKVNFKIRHNIEDGSVQLADH
mCitrine .....

430     440     450     460     470     480
Ddx4WT1-236mCitrineDdx4WT676-724 YQONTPIGDGPVLLPDNHLYLSYQSKLSKDPNEKRDHMLLEFVTAAGIT...TSTYIPGFSGS
Ddx4WT1-234mCitrineDdx4WT676-724 YQONTPIGDGPVLLPDNHLYLSYQSKLSKDPNEKRDHMLLEFVTAAGIT...TSTYIPGFSGS
Ddx4WT1-231mCitrineDdx4WT676-724 YQONTPIGDGPVLLPDNHLYLSYQSKLSKDPNEKRDHMLLEFVTAAGIT...TSTYIPGFSGS
Ddx4WT1-229mCitrineDdx4WT676-724 YQONTPIGDGPVLLPDNHLYLSYQSKLSKDPNEKRDHMLLEFVTAAGIT...TSTYIPGFSGS
mCitrine .....

490     500     510
Ddx4WT1-236mCitrineDdx4WT676-724 TRGNVFASVDTRKGGKSTLNTAGFSSSSQAPNPVDDDESVD
Ddx4WT1-234mCitrineDdx4WT676-724 TRGNVFASVDTRKGGKSTLNTAGFSSSSQAPNPVDDDESVD
Ddx4WT1-231mCitrineDdx4WT676-724 TRGNVFASVDTRKGGKSTLNTAGFSSSSQAPNPVDDDESVD
Ddx4WT1-229mCitrineDdx4WT676-724 TRGNVFASVDTRKGGKSTLNTAGFSSSSQAPNPVDDDESVD
mCitrine .....

```

Figure 2.3: Sequence alignments for the Ddx4 sequences used in the *in vivo* work in Chapter 3. Figure generated using ESPrpt.

2.5 Molecular cloning

The condensate forming proteins studied in this work were obtained through expression and purification from bacterial cell culture. This is enabled through standard molecular biology techniques, where the genes encoding the protein are delivered to *Escherichia coli* (*E. coli*) cells as plasmids, which are transcribed and translated by the bacteria as they proliferate. Nuclease free water was provided by Invitrogen™. All procedures involving cell culture were performed under a flame, to minimise contamination.

2.5.1 Generation of chemically competent cells

E. coli cells were made chemically competent (able to uptake DNA plasmids) by inoculating 5 mL LB high salt with 5 μ L of cell culture. This was incubated overnight at 37 °C with 200 rpm shaking. 100 μ L LB high salt was inoculated with 1 mL of overnight culture and incubated at 37 °C with 200 rpm shaking. The optical density was monitored at 600 nm (OD600), using fresh LB high salt to blank the measurement, until the OD600 was in the range 0.3-0.5. The culture was then placed on ice for 20 min before centrifugation in two pre-chilled conical tubes at 1962 rcf for 10 min at 4 °C. The supernatant was discarded and the pellets drained for 2 min before resuspension in 100 μ L CaCl₂ 15% (v/v) glycerol (10 mL per tube). Resuspended pellets were incubated on ice for 20 min and centrifuged at 1962 rcf for 10 min at 10 °C. Again, supernatants were discarded and pellets drained for 2 min. Pellets were resuspended in 100 μ L CaCl₂ 15% (v/v) glycerol (2 mL per tube) and 100 μ L aliquots in 1.5 mL Eppendorf® tubes were flash frozen in liquid nitrogen for storage at -80 °C.

2.5.2 Transformation of *E. Coli*

Plasmids used for protein expression carried a kanamycin resistance gene, serving to ensure only bacteria that have been successfully transformed are able to proliferate in the presence of the antibiotic. 50 ng plasmid was added to 50 μ L competent cells. After 30 min on ice, cells were subject to a 45 s heat shock at 42°C. Immediately following the heat shock, cells were returned to ice for 2 min, before incubation at with 900 μ L LB high salt at 37°C with 200 rpm shaking. 150 μ L culture was spread onto a 50 μ g mL⁻¹ kanamycin LB high salt agar plate. 150 μ L was added to another, equivalent plate, serving as a control. Both plates were incubated overnight at 37°C. A successful transformation shows colonies on the plate inoculated with *E. coli* and none on the control. Successful transformations were stored at 4°C until use.

Two *E. Coli* cell lines are transformed in this work. DH5 α cells, engineered by Douglas Hanahan, show a high transformation efficiency [153] and reduced degradation of inserted plasmids, making them suitable for molecular cloning. BL21(DE3) cells, growing to high densities and deficient in proteases [154,155], are used for protein expression and purification.

2.5.3 Gene amplification

A Ddx4^{N*} 1-236 gene was synthesised by GenScript, codon optimised for bacterial expression, in a pUC57 vector. The double stranded DNA encoding the Ddx4^{N*} length variants were obtained through amplification by the polymerase chain reaction (PCR), with primers synthesised by Merck. Four PCRs were set up, each with a 5' primer encoding the DNA sequence targeted by the BamHI restriction enzyme, and 3' primer, with overhang encoding the stop codon (ATT) and DNA sequence targeted by the XhoI restriction enzyme. Reactions were performed following the protocol detailed at <https://international.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase->

m0491, using a 25 μL reaction volume with 10 ng of template DNA. After completion, 1 μL DpnI was added to each PCR mixture to digest the methylated template DNA.

A gene encoding Ddx4^{N*} 1-236 F $\rightarrow$ Y, also synthesised by GenScript was amplified by PCR. The primers used bound to the 5' and 3' ends of the gene with overhangs encoding the BamHI and XhoI restriction sites respectively.

2.5.4 Restriction digest & ligation

The host vector was digested by making a solution containing 20 μL pET-SUMO 2HTB 800 ng μL^{-1} , 1 μL BamHI-HF, 1 μL XhoI, 3 μL CutSmart[®] Buffer (New England Biolabs[®] inc.) and 5 μL nuclease free water. The target gene was digested by preparing a reaction containing 5 μL PCR amplified DNA, 3 μL CutSmart Buffer[®], 1 μL BamHI-HF, 1 μL and 20 μL nuclease free water.

After 1 h, restriction digests were run down a 1% agarose gel at 100 V for 40 min, separating the nucleic acid reaction products. The bands containing the digested pET-SUMO 2HTb and target gene were cut out and purified using a QIAquick Gel Extraction Kit. The ligation reaction was set up containing 80 ng digested pET-SUMO 2HTb, 10 $\times$ T4 DNA Ligase Buffer (Thermo Scientific[™]), 1 μL T4 ligase, and sequence to be inserted at a 3:1 molar ratio to the pET-SUMO 2HTb template, and made up to 10 μL with nuclease free water. After 1 h at room temperature, the ligation reaction mixture was transformed into competent DH5 α cells.

5 mL LB high salt containing 5 μL 50 mg mL^{-1} kanamycin was inoculated with one colony of transformed DH5 α cell and incubated overnight at 37 °C with 200 rpm shaking. Overnight starter cultures were centrifuged at 2800 rcf for 10 min, discarding the supernatant. The ligation product was purified from the pellet using a QIAprep[®] Spin Miniprep Kit. The Source BioScience sequencing service was used to verify plasmid DNA through Sanger sequencing [156, 157].

2.6 Protein expression

Figure 2.4 (a) shows the pET-SUMO 2HTb plasmid, encoding the His-tagged ($6\times$ histidine at the N-terminus) Ddx4^{N*} SUMO fusion protein, under the control of the T7 expression system [153, 158]. BL21 cells carry the T7 RNA polymerase gene, whose gene product transcribes several times faster than endogenous *E. coli* RNA polymerase, and is highly specific for its promoter DNA sequence. The DNA sequence encoding both the T7 RNA polymerase gene and the Ddx4^{N*} fusion protein on the pET-SUMO 2HTb plasmid is immediately preceded by the lac operator, shown adjacent to the ribosome binding site (RBS) in Figure 2.4 (a). The lac repressor, a DNA binding protein encoded by the pET-SUMO 2HTb plasmid, binds tightly to the lac operator sequence, repressing transcription by allosterically inhibiting the binding of RNA polymerase. Thus, whilst the lac repressor is bound, there is effectively no transcription of T7 polymerase RNA and the Ddx4^{N*} fusion protein.

When *E. coli* cells grow to a high cell density in auto induction media [159] and the glucose feedstock is depleted, their metabolic profile switches to α -lactose. The uptake of α -lactose binds to the lac repressor, triggering a conformational change in the DNA binding protein, preventing it from repressing the transcription of T7 polymerase and the Ddx4^{N*} fusion protein. mRNA encoding the fusion protein is then synthesised by T7 polymerase, which is subsequently translated from the ribosome binding site (RBS) to the stop codon (STP), producing the Ddx4^{N*} fusion protein. T7 is such an active enzyme, that once expressed, the translational profile of the cell is dominated by mRNA produced by the enzyme, inhibiting further culture proliferation [153]. Triggering T7 expression and transcription at high cell densities therefore gives the greatest yield of target proteins.

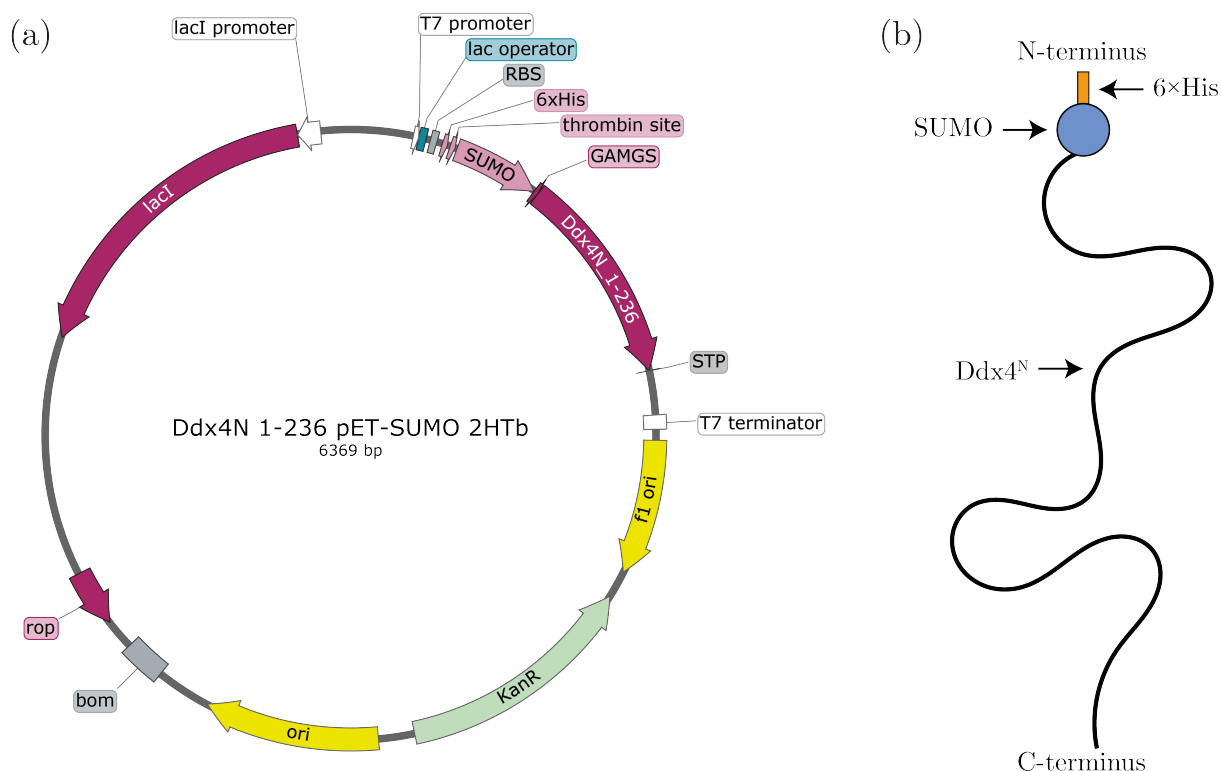


Figure 2.4: (a) Vector map of the plasmid, produced by restriction cloning, used to express Ddx4^N and Ddx4^{N*} proteins. (b) Cartoon of the gene product of (a), showing the 6×His-tag, the folded small ubiquitin-like modifier (SUMO) and the Ddx4^{N*} disordered chain. During protein purification, ULP1 cleaves at the C-terminus of SUMO.

2.6.1 Protocol

50 mL LB High Salt with 50 μ L 50 mg mL⁻¹ kanamycin was inoculated with 1 colony of BL21(DE3) cells transformed with the pET-SUMO 2HTb plasmid encoding the target protein. The inoculated media was incubated at 37°C with 200 rpm shaking overnight, generating a starter culture. To 1 L auto induction media in sterile 2.5 L Ultra Yield[®] grow-up flasks (Thomson), 1 mL 50 mg mL⁻¹ kanamycin was added, along with 25 mL starter culture (2 L of grow-up per starter culture). The flasks were incubated at 37°C with 200 rpm shaking until the OD₆₀₀ reached a value of 2 (roughly 3 h), at which point the temperature on the incubator was reduced to 18°C and cells were left to grow and express overnight. Grow-ups were decanted into a 1 L centrifuge tube and spun at 4000 rcf for 15 min in an Avanti[®] J-26 XP Centrifuge (Beckman Coulter[®]). The supernatant was decanted and cell pellets were scraped into freezer bags and stored at -80°C.

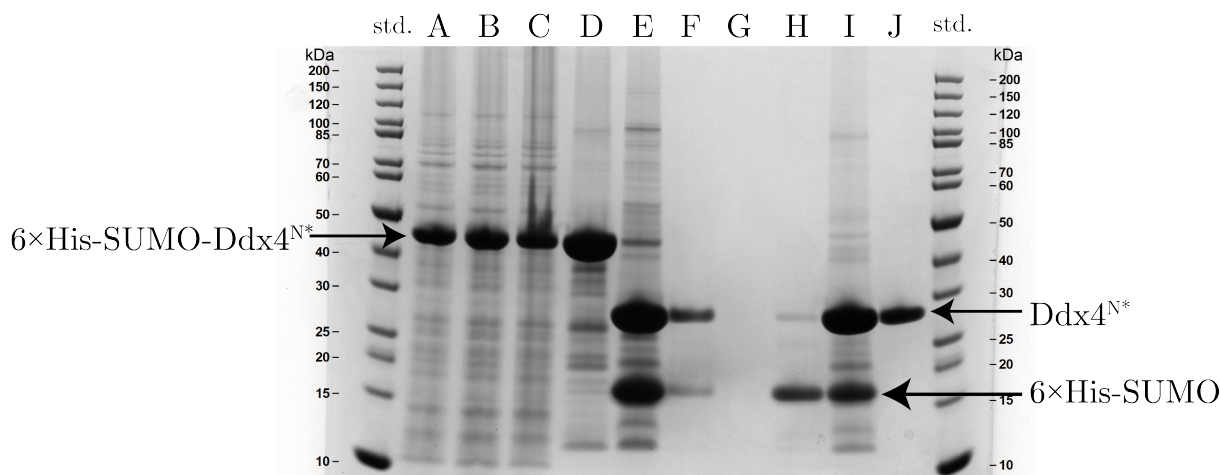


Figure 2.5: SDS-PAGE gel showing the purification of Ddx4^{N*} 1-231. Lane labels correspond to 9 μ L taken at sampling points during the protocol (see text). Lane J contains 1.5 μ g purified protein.

2.7 Protein purification

The Ddx4^{N*} fusion protein displayed in Figure 2.4 (b) carries a 6 $\times$ His-tag and a small soluble folded domain, SUMO. The 6 $\times$ His-tag chelates Ni(II) centres bound to agarose beads during the purification process, allowing all other proteins in the cell lysate to flow over the beads. Imidazole is used to outcompete the 6 $\times$ His-tag for the Ni(II), liberating bound species. The SUMO domain inhibits protein aggregation during the purification procedure. The target Ddx4^{N*} protein is obtained by cleaving at the C-terminus of SUMO with 6 $\times$ His-tagged ubiquitin-like-specific protease 1 (ULP1), previously recombinantly expressed and purified by other Nott lab members.

Samples were taken for SDS-PAGE throughout the purification, at points labelled **A**, **B**, **C** etc.. The labels correspond to the lanes in the exemplary gel displayed in Figure 2.5. The protocol below details purification of Ddx4^{N*}, purification of Ddx4^N (containing 4 cysteine residues) follows the same procedure, except DTT is added to the lysis buffer, Nickel-A and Nickel-B buffers to a concentration of 10 mM. Ddx4^N is also purified into Ddx4^N reducing storage buffer.

2.7.1 Protocol

6-8 g BL21(DE3) cells expressing the target protein were resuspended in 60 mL lysis buffer, stirring in a beaker until no chunks of cell mass remained. The beaker containing the lysate was placed in a larger beaker full of ice. Cells were then further lysed by probe sonication, using a Fisherbrand® Model 705 Sonic Dismembrator with process settings: amplitude 60, process time 30 s, pulse-on time 3 s, pulse-off time 7 s. The sonication procedure was repeated three times, stirring the lysate for 30 s between each cycle (**A**). The sonicate was centrifuged at 16.5 krpm, 4 °C for 60 min in an Avanti® J-26 XP Centrifuge (Beckman Coulter®). The supernatant was decanted (**B**) and sediment discarded.

10 mL Nickel NTA Agarose Resin (Agarose Bead Technologies) combined with the lysate was left on a roller for 60 min, allowing the Ddx4^{N*} fusion protein to bind to the Ni(II). The solution was centrifuged at 500 ref for 5 min, decanting the supernatant (**C**). The sediment was resuspended in 30 µL Nickel-A and added to a sterile Econo-Column® chromatography column (Bio-Rad). The lysate was eluted, leaving the resin in the column, at 3 mL min⁻¹ and discarded. 60 mL Nickel-A buffer was flowed through the column to remove any unbound species, leaving behind 1 mL of buffer with the slurry (**D**). 2 µL Nickel-A, 10 µL 1 M DTT, 200 µL high imidazole buffer and 20 µL ULP1 were combined and added to the column. Cleavage was allowed to proceed for 20 min (**E**), after which 60 µL Nickel-A was added to the column, which was transferred to a rocker at 4 °C for 90 min to allow binding of the His-tagged SUMO and ULP1 to the resin.

Cleaved protein was eluted (**F**) and concentrated using 10 kDa Merck Amicon™ Ultra-15 Centrifugal Filter Units to a volume of 5 mL, retaining the flow (**G**) in case of centrifugal filter permeability issues. Proteins bound to the Nickel-NTA resin were eluted and collected using 30 mL Nickel-B (**H**). Concentrated cleaved protein (**I**) was run over a HiLoad 26/600 Superdex 75 pg gel filtration column at a rate of 1.5 mL min⁻¹ into Ddx4^{N*} storage buffer, using an Akta Pure high performance liquid chromatography (HPLC) system.

Ddx4^{N*} variants eluted at 150 mL, shown for Ddx4^{N*} 1-231 in Figure 2.6 (a), with a UV 260/280 absorbance ratio of roughly 0.6, consistent with minimal nucleic acid contamination [160]. Eluted 1 mL fractions deemed to be pure enough by SDS-PAGE (see Figure 2.6 (b)), were pooled and concentrated in a 10 kDa Merck AmiconTM Ultra-15 Centrifugal Filter Unit to a protein concentration that showed phase separation when diluted 1:1 with 20 mM TRIS pH 8.0 at room temperature. The purified protein(**J**) syringe filtered through a 0.22 μ m membrane before division into 11 μ L aliquots in PCR tubes. Aliquots were snap frozen in liquid nitrogen and stored at -80°C .

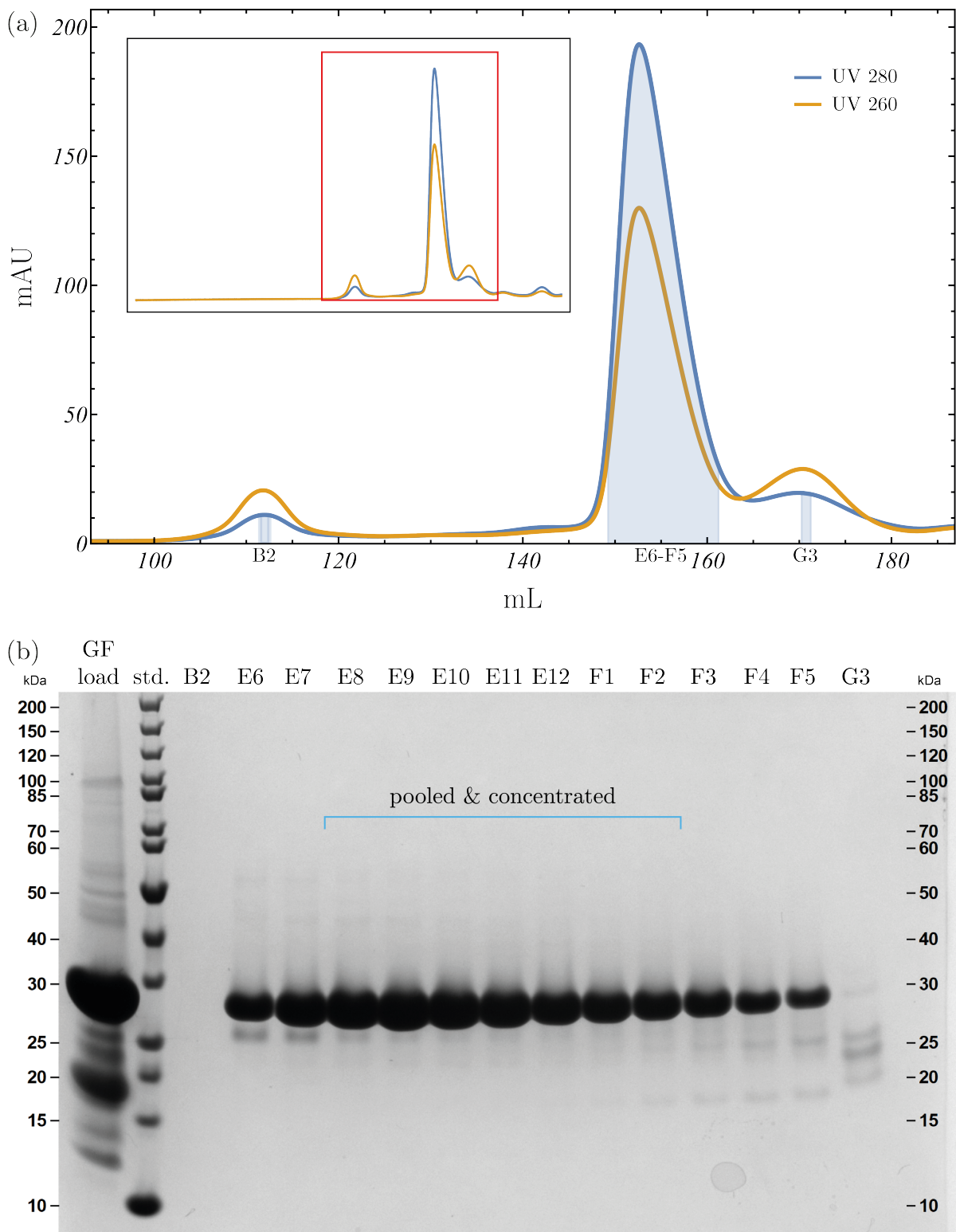


Figure 2.6: (a) Chromatogram obtained from HPLC run of concentrated cleaved Ddx4^{N*} 1-231 (see text) over a HiLoad 26/600 Superdex 75 pg gel filtration column. UV280 and UV260 absorbance (mAU) is plotted against the elution volume in mL. (b) SDS-PAGE gel of 10 μ L samples taken from concentrated cleaved Ddx4^{N*} 1-231 (GF) load and the corresponding eluted fractions.

2.8 Sample preparation

Samples for *in vitro* phase separation and drop shape analysis experiments were prepared by heating using the heating block of a thermomixer before combining and sealing between two 22 mm diameter glass coverslips, separated and sealed by a 0.12 mm imaging spacer (Sigma). This procedure, or variants of it, are used throughout this thesis and is described verbatim below. The base of the dilution buffer consisted of 20 mM Tris pH 8.0, generated by diluting the 2× TRIS buffer 1:1 with filtered MilliQ ultrapure water. For confocal fluorescence experiments, the dilution buffer was prepared by diluting the 2× TRIS buffer 1:1 with the appropriate concentration of Alexa-488 or Cy3B.

1. Remove a 11 μ L aliquot of protein from cryogenic storage and place in a rack on at room temperature. Allow the solution to defrost without any agitation.
2. Dilute the protein to the desired concentration with storage buffer.
3. Heat the protein solution and dilution buffer to 50 °C on a thermomixer for 3 min.
4. Combine 4.7 μ L protein with 4.7 μ L buffer, mix by pipetting and return to the thermomixer.
5. Using scissors, trim each corner off the imaging spacer so the spacer fits entirely within the diameter of the siliconized glass coverslips, as seen in Figure 2.7.
6. Attach the imaging spacer to a siliconized glass coverslip by using tweezers to peel off the opaque layer. Then press the adhesive transparent layer against the coverslip.
7. Peel off the top layer from the imaging spacer, revealing the second adhesive layer, and place the coverslip onto the 50 °C heating block in the thermomixer.
8. Pipette 9.1 μ L diluted phase-separating protein solution onto the area in the center of the imaging spacer on the coverslip, as in Figure 2.7. Wait for any air bubbles to pop.

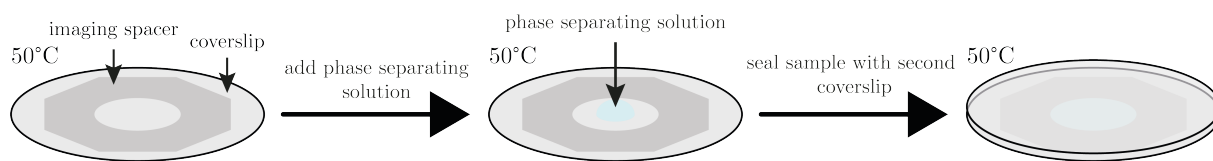


Figure 2.7: Schematic of sample preparation using siliconized glass coverslips and an imaging spacer. All steps should be carried out with the bottom coverslip kept at 50 °C by placing it on a heating block. The top coverslip should also be preheated to 50 °C, before being brought into contact with the phase-separating solution.

9. Carefully place a siliconised glass coverslip on top of the coverslip carrying the phase separating solution, as in Figure 2.7.
10. Seal the sample by applying light pressure onto the top coverslip with a pipette tip, pressing down along the region above the imaging spacer.

Throughout this thesis we use siliconized glass (Hampton research 22 mm 0.19-0.22 mm thick) and borosilicate glass (Agar Scientific Ltd. 22 mm No. 1.5) coverslips. It was found that cleaning the borosilicate glass coverslips with nitrogen gas, isopropanol, nitrogen gas again, followed by a wipe with lens paper, gave a clean surface with no dust, and allowed for reproducible measurements of contact angles and capillary lengths. Siliconized glass coverslips required no cleaning before use, giving reproducible transition temperatures, contact angles and capillary lengths.

Acknowledgments

We thank the Barr lab for use of their equipment and general consumables.

Chapter 3

Tuning Ddx4^{N*} Phase Separation

ABSTRACT

This chapter investigates the tuning of protein-protein interactions driving the phase separation Ddx4^{N} condensates through mutational studies. The introduction serves to motivate the subsequent investigations, whilst also detailing the rationale and form of the Ddx4^{N*} mutations effecting its cation- π and electrostatic interactions. The theoretical background that follows explains the Flory-Huggins theory for phase separating polymer solutions, concluding with a review of its applicability to Ddx4^{N*}. Through using transition temperature measurements to draw phase diagrams, as well as in cell fluorescence imaging, we reveal the sequence context dependent deleterious impact of increased net charge on condensate formation. We also demonstrate a strengthening of the cation- π interaction between Ddx4^{N*} chains, through a sequence-wide phenylalanine to tyrosine substitution.*

3.1 Introduction

A prerequisite for any functional phenomena of intracellular biomolecular condensates are their formation by their constituent molecules. It has been hypothesised that many of these membraneless compartments form spontaneously by liquid-liquid phase separation [9], driven by transient interactions between multivalent biomolecules [116]. Over the last decade, phase separating regions of many condensate associated proteins have been identified, with intrinsically disordered regions commonly implicated [11, 27, 57–59, 116, 161]. Ddx4^N is a notable phase separating intrinsically disordered protein, brought to light by Nott *et al* in 2015. This protein phase separates homotypically in aqueous buffer, as well as forming condensates when exogenously expressed in mammalian cells [59, 162]. Its phase separation is known to be driven by patterned electrostatic interactions between charged residues, $\pi - \pi$ interactions between aromatic residues and by cation- π interactions between arginine and phenylalanine/tyrosine residues. These sequence features are common amongst many other condensate forming proteins [57, 58, 63–67, 75–77]. As such, Ddx4^N has become an established model system in the biomolecular condensate field.

Here, we advance previous work on Ddx4^N, investigating the effect of net protein charge on its phase separation, as well as modulating its cation- π interaction. We base our study around a mutant Ddx4^N, wherein all cysteine residues have been swapped for serine, alleviating any propensity for disulfide bond formation (see Figure 2.1). This construct, Ddx4^{N*}, permits investigations into interfacial phenomena in later chapters in the absence of multimerisation. Condensate stabilities are assessed by recording the protein concentrations and temperatures at which they form, using these data to construct phase diagrams. The intrinsically disordered nature of Ddx4^N has facilitated previous modelling of its phase diagrams using Flory-Huggins theory [59, 62]. This thermodynamic framework is predicative of liquid-liquid phase separation of polymer solutions, permitting the extraction of mean-field interaction parameters through fitting to experimental phase di-

agrams. These parameters, analogous to the second virial coefficient describing pairwise interactions [163], reveal the nature of the effective enthalpic and entropic interactions between Ddx4^{N*} chains driving their phase separation. *In cell* phase separation experiments are presented alongside *in vitro* data, allowing for a direct comparison between phase separation in aqueous buffer and that in mammalian cellular milieu.

3.1.1 Design of constructs

Modulation of net charge

Electrostatic interactions have been highlighted as drivers of the phase separation of archetypal homotypically phase separating intrinsically disordered protein regions of Ddx4, LAF-1, FUS and hnRNPA1 [57–59, 70]. These sequences are enriched in charged amino acids, yet carry an overall low net charge per residue (NCPR). Figure 3.1 displays residues of Ddx4^{N*} as beads on a string, highlighting positively and negatively charged amino acids at pH 8.0, the *in vitro* pH used in this thesis. NCPR is known to influence conformational properties of IDPs [128], increasing their radii of gyration, as well as general protein solubility [164, 165], with proteins tending to aggregate as solution pH approaches their isoelectric point. We hypothesise that a decreased NCPR favours phase separation by decreasing repulsive electrostatic interactions between protein chains.

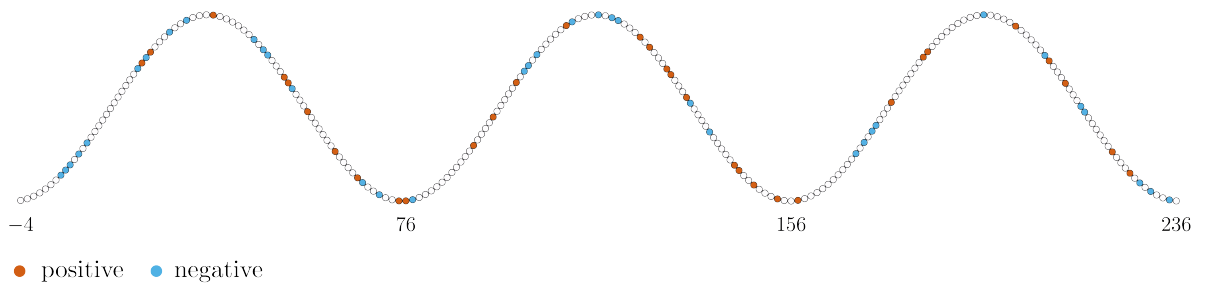


Figure 3.1: Cartoon of Ddx4^{N*} 1-236 highlighting anionic and cationic residues. Note that the amino and carboxyl groups of the N and C termini also carry positive and negative charges respectively. The first five residues are an artifact from the restriction cloning used to make the construct, index labels correspond to the wildtype amino acid sequence.

Ddx4 ^{N*} construct	chain length	net charge	R+K	D+E	κ
1-229	234	-1.1	32	33	0.245
1-231	236	-2.1	32	34	0.241
1-234	239	-3.1	32	35	0.240
1-236	241	-4.1	32	36	0.242

Table 3.1: Sequence properties of the net charge mutants of Ddx4^{N*}. Net charge is calculated at pH 8.0 using the Henderson-Hasselbalch equation [167, 168] with side-chain pKa values taken from Grimsley *et al* [169]. κ is calculated using the online CIDER tool [170].

To investigate the effect of NCPR on the phase separation of Ddx4^{N*}, we recall that previous work on Ddx4^{N*} and other systems, has emphasised the importance of charge patterning for facilitating electrostatic interactions between IDP chains [59, 62–67]. Therefore it is desirable to alter the NCPR without disturbing the charge patterning. To do so, four variants of Ddx4^{N*} were generated by truncating the C-terminus, resulting in a stepwise decrease in the number of acidic residues, leaving basic residues unchanged, and also leaving the cation- π interaction unaffected. This degree of truncation, from a chain length of 241 to 234, is expected to have minimal impact on phase separation propensity, as covered in the subsequent theory section.

Table 3.1 displays the sequence properties of the truncated sequences of Ddx4^{N*}. The full sequences can be found in Figure 2.2. The construct name has been chosen to refer to the length of sequence originating from the canonical sequence, as the first five residues, GAMGS, are an artefact of recombinant protein expression. The final column displays the construct’s κ value, a metric developed by Das & Pappu to quantify the degree of charge patterning of IDP sequences [166]. We see that the charge patterning does not vary by more than 2%.

Modulation of cation- π Interactions

Previous work has established that the short-range, cation- π interaction between arginine and aromatic residues, formed by overlapping electron clouds and augmented by flanking glycines/serines, is a key driver of IDP phase separation [59, 63, 65, 70, 72, 73]. Figure 3.2

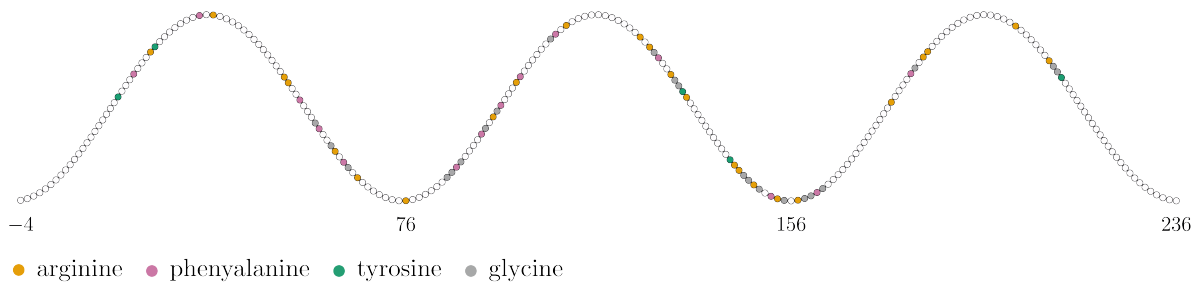


Figure 3.2: Cartoon of Ddx4^{N*} 1-236 highlighting the sequence features involved in the cation- π interaction. The first five residues are an artifact from the restriction cloning used to make the construct, index labels correspond to the wildtype amino acid sequence.

shows the sequence features involved in its cation- π interaction, illustrating the repeated occurrence of the [F/Y]G and RG motifs. Nott *et al* previously showed that the cation- π interaction between Ddx4^{N*} chains is inhibited by arginine methylation [59], reducing its phase separation propensity. Subsequently, Wang *et al* demonstrated in 2018 a reduction in the phase separation of the prion-like domain of FUS upon Y $\rightarrow$ F amino acid substitutions, also attributed to the cation- π interaction. Based on this result, we hypothesise that F $\rightarrow$ Y substitutions will increase the phase separation propensity of Ddx4^{N*}. To test this, we have generated a mutant construct where all 14 phenylalanines of the Ddx4^{N*} 1-236 sequence are substituted with tyrosine, referred to as Ddx4^{N*} F $\rightarrow$ Y, which contains 19 tyrosines and 24 arginines. Its sequence is given in full in Figure 2.2.

3.2 Theoretical background

Our *in vitro* experiments comprise solutions of Ddx4^{N*} suspended in aqueous buffer. These are multicomponent liquids whose constituent molecules are bound together by intermolecular cohesive forces. The nature of these intermolecular interactions determines the structure of the liquid, and its tendency to phase separate. Here we present a theory developed by Flory and Huggins in the 1940s [171, 172] for polymers in solution, which considers these cohesive forces and is predictive of liquid-liquid phase separation. This theory invokes a treatment of intrinsically disordered protein chains as homopolymers,

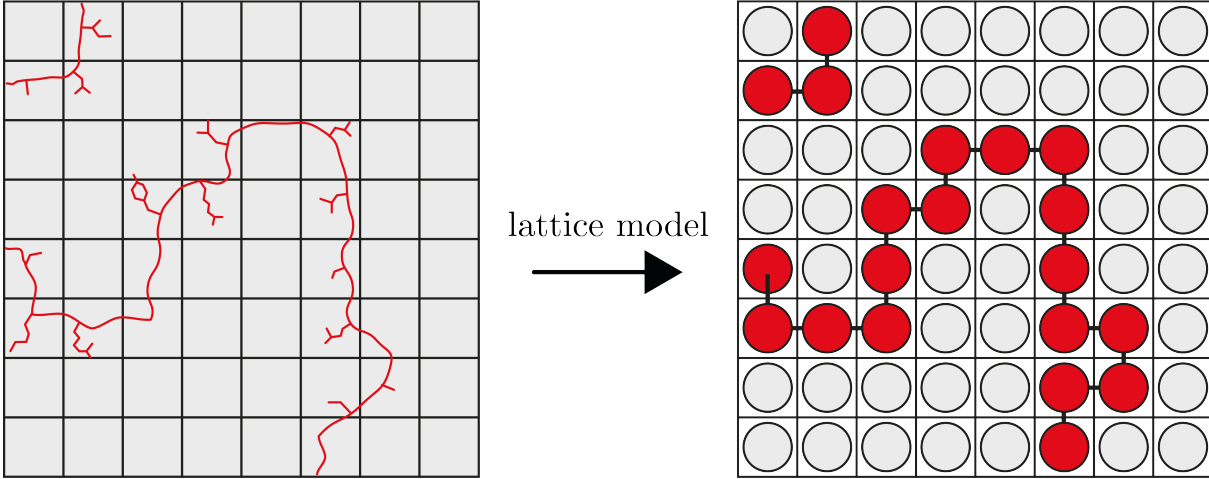


Figure 3.3: Coarse graining a protein in solution into a lattice of solvent and polymer sites allows for a soluble expression for its entropy. Left: cartoon snapshot of regions of Ddx4^{N*} in solution, with the protein structure shown in red and solvent in grey. Right: The coarse grained protein conformation according to the lattice model, red protein sites show connectivity and grey solvent sites do not. Each lattice site fits, on average, one amino acid and 4.5 water molecules.

and the multicomponent buffer as a single solvent species. Despite these shortcomings, Flory-Huggins theory has been successfully applied to phase diagrams of Ddx4^{N*}, revealing molecular details of its phase separation.

3.2.1 Mixing & the lattice model

The equilibrium state of a system is one that minimises its free energy. To derive an expression for the free energy of a solution, we consider the Helmholtz free energy change associated with mixing polymer and solvent components to make a solution,

$$\Delta_m F = F_{\text{mixed}} - F_{\text{unmixed}}. \quad (3.1)$$

Our reference state is the total free energy of the pure chemical components in the solution, at the same temperature and pressure as the solution. As $F = U - TS$, we require expressions for $\Delta_m U$ and $\Delta_m S$. To derive these we follow the 1942 treatment of Flory [171].

We approximate our aqueous solutions of Ddx4^{N*} in 150 mM NaCl 20 mM Tris pH

8.0 buffer as consisting of two components, protein and solvent. To allow a soluble calculation of its free energy of mixing, the protein is considered as polymer p , taken to be composed of r , linearly connected, identical *effective* amino acids, dissolved in solvent s . The phase behaviour of the protein in solution can then be modeled as a homopolymer, using the Flory-Huggins expressions for mixing entropy and internal energy by considering the lattice model. The total volume of the solution is divided into a uniform lattice, with $N = rN_p + N_s$ sites. N_p is the number of polymer molecules and N_s is the number of sites occupied by solvent molecules, given by

$$N_s = \frac{N_s^* \nu_s}{\nu_p / r}, \quad (3.2)$$

with respective molecular volumes ν_p and ν_s , and N_s^* the number of solvent molecules on the lattice. The fixed molecular volumes ν_p and ν_s do not allow for volume changes upon mixing, making Helmholtz free energy the natural choice of thermodynamic potential.

3.2.2 Entropy of mixing

The lattice model allows us to calculate the configurational or ‘lattice’ entropy by using Boltzman’s formula,

$$S = k_B \ln W, \quad (3.3)$$

where W is the weighted number of microscopic realisations of the lattice. Populating the lattice with randomly placed polymer segments and solvent molecules gives a mixed entropy of

$$S_{\text{mixed}} = k_B \ln \frac{N!}{(rN_p)!N_s!}. \quad (3.4)$$

The numerator in the logarithm stems from there being N ways to arrange N distinguishable objects on the lattice, whereas the denominator accounts for the indistinguishable arrangements. In reality, the polymer segments are covalently linked and only the first segment of a chain is randomly placed. Consecutive segments are then restricted to no less than $z - 1$ sites, where z is the coordination number of the lattice. This amounts to a correction of $(z - 1)/N$ per segment, and for the $(r - 1)N_p$ restricted segments, a correction of

$$\left(\frac{z - 1}{rN_p + N_s} \right)^{(r-1)N_p}. \quad (3.5)$$

The $(rN_p)!$ term in the denominator of Equation 3.4 accounts for the randomly placed indistinguishable arrangements of polymer segments. As only the first segment of a linked chain is randomly placed, we replace $(rN_p)!$ by N_p . The expression for the mixed entropy of the polymer solution is then

$$S_{\text{mixed}} = k_B \ln \frac{N!}{N_p! N_s!} \left(\frac{z - 1}{rN_p + N_s} \right)^{(r-1)N_p}. \quad (3.6)$$

This expression for the mixed entropy allows aphysical configurations where segments on the same polymer, separated by two or more intervening segments, occupy the same lattice point. S_{unmixed} is given by setting $N_s = 0$ in Equation 3.6 to give

$$S_{\text{unmixed}} = k_B \ln \frac{(rN_p)!}{N_p!} \left(\frac{z - 1}{rN_p} \right)^{(r-1)N_p}. \quad (3.7)$$

The unmixed entropy is the sum of the lattice entropy of pure polymer and solvent. For the latter, $W = 1$, as there is only one way to arrange N_s indistinguishable objects on a lattice with N_s points and there is no contriution to S_{unmixed} . The mixing entropy $\Delta_m S$ is given by subtracting Equation 3.7 from Equation 3.6 and applying Stirlings formula,

$\ln x! \approx x \ln x - x$, yielding

$$\Delta_m S = -k_B [N_s \ln \frac{N_s}{N} + N_p \ln \frac{N_p}{N}]. \quad (3.8)$$

The factor of $z-1$ in Equation 3.6 is not present in Equation 3.8, as the aphysical entropic contribution of intra-chain overlaps is present in both S_{mixed} and S_{unmixed} and cancels out in the expression for $\Delta_m S$. Introducing the notion of volume fractions ϕ where

$$\phi_p \equiv \frac{rN_p}{N} \quad \text{and} \quad \phi_s \equiv \frac{N_s}{N} \equiv \frac{N_s^* \frac{\nu_s r}{\nu_p}}{N}, \quad (3.9)$$

are the fractional populations of the lattice by polymer and solvent respectively, we rewrite 3.8 as

$$\Delta_m S = -nR [\phi_s \ln \phi_s + \frac{\phi_p}{r} \ln \phi_p], \quad (3.10)$$

where n is the molar number of lattice sites. $\Delta_m S$ is always positive and favours mixing. As $\phi_p + \phi_s = 1$, we can rewrite Equation 3.10 as a function of only ϕ_p

$$\Delta_m S = -nR [(1 - \phi_p) \ln(1 - \phi_p) + \frac{\phi_p}{r} \ln \phi_p]. \quad (3.11)$$

Figure 3.4(a) plots $\Delta_m S$ against ϕ_p for multiple values of r . Increasing the chain length r , decreases the maximum value of $\Delta_m S$, as well as shifting its maxima towards the right. Increasing the length of the polymer chain therefore disfavors mixing, by making the mixing entropy less positive.

3.2.3 Internal & free energy of mixing

Let w_{ij} denote the interaction energy between species i and j occupying neighbouring sites on the lattice. Each site has z nearest neighbours and is populated by solvent with mean probability ϕ_s , and polymer segment with mean probability ϕ_p . The mean

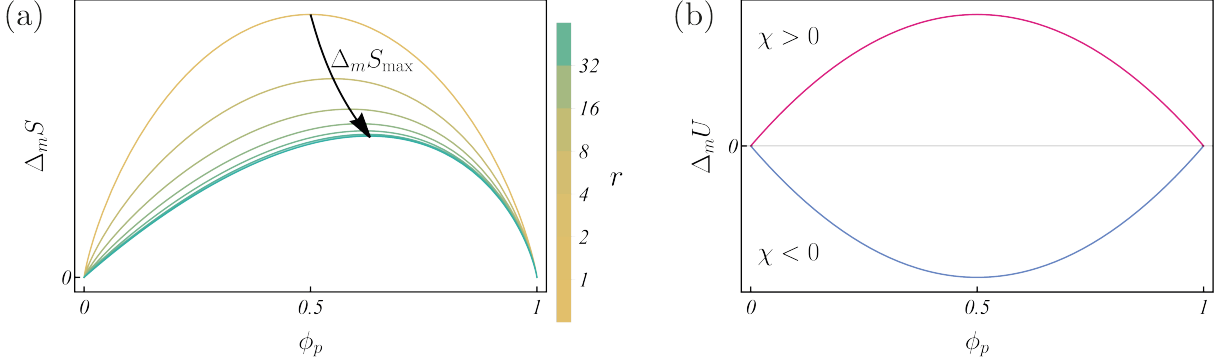


Figure 3.4: (a) Entropy of mixing over the composition range and its dependency on chain length, r . The contribution to $\Delta_m S$ from the protein chain decreases asymptotically r increases. (b) Internal energy of mixing over the composition range. A positive interaction parameter χ favours demixing and a negative interaction parameter favours mixing.

interaction energy per solvent site is $z(\phi_p w_{sp} + \phi_s w_{ss})$, and the mean energy per protein site is $z(\phi_p w_{pp} + \phi_s w_{sp})$. The total mixed internal energy for n moles of lattice sites is

$$U_{\text{mixed}} = \frac{1}{2} n z [\phi_s (\phi_p w_{sp} + \phi_s w_{ss}) + \phi_p (\phi_p w_{pp} + \phi_s w_{sp})], \quad (3.12)$$

with factor of 1/2 accounting for the pairwise nature of the interactions. The internal energy of the pure components is simply given by

$$U_{\text{unmixed}} = \frac{1}{2} z [N_s w_{ss} + N_p w_{pp}]. \quad (3.13)$$

The internal energy of mixing is the difference of Equations 3.12 and 3.13,

$$\Delta_m U = n R T \chi \phi_s \phi_p, \quad (3.14)$$

with χ being the Flory-Huggins interaction parameter, defined by

$$\chi \equiv \frac{N_A z}{R T} \left[w_{sp} - \frac{w_{ss} + w_{pp}}{2} \right]. \quad (3.15)$$

This is the sum over nearest neighbour $s - p$ interactions, relative to the self-self nearest neighbour interactions, scaled by thermal energy $R T$ [173]. Figure 3.5 gives a

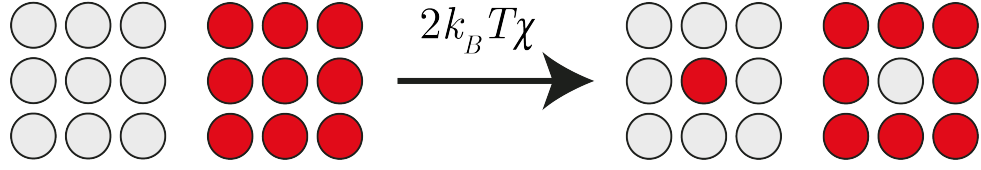


Figure 3.5: Diagrammatic definition of the Flory-Huggins interaction parameter χ describing interactions within the lattice model (see Equation 3.15). Note that $R = k_B N_A$.

diagrammatic representation of the energy changes accounted for by χ .

As with our expression for $\Delta_m S$, we rewrite Equation 3.14 by substituting $\phi_s = 1 - \phi_p$, yielding

$$\Delta_m U = nRT\chi\phi_p(1 - \phi_p). \quad (3.16)$$

Our expression for $\Delta_m U$ is based on the **mean** probabilities of interactions, therefore χ is a **mean-field** interaction parameter. It represents interactions in the system assuming a mean molecular field; deviations in U_{mixed} due to local variations of polymer and solvent volume fractions from the bulk values, ϕ_p and ϕ_s , are assumed to cancel each other out. Equation 3.16 is plotted in Figure 3.4 (b) for positive and negative values of χ . The mixing energy is quadratic in ϕ_p and symmetric about $\phi_p = 0.5$.

The free energy of mixing is found by substituting Equations 3.10 and 3.14 into

$$\Delta_m F = \Delta_m U - T\Delta_m S, \quad (3.17)$$

yielding

$$\Delta_m F = nRT\left[\chi\phi_p\phi_s + \frac{\phi_p}{r} \ln \phi_p + \phi_s \ln \phi_s\right], \quad (3.18)$$

or as a free energy per lattice site in terms of only ϕ_p ,

$$\frac{\Delta_m F}{n} = RT\left[\chi\phi_p(1 - \phi_p) + \frac{\phi_p}{r} \ln \phi_p + (1 - \phi_p) \ln(1 - \phi_p)\right]. \quad (3.19)$$

3.2.4 Phase separation

A system reaches equilibrium by minimizing its free energy. For phase separation, the relevant free energy is the mixing free energy, with equilibrium condition $d\Delta_m F_{total} = 0$. Ignoring interfacial contributions, the Helmholtz free energy of a two phase solution of polymer and protein, composed of phases α and β , can be written as

$$\Delta_m F_{total}(T, P, n_p, n_s) = \Delta_m F_\alpha(T, P, n_p^\alpha, n_s^\alpha) + \Delta_m F_\beta(T, P, n_p^\beta, n_s^\beta). \quad (3.20)$$

At a fixed temperature and pressure, the free energy of the system, F_{total} , is dependent only on the compositions, n_p and n_s , of each phase. The infinitesimal free energy change associated with the movement of dn_p protein molecules, represented by lattice sites in the Flory-Huggins scheme, between phases is

$$d\Delta_m F_{total} = \left(\frac{\partial \Delta_m F_\alpha}{\partial n_p} \right) dn_p^\alpha + \left(\frac{\partial \Delta_m F_\beta}{\partial n_p} \right) dn_p^\beta. \quad (3.21)$$

The derivatives of free energy with respect to composition, are the (mixing) chemical potentials of p in each phase,

$$\left(\frac{\partial \Delta F_{\alpha/\beta}}{\partial n_p} \right) = \mu_p^{\alpha/\beta}. \quad (3.22)$$

The total n_p in the system is fixed, meaning $dn_p^\beta = -dn_p^\alpha$. Equation 3.21 is now written

$$dF_{total} = (\mu_p^\alpha - \mu_p^\beta) dn_p^\alpha, \quad (3.23)$$

and with the equilibrium condition $dF_{total} = 0$,

$$\mu_p^\alpha = \mu_p^\beta. \quad (3.24)$$

Equation 3.24 is our first equilibrium constraint. Applying the same argument to the movement of dn_s solvent molecules between phases gives:

$$\mu_s^\alpha = \mu_s^\beta. \quad (3.25)$$

Taking Equations 3.24 and 3.25 together, the equilibrium constraint on phase separation is the chemical potentials of protein and solvent are simultaneously equal between the two phases. Within the Flory-Huggins model Equations 3.24 and 3.25 form a pair of simultaneous equations with two unknowns, ϕ_p^α and ϕ_p^β for a given value of χ . For polymers with $r > 1$, these equations have no analytical solutions. Practically they can be solved numerically, or as is illustrated below, graphically, by common tangent construction.

Common tangent construction

The mixing free energy of a solution containing the two equilibrium phases can be written

$$\Delta_m F = n_s \mu_s + n_p \mu_p, \quad (3.26)$$

when μ_p and μ_s satisfy Equations 3.24 and 3.25. Substituting $n_s = n(1 - \phi_p)$ and $n_p = n\phi_p/r$, allows us to linearize Equation 3.26 in ϕ_p and write as a free energy per lattice site

$$\frac{\Delta_m F}{n} = \mu_s + \phi_p \left(\frac{\mu_p}{r} - \mu_s \right). \quad (3.27)$$

Within the Flory-Huggins model, μ_p and μ_s are both determined by the ϕ_p^α and ϕ_p^β that describe the coexisting phases for a given value of χ . The equilibrium constraints are satisfied when Equation 3.27 makes a double tangent with Equation 3.19. Figure 3.6 (a) plots both Equation 3.27 and Equation 3.19, rescaled by $1/nRT$, illustrating the ‘common tangent’ construction and its relation to the composition of the two coexisting phases. The coexistence compositions, ϕ_p^α and ϕ_p^β , depend only on the intrinsic properties of the system; the interaction parameter, χ , and the chain length, r .

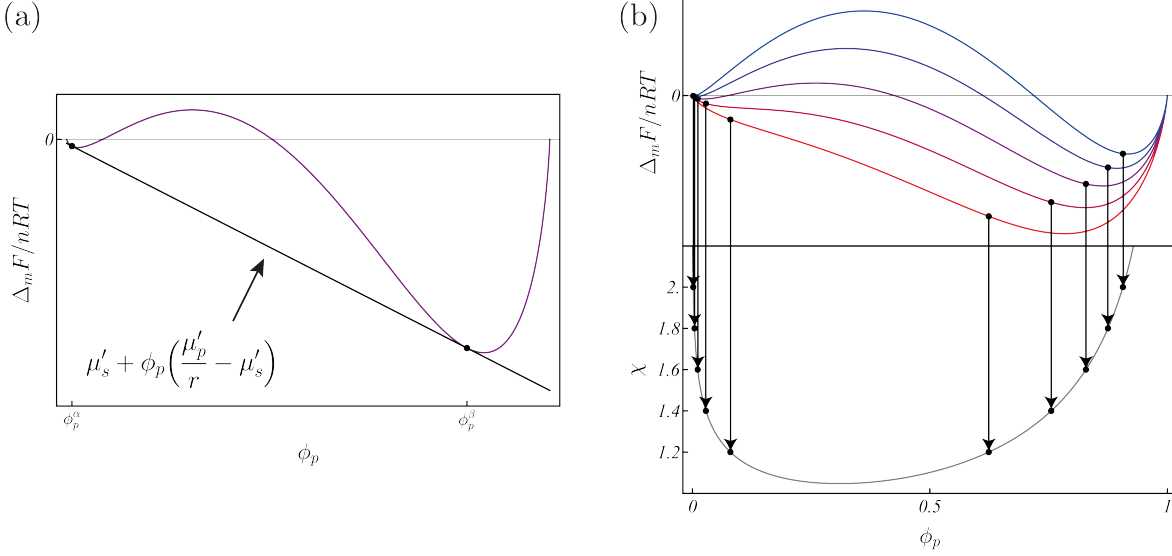


Figure 3.6: (a) Common tangent construction within the Flory-Huggins model for $r = 5$ and $\chi = 1.6$. Rescaled Equation 3.27 (black), with $\mu' = \mu/RT$, makes a double tangent with rescaled Equation 3.19 (black) at the equilibrium compositions ϕ_p^α and ϕ_p^β . (b) Relationship between $\Delta_m F/nRT$ and the binodal for $r=5$. Black points on the blue to red curves show coexistence values of ϕ_p , for decreasing values of χ , and their corresponding position on the binodal.

3.2.5 Phase diagrams

The binodal & spinodal

Solving Equations 3.24 and 3.25 while varying χ , as in Figure 3.6 (b), gives a line in the (ϕ_p, χ) plane termed the binodal. This line is a key feature of a phase diagram describing liquid-liquid phase separation, separating the state points stable with respect to phase separation, from those that are not. The equilibrium compositions of the two phases present after phase separation for a given χ can be read off the binodal.

When a system is prepared at a (supersaturated) composition ϕ_p , inside the binodal, its driving force for phase separation per unit volume V is given by the excess free energy density

$$W(\phi_p) = \frac{1}{V} \left(\Delta_m F - n \left[\mu_s + \phi_p \left(\frac{\mu_p}{r} - \mu_s \right) \right] \right), \quad (3.28)$$

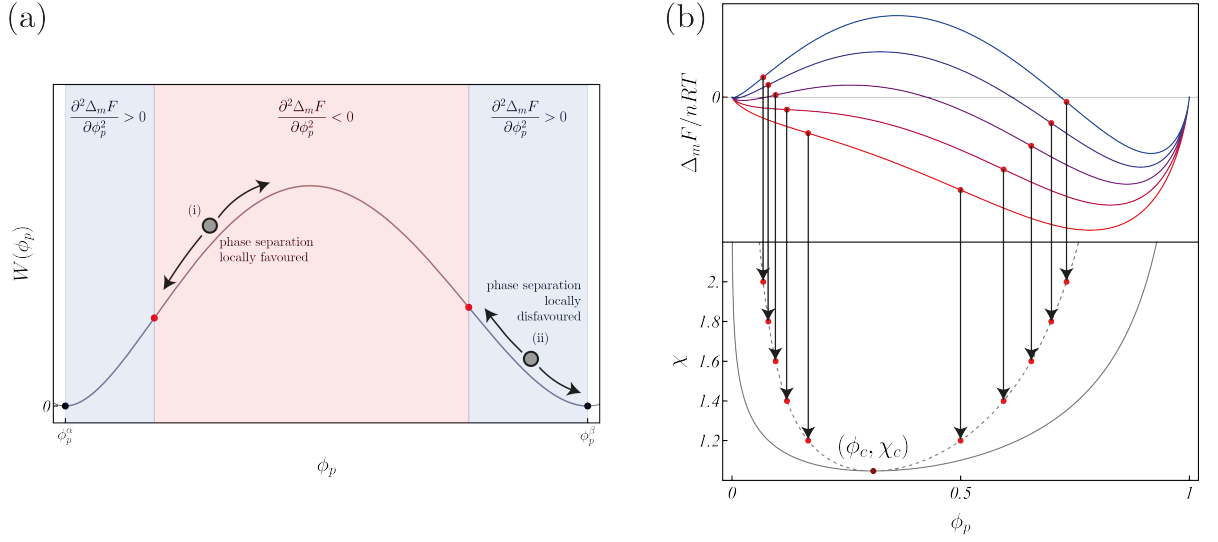


Figure 3.7: (a) Excess free energy density $W(\phi_p)$ - the difference between Equation 3.27 and Equation 3.19, as a function of composition. The blue regions highlight locally stable compositions, the red region highlights locally unstable compositions. (b) Spinodal (dashed line) constructed from the locus of points where $\frac{\partial^2 \Delta_m F}{\partial \phi_p^2} = 0$ as χ is decreased (blue to red free energy curves). The critical point is marked as (ϕ_c, χ_c) .

where μ_s and μ_p take their equilibrium values. The path to equilibrium involves contacting regions in space continuously changing from ϕ_p to ϕ_p^α and ϕ_p^β , achieving phase separation. If ϕ_p lies at point (i) in Figure 3.7 (a), then the initial free energy released on the path to ϕ_p^α is greater than the initial cost of the path from (i) to ϕ_p^β . Phase separation is favoured locally and globally, characterised by $\frac{\partial^2 \Delta_m F}{\partial \phi_p^2} < 0$. Any fluctuation in ϕ_p leads to phase separation by spinodal decomposition, forming a bicontinuous network [174].

Conversely, a system prepared at point (ii) in Figure 3.7 (a) faces an uphill struggle. The initial path to ϕ_p^α costs more free energy than the initial path to ϕ_p^β - phase separation is disfavoured locally, despite being favoured globally. The system is metastable, characterised by $\frac{\partial^2 \Delta_m F}{\partial \phi_p^2} < 0$, requiring a finite fluctuation to initiate phase separation. Physically, this manifests as phase separation by nucleation and growth, an activated process leading to the formation of discrete droplets which grow as phase separation evolves. This is the predominant mechanism of phase separation leading to intracellular condensates.

The points of inflexion of $\Delta_m F$ enclose the compositions where spinodal decomposition is favoured. The locus of these points as χ is varied gives the spinodal, illustrated

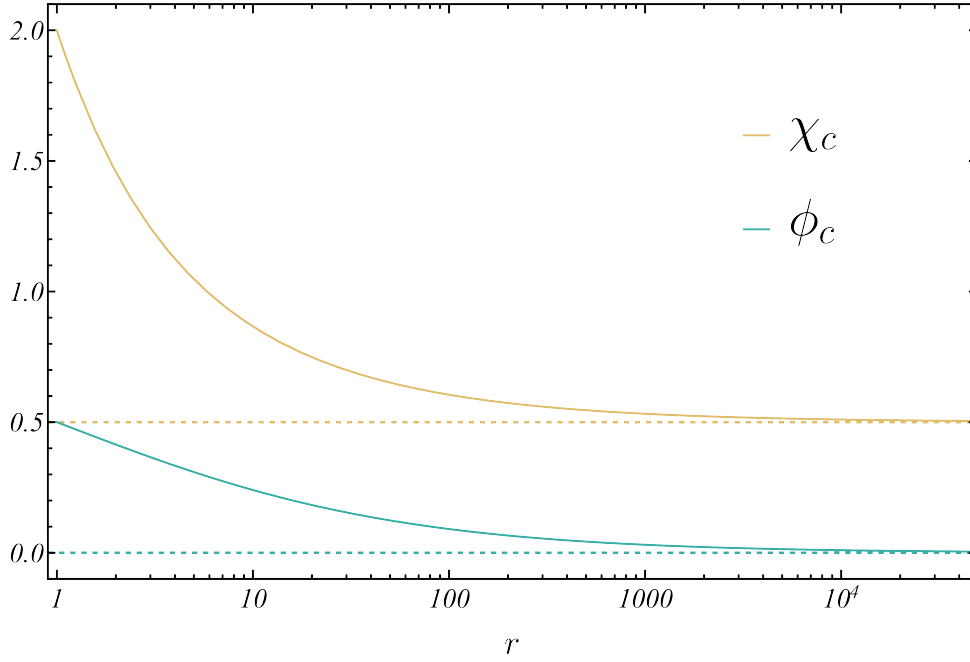


Figure 3.8: Dependency of the critical composition and critical interaction parameter on chain length.

in Figure 3.7 (b). Unlike the binodal, the spinodal has an analytical form,

$$\chi = \frac{1}{2} \left(\frac{1}{1 - \phi_p} + \frac{1}{r\phi_p} \right). \quad (3.29)$$

The critical point

Figure 3.7 (b) shows that the intersection of the spinodal with the binodal, (ϕ_c, χ_c) , is given by the minimum of the spinodal. This point on the phase diagram, the critical point, is given by

$$\phi_c = \frac{1}{1 + \sqrt{r}} \quad \text{and} \quad \chi_c = \frac{(1 + \sqrt{r})^2}{2r}. \quad (3.30)$$

As $\chi \rightarrow \chi_c$ from above, the equilibrium compositions tend to the same value until they are indistinguishable. Conversely, the width of the binodal and spinodal increase with $\chi - \chi_c$. The critical point therefore serves as a natural reference point in the phase diagram. Figure 3.8 shows that with increasing chain length r , ϕ_c decreases. This is owed to Equation 3.10,

as r increases, the contribution to $\Delta_m S$ from the chain decreases. This asymmetry in the phase diagram favours demixing at lower concentrations of polymer, with a diminishing return in r . A diminishing return in χ_c with increasing r is also manifest. Applied to phase separating intrinsically disordered proteins, these relationships suggest a reducing evolutionary pressure on increasing chain lengths for longer chains.

Interaction parameters

The interaction parameter from the Flory-Huggins mixing model is inversely proportional to temperature. This reflects the fact that increasing temperature decreases the significance of energetic interactions, by giving more weight to the lattice entropy. A purely Flory-Huggins solution can be transformed from the χ domain to the T domain by considering Equation 3.15, where the numerator represents the relative strength of the pairwise energetic interactions in the system. We rewrite this equation here, replacing the numerator with ΔU , yielding

$$\chi(T) = \frac{\Delta U}{RT}. \quad (3.31)$$

Rearranging this to make T the subject has the effect of inverting the vertical axis on the (ϕ_p, χ) phase diagram, with χ_c being replaced by a critical temperature, T_c . Provided the interactions in the system favour phase separation $\Delta U > 0$, the critical point is maximum temperature at which phase separation can be observed, and is termed the upper critical solution temperature. Equation 3.31 is analogous to Equation 3.15, where we have replaced the numerator with ΔU .

It is often the case that pairwise interactions in solution carry entropic components [175]. The Flory-Huggins interaction parameter can be augmented to include these, by writing

$$\chi(T) = \frac{\Delta U}{RT} - \frac{\Delta S}{R}. \quad (3.32)$$

Now $RT\chi$ is a free energy, rather than an internal energy, with ΔS describing excess entropy changes upon phase separation [59,176]. This expression for χ has been shown to be sufficient to describe the phase behavior of Ddx4^N [59,62]. In the case of negative ΔS and ΔU , this expression predicts increased phase separation as temperature **increases**. In this case, T_c describes the minimum temperature at which phase separation is observed, and the solution is said to have a lower critical solution temperature.

3.2.6 Applicability of Flory-Huggins theory to Ddx4^{N*} condensates

Flory-Huggins theory is derived for homopolymers, wherein all p sites are identical. However, the invocation of the mean-field approximation allows for heteropolymers, providing enough mixing is present to ensure the placement of differing monomers is uncorrelated. We also note that the binary theory presented here is for only two components, whereas our experimental buffer solution contains at least six (Ddx4^{N*}, H₂O, Na⁺, Cl⁻, Tris⁺, Tris). The successful application of Flory-Huggins theory to Ddx4^N phase separation under nearly identical buffer conditions suggests this is not an issue [59,62], and that the non protein components can be grouped together and considered as a uniform solvent. More recent work on the formation of P-granules in living *Caenorhabditis elegans* embryos through Flory-Huggins liquid-liquid phase separation, has in fact demonstrated the successful modeling of the embryonic cytoplasm as a two component mixture, containing protein and solvent [177].

At a glance, the homopolymer approximation, where each protein site is taken as an equal contribution from each amino acid, does not rationalise the difference in measured interaction parameters for Ddx4^N and its charge scrambled mutant, as shown by Brady *et al* [62]. Both of these sequences have the same mean amino acid composition, suggesting that their Flory-Huggins interaction parameters are sensitive to cooperative interactions, enabled by sequence motifs, particularly that introduced through charge patterning. Lin *et*

al introduced the random phase approximation [178], calculating electrostatic interactions assuming a Gaussian chain to explain the sequence dependent charge-charge interactions of Ddx4^N. However, the core of this method is still based on a mean-field Flory-Huggins χ parameter, and does not conflict with the methods presented in this chapter.

We also note that Flory-Huggins theory is best applied for short ranged interactions, such as dipole-dipole, van der Waals and cation- π , existing only between directly adjacent, contacting lattice sites [173,179]. Electrostatic interactions are typically long ranged, but are screened by electrolytes to a range given by the Debye length [180]. For Ddx4^{N*} in a solution of 150 mM NaCl 20 mM Tris, this amounts to 7.9 Å. The spacing between the centres of adjacent lattice sites is given by the cube root of the site volume, which for the Ddx4^{N*} constructs presented in this work is 5.125 ± 0.004 Å (see Section 3.3.3). Therefore, electrostatic interactions are expected to cause weak correlations, extending only slightly further than the first coordination shell of lattice sites. The aforementioned success of the random phase approximation suggests weak or no correlation between charged protein monomers, strengthening the well mixed approximation of Flory-Huggins theory.

3.3 Methods

The nucleation and growth of condensates at the onset of phase separation causes a stark change in the optical properties of the solution. Microscopic proteinacious condensates, with refractive index different to that of the surrounding solvent, scatter visible light. From a macroscopic viewpoint, this causes the solution to turn cloudy, and can be easily monitored using optical microscopy. Here, the experimental procedure to determine transition points, (c_p, T) , by cloud point measurement is described.

A phase-separating solution of Ddx4^{N*} is prepared by diluting the protein solution 1:1 with a low ionic strength solution at high temperature, yielding a protein concentration c . Owing to the greater dependency of the Ddx4^{N*} transition temperature on ionic

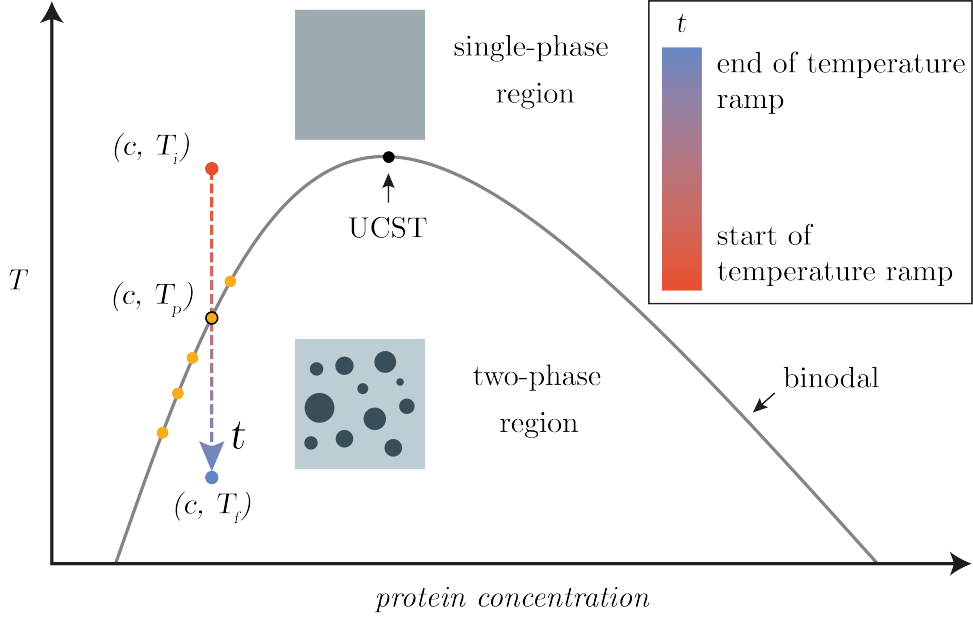


Figure 3.9: Sketch of a phase diagram for a phase separating protein with an upper critical solution temperature (UCST). Data points obtained by transition temperature measurement are shown in orange, with the outlined orange point generated by the trajectory indicated by the dashed line. The experiment starts at protein concentration c and initial temperature T_i which is then lowered as a function of time (t) (see text), passing through the transition point T_p , indicated by condensate formation, and finishing at final temperature T_f .

strength [59] over protein concentration, this increases the phase separation propensity of the solution. Differential interference contrast (DIC) microscopy is used to take greyscale images of the phase-separating protein solution. Images are recorded as a temperature-controlled microscope stage is used to slowly decrease the temperature of the sample. Automated image analyses extract the transition temperature, T_p , as the onset of condensate nucleation.

Repetition of the procedure for different values of protein concentration c determine multiple points along the dilute arm of the binodal. These data points are then fit to Flory-Huggins theory, from which the Flory-Huggins interaction parameter is extracted. The trajectory through the phase diagram taken during the course of a transition point measurement is shown by the dashed line in Figure 3.9.

3.3.1 Microscopy & thermal stage setup

An Olympus BX43 microscope with a 10 $\times$ objective and differential interference contrast (DIC) optical set up, illustrated in Figure 3.10, is used to image the onset of phase separation. Compared to bright-field illumination, DIC optics enhance contrast across interfaces of differing refractive indices, generating an artificial shadow in the image [181].

The light path from lamp to camera is illustrated in Figure 3.10. Incident light is first polarised by the polarising filter, before the condenser Wollaston prism splits the light into two perpendicularly polarised rays with a phase offset. The rays are then focused by the condenser lens, passing through the sample with an offset of roughly 0.2 μm . Refractive index gradients, on the order of 0.2 μm , in the focal plane cause a differential phase shift of the two rays. The images generated by the two light paths are focused by the objective lens and onto objective Wollaston prism, which resets the polarization of the two rays, allowing them to interfere. The constructive and destructive interference of the two rays gives a final image with contrast generated by interfaces in the sample. This makes DIC microscopy suited to the detection of microscopic proteinaceous condensates [182].

The sample is mounted onto a Linkam PE120 thermoelectric stage, allowing control of the temperature of the sample to within $\pm 0.1^\circ\text{C}$ over the range -20°C to 120°C . The temperature of the stage is stabilised by a constant flow of water, provided by connecting the stage to a 2L water reservoir via a peristaltic pump as in Figure 3.10. LINK software is used to program temperature profiles with ramp speeds ranging from 0.01°C to 20°C .

3.3.2 Data collection & image analysis

Samples were prepared as described in Section 2.8, using siliconized glass coverslips. Before loading the sample, the thermal stage was heated to the decided initial temperature,

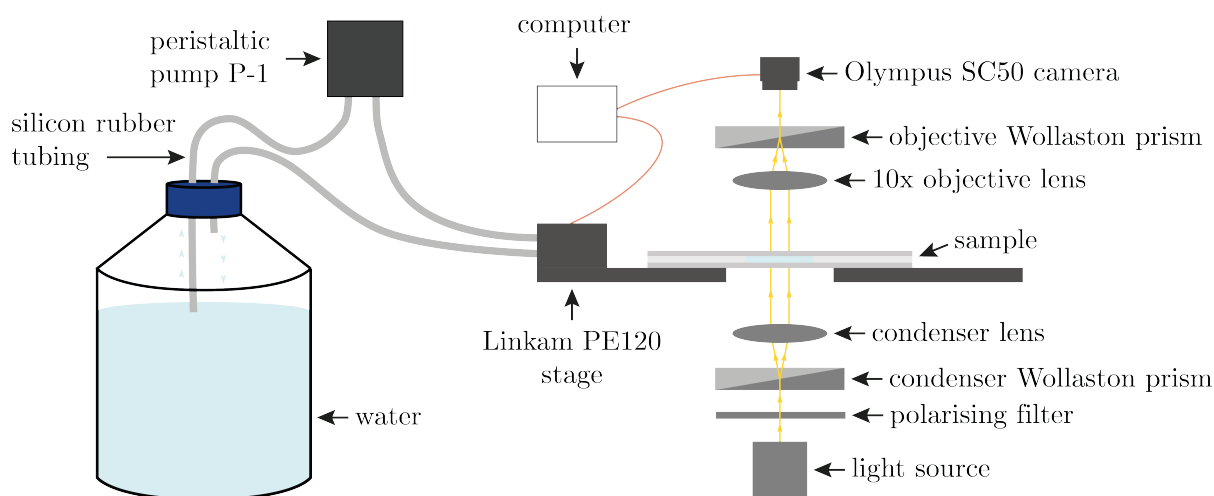


Figure 3.10: Schematic of the DIC microscopy and thermal stage setup. The water reservoir in the Duran bottle acts as a heat sink and is kept at room temperature.

typically 50°C. For transition point measurements representative of the bulk solution, it is necessary to image the sample far away from its edges. This was achieved by placing ink dots on the inner surfaces of the coverslips, outside the imaging spacers. Figure 3.11 shows the function of these marks in guiding the focal plane to the centre of the sample. Once the central focal plane was identified, the stage was translated in x and y to focus at the centre of the phase separating solution.

A temperature profile is programmed onto the stage consisting of 2 min at 50°C followed by a ramp down to 10°C¹. If condensates were already visible at this point, the sample was prepared again at either a higher temperature, or a lower concentration of phase separating protein. Greyscale images were recorded every 10 s using CellSens software, commencing from the start of the temperature profile. Images were captured using 2×2 pixel binning and an exposure time of 20 ms.

For transition temperature measurement, ImageJ was used to extract pixel data from image series which were subsequently analysed by a Python 3 script, outputting the transition temperature. Firstly, the standard deviation in pixel intensity, σ , was measured for 5 circular regions of interest (ROIs) in each image in a series as in Figure 3.12 (a).

¹If the transition point of the sample was roughly known, the initial and final temperatures of the ramp were set to 10°C higher and 5°C lower than the expected T_p respectively.

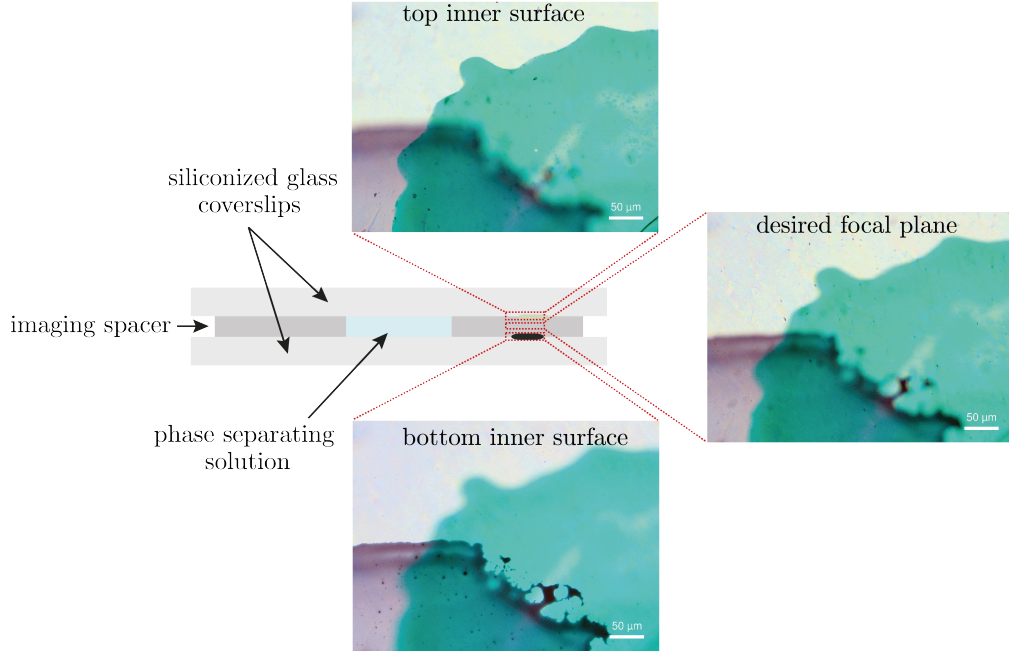


Figure 3.11: Using ink dots on the inn surfaces of coverslips aids in focusing the microscope to the central focal plane of the sample.

Then the timestamp for each image are mapped to a temperature T , using the formula

$$T(t) = \begin{cases} T_i, & t \leq 120 \text{ s} \\ T_i - (t - 120) \times \text{ramp speed}, & t > 120 \text{ s} \end{cases}, \quad (3.33)$$

where T_i is the initial temperature, t is time and the ramp speed is in units of $^{\circ}\text{C s}^{-1}$. We then define a baseline σ value, $E[\sigma(t < 2 \text{ min})]$, for a given ROI as the arithmetic mean of all data points collected in the first two minutes of the experiment, while the temperature is held at T_i . The baseline variability is then measured as the standard deviation of the same set of data points, $SD[\sigma(t < 2 \text{ min})]$.

A ROI's transition temperature is then measured as the first (descending) temperature T that satisfies

$$\frac{\sigma(T) - E[\sigma(t < 2 \text{ min})]}{SD[\sigma(t < 2 \text{ min})]} \geq 10. \quad (3.34)$$

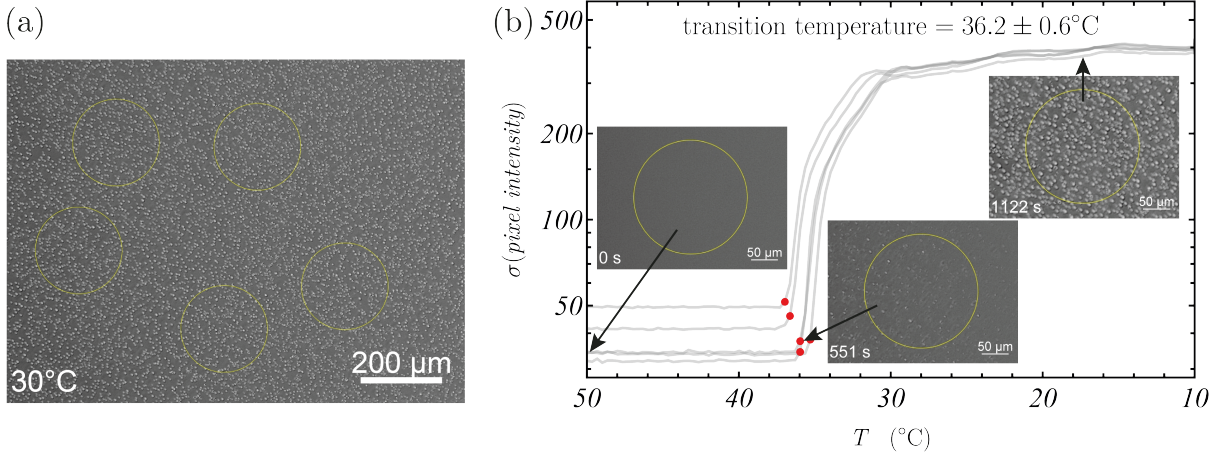


Figure 3.12: (a) Micrograph from a transition temperature measurement experiment with a transition temperature of $36.2 \pm 0.6^\circ\text{C}$ showing Ddx4^{N*} condensates at 30°C , 3 min after phase separation. The regions of interest (ROIs) used for transition temperature determination are shown as yellow circles. (b) Plot of the pixel intensity standard deviation on a per ROI basis from the experiment shown in (a). The ramp speed in this experiment was $2^\circ\text{C}/\text{min}$. Inset images show the state of one ROI throughout the course of the experiment. The first image is taken from the baseline measurement period and shows a clear sample. The onset of condensate nucleation is visible at 551 s. The sample is fully populated with condensates after 1122 s. Red dots indicate the cloud point for each ROI, determined using a threshold of $10\sigma_0$ (see text)).

The threshold of $10SD[\sigma(t < 2\text{ min})]$ is found to be sufficiently larger than the noise present in images before phase separation across multiple experiments, and small enough to coincide with the onset of condensate nucleation, generating reproducible transition temperatures. The mean of the set of ROIs transition temperatures is taken to be the transition temperature of the sample. Figure 3.12 (b) shows the pixel intensity standard deviation throughout the course of a temperature ramp, with images cropped around a ROI and each ROIs transition temperature highlighted by red dots.

As phase diagrams represent equilibrium phenomena, it is crucial that any effects of kinetics are removed from transition point measurements. Firstly, there is a delay between the thermostat reading on the thermal stage and the temperature of the sample. Secondly, there is a lag time between crossing the binodal and the nucleation and growth of condensates. Both of these effects can be alleviated by a temperature ramp speed that is much slower than both the rate of heat transfer between the sample and stage, and the lag time delaying the formation of condensates. When both of these conditions are

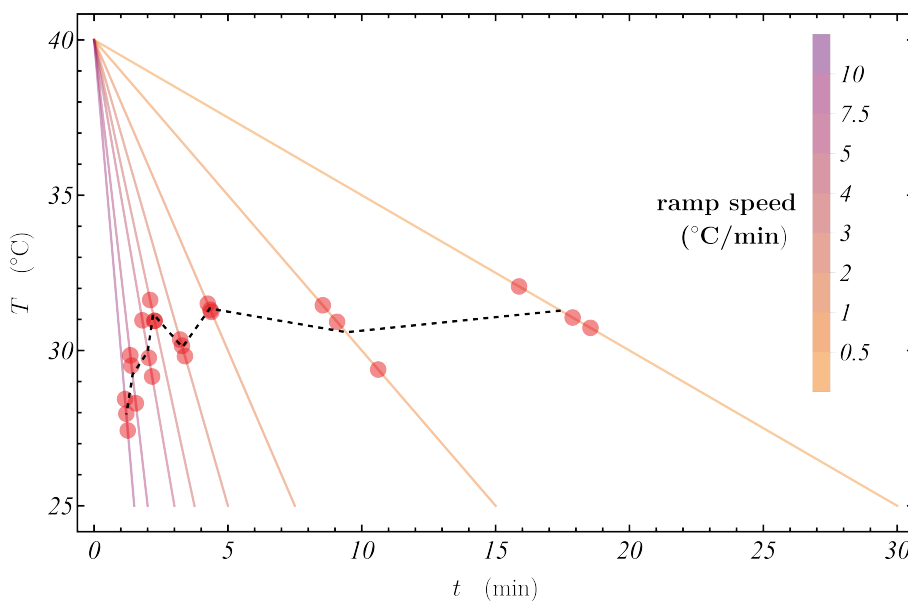


Figure 3.13: The effect of altering the temperature ramp speed on the measured transition temperature. The horizontal axis displays the elapsed time following the start of the ramp. Measurements were performed in triplicate for each ramp speed, with the respective transition temperatures indicated by red dots. The mean transition temperature for each ramp speed are joined by the dashed line.

satisfied, the recorded transition temperature will be representative of the true transition temperature. In order to determine an appropriate temperature ramp speed, transition temperature experiments were performed using 172.5 μM Ddx4^{N*} CtoS 1-236 in 150 mM NaCl 20 mM Tris pH 8.0, varying the temperature ramp speed.

Figure 3.13 shows a clear dependency of the measured transition temperature on ramp speed. As the ramp speed is decreased from 10 °C/min to 0.5 °C/min, the mean measured T_p increases from 27.9 °C, before plateauing around 31 °C for ramp speeds less than 4 °C/min. Therefore, performing transition temperature measurements with ramp speeds less than 4 °C/min will yield equilibrium T_p values. It is noted that only nucleation and growth, not spinodal decomposition, was observed in all of these experiments. Unless stated otherwise, all transition point measurements in this thesis are performed using a ramp speed of 2 °C/min. This value is chosen as the optimum payoff between potential variable nucleation rates between Ddx4^{N*} constructs, and experimental time.

3.3.3 Fitting to Flory-Huggins theory

Phase diagrams are fit by transforming Flory-Huggins binodals from χ to T , using Equation 3.32. For each value of r , binodals are generated by simultaneously solving the equilibrium constraints derived in Section 3.2.4,

$$\mu_p^\alpha(\phi_p^\alpha, \chi) = \mu_p^\beta(\phi_p^\beta, \chi) \quad \text{and} \quad \mu_s^\alpha(\phi_s^\alpha, \chi) = \mu_s^\beta(\phi_s^\beta, \chi), \quad (3.35)$$

as χ is varied. This is performed numerically, using the FindRoot function in Mathematica 13 [183], starting at $\chi = 3$ and decreasing in steps of 5×10^{-5} to χ_c . The initial ϕ_p^α value for optimisation at $\chi = 3$, is found by considering

$$\frac{\mu_p(\phi_p, \chi)}{RT} = \frac{1}{r} \ln \phi_p + \left(\frac{1}{r} - 1\right)(1 - \phi_p) + \chi(1 - \phi_p)^2, \quad (3.36)$$

in the limit of large χ . For strong phase separation $\phi_p^\beta \rightarrow 1$, so μ_p^β approaches 0. Combining this with Equation 3.35, as $\phi_p^\alpha \rightarrow 0$ gives

$$\phi_p^\alpha = \exp\{[r(1 - \chi) - 1]\}. \quad (3.37)$$

The starting point for ϕ_p^β is found by applying the same argument to μ_s , yielding

$$\phi_p^\beta = 1 - \exp\{[-\chi + 1 - 1/r]\}. \quad (3.38)$$

The optimum values for ϕ_p^α and ϕ_p^β at $\chi = 3$ are taken as the initial values for optimisation at subsequent χ values.

Experimental data points are transformed into the (ϕ_p, T) plane by multiplying protein concentrations in mol dm^{-3} , by their molar volume in dm^3 . Unique for each construct, the molar volumes were calculated based on their sequence, using the selected averages for each amino acid reported by Zhao [184]. For fitting, the chain length r is taken to be the

number of amino acids comprising the protein construct. The binodal in the (ϕ_p, χ) plane for the construct is fit by applying Equation 3.32. The NMinimize function in Mathematica 13 is used to find the ΔU and ΔS that minimize the mean squared displacement between the transformed binodal, and the experimental data points.

Bootstrapped error calculations

As there exists no analytical solution for the Flory-Huggins binodals, there is also no analytical form for the errors associated with the best-fit parameters. In this case, it is appropriate to perform a bootstrap analysis. The parameters ΔU and ΔS are recalculated a (large) number of times using data points obtained by resampling, with replacement, from the full set of experimental data points. The resultant distribution of the fitting parameters approximates their true distribution, given the input data. The validity of this increases with sample size, and the number of resamples included in the bootstrap. A good bootstrap will have a normally distributed resampled distribution of statistics. From the resampling distribution, statistical parameters can be calculated. In this work, the error in ΔU and ΔS is calculated as the standard deviation of their resampled distributions.

3.3.4 *In cell* partitioning measurements

The experimental work and analysis in this section was performed by Dr TJ Nott & Dr MD Crabtree.

HeLa cells expressing Ddx4^N tails fused to YFP form exogenous nucleoplasmic Ddx4^N-YFP condensates. Comparing the fluorescence intensity inside condensates, with the intensity outside condensates, gives a measurement of the degree of phase separation. This provides a platform to measure the effect of Ddx4^{N*} mutations on its intracellular phase separation.

HeLa cell culture and transfection

HeLa cells were cultured as previously described [59, 91]. Briefly, HeLa cells were grown on 22 mm diameter #1.5 glass coverslips (Agar Scientific) in growth media (high glucose DMEM (Gibco™) containing 10% FBS (Sigma)) at 37°C and 5% CO₂. Protein constructs were expressed in HeLa cells from pcDNA 3.1+ (Invitrogen) plasmids by transient transfection using the TransIT-LT1 Transfection Reagent (Mirus). Transfections used 0.5 – 1 µg plasmid DNA per coverslip and were carried out according to the manufacturer's instructions. Transfected cells were fixed approximately 24 hours after transfection.

Imaging fixed HeLa cells

HeLa cells expressing fluorescent proteins were grown on 22 mm diameter glass coverslips, washed twice with phosphate buffered saline (PBS) at 37°C and fixed with 4% paraformaldehyde (PFA) in PBS (Alfa Aesar) at 37°C, for 5 minutes. Cells were then washed three times with PBS to remove excess PFA. Cells were incubated for 5-10 minutes in the wash solution at each step. Coverslips were mounted on microscope slides (Fisher Scientific™) using Immu-Mount (Thermo Scientific™ Shandon™) mounting medium.

Cells were imaged on an Olympus FV1000 Laser Scanning Microscope based on an Olympus IX81 inverted microscope with a motorised stage, and equipped with an Olympus PLAPON60XOSC2 (NA 1.4) oil immersion objective. YFP (mCitrine) was excited with an argon 515 nm laser and YFP fluorescence was collected between (530 - 650 nm). DIC images were collected using illumination from the 515 nm laser.

Unless otherwise stated, imaging data were collected as stacks of 86 Z-slices (256 x 256 pixels (xy), 0.1 µm spacing (z), 2 µs per pixel scanning speed, 12 bit depth, 2 x line averaging). Image analysis was performed using ImageJ [185].

***In cell* determination of fluorescence intensity and partitioning**

Imaging data was collected with identical laser power and detector sensitivity so that fluorescence intensities across different samples were directly comparable. Image analyses to extract intensities for Ddx4^{N*}-YFP condensates and the nucleoplasm (excluding nucleoli) were performed using Fiji. First, maximum intensity z-projections were made from each stack and a histogram of pixel intensity (64 bins) was generated. The peak of the histogram (log10 y-axis) in the high-intensity range (i.e. close to the maximum value) was taken as a proxy for mean condensate fluorescence intensity in that stack. Nucleoplasmic fluorescence intensity was determined as the highest frequency bin following manual removal of regions corresponding to nucleoli, Ddx4^{N*}-YFP condensates and extra-nuclear regions. Fluorescence intensity values for Ddx4^{N*}-YFP condensates and the nucleoplasm were then averaged from multiple stacks for the same construct. Data represent the mean of ≥ 10 cells from a single experiment.

3.4 Results & discussion

3.4.1 Phase diagram fits & interaction parameters

Transition temperature measurements were performed in triplicate for 5-7 concentrations of each Ddx4^{N*} construct in buffer A. After removal from cryogenic storage, proteins were diluted to twice the values on the horizontal axes of Figure 3.14, measured by taking the mean of five nanodrop measurements. These transition point measurements are plotted on a linear scale in Figure 3.14 (a) and a linear-log scale in Figure 3.14 (b), showing the full width of the predicted phase diagram. The mean squared error of all fits is below 10^{-9} .

Common amongst all constructs investigated is a spinodal arm at Ddx4^{N*} concen-

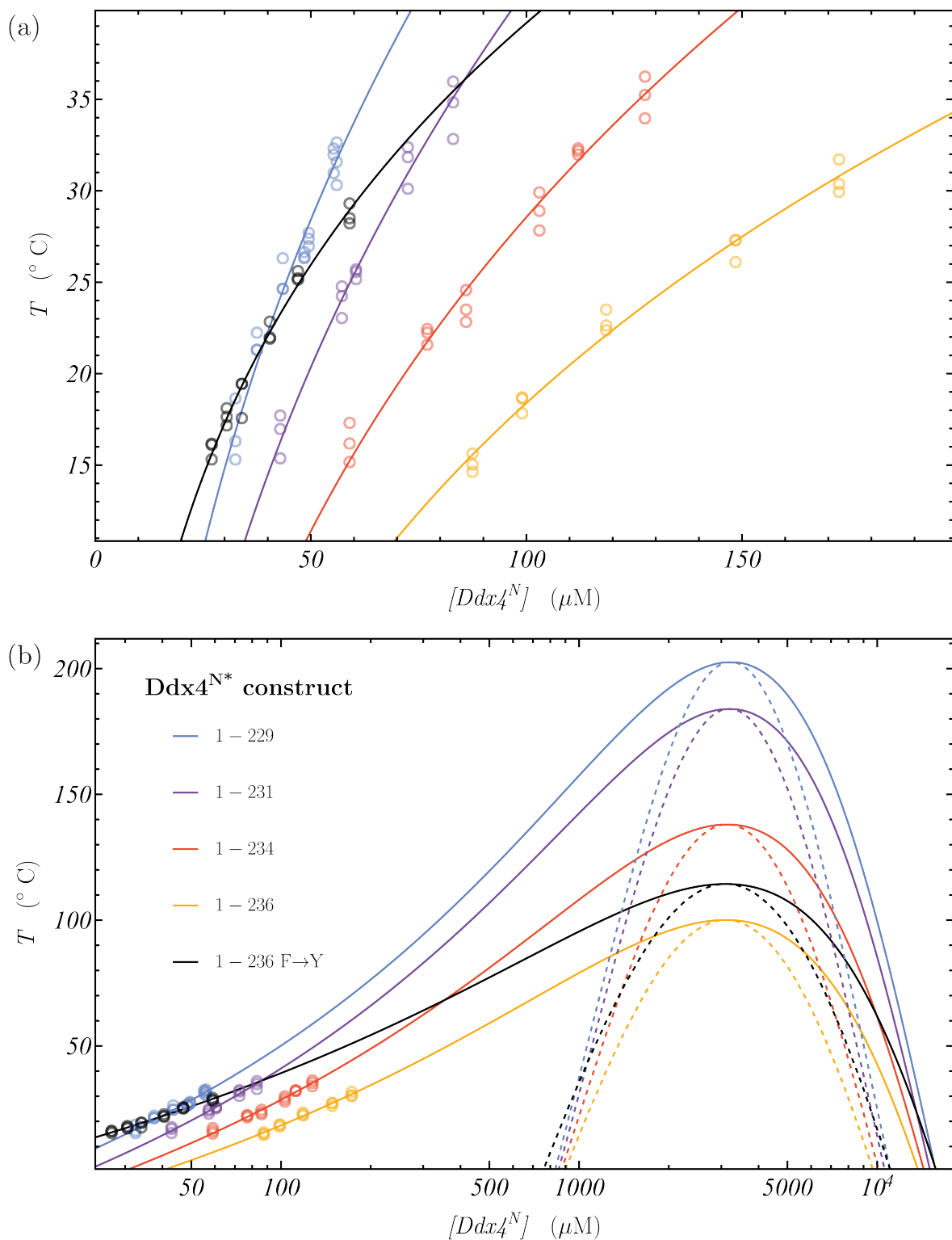


Figure 3.14: (a) Transition temperature data for the five Ddx4^{N*} constructs studied in this work. The legend is common amongst (a) and (b). The solid lines are the best-fit binodals. (b) Transition temperature data for the five Ddx4^{N*} constructs studied in this work. The solid and dashed lines are the best-fit binodals and spinodals respectively.

trations around 1 mM for ambient temperatures. This agrees with the lack of spinodal decomposition at any of the ramp speeds investigated for Ddx4^{N*} 1-236 in Figure 3.13. Phase separation through spinodal decomposition for Ddx4^{N*} 1-236 at 1 mM would require a rapid quench from 90 °C to 10 °C, and higher still for the other constructs. These conditions are far from physiological, both in terms of protein concentration and temperature. The gap between the spinodals and binodals in Figure 3.14 (b) at ambient temperatures suggest the sequence of Ddx4^{N*} has evolved to produce discrete condensates by nucleation and growth. To the authors knowledge, there have not been any *in cell* observations of intracellular spinodal decomposition forming endogenous condensates, and *in vitro* observations are rare [62].

Figure 3.14 (a) displays the enthalpic and entropic components of the interaction parameters for each construct. The error bars display their standard deviations, calculated by performing a bootstrap by resampling with replacement 1000×. The bootstrap distribution is displayed as probability density functions of their Z scores in Figure 3.15 (b), where

$$Z(x) = \frac{x - \mu}{\sigma}. \quad (3.39)$$

This allows a comparison to the normal distribution, shown by the grey area in Figure 3.15 (b). With 1000 samples, the bootstrap distribution of ΔU and ΔS have tended to the normal distribution. The bootstrap distributions for ΔU and ΔS are nearly perfectly correlated; the errors are therefore displayed as diagonal lines in Figure 3.15 (a), reflecting this correlation.

Due to the similarity in the length of the constructs, their critical interaction parameters (Equation 3.30) are within 0.1% of 0.567. The contours in Figure 3.15 (a) give the interaction parameter at 22 °C, χ^* , relative to $\chi_c = 0.567$ as a function of ΔH and ΔS . The distance of χ^* from the region marked in black gives a measure of the phase separation propensity of each construct at room temperature. All constructs in Figure

3.15 (a) have a positive energetic contribution to χ and a negative entropic contribution to χ , in agreement with previous measurements of Ddx4^{N*} interaction parameters [59,62].

The experimental methods presented in this thesis address only the left-hand ‘dilute’ arm of the binodals. Measurements of the right-hand side were attempted by pipetting sub μL volumes from $\sim 1\mu\text{L}$ of the condensed phase (obtained by centrifuging phase separated samples for 1 min at 1000 rcf) into a 500 mM NaCl 20 mM TRIS pH 8 buffer at a 1:20 dilution ratio. The concentration of this solution was then measured using spectrophotometry, returning in values with a high variance within samples, and even higher variance between samples; $2324 \pm 532\mu\text{M}$, $1627 \pm 496\mu\text{M}$ and $115 \pm 3\mu\text{M}$, for Ddx4^{N*} 1-236 samples where concentrations were measured in triplicate. As such, based on previously reported Flory-Huggins phase diagrams well fit to both the dilute and dense proteinacious arms of the binodal for Ddx4^N [62], we decided to proceed with only the accurate measurements of the left-hand arm of binodals. Here, the phase diagram fits based on only this section of the binodal, in Figure 3.14, are enough to resolve significant differences between the five Ddx4^{N*} constructs.

3.4.2 Release of water & counterions drives Ddx4^{N*} phase separation

Examining Figure 3.15 (a), we see the entropic part of χ (ΔS) for all Ddx4^{N*} constructs, falls between $-3\text{ J K}^{-1}\text{ mol}^{-1}$ and $-4\text{ J K}^{-1}\text{ mol}^{-1}$. These values are measurements of the entropy change associated with bringing the protein into contact with the solvent, from a pure protein phase (see Figure 3.5). It has recently been alluded that the entropic release of protein-associated water is a driving force of phase separation [186,187]. The lattice sites in the models used to fit the phase diagrams in Figure 3.14 contain roughly 4.5 water molecules or 1 amino acid. Therefore, solvent sites can contain many configurations of water, unaccounted for by Equation 3.11, whereas protein sites have only one trans-

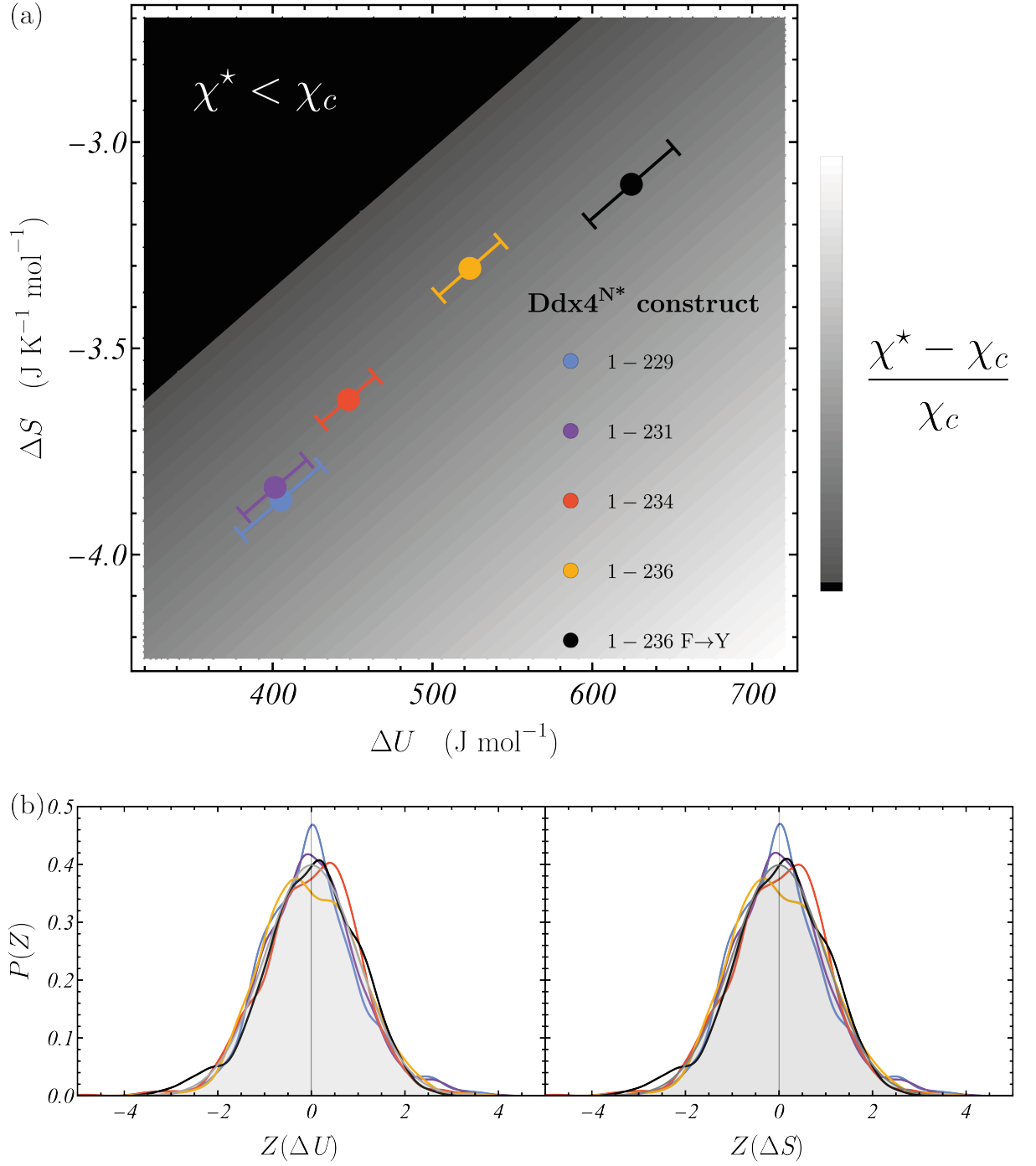


Figure 3.15: (a) Enthalpic and entropic contributions to the interaction parameter for each construct. Contours display lines of equal interaction parameter at 22 °C. The black region represents the parameter space where no phase separation is observed at 22 °C as $\chi^* < \chi_c$. (b) Histograms of z-scores for ΔU and ΔS statistics, generated by fitting phase diagrams to transition point data generated by resampling, with replacement, experimental data points 1000×. The gray area represents the normal distribution. The plot legend is as in (a).

lational configuration available. In the vicinity of hydrophobic residues, the number of configurations available to water that maximise the number of hydrogen bonds decreases.

The associated decrease in entropy, relative to bulk water, is the entropic hydrophobic effect.

The impact of this hydrophobic effect can be estimated using [173]

$$\Delta S = S_{ps} - \left(\frac{S_{pp} + S_{ss}}{2} \right) \approx \Delta S_{water}. \quad (3.40)$$

The entropy associated with *s-s* interactions contributes half as much as *p-s* interactions to the entropic contribution to χ . Taking Phe, Tyr, Trp, Ala, Val, Ile, Leu, Met and Pro residues to be hydrophobic, these account for 23.7% of Ddx4^{N*} 1-236. The change in Equation 3.40 concerns the rearrangement of 4.5/2 water molecules. Assuming that the configurations available to water are reduced by a factor of 2 in the vicinity of a hydrophobic residue [173], and that 23.7% of protein-solvent contacts are hydrophobic, we can apply Equation 3.3 to give

$$\Delta S_{water} = 0.237R \ln (1/2)^{4.5} = -3.07 \text{ J K}^{-1} \text{ mol}^{-1}. \quad (3.41)$$

This is remarkably close to the ΔS values plotted in 3.15 (a). This calculation assumes a constant entropic hydrophobic effect over the temperature ranges investigated, 15-37 °C, as expected according to references [173, 188].

The calculation in Equation 3.41 does not consider rearrangement of the other solutes, 150 mM NaCl and 20 mM Tris. In 2015, Nott *et al* observed that increasing [NaCl] from 100 mM to 200 mM was associated with a $\Delta\Delta S$ of $0.5 \text{ J K}^{-1} \text{ mol}^{-1}$, and ascertained a logarithmic dependency [59]. The entropically driven attraction between charged polymers, is due the release of counterions upon their association [67, 189, 190]. At pH 8.0, Ddx4^{N*} 1-236 carries 70 charged residues, which, at 150 mM NaCl will be associated with counterions in the mixed state. The discrepancy of the value calculated by Equation 3.41 and the measured values in Figure 3.15 is therefore likely due to the liberation of these Na⁺ and Cl⁻ counterions. These arguments are consistent with the notion that entropy changes

are dominated by changes in translational entropy [180].

3.4.3 F→Y substitution enhances Ddx4^{N*} phase separation

Previous research by Wang *et al* identified Y→F as a deleterious mutation for FUS phase separation. Here, we discuss the effect of the reverse mutation, F→Y, on Ddx4^{N*} phase separation. Figure 3.14 shows that the Ddx4^{N*} F→Y mutation decreases the saturation concentration by a factor of four at room temperature, reflected also by the proximity to χ^* to χ_c in Figure 3.15 (a). Accompanying this increased phase separation propensity, is a change in the enthalpic contribution to the interaction parameter from $523 \pm 19 \text{ J mol}^{-1}$ to $624 \pm 26 \text{ J mol}^{-1}$. Relative to Ddx4^{N*} 1-236, Ddx4^{N*} F→Y increases the probability of a protein site in the lattice being occupied by tyrosine by $14/241 = 0.058$. The energetic stabilisation per tyrosine, relative to phenylalanine, is therefore given by $101/0.058 \text{ kJ mol}^{-1} = 1.739 \text{ kJ mol}^{-1}$, comparable to a weak hydrogen bond. This stabilisation follows from the electron donating power of the para hydroxyl substitution on the phenyl ring of tyrosine, compared to phenylalanine, increasing the electron density on the ring and strengthening the electrostatic attraction with the electron-poor arginine.

Figure 3.15 (a) shows that the F→Y mutation increases the entropic contribution to the interaction parameter by $\Delta\Delta S = 0.2 \text{ J K}^{-1} \text{ mol}^{-1}$. The modest increase in ΔS is attributed to the decreased hydrophobicity of tyrosine relative to phenylalanine, resulting in increased water entropy on solvent sites adjacent to protein sites. The associated change in water configurations can be approximated using the same logic as in Section 3.4.2, where

$$\Delta\Delta S = 0.2 \text{ J K}^{-1} \text{ mol}^{-1} \approx \Delta\Delta S_{\text{water}}. \quad (3.42)$$

Again, we apply Boltzmann's formula (Equation 3.3), with x representing the fold change

in configurational freedom per water molecule,

$$\Delta\Delta S = 0.058R \ln x^{4.5} = 0.2 \text{ J K}^{-1} \text{ mol}^{-1}. \quad (3.43)$$

Equation 3.43 has solution $x = 1.1$, reflecting a small increase in the number of available water configurations on a solvent site adjacent to tyrosine, relative to phenylalanine. These configurations are stabilised through hydrogen bonding to the para hydroxyl on tyrosine. Only a slight increase in configurations is observed, as π conjugation of the para hydroxyl on tyrosine with its phenyl ring inhibits its ability to hydrogen bond, compared to aliphatic residues serine and threonine, which are typically classed as hydrophilic. The decreased hydrophobicity of Tyr compared to Phe is expected to have minimal impact on ΔU , as the hydrophobic effect in water far from its boiling point is predominantly entropic [188].

These data demonstrate the ability of F→Y substitutions in the stabilisation of intrinsically disordered phase separation, concurrent with those published on the prion-like domain of FUS [70]. More recently, the same strategy has been by Bremer *et al* applied to tune the phase separation of a human isoform of hnRNPA1, where they demonstrated an increased phase separation propensity with the equivalent substitution [73]. Taken together, these data indicate tyrosine may evolutionary selected for, due to its ability to enhance IDP phase separation.

3.4.4 Reduced net charge favours phase separation in a sequence context dependent manner

Charged polymers interact directly through energetic coulombic electrostatic interactions, and indirectly through their effect on the local distribution of counterions, constituting an effective entropic interaction [191]. In the case of like charges, both of these interactions are repulsive, with reducing absolute Ddx4^{N*} net charge expected to increase the energetic

part of χ and favour phase separation. Figure 3.14 (a) and Figure 3.14 (b) shows that decreasing Ddx4^{N*} net (negative) charge in the series 1-236, 1-234, 1-231, 1-229, favours phase separation, decreasing the saturation concentration and increasing the upper critical solution temperature.

Figure 3.15 (a) shows reducing the absolute net charge per residue (NCPR) alters both the energetic and entropic contributions to the interaction parameter, in a highly correlated fashion ($r = 0.996$), with monotonically increasing ΔU and ΔS , shown in Figures 3.16 (a) and (b). Increasing ΔU with Ddx4^{N*} NCPR is contrary to what is expected for the introduction of ‘excess’ charged amino acids with no complementary charged partners. In 2020, Zheng *et al* [192] performed atomistic simulations of the phase separation of FUS and Laf-1 IDPs, carrying net charges of -2 and +4 respectively. They saw preferential inclusion of Na⁺ into FUS condensates, and preferential inclusion of Cl⁻ into Laf-1 condensates, with the degree of ion partitioning scaling with the absolute net charge of the protein, conforming to the electroneutrality principle. As discussed in Section 3.4.2, the release of counterions accompanying Ddx4^{N*} phase separation, decreases to the entropic contribution to χ . The introduced excess charges likely decrease the entropic gain of counterion release.

Upon increasing NCPR from 0.0047 to 0.0089, from Ddx4^{N*} construct 1-229 to 1-231, adding Glu-Ala to the C-terminus, ΔU and ΔS remain unaffected. In 2020, Madinya *et al* presented work on the effect of adjacent positively and negatively charged monomers along polyampholyte sequences. These ‘charge-sign’ interfaces undergo favourable intramolecular coulombic interactions [67]. The sequence of Ddx4^{N*} contains many such charge-sign interfaces, with one positioned near the C-terminus of Ddx4^{N*}, between K228 and E230. The backbone distance between K228 and E230 is roughly 7 Å, less than the Debye length at 150 mM NaCl - 7.9 Å. An intramolecular interaction between these two oppositely charged residues, exchanged for an intermolecular interaction in the phase separated state, would result in little to no net ΔU . Moving from 231 to 229, the K228-E230 intramolecular interaction is replaced by an intramolecular interaction between K228 and

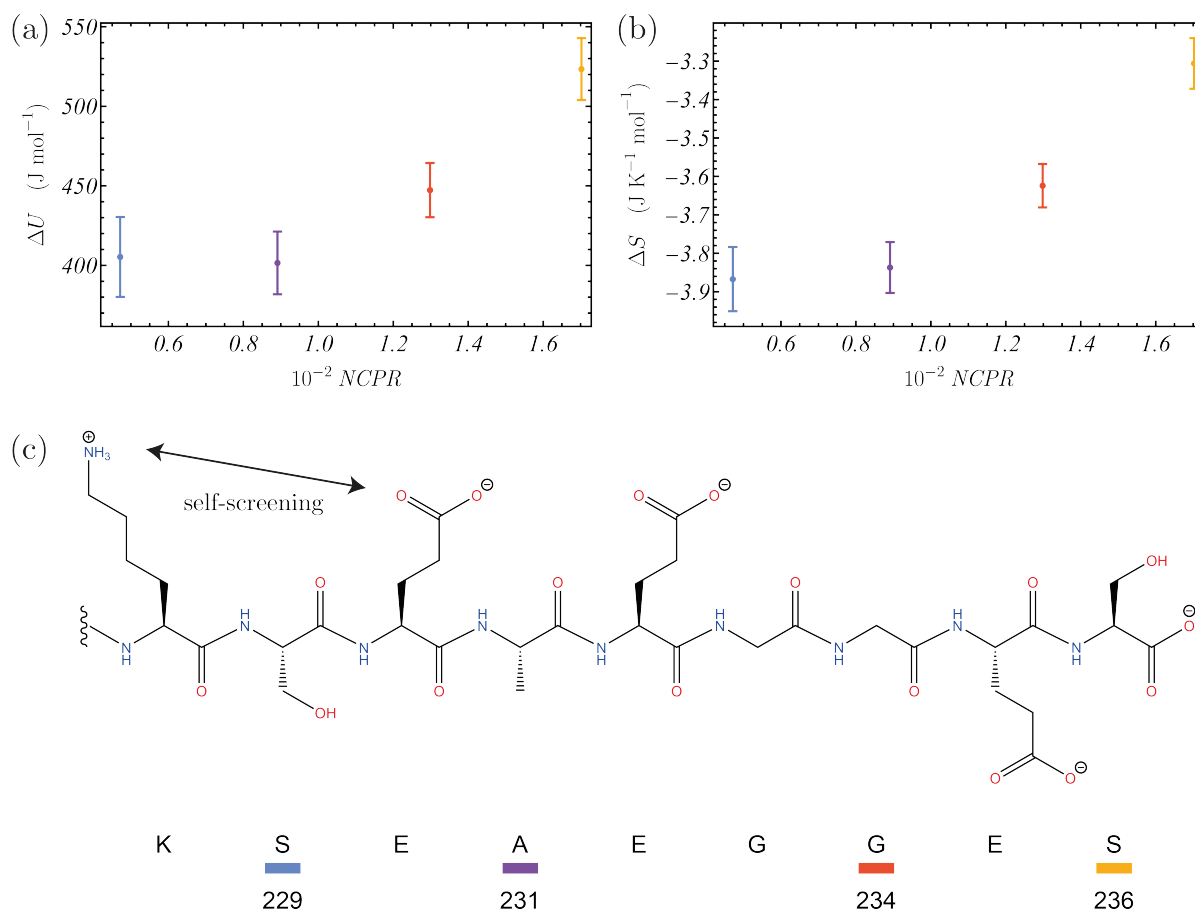


Figure 3.16: (a) Absolute net charge per residue (NCPR) plotted against the internal energy contribution to the interaction parameter for Ddx4^{N*} length mutants. (b) NCPR plotted against the entropic contribution to the interaction parameter for Ddx4^{N*} length mutants. (c) Skeletal formula of the C-terminus of Ddx4^{N*} 1-236, highlighting the C-termini of the four length mutants. The self-screening attributed to the charge-sign interface between K228 and E230 is illustrated.

the terminal carboxyl at position 229. Therefore the $\Delta\Delta U$ associated with this mutation is roughly 0.

The mirrored trend in ΔS is likely due to a degree of self-screening between K228 and E230, depleting the concentration of counter ions surrounding both residues. This self-screening is maintained in the 1-229 Ddx4^{N*} construct, instead occurring between K228 and the terminal carboxyl at position 229. The reduced counter-ion concentration at the charge-sign interface [67] between K228 and E230 explains the suppressed effect of increased NCPR on construct 231 relative to construct 229.

The strong correlation between ΔS and ΔU implies the proposed increased ion par-

titutioning with increasing NCPR also causes the increased ΔU observed in Figure 3.16 (a). Upholding the electroneutrality principle, the condensates formed by the Ddx4^{N*} mutants with greater (negative) NCPR must contain more free cations, with Na⁺ the dominant cationic species in the solution. Previous work has suggested a decreased dielectric constant in the condensed phase of Ddx4^{N*} [59], as well as other model biomolecular condensate systems [193, 194], relative to bulk water, due to decreased solvent polarity in condensates. A decreased dielectric constant augments coulombic interactions between complementary charges in solution, and would strengthen interactions between excess charged residues and their complementary counterions.

Here we have demonstrated reducing absolute net charge per residue as a method to increase χ , favouring phase separation. The effect is shown to be sequence-context dependent, suppressed by the presence of charge-sign interfaces. The augmentation of phase separation seems to be driven not by decreased electrostatic repulsions, but rather through decreased partitioning of counterions into the condensed protein phase. We acknowledge the recent (2022) article from Bremer *et al* [73], showing decreased NCPR augmenting the phase separation of a human isoform of hnRNPA1, mirroring the trend presented here, broadening the importance of our observations.

3.4.5 Modulation of intracellular electrostatic interactions

Experimental work in this section was carried out by Dr TJ Nott & Dr MD Crabtree.

Riback *et al* showed that the *in cell* formation of exogenous Ddx4^N-based condensates behaves as a two component phase separation event, consisting of Ddx4^{N*} and solvent [195]. This affords a direct comparison between *in vitro* and *in cell* Ddx4^N phase separation. To facilitate *in cell* imaging of condensates, DNA encoding the (folded) yellow fluorescent protein mCitrine, concatenated with the intrinsically disordered C-terminal tail of wild-type (WT) Ddx4 fused to the C-terminus of Ddx4^N WT length mutants, as illustrated in Figure 3.17 (a). This permits a study of the effect of altering net charge per residue

on phase separation in cells. The method outlined in Section 3.3.4 was used to prepare and image HeLa cells expressing Ddx4^N charge mutants through transient transfection. Figure 3.17 (a) shows confocal fluorescence microscopy images of representative cells, with an inverted greyscale lookup table, highlighting Ddx4^N condensates in black.

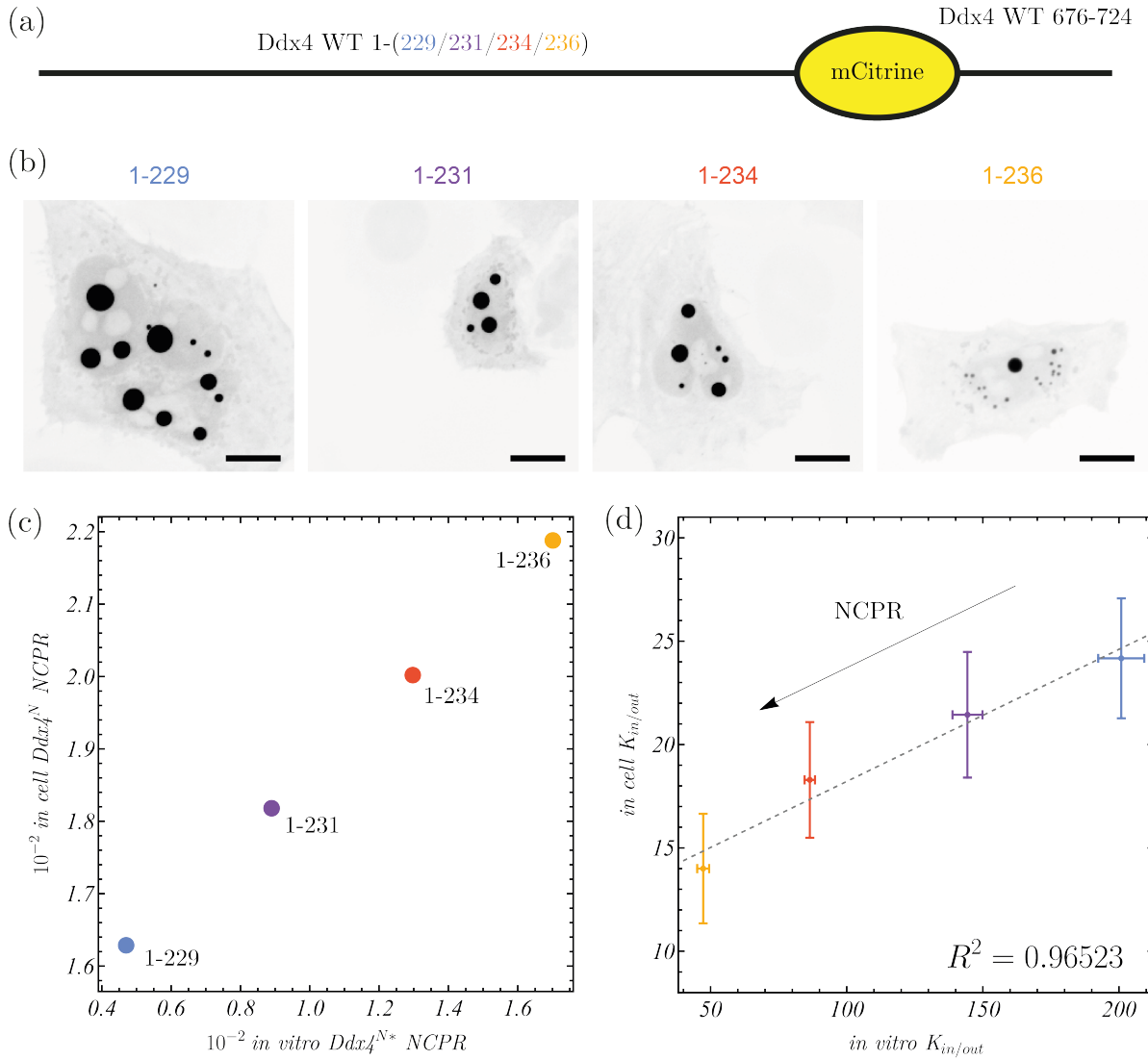


Figure 3.17: (a) Architecture of the Ddx4^N fusion proteins expressed in HeLa cells. (b) Negative greyscale images of HeLa cells expressing Ddx4^N charge mutants, obtained using confocal fluorescence microscopy. The scale bar represents 10 μm . (c) The (negative) net charge per residue (NCPR) for the *in vitro* Ddx4^{N*} constructs plotted against the NCPR for the *in cell* Ddx4^N constructs. (d) *In cell* partitioning at 37°C taken from intracellular fluorescence measurements plotted against *in vitro* partitioning, derived from the equilibrium concentrations at 37°C displayed on the phase diagrams in Figure 3.14. Vertical error bars are calculated as explained in Section 3.3.4, horizontal errors are taken from equilibrium concentrations calculated using the upper and lower errors in χ , corresponding to the errors in the fit *in vitro* binodals.

The ratio of the mean pixel intensity inside the condensates, to that measured outside the condensates, is directly proportional to the concentration of Ddx4^{N*} in those two intracellular regions. This parameter, $K_{in/out}$, increases with the distance from the critical point on the phase diagram, facilitating its utility as a measure of phase separation propensity. $K_{in/out}$ for *in vitro* Ddx4^{N*} phase separation is easily obtained from the binodals presented in Figure 3.14 (b). The *in cell* and *in vitro* $K_{in/out}$ are plotted against each other in Figure 3.17 (b), showing a strong linear correlation between the two datasets. These data show that modulation of Ddx4^{N*} net charge can be used to control its intracellular phase separation, through the same mechanisms discussed for *in vitro* studies in Section 3.4.4. This indicates the effect of altering net charge per residue on protein phase separation is not suppressed by the intracellular solvent environment, raising the possibility that net charge per residue may be subject to evolutionary pressure, and low net charge of phase separating IDPs is an evolved feature.

3.5 Conclusions & outlook

The fitting of Flory-Huggins theory to experimentally determined phase diagrams of Ddx4^{N*} facilitated an investigation into the entropic and energetic components of the effective interactions between Ddx4^{N*} chains. A rough calculation supported the notion that hydrophobic interactions are significant drivers of phase separation, manifesting as entropic forces - as expected around room temperature. This is consistent with recent work by Kranier *et al*, where they emphasised the importance of hydrophobic contacts in driving the phase separation of a diverse range of sequences, including FUS, TDP-43, Brd4 and Sox2 [187]. Combined with the data presented in this thesis, these works suggest an overlooked importance of aliphatic residues as drivers of condensate formation. Future work may focus on mutations interchanging Ile, Leu, Val and Ala residues, as well as bioinformatics studies on any sequence patterning of these residues.

Two strategies for tuning the phase separation of Ddx4^{N*} have been presented. The first, performing a sequence-wide phenylalanine to tyrosine substitution, promoted phase separation by increasing the energetic component of the interaction parameter, attributed to a strengthening of cation- π interactions, in agreement with studies on FUS and hn-RNPA1 proteins [70, 73]. The second involved reducing the net charge per residue. It is pertinent that at room temperature, a decrease in the magnitude of the net charge of Ddx4^{N*} by 3, has a similar effect on the phase separation propensity of Ddx4^{N*} as does swapping 14 phenylalanine residue for tyrosine. This indicates net charge per residue has a stronger effect than F $\rightarrow$ Y substitution, with the latter offering a finer control over phase separation propensity. The Flory-Huggins interaction parameters measured in this chapter are carried forwards in subsequent chapters, for mean-field calculations of interfacial tension.

The mechanism through which decreasing net charge per residue influences phase separation has been demonstrated to be entropic in nature, hypothesised to be driven by decreased counterion partitioning. Further research on the effect of salt concentration on this trend may be used to falsify this hypothesis. The effect of net charge per residue on phase separation is also shown to be dependent on the sequence context, with the presented explanation invoking the formation of intramolecular electrostatic interactions at charge-sign interfaces. The influence of charge-sign interfaces on Ddx4^{N*} phase separation may be further probed by separating the adjacent oppositely charged residues, by introducing serines or glycines, inhibiting the formation of intramolecular electrostatic interactions across the interface.

Expression of Ddx4 net charge per residue mutants in HeLa cells formed nuclear condensates, whose innate partitioning of Ddx4 follows the same trend as the equivalent *in vitro* Ddx4^{N*} mutational series. This indicated that the mechanism through which net charge per residue affects phase separation is reproduced in the highly complex environment of living cells.

Acknowledgments

We thank Dr Timothy James Nott & Dr Michael David Crabtree for performing the *in cell* experiments.

Chapter 4

Implementation of Axisymmetric Drop Shape Analysis

ABSTRACT

This chapter is focused on the development of an implementation of axisymmetric drop shape analysis (ADSA), for the in vitro inference of interfacial phenomena of microscopic biomolecular condensates. This forms the basis for investigations presented in Chapters 5 and 6. Here, we ground this methodology in theory, based around the Young-Laplace equation. The generation and confocal imaging of sessile condensates is explained, detailing corrections for aberrations present in our optical setup. Then we discuss the bespoke image analysis and subsequent fitting protocol, enabling the extraction of condensate contact angles and capillary lengths. This chapter concludes with a short investigation comparing ADSA measurement of biomolecular condensate contact angles, with a manual method used by Feric et al [11] to measure contact angles of model nucleolar condensates.

4.1 Introduction

The shape of a sessile liquid drop on a solid surface is dependent on its tendency to wet (spread over) the surface, its interfacial tension and its weight. Precise analysis of the shape can be used to measure these factors, the first through the contact angle at the base of the droplet, and the latter two via the capillary length. For axisymmetric sessile drops, there exists a convenient form of the Young-Laplace equation, which can be fit to images of drops, yielding measurements of capillary lengths and contact angles. Commonly known as axisymmetric drop shape analysis (ADSA) [144–148], this technique is potentially well suited to *in vitro* specimens of biomolecular condensates, affording measurements of contact angles and capillary lengths. Its application to biomolecular condensates has only recently, in 2021, been reported in the literature by Ijavi *et al* [136], where they imaged condensates roughly 500 μm tall using brightfield microscopy, focusing entirely on capillary length measurements.

The formation of Ddx4^{N*} condensates through nucleation and growth presented in Chapter 3, typically produces thousands of condensates per μL of sample. These μm sized drops proceed to grow and sediment to the bottom surface of the sample, generating vast fields of highly symmetric condensates. In this chapter, we focus on the implementation of ADSA, to allow measurement of condensate interfacial tensions and contact angles for investigations presented in Chapters 5 and 6.

A similar experimental system has been used by Feric *et al* to measure the contact angles of condensates formed by nucleolar components [11], relating them to the architecture of nucleolar subcompartments. They refrained from ADSA, instead measuring contact angles by manually drawing tangents on condensate xz projections captured with 3D confocal microscopy. Compared to an automated ADSA implementation, this method introduces potential for human error through manual drawing of tangents, as well as requiring more hours on the part of the scientist.

Here, we describe the theoretical background for axisymmetric drop shape analysis, as well as methods to prepare samples of axisymmetric sessile Ddx4^{N^*} condensates of varying sizes. The imaging of these condensates, obtained using 3D confocal microscopy, and subsequent drop-shape analysis, implemented using bespoke image analysis protocols implemented in Mathematica 13 [183] are then described. This chapter concludes with a comparison of our ADSA implementation with the manual method presented by Feric *et al*, on two Ddx4^{N^*} condensate setups with markedly different drop shapes.

4.2 Theoretical background

4.2.1 Curved surfaces

Away from the base of the sessile droplet, fluid-fluid interface is necessarily curved. The thermodynamic penalty of curvature is well described by the Young-Laplace equation which we will now derive [196].

The free energy of system composed of two bulk phases, α and β , separated by an interface with interfacial tension γ is

$$F_{total} = F_{\alpha} + F_{\beta} + \gamma A. \quad (4.1)$$

In the case of a small perturbation of the interface by δz in the direction normal to the equilibrium surface, we have

$$dF_{total} = (P_{\beta} - P_{\alpha})\delta V + \gamma\delta A = 0, \quad (4.2)$$

where δV and δA are the associated respective volume and area changes. The goal is to

relate δA and δV to δz . For a curved surface,

$$\delta A = \left(1 + \frac{\delta z}{R_1}\right)dl_1\left(1 + \frac{\delta z}{R_2}\right)dl_2 - dl_1dl_2, \quad (4.3)$$

where dl_1 and dl_2 are orthogonal length elements along the surface, with principle radii R_1 and R_2 . Expanding the brackets gives

$$\delta A = \left(\frac{\delta z}{R_1} + \frac{\delta z}{R_2} + \frac{\delta z^2}{R_1R_2}\right)dl_1dl_2, \quad (4.4)$$

with the final term on the right hand side equal to zero, as $\delta z^2 \sim 0$. Noting that $dl_1dl_2 = dA$ and factoring out δz from Equation 4.4 we have

$$\delta A = \left(\frac{1}{R_1} + \frac{1}{R_2}\right)\delta z dA, \quad (4.5)$$

The volume change can be rewritten as the product of δz with the surface element, dA . Taking $\delta z dA = dV$ and substituting Equation 4.5 into Equation 4.2, yields

$$dF = \left[P_\beta - P_\alpha + \gamma\left(\frac{1}{R_1} + \frac{1}{R_2}\right)\right]dV = 0. \quad (4.6)$$

As $dV \neq 0$, we obtain the Young-Laplace equation [197, 198]:

$$\Delta P_{\beta\alpha} = \gamma\left(\frac{1}{R_1} + \frac{1}{R_2}\right). \quad (4.7)$$

Equation 4.7 states that the pressure across a convex surface greater on its interior. This pressure difference is the force acting on the surface to reduce the proportion of molecules at the interface by reducing the surface area to volume ratio of the system.

4.2.2 The Young-Laplace equation for a sessile drop

Drops sitting on a solid substrate are subject to both gravitational and capillary (interfacial) forces. The former drives a depression of the droplet, while the latter works to minimize the droplet surface area. Balancing these two forces gives the shape of the equilibrium fluid-fluid surface for a sessile droplet of given density and surface tension.

Application of the Young-Laplace equation, Equation 4.7, to the the sessile drop in Figure 4.1 (a), we see that

$$P_1 = P_2 + \frac{2\gamma}{R_0}, \quad (4.8)$$

where R_0 is the local curvature of the interface at the apex of the drop. Equally,

$$P_3 = P_4 + \gamma \left(\frac{1}{R_1} + \frac{1}{R_2} \right). \quad (4.9)$$

Taking the difference of these two equations we have,

$$g\rho_\alpha h = g\rho_\beta h + \frac{2\gamma}{R_0} - \gamma \left(\frac{1}{R_1} + \frac{1}{R_2} \right), \quad (4.10)$$

where the left hand side and the first term on the right hand side, account for the hydrostatic pressure difference over a height, h , in phases with density ρ . Rearranging Equation 4.10 and collecting like terms gives

$$g\Delta\rho h = \gamma \left[\frac{2}{R_0} - \left(\frac{1}{R_1} + \frac{1}{R_2} \right) \right]. \quad (4.11)$$

Equation 4.11 expresses mechanical equilibrium between gravitational and capillary forces at each point in the droplet.

Droplets resting on a homogeneous surface, with a constant contact radius, are rotationally symmetric about their principle axis - they are axisymmetric. The fluid-fluid

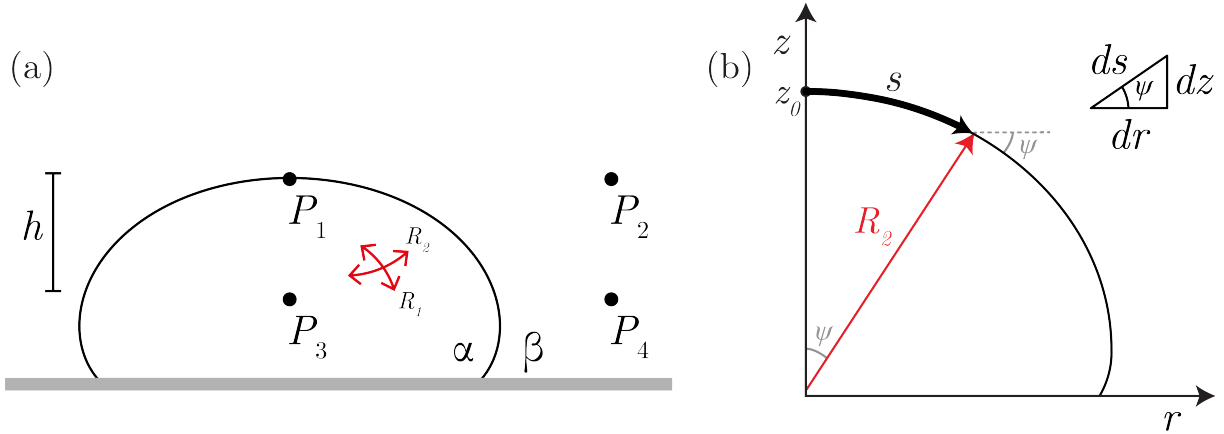


Figure 4.1: (a) Schematic of a sessile drop of phase α in coexistence with phase β resting on a solid surface. P represents pressure at different points, R_1 and R_2 are the principle radii of curvature at a given height h , measured from the apex of the drop. (b) Defining the parameters in Equation 4.12 for an axisymmetric droplet with height z_0 , measured from its base.

surface of axisymmetric droplets can be fully parameterised by $r = r(z)$, where r is the droplet radius at a height z . Figure 4.1 (b) shows the distance along the surface, s , and its angle of incidence, θ , are parameterized by

$$ds^2 = dr^2 + dz^2 \quad \frac{dr}{ds} = \cos \psi \quad \frac{dz}{ds} = \sin \psi. \quad (4.12)$$

The axial and circumferential radii of curvature are then given by

$$R_1 = \frac{ds}{d\psi} \quad \text{and} \quad R_2 = \frac{r}{\sin \psi}, \quad (4.13)$$

Taking h as $z_0 - z$, where z_0 is the z coordinate of the apex of the drop, we can now rewrite Equation 4.11 as

$$\frac{d\psi}{ds} = \frac{2}{R_0} + \frac{(z - z_0)}{\ell_c^2} - \frac{\sin \psi}{r}. \quad (4.14)$$

The second term in the denominator, the capillary length, is given by

$$\ell_c = \sqrt{\frac{\gamma}{g\Delta\rho}}. \quad (4.15)$$

The capillary length ℓ_c , gives the length scale over which capillary forces dominate over gravitational forces. When $|z - z_0| \ll \ell_c$, Equation 4.14 describes a spherical cap, a minimal surface for the sessile drop. Therefore, in the absence of gravitational effects, the Young-Laplace equation predicts spherical cap shaped droplets.

Integrating Equation 4.14 gives the equilibrium profile of the fluid-fluid interface, $\psi(s)$, where ψ and s depend parametrically on r and z . Defining s_{max} as the first point where the fluid-fluid surface meets the solid surface, the contact angle is simply

$$\theta = -\psi(s_{max}), \quad (4.16)$$

The fitting of Equation 4.14 to images of droplet forms the basis of axisymmetric drop shape analysis (ADSA), used to measure contact angles and capillary lengths.

4.3 Methods

4.3.1 Sample preparation

Sessile condensates were prepared as detailed in Section 2.8, including either Cy3B or Alexa-488 in the dilution buffer to give final concentrations of 5 μM and 2.5 μM respectively. These concentrations were found not to influence the phase diagram of Ddx4^{N*}, supporting their function as an inert contrast agent. Following the protocol to completion and allowing samples to cool to room temperature resulted in nucleation of condensates as the sample passed the transition temperature. These condensates proceeded to sediment over the course of 10 min, as in Figure 4.2 (a). After 1 h, all condensates had long sedimented, leaving a field of sessile droplets resting on the bottom surface of the sample, as in Figure 4.2 (b).

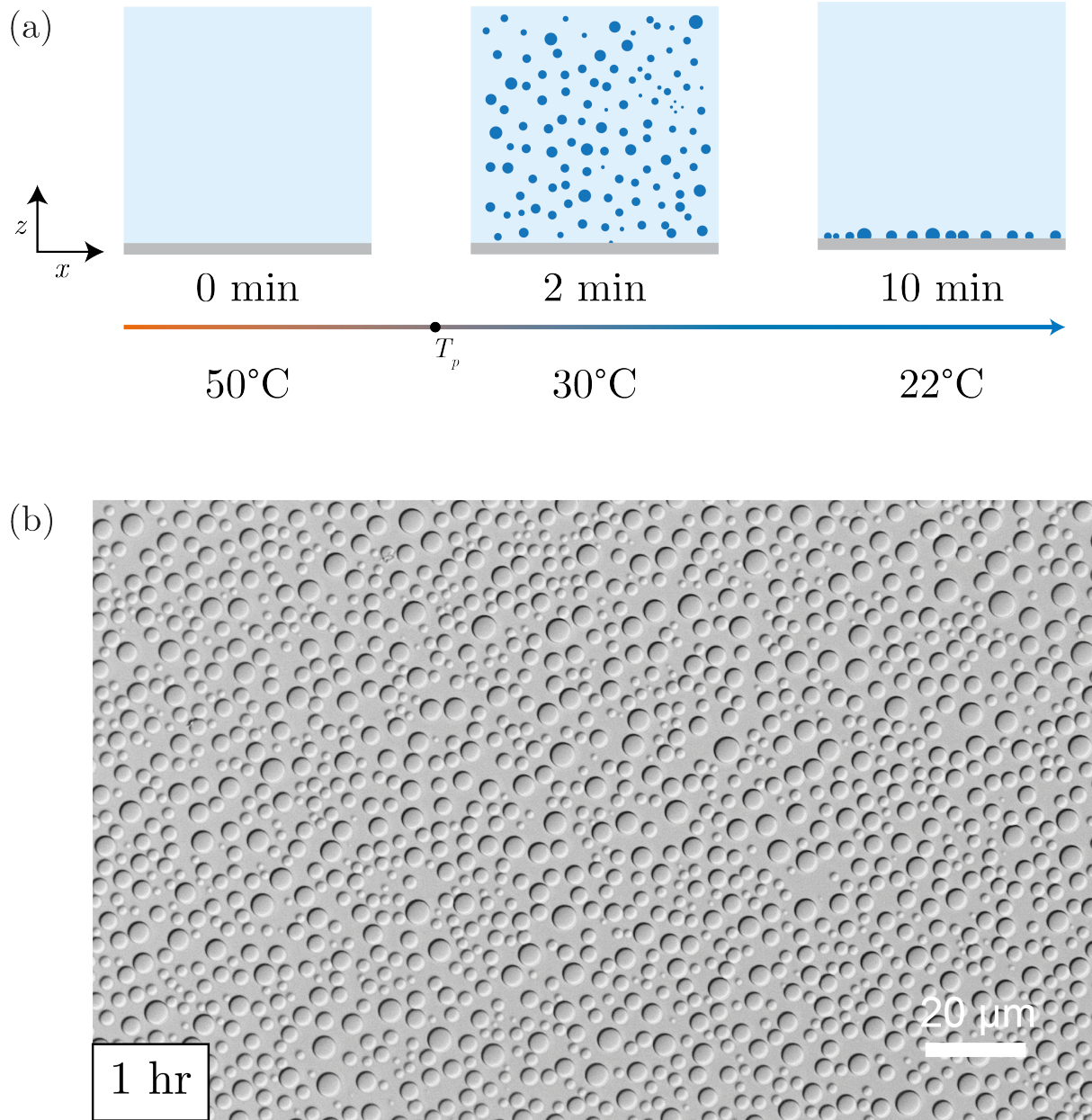


Figure 4.2: (a) As samples cool down from 50 °C towards room temperature, they pass through the transition point T_p , resulting in the nucleation of condensates which proceed to sediment. (b) xz image of sessile Ddx4^{N*} 1-236 condensates resting on siliconized glass imaged using DIC one hour after sample preparation.

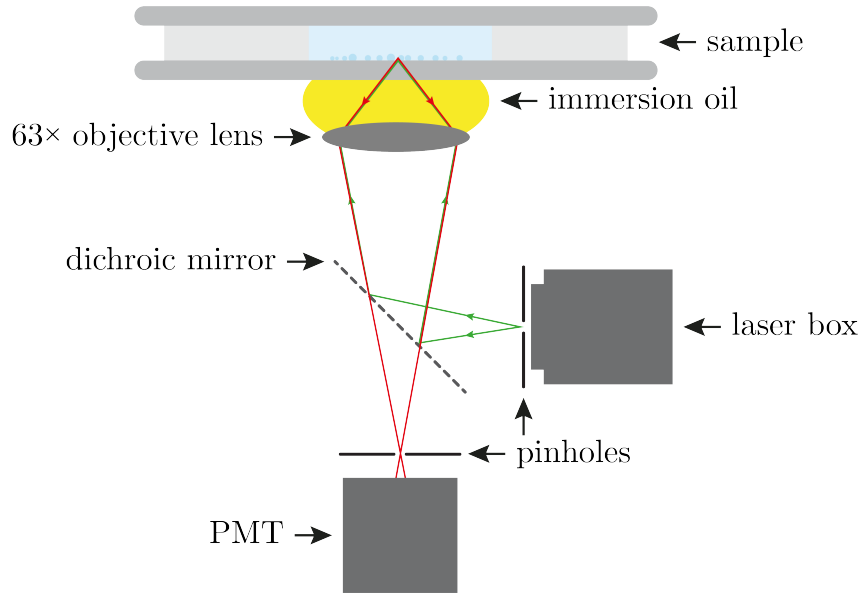


Figure 4.3: Schematic of the confocal microscopy setup showing the incident (green) light path and imaged (red) light path.

4.3.2 Confocal imaging

Three dimensional imaging of micrometre-scale condensates formed by Ddx4^{N*} phase separation was achieved through the use of laser scanning confocal microscopy, invented in 1955 by Minsky [199]. In this thesis we use a Leica TCS SP5 machine, with the optical setup as shown in Figure 4.3.

Monochromatic light, generated by a laser box, passes through the first pinhole and onto the dichroic mirror. All experiments in this thesis use a RT 30/70 dichroic mirror, reflecting 30% and transmitting 70% of incident light respectively. Light reflected by the dichroic mirror is focused onto the sample, through the immersion oil, by the 63x lens. The immersion oil between the objective lens and sample minimizes scattering of light caused by stark changes in refractive index. Incident light at the focal point excites fluorescent molecules in the sample, with a probability given by the extinction coefficient of the molecule. Fluorescence of excited molecules results in the isotropic release of photons, whose wavelength is greater than the incident laser light. Photons that travel along the reverse trajectory of the incident light are refocussed by the objective lens, through the

dichroic mirror, onto the photomultiplier tube (PMT).

Incident photons on the PMT eject electrons due to the photoelectric effect. These electrons, accelerated by voltage, collide with an electrode, causing the release of further electrons, which are then further accelerated. This procedure is repeated, amplifying the current to give a measurable signal. The amplitude of this signal is converted to a pixel intensity. Images are constructed by scanning the focal point over a region of interest, collecting photons along the way and storing the intensity data as pixel values. The range of wavelengths to accept into the PMT - the collection bandwidth, is set by the user.

The dual pinhole system provides the increased resolution of confocal microscopy. The first pinhole generates a small laser spot which is focused onto the sample. The second pinhole ensures only light from this focused spot reaches the PMT. This augments the axial and lateral resolution of images, and minimizes blur in the image due to out of focus light. Confocal images recorded at constant z increments form a z -stack, a three dimensional image whose voxels represent intensities given by the point spread function of the optical setup.

Correcting aberrations

Objectives are typically designed to work with coverglasses 0.17 mm thick with a refractive index of 1.515. It is with these conditions that the objective offers maximum confocality. The objective used in this work, a HCX PL APO CS 63.0x1.40 OIL UV, includes a correction collar. When preparing samples there is some inherent variation in the thickness of the coverglass, the correction collar permits the adjustment of lenses inside the objective, to account for this variation.

A fraction of light incident normal to an interface separating media of differing refractive index is reflected [200]. This reflected light can be imaged by setting the collection bandwidth to the incident light ± 5 nm. Figure 4.4 shows how the sharpness of the reflected

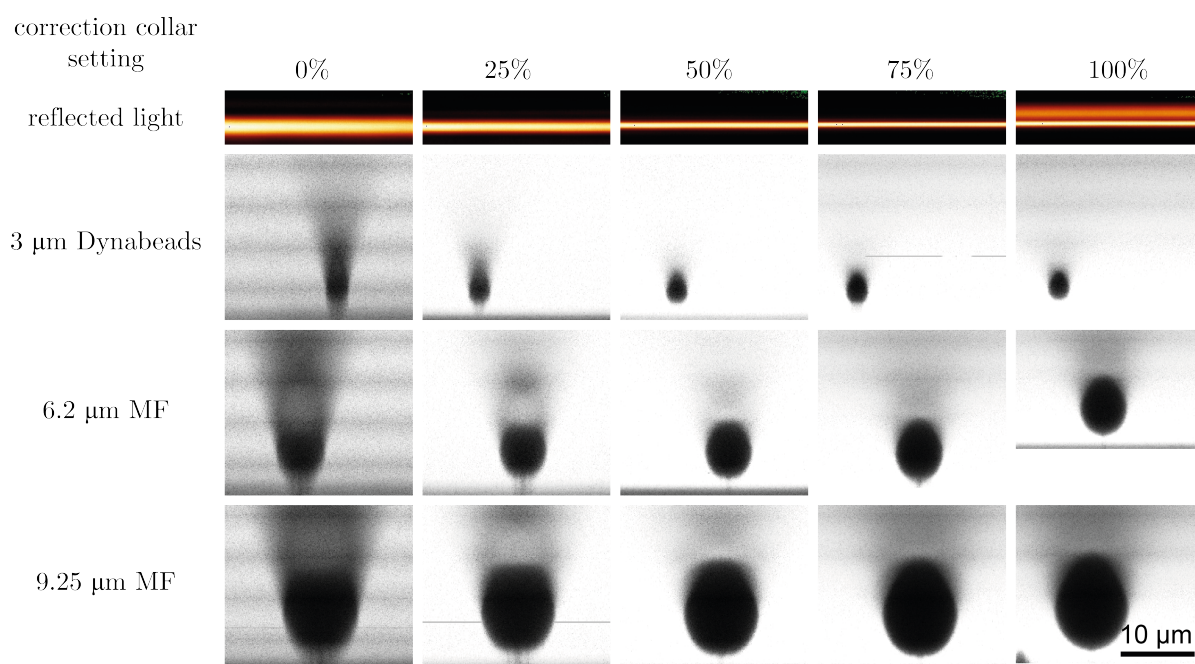


Figure 4.4: The effect of adjusting the correction collar on xz scans of 543 nm reflected light at the siliconized glass-water interface, and colloidal particles resting on siliconized glass. Colloidal samples contained particles suspended in 5 μM Cy3B dye. The colloids were imaged by exciting Cy3B at 543 nm and recording the emission at 560-630 nm as an xyz stack. As colloids excluded dye molecules, images are presented as minimum xz projections, colloids are visible as dark regions devoid of fluorescence intensity.

light band in xz scans of the glass-liquid interface can be used to optimise the correction collar. Here, fluorescent xz -stacks images of colloids with diameters¹ 3 μm , 6.2 μm and 9.25 μm resting on a siliconized glass coverslip are presented as the correction collar is adjusted. The images of the colloids are sharpest at a correction collar setting of 75%, also where the reflected light band is the sharpest. All further confocal z -stacks were collected using a correction collar setting that optimised the sharpness of the reflected light band at the glass-liquid interface.

Axial distortion is clearly visible in Figure 4.4, even at the optimum correction collar setting of 75%, causing spherical colloids to appear ovular. Figure 4.5 (a) shows the measured aspect ratios of these colloidal particles, determined using the Leica TCS SP5 internal pixel calibration. The width of each colloid closely matches that of the value measured by electron microscopy, but the heights are roughly $1.2\times$ their expected value. The $63\times$ objective used in this work is calibrated for Type-f immersion oil, index matched to borosilicate glass with a refractive index of 1.51. Refractive index mismatch between the immersion oil and the aqueous sample, with $n = 1.33$, causes an offset between the nominal focal point, and the actual focal point of the objective, by refraction of light at the glass-aqueous interface. This is illustrated in Figure 4.5 (b). In 1993, Hell *et al* showed that the confocal axial offset when imaging aqueous samples using immersion oil, with a high numerical aperture objective, is 1.2 [201], as is observed in Figure 4.5 (a).

McCall *et al* have reported a refractive index in PGL3, TAF15(RBD) and FUS proteinaceous condensates of roughly 1.36 [202]. This effect is likely present in Ddx4^{N*} condensates, owing to its sequence similarity with the aforementioned proteins. We do not account for axial aberrations due to refractive index mismatch with Ddx4^{N*} in this thesis. All confocal z -stacks of sessile condensates in this thesis are therefore rescaled by a factor of $1/1.2$ in z for drop shape analysis. All imaging was performed at room temperature, $\sim 22^\circ\text{C}$.

¹Verified by Prof DGAL Aart's and Prof R Dullens' (University of Oxford chemistry department) lab members using electron microscopy.

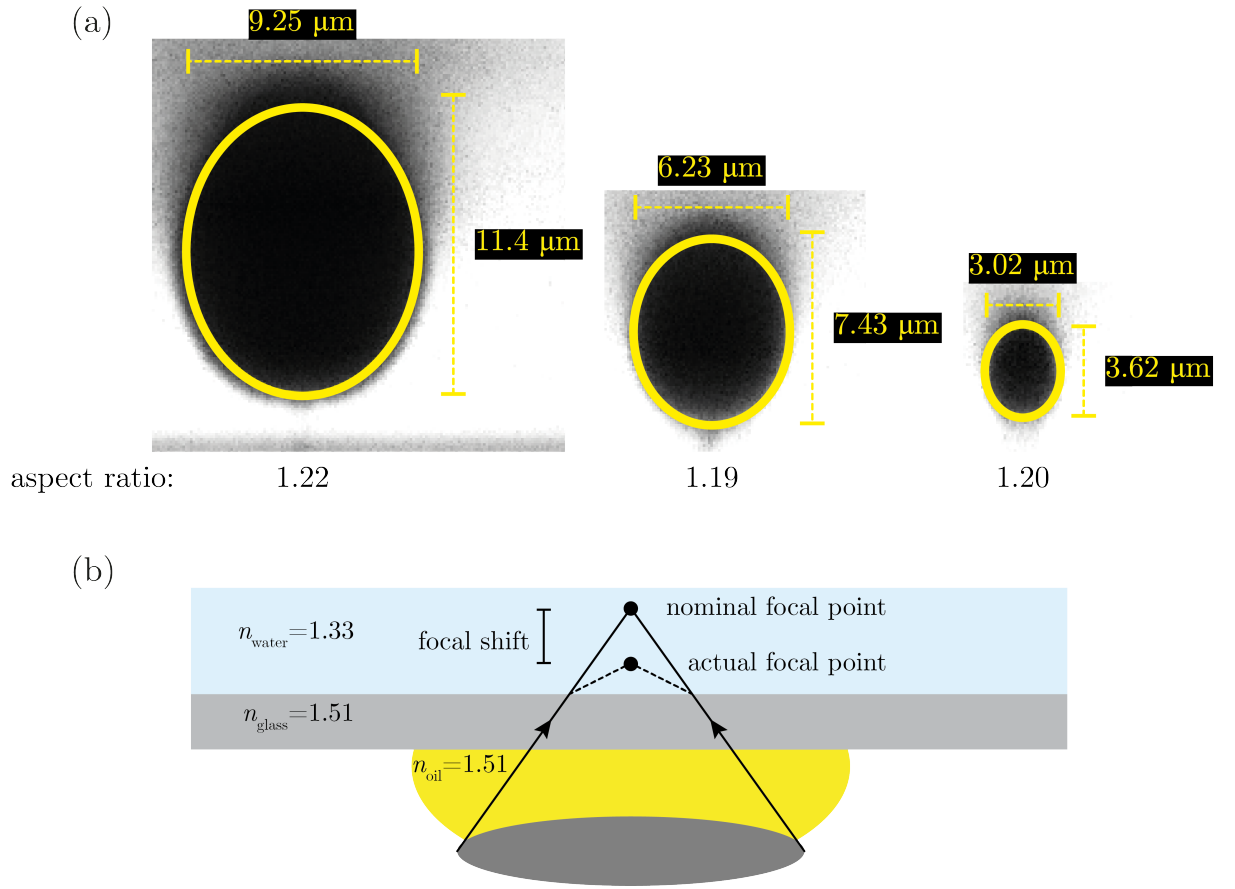


Figure 4.5: (a) xz scans of colloidal particles imaged in a suspension of 5 μm Cy3B dye, annotated with ellipses emphasising axial distortion. (b) Illustration of focal shift in our confocal microscopy setup induced by the refractive index mismatch between our aqueous samples and the immersion oil coating the objective lens [201]. Dashed black lines indicate the divergence from the expected light path (solid lines).

Imaging settings

Fluorophores Alexa-488 and Cy3B partitioned into Ddx4^{N*} condensates provided the contrast necessary for fluorescence imaging. For condensates imaged using Alexa-488, an excitation wavelength of 488 nm and collection bandwidth of 530-700 nm was used. For condensates imaged using Cy3B, an excitation wavelength of 543 nm and a collection bandwidth of 560-630 nm was used. Laser power and PMT voltage were set to maximise contrast, whilst avoiding empty or saturated pixels, between the condensate interior and exterior, generating fluorescent images allowing the identification of condensate interfaces.

Accurately defining contact angles from ADSA profiles requires reliable measurement of the position of the solid-liquid interface. It was found that imaging the reflected light, rather than fluorescent light provided a sharper image of this interface. For both fluorophores, reflected light was collected at ± 5 nm from the excitation wavelength, with laser power and PMT voltage were set to maximise contrast.

All images in both the fluorescence and reflected light channels were collected with a line averaging of 2. The lateral resolution for our sample setup is $0.2 \mu\text{m}$, and the axial resolution is $0.5 \mu\text{m}$ near the glass, dropping to $1 \mu\text{m}$ at $25 \mu\text{m}$ away from the glass [201]. z -stacks were constructed with a minimum xy pixel dimension of 100.5 nm for the smallest condensates, and increased for larger condensates accordingly. The z increment was set to 98.8 nm for all z -stacks, 82.3 nm in real space, always oversampling [203]. Full z stacks consisted of collected xy images from below the glass-liquid interface, up to a few μm above the apex of the largest drop in the field of view.

4.3.3 Image analysis

Figure 4.6 shows sessile Ddx4^{N*} condensates in the fluorescence emission channel, and the reflected light channel. The base of the droplets clearly reduce the reflected light intensity, indicating a reduced refractive index relative to the surrounding protein poor

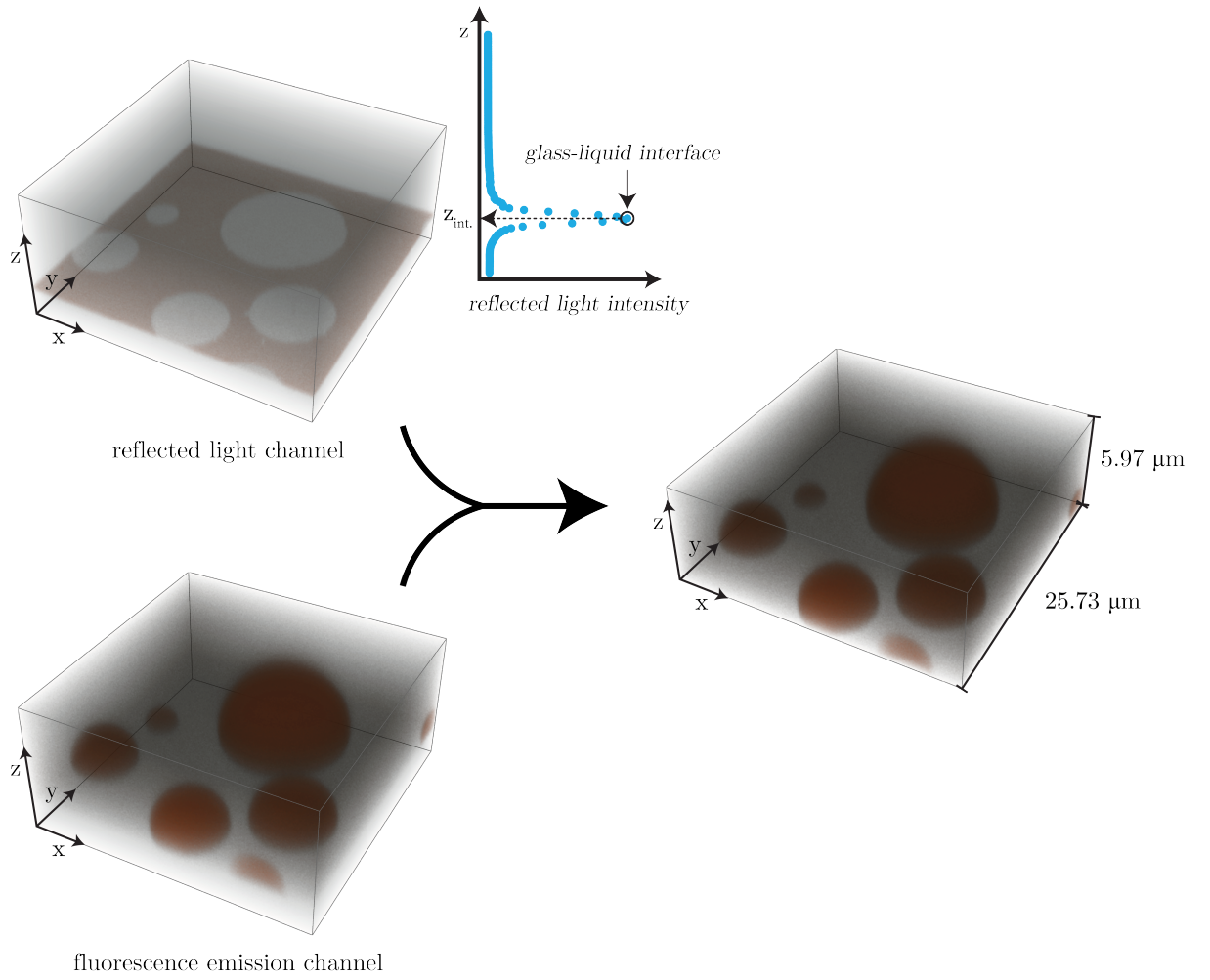


Figure 4.6: The reflected light channel from z -stacks of sessile condensates is used to identify $z_{\text{int.}}$ - the glass-liquid interface. xy slices with $z \geq z_{\text{int.}}$ in the fluorescence emission channel are carried forwards.

phase. xy slices of the fluorescence emission z -stack below the z position at which the mean reflected light intensity is greatest are taken to be inside the glass, and are removed from the image as illustrated in Figure 4.6.

The resultant image is then slightly blurred by applying the `GaussianFilter` function, with pixel radius 2, to remove image noise and increase contrast. The image histograms in Figure 4.7 (a) show the blur producing more defined maxima corresponding to increased contrast between the condensates and background. Otsu's algorithm [204] is then applied to the blurred image, partitioning its histogram into two regions as in Figure 4.7. Pixels whose value is above the threshold determined by Otsu's algorithm are set to 1, all others

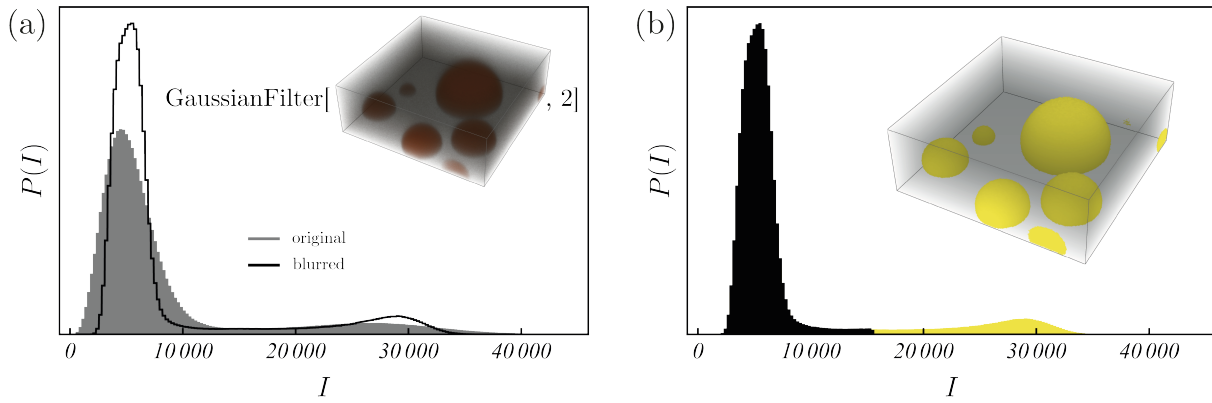


Figure 4.7: (a) Slightly blurring 3D confocal images of sessile condensates with a GaussianFilter of radius 2 [183] increases the separation of the population of bright pixels from the condensates, from the population of dark pixels originating from the protein poor phase. (b) Applying Otsu's algorithm [204] binarizes blurred 3D confocal images of sessile condensates, separating bright pixels from dark.

are set to 0, generating the binary image inset in Figure 4.7 (b).

Mathematica 13 [183] is now able to recognise condensates as independent regions of interconnected pixels with a value of 1. The SelectComponents function is applied to the binary image, filtering out any regions not in contact with only the bottom surface of the sample. Masks of the remaining sessile condensates are generated by the ComponentMeasurements function, returning the masks as SparseArray objects. A condensates mask is transformed by mapping the Image function onto the mask, turning each z level of the array into an xy binary image of the condensate's cross-section. Any holes in the binary cross sections are removed by applying the FillingTransform function. The equivalent disk radius of each xy cross-sectional binary image of the condensate is then measured using the ComponentMeasurements function. This radius is then augmented by the displacement of the best-fit disk's centroid from the condensate centroid², giving the radius of the condensate at each height z .

Figure 4.8 (a) shows the original fluorescence channel image, with three sessile condensates away from the sample edges reconstructed as towers of best fit circles obtained from their (R, Z) coordinates, shown in Figure 4.8 (b). At the base of condensate profile

²This step is necessary to obtain meaningful radii near the apex of drops, where the cross sections display more noise.

coordinates, there is a sharp drop in the measured radius. Figure 4.8 (c) displays the binary images, with the best fit circles used to obtain condensate radii at the base of the condensates shown in Figures 4.8 (a-b). The grainy binary images at $z = 0.00 \mu\text{m}$ is likely due to scattering from the out of focus solid-liquid interface caused by the stark change in refractive index, causing axial blurring in the 3D confocal image. This optical artefact was present in all confocal z -stacks collected in this thesis. To alleviate this, the first 3 (R, Z) coordinates from the base, are omitted from any fitting procedure.

4.3.4 Droplet profile fitting

Equations 4.12 and 4.14 form a system of ordinary differential equations, which can be written in vector form

$$\dot{\mathbf{v}} = \begin{pmatrix} \dot{\psi}(s) \\ \dot{r}(s) \\ \dot{z}(s) \end{pmatrix} = \begin{pmatrix} \frac{2}{R_0} + \frac{(z-z_0)}{\ell_c^2} - \frac{\sin \psi}{r} \\ \cos \psi \\ \sin \psi \end{pmatrix}, \quad (4.17)$$

where the over dot represents the first derivative with respect to s . The initial values for 4.17 are

$$\psi(0) = 0, \quad (4.18)$$

$$r(0) = 0, \quad (4.19)$$

$$z(0) = z_0. \quad (4.20)$$

At the apex of the drop, R_1 and R_2 are simultaneously equal to R_0 . Combining this with Equation 4.13 gives us

$$\left. \frac{d\psi}{ds} \right|_{s=0} = \frac{1}{R_0}, \quad (4.21)$$

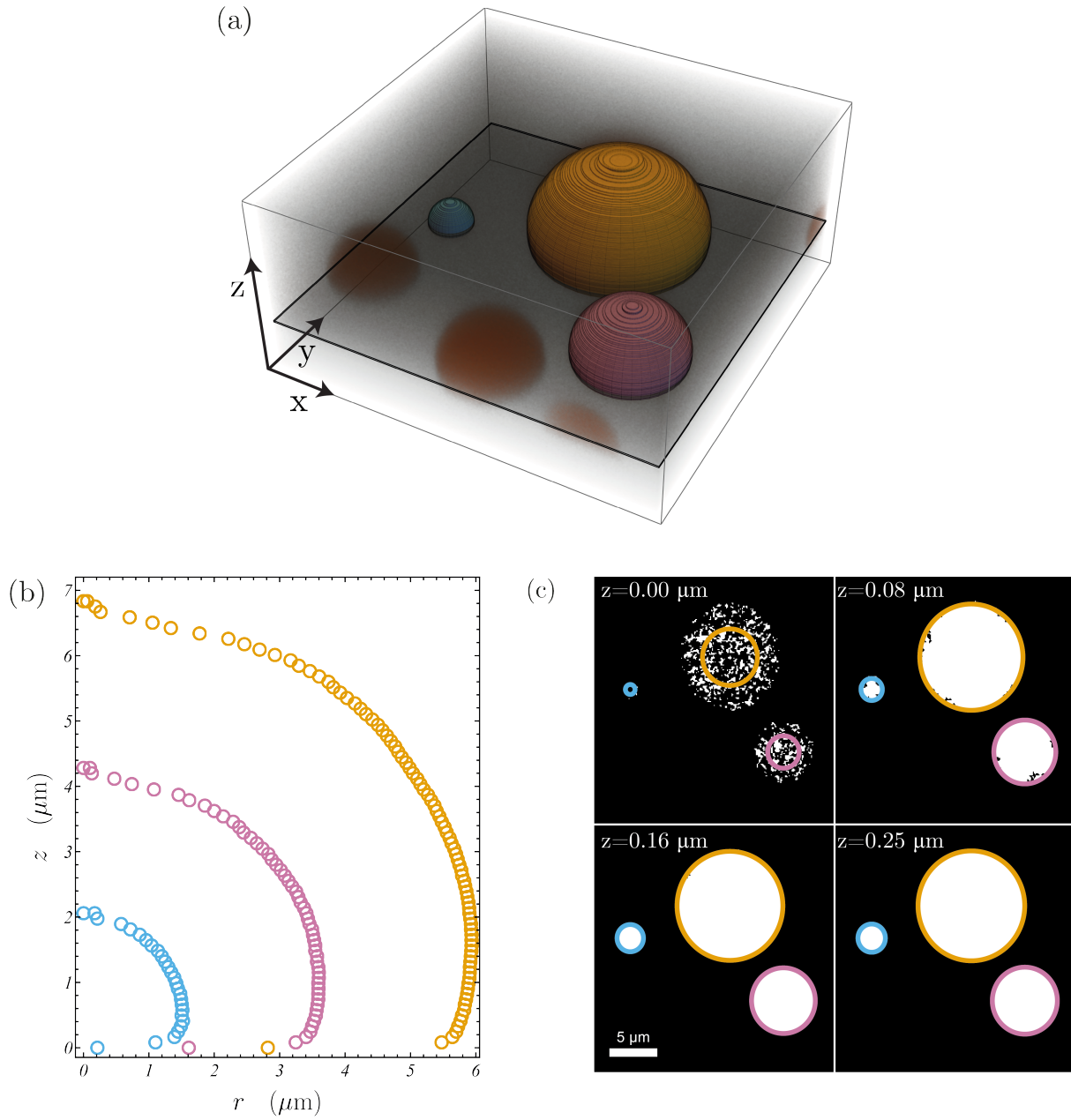


Figure 4.8: (a) Condensates identified as towers of best fit circles resting on a the glass-liquid interface, highlighted in black. (b) Radii r at height z for the sessile condensates in (a). (c) Binary images used to find radii for condensates in (a).

which prevents dividing by 0 in Equation 4.17 at $s = 0$. With these initial conditions, Equation 4.17 can be integrated numerically up to $s = s_{max}$ for a given set of R_0 , ℓ_c and z_0 , defining a sessile drop profile in the (r, z) plane from $s = 0$ to $s = s_{max}$.

The best fit curve for an experimentally determined set of profile coordinates, is found by minimizing the following objective function by varying R_0 , ℓ_c and z_0 :

$$\lambda(R_0, \ell_c, z_0, s_{max}) = \frac{1}{N} \sum_{i=1}^N [(r_i - R_i)^2 + (z_i - Z_i)^2], \quad (4.22)$$

where (R_i, Z_i) are experimental coordinates, and (r_i, z_i) are points closest to (R_i, Z_i) on the generated curve. This is implemented in Mathematica 13 [183] by integrating 4.17 using the NDSolve function. 1000 points along the trial curve in the (r, z) plane, evenly spaced from 0 to s_{max} are generated using the Interpolation function. The minimum distance between these trial points and each data point, is found using the DistanceMatrix function. The mean of the sum of the square of these minimum distances is the objective function, Equation 4.22.

Minimization is performed using the NMinimize function, with the method option set to NelderMead, implementing the Nelder-Mead algorithm [205]. The WorkingPrecision is set to MachinePrecision and the PrecisionGoal is set to ∞ , maximising the numerical accuracy of the search. Necessarily, $R_0 < 0$ and $\ell_c > 0$, restricting the search region for R_0 and ℓ_c .

Careful choice of s_{max} is required when fitting. Too small a value of s prevents generated curves from extending the full length of the profile, artificially raising the contribution to λ from points near the base of the droplet. Too large a value results in aphysical profiles. As s increases from 0, ψ decreases monotonically, eventually passing a critical value of s where $\psi > \pi$, where the curve no longer describes a physically realisable sessile droplet. The s_{max} used to generate trial curves is calculated from N data points

as follows:

$$s_{max} = 1.2 \sum_{i=4}^{N-1} \sqrt{(\bar{R}_{i+1} - \bar{R}_i)^2 + (Z_{i+1} - Z_i)^2}, \quad (4.23)$$

with

$$\bar{R}_i = \sum_{j=1}^7 \frac{R_{j-3}}{7}. \quad (4.24)$$

Equation 4.23 calculates $1.2\times$ the smoothed distance between consecutive coordinates along an experimental condensate profile.

Once the best fit parameters are obtained, the best fit value of s_{max} is given by

$$z(s_{max}) = 0, \quad (4.25)$$

which in conjunction with the fit parameters R_0 , ℓ_c and z_0 , give the best fit profile. The contact angle is trivially obtained from the best fit profile using Equation 4.16. Figure 4.9 (a) shows the best fit droplet profiles for the condensates displayed in Figure 4.8. Their residuals, displayed in Figure 4.8 (b), show that the fitting quality around the base of the profile is poor. This is attributed to the aforementioned axial distortion.

After manually inspecting populations of fit profiles, we defined the threshold

$$\frac{\lambda N}{s_{max}} > 0.5 \mu\text{m}, \quad (4.26)$$

for condensates considered to be poorly fit, where N is the number of experimental profile coordinates. Profiles with errors satisfying Equation 4.26 were discarded. We also discard condensates with heights less than $1.2 \mu\text{m}$. as we found these to be poorly binarized, resulting in sporadic profile fitting.

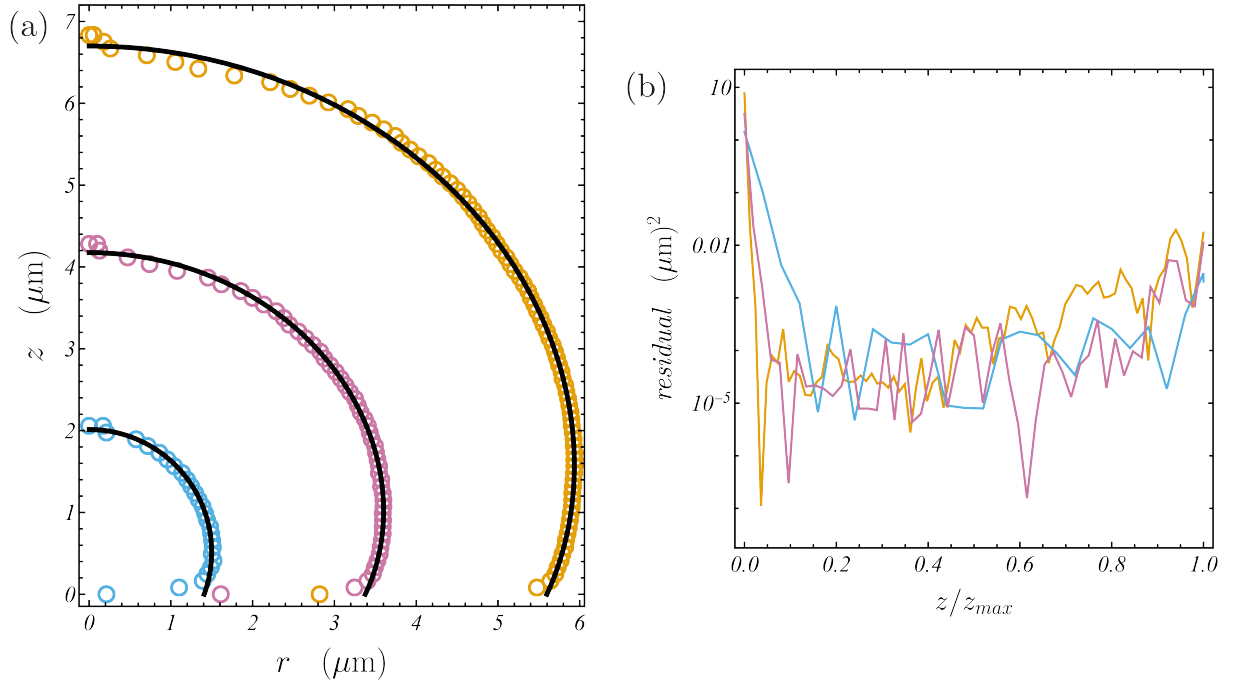


Figure 4.9: (a) Experimental profile coordinates overlaid with their best fit ADSA profiles (solid black lines) for three Ddx4^{N*} 1-236 condensates resting on siliconized glass coverslips. (b) Residuals calculated as the squared distance from profile coordinates in (a) to their best fit profiles, plotted against the height above the base of the condensate, normalised by the height of the condensate (z/z_{max}).

4.3.5 Manual measurement of contact angles using ImageJ

3D confocal images of condensates were imported into ImageJ software [206]. z -stacks were resliced starting at "top" to give a stack of xz images, which was used to generate a maximum intensity xz projection. The resultant image was then rescaled in z by a factor of $1/1.2$ to account for the refractive index match. By eye, an angle was drawn at one of the two visible contact points using the angle tool. The measured value was recorded as the contact angle.

4.4 Results & discussion

4.4.1 Comparison with manual measurements of contact angle

Previously published measurements of biomolecular condensate contact angles from Feric *et al* were obtained by manually drawing angles on confocal images of xz cross sections of sessile condensates, taken across their diameter [11]. To compare this method with the automated ADSA procedure presented in this work, Ddx4^{N*} 1-229 and Ddx4^{N*} 1-236 condensates were prepared on siliconised glass, showing markedly different contact angles when analysed by ADSA (see Section 6.4.3). For fair comparison with ADSA, xz cross sections for manual measurement were saved as .tifs, renamed with a random number, and given to Nott lab member Dr Purnima Kompella, who proceeded to perform the manual contact angle measurements as a blind control.

Figure 4.10 (a) shows the results of the blinded manual measurements for the 29 Ddx4^{N*} 1-236 and 23 Ddx 1-229 condensates resting on siliconized glass, plotted against their ADSA measurements. The mean $\pm$ standard deviation for the respective manual measurements are $96.4\pm10.3^\circ$ and $67.8\pm11.0^\circ$. For the ADSA measurements, these are $107.4\pm6.5^\circ$ and $41.9\pm8.3^\circ$. In both cases, the standard deviation is greater for the manual measurement, indicating reduced accuracy. Manual measurement of contact angles also involves choosing a single cross section of the droplet out of 360° of possible cross sectional profiles, capturing any noise inherent to the imaging setup. ADSA measures contact angles based on a mean of condensate profiles from all perspectives, averaging out associated imaging noise. For surfaces with small (compared to the condensate contact area) inhomogeneities, there may be slight deviations in the contact angle around the base of the droplet. Again, these inhomogeneities would serve to increase the experimental noise of manual measurement, whilst being averaged out in ADSA measurement of contact angles.

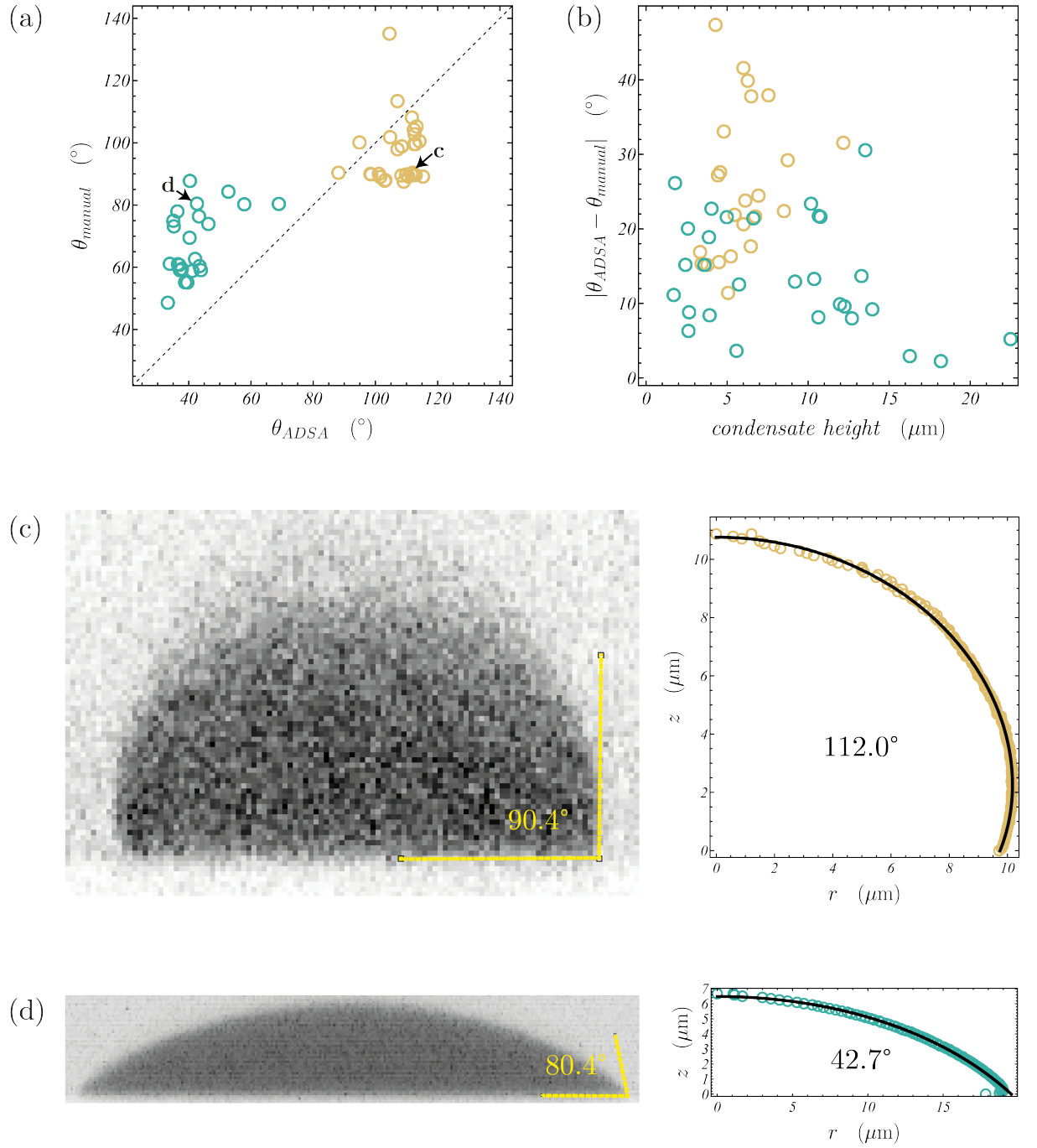


Figure 4.10: (a) ADSA measurements of contact angle plotted against manual measurements of contact angles for Ddx4^{N*} 1-236 condensates on siliconised glass (yellow) and Ddx4^{N*} 1-229 condensates on siliconised glass (green). The dashed line represents $\theta_{\text{ADSA}} = \theta_{\text{manual}}$. (b) Absolute difference between manual and ADSA measurements plotted as a function of condensate height. (c) Manual and ADSA measurement of contact angle for the condensate marked by **b** in (a). (d) Manual and ADSA measurement of contact angle for the condensate marked by **c** in (a)

The mean contact angle for the manual measurements for both Ddx4^{N*} 1-236 and Ddx4^{N*} 1-229 is shifted towards 90° relative to equivalent ADSA measurements. This is reflected by the plot in Figure 4.10 (a), which shows a vertical skew of points away from the dashed line, where the two measurements are equal, towards 90°. One reason for the discrepancy between contact angles obtained through ADSA and manual measurement may be the difficulty of accurately drawing angles on smaller condensates. To investigate this, we plot condensate heights against the absolute differences between measured angles measured by the two methods in Figure 4.10 (b). There is no clear relationship between contact angle offset and height, indicating the discrepancy is not due to condensate size.

In Figures 4.10 (c) and (d) we more closely examine two condensates with a large discrepancy in their contact angles measured through ADSA and manual measurement. In Section 4.3.3 we discussed the axial blurring near the base of droplets, skewing the measured mean radii of condensate profiles towards zero. This blurring introduces artificial right angles into the condensate profile, which may tend the user to perform a manual measurement closer to 90°. This effect was circumvented in ADSA by omitting the first three radii from the base of the droplet from profile fitting.

4.5 Conclusions & outlook

Axisymmetric drop shape analysis (ADSA) allows the automated measurement of shape parameters describing axisymmetric drops, including capillary lengths and contact angles. We have presented an implementation of ADSA for *in vitro* Ddx4^{N*} condensates resting on solid surfaces, relying on the collection of high quality 3D confocal microscopy images of sessile condensates. The importance of correctly setting the correction collar on the microscope objective was demonstrated using colloidal particles of known diameter. These particles were also used to correct for axial distortions introduced through refractive index mismatch between the aqueous sample and the immersion oil coating the microscope

objective.

The solid-liquid interface present in samples was identified by imaging reflected light, defining the base of condensates, whereas their fluid-fluid interfaces were identified through image analyses of fluorescent light. Experimental sessile condensate profiles were then fit to the Young-Laplace equation for sessile drops, allowing the measurement of contact angles and capillary lengths. ADSA contact angle measurements were then compared to a manual method, based on inspecting images of sessile condensates and eyeballing the condensate interface. Automated ADSA measurements showed reduced spread, also removing any subjective bias associated with manual contact angle measurement.

ADSA is a readily implementable method, enabling the characterisation of biomolecular condensate wetting phenomena. Adoption of the protocols detailed in this chapter by other research groups will allow the introduction of a standard measurement of contact angles and capillary lengths of microscopic biomolecular condensates. We recognise our method was not tested on a system of known capillary length and contact angle, due to difficulty in obtaining a well characterised aqueous phase separated system. This should form the basis of future work, allowing further standardization, as well as revealing the effects of unforeseen aberrations in our system on drop profiles obtained by ADSA.

Acknowledgments

We thank Dr Joseph Hutchinson for the colloidal particles and Dr Purnima Kompella for performing manual measurements of contact angles.

Chapter 5

Calculation & Measurement Of Fluid-Fluid Interfacial Tensions

ABSTRACT

Working with $Ddx4^{N^}$ condensates in vitro, this chapter is focused on advancing the understanding of biomolecular condensate fluid-fluid tension. We begin with a review of condensate fluid-fluid interfacial tension measurements presented in the field, followed by a discussion of the experimental techniques used in their acquisition. Theoretical background is then given on van der Waals theory for fluid-fluid interfacial tension, which is then extended to Scheutjens-Fleer theory, subsequently applying this to our $Ddx4^{N^*}$ condensates. Further background explains the effect of gravity on sessile condensates, necessary for the experimental section, where we use axisymmetric drop shape analysis to measure condensate capillary lengths. This is with a view to indirectly measure their fluid-fluid interfacial tensions, allowing a comparison with our experimental values.*

5.1 Introduction

The formation of biomolecular condensates through liquid-liquid phase separation necessitates the formation of a fluid-fluid interface separating the coexisting phases. This interfacial region has associated interfacial tension, defined as [207]

$$\gamma = \left(\frac{\partial G}{\partial A} \right)_{T,P,\{N\}} = \left(\frac{\partial F}{\partial A} \right)_{T,V,\{N\}}, \quad (5.1)$$

where G and F are the Gibb's and Helmholtz free energies of the system and A is the area of the interface. The unambiguously positive value of γ drives fluid-fluid interfaces towards spherical geometries, minimizing their interfacial energy, as is typical of intracellular condensates [9, 18, 24, 30, 103].

Aside from driving biomolecular condensates to be spherical, according to Equation 5.1, fluid-fluid interfacial tension is involved in **any** process that involves a change in the area of the interface(s). These include (but are not limited to) condensate nucleation - whereby a decreasing γ increases the rate of condensate formation [105, 109, 110], condensate coalescence - whose rate proportional to the ratio of interfacial tension to viscosity [9, 57, 70, 95, 104], and the interior organisation of the multiphase nucleolus [11, 32]. Recently, the physiological regulation of condensate interfacial tension has been demonstrated, whereby P-granules are stabilised with respect to coalescence by adsorption of MEG-3 protein clusters at their interface [108]. An understanding of the fluid-fluid interfacial tension(s) of biomolecular condensates, especially their relation to the sequences of their constituent proteins, is therefore fundamental to explaining their dynamics and expanding our understanding of their structure.

The following scaling relation,

$$\gamma \sim \frac{k_B T}{d^2}, \quad (5.2)$$

Table 5.1: Reported fluid-fluid interfacial tensions for biomolecular condensates measured *in vitro* (unless stated otherwise). ^a experimental radius of gyration. ^b simulated hydrodynamic radius. ^c hydrodynamic radius based on molecular weight assuming a fully unfolded sequence, calculated using <http://www.fluidic.com/toolkit/hydrodynamic-radius-converter/>. ^d protein contains disordered and folded regions, lower and upper bounds correspond to folded and unfolded hydrodynamic radii calculated as in ^c.

condensate	γ (J m ⁻²)	molecular lengthscale
NPM1	8 ± 2 E-7 [11]	~ 5 nm ^a [213]
GC (NPM1) <i>in vivo</i>	4 ± 1 E-7 [11]	~ 5 nm ^a [213]
PGL-3	1.94 E-5, 1-5 E-6 (salt dependent) [98, 108]	9.11 nm ^c
LAF-1	1.9 E-4, 6.8 E-4, 1.6 E-4 [57, 135, 214]	4 nm ^c
FUS	3.1 E-3, 9 E-5 [135]	~ 5 nm [215] ^b
FUS ₂₆₇	9 E-5 [136]	5 nm ^c
WHI3	4 E-6 [214]	3.6-8.9 nm ^d
nucleolus <i>in vivo</i>	1.5 ± 0.5 E-6 [95]	-

where d is a characteristic lengthscale of the interface and $k_B T$ approximates the strength of intermolecular interactions in the fluid, can be used to approximate interfacial tensions [102, 163]. Far away from the critical point, d is expected to be on the order of the molecular diameter. For simple liquids at room temperature (20 °C), with $d = 2\text{--}5$ Å, Equation 5.2 predicts interfacial tensions in the range 10-100 mJ m⁻². This holds well for a range of interfaces [208], including water-air ($\gamma = 72$ mJ m⁻²), benzene-air (28.88 mJ m⁻²), ethanol-air (22.39 mJ m⁻²) and dimethyl sulfoxide-air (43.54 mJ m⁻²), correctly predicting the order of magnitude of γ . The relation also holds for more complex systems, such as multicomponent, phase-separated, colloid-polymer solutions with particle sizes of ~ 500 nm, with interfacial tensions $\sim 10^{-6}$ J m⁻² [209–212].

Table 5.1 collates measurements of fluid-fluid interfacial tensions for a range of condensates. Included in the table is a measure of their molecular lengthscale, reported as their experimentally verified or, in the absence of experimental data, calculated radius of gyration or hydration. Despite the molecular lengthscales all falling in the range 3-10 nm, we see a much greater than expected variance in γ according to Equation 5.2, ranging from 10^{-7} for NPM1 to 10^{-3} for FUS. This is emphasised by Figure 5.1, where we plot the molecular lengthscales against γ for the values from Table 5.1, also showing the expected value of γ according Equation 5.2. We see that LAF-1, WHI3 and PGL-3

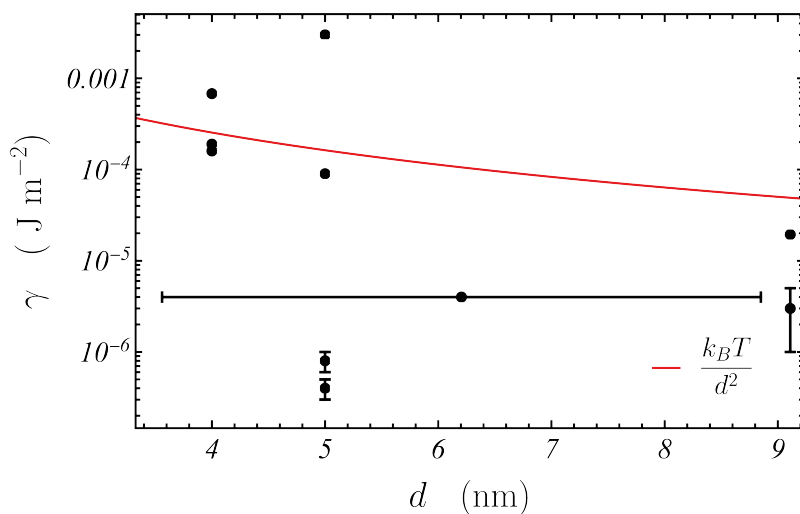


Figure 5.1: Surface tensions of intrinsically disordered protein condensates plotted against their molecular lengthscales from Table 5.1. Equation 5.2 is plotted in red for 22 °C.

fluid-fluid interfacial tensions are well described (to within an order of magnitude) by the scaling relation (Equation 5.2), whereas the relation is a poor descriptor of the remaining condensates, with the interfacial tension of NPM1 condensates being as much as 3 orders of magnitude lower than expected.

Comments on the molecular understanding of condensate fluid-fluid interfacial tensions are usually limited to a brief comparison with values expected for macromolecular liquids, often with reference to Equation 5.2 [11, 57, 136]. Wang *et al* compared their measurements on FUS₂₆₇ and LAF-1 condensates with water-oil interfaces, with the latter 300× greater, hypothesizing the low interfacial tension of condensates reflects the larger size and relative hydrophilicity of their constituent proteins [135]. Jawerth *et al* showed the fluid-fluid interfacial tensions of PGL-3 condensates decrease with increasing salt concentrations [98], in a manner well described by the mean-field theory of Overbeek and Voorn [216, 217], emphasising the influence of electrostatic interactions. Salt dependent electrostatic interactions have been demonstrated in the phase separation of many intrinsically disordered proteins [57–59, 75–77], the work of Jawerth *et al* suggests a similar set of interactions may modulate condensate interfacial tensions.

This chapter concerns the calculation and measurement of the fluid-fluid interfa-

cial tension of Ddx4^{N*} condensates. By investigating the Ddx4^{N*} variants introduced in Chapter 3, we aim to elucidate a relationship between phase separation propensity and interfacial tension. Furthermore, we hope to reveal the contribution of cation- π and net charge per residue to condensate fluid-fluid interfacial tension. We now proceed to summarise the techniques used thus far in the investigation of the fluid-fluid interfacial tension of condensates, followed by an outline of the methodology employed in this chapter.

5.1.1 Methods to study fluid-fluid interfacial tension

Experimental techniques to measure biomolecular condensate interfacial tension often rely on indirect measurements, such as studying droplet coalescence rates and relating them to viscosity and interfacial tension [9, 11, 57, 95, 214]. Jawerth *et al* measured the interfacial tension of PGL-3 condensates using a dual optical trap setup, recording the response of their interface to oscillatory forces [98]. In 2021, Wang *et al* demonstrated the use of micropipette aspiration, as well as condensate coalescence, to measure the interfacial tensions of LAF-1. While able to provide accurate values of γ , these techniques are prone to complication by non-Newtonian, time-dependent, rheological properties of condensates [70, 75, 99].

The direct observation of thermal fluctuations of fluid-fluid interfaces, and subsequent characterisation using capillary wave theory provides an accurate measure of γ [218]. This technique has been applied to *in vitro* PGL-3 condensates [98], and *in cell* to nucleoli [95], but is limited to ultralow interfacial tensions ($\sim 10^{-6} \text{ J m}^{-2}$ and lower), where interface fluctuations are optically resolvable [218]. Alternatively, one may observe the gravity-induced deformation of interfaces, through measurement of capillary lengths. This has been employed by Feric *et al* in their study of nucleolar components, where they examined the shapes of NPM1 condensates and fit them to a semi-empirical relationship, allowing a measurement of capillary length and interfacial tension [11]. Recently (2021), Ijavi *et al* performed axisymmetric drop shape analysis (ADSA) on Bik1 and FUS₂₆₇ condensates,

allowing accurate measurement of capillary lengths on a per-condensate basis [136]. Both based on the static geometry of condensates, these measurements of interfacial tension through capillary length are unaffected by the rheological properties of condensates, but do require optically resolvable deformation of the interface by gravity.

Computational efforts to understand the fluid-fluid interfaces of biomolecular condensates have been limited to explaining phenomenology. Feric *et al* utilised a coarse grained model to show differential interactions between condensate components leads to the multiphase architecture seen in nucleoli [11]. To the authors knowledge, there have been no reported calculations of condensate interfacial tensions, beyond the use of Equation 5.2. The lack of computational and theoretical studies of the interfaces of biomolecular condensates likely reflects their complexity, being defined by concentrated regions of complex macromolecules.

In this chapter we look to alternative systems for inspiration, namely the fluid-fluid interface in phase separated colloid-polymer systems. Aarts has shown their phase behaviour to be well described by the mean-field free volume theory, while their capillary lengths may be calculated using van der Waals theory [219]. Analogously, the phase behaviour of the Ddx4^{N*} model condensate system used in this thesis is well described by the mean-field Flory-Huggins theory. Therefore we explore the application of van der Waals theory, facilitated by the intermediate Scheutjens-Fleer theory, to calculate the interfacial tensions and capillary lengths of Ddx4^{N*} condensates, describing the pairwise interactions present using the Flory-Huggins interaction parameters presented in Section 3.4. We then explore the utility of ADSA to measure Ddx4^{N*} condensate capillary lengths, with a view to verifying our calculations. At the undertaking of this project, there was no published demonstration of (ADSA) on biomolecular condensates - this work was intended to also be a proof-of-principle. By the time of writing, the 2021 work of Ijavi *et al* remains the only published example of ADSA applied to biomolecular condensates [136].

5.2 Theoretical background

5.2.1 Mean field calculation of Interfacial Tensions

Van der Waals interfacial tension

For a two phase $\alpha - \beta$ system, there exists a density profile $\phi(z)$ that describes the composition along a direction z , perpendicular to the $\alpha\beta$ interface. Far away from the interface, $\phi(z)$ is flat and equal to either ϕ_α or ϕ_β . Through the interface, $\phi(z)$ changes continuously from α to β . To calculate the interfacial tension associated with this interface, van der Waals proposed the following functional [220]:

$$\gamma[\phi(z)] = \int_{-\infty}^{\infty} \left[W(\phi) + m \left(\frac{d\phi}{dz} \right)^2 \right] dz. \quad (5.3)$$

The first term in the integral, $W(\phi)$, was introduced in Section 3.2.5 and describes the excess free energy density relative to the phase separated state. The second term, known as the square gradient approximation, accounts for gradients in composition and is valid away from the critical point, where density fluctuations in the fluid are short-ranged [221]. The factor m is given by the second moment of the direct correlation coefficient, a generally non-trivial calculation. The density profile $\phi(z)$ which minimises Equation 5.3 is the equilibrium interfacial tension. For our binary phase separated system of Ddx4^{N*} and solvent, $W(\phi_p)$ is easily obtained from the Flory-Huggins free energy of mixing via Equation 3.28. To find m , we look to the work of Scheutjens and Fleer.

Scheutjens-Fleer theory

Scheutjens and Fleer extended the Flory-Huggins lattice theory for polymers to include gradients in ϕ , permitting the evaluations of interfacial tensions [150, 151]. Consider

the true interface between the protein dense and poor phases, represented by the black line in Figure 5.2 (a). Discretization of the interfacial profile onto a lattice, where the occupation of each site is the equilibrium ϕ_p , allows application of the Flory-Huggins free energy of mixing expression. Figure 5.2 shows a 2-dimensional representation of this lattice, where the energy of mixing per of site x is taken as the mean of its interaction with the neighbouring sites,

$$\Delta_m U(z) = \chi(1 - \phi_p)[\lambda\phi_p(z-1) + (1 - 2\lambda)\phi_p(z) + \lambda\phi_p(z+1)], \quad (5.4)$$

where λ is the inverse coordination number of the lattice. For a three dimensional fluid, λ is typically set to $1/6$, representing a cubic lattice. Introducing $\langle\phi_p(z)\rangle$ to represent the term in square parentheses on the right hand side of Equation 5.4, the mixing free energy per unit area of the system is then

$$\frac{F_{tot}}{A} = \sum_z \frac{\phi_p(z)}{r} \ln \phi(z) + (1 - \phi_p(z)) \ln (1 - \phi_p(z)) + \chi\lambda(1 - \phi_p(z))\langle\phi_p(z)\rangle. \quad (5.5)$$

In the continuous limit, the sum in Equation 5.5 becomes

$$\frac{F_{tot}}{A} = \int_{-\infty}^{\infty} \left[\Delta_m F + \chi\lambda(1 - \phi_p) \frac{d^2 \phi_p}{dz^2} \right] dz. \quad (5.6)$$

The total free energy of a two phase system with an interface of area A is given by

$$F_{tot} = F_\alpha + F_\beta + A\gamma. \quad (5.7)$$

Subtracting $\frac{1}{A}(F_\alpha + F_\beta)$ from Equation 5.6 therefore gives the interfacial tension as

$$\gamma[\phi_p(z)] = \int_{-\infty}^{\infty} \left[W(\phi) + \chi\lambda(1 - \phi_p) \frac{d^2 \phi_p}{dz^2} \right] dz. \quad (5.8)$$

Integration by parts of the final term shows Equation 5.8 is equivalent to Equation 5.3, with $\lambda\chi = m$. Equation 5.5 can be used to numerically calculate the free energy of a phase

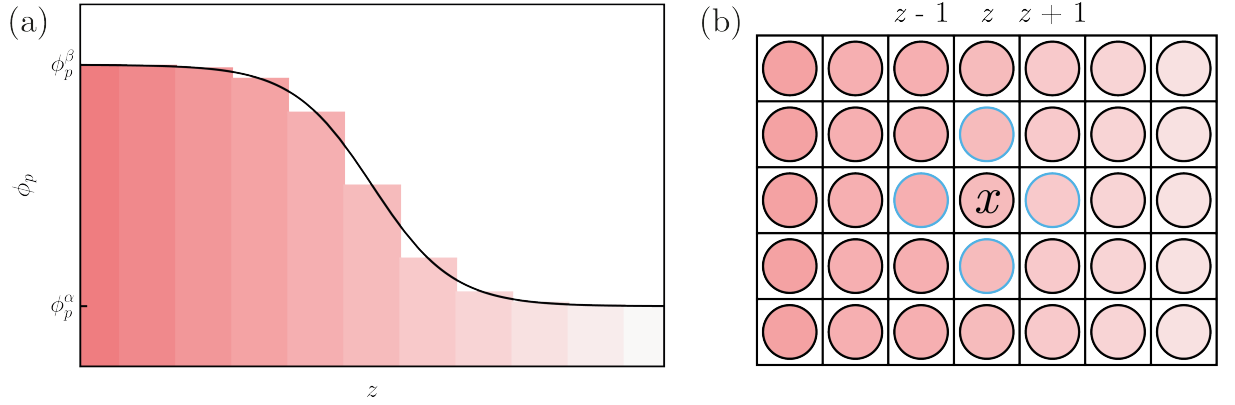


Figure 5.2: (a) Continuous interfacial profile (black) approximated by a discretisation in z . (b) 2D Scheutjens-Fleer lattice with each site populated by volume fractions given in (a). Blue circles indicate the sites x is considered to interact with through χ .

separated polymer system, including its interface, therefore also the interfacial tension, without knowledge of the squared gradient coefficient m .

5.2.2 The capillary length & sessile drops

The equilibrium geometries of the surfaces of sessile droplets resting on smooth surfaces is determined by surface and gravitational forces. Here, we investigate the utility of axisymmetric drop shape analysis in measuring the fluid-fluid surface tensions of Ddx4^{N^*} , through measurement of their capillary lengths. The capillary length describes the length-scale at which the geometry of surfaces are deformed by gravitational forces. It is defined by

$$\ell_c = \sqrt{\frac{\gamma}{g\Delta\rho}}, \quad (5.9)$$

where $g = 9.81 \text{ ms}^{-2}$, $\Delta\rho$ is the density difference of the two coexisting phases and γ is the interfacial tension between the two phases. Through Equation 5.9, knowledge of the capillary length can be used to obtain the interfacial tension.

The effect of gravity on the shapes of sessile droplets is illustrated, Figure 5.3, by the familiar example of a sessile water droplets in air. As droplets increase in volume

and their height is pushed through the capillary length of 2.7 mm, they transition from a spherical cap geometry, to a puddle-like shape. This behaviour is fully described, for axisymmetric drops, by Equation 4.14.

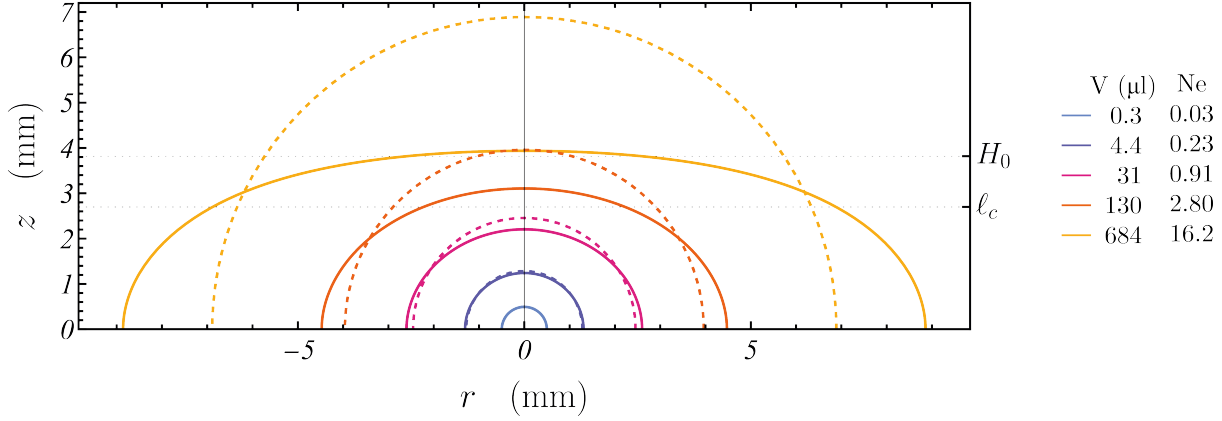


Figure 5.3: Solid lines: equilibrium sessile drop cross sections for a system with $\ell_c = 2.7$ mm (eg. water in air) and a contact angle of 90° . Dashed lines: spherical cap profiles for equivolume droplets as represented by solid lines, also with a contact angle of 90° .

The degree to which a droplet is deformed under gravity is quantified by the Neumann number [222, 223],

$$\text{Ne} = \frac{R_0 H_0}{\ell_c^2}, \quad (5.10)$$

where R_0 and H_0 are the radius of curvature the apex of the drop and it's height respectively. Ne is a modification to the classic Bond number [224], used to quantify the scaling of gravitational effects on interfaces with system dimensions, specific for sessile drops. Larger values of Ne correspond to greater gravitational distortion of the droplet interface, as demonstrated by the axisymmetric surfaces plotted in Figure 5.3. Generally, a sessile drop profile with $\text{Ne} > 0.3$ is considered to provide a reliable measurement of the capillary length [136, 222, 223].

In the limit of large Ne, sessile drops form puddles with flat tops. The height of these puddles is found by equating the horizontal forces illustrated in Figure 5.4 [225],

$$\gamma_{s\beta} + \frac{1}{2}g\Delta\rho H_0^2 = \gamma + \gamma_{s\alpha}. \quad (5.11)$$

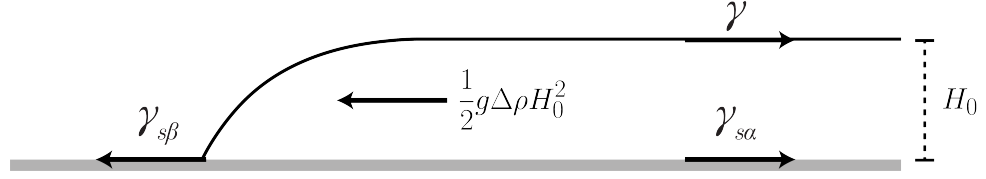


Figure 5.4: Puddle configuration of a sessile drop illustrating the origin of the horizontal forces in Equation 5.11. $\gamma_{s\alpha}$ and $\gamma_{s\beta}$ are the interfacial tensions between phases α and β and the solid surface respectively. The hydrostatic pressure contribution is given by the solution to $\int_0^{H_0} g\Delta\rho(H_0 - z)dz$.

The second term on the left-hand side is the hydrostatic pressure for the puddle integrated over its height H_0 . Equation 5.11 is valid when the surface area of the curved region near the edge of the drop is insignificant compared to the total surface area of the drop. The definition of Young's angle (Equation 6.1) allows us to use Equation 5.11 to write

$$H_0 = 2\ell_c \sin \frac{\theta_0}{2}. \quad (5.12)$$

The largest drop profile in Figure 5.3 is approaching the limit set by Equation 5.12, slightly overshooting it due to the finite curvature at its apex. Drops much larger than H_0 are not physically realisable in an equilibrium system.

Hansen has shown [226] that small deviations from the spherical cap shape, for sessile drops with contact angles greater than or equal to 90° , are described by the semi-empirical relation

$$\ell_c^2 = \frac{H^2}{4.308(1 - H/R)}. \quad (5.13)$$

R and H are the maximum radius of the droplet and the distance from the z coordinate of that radius to the apex of the drop, as illustrated in Figure 5.5. Equation 5.13 is quadratic in H and can be rearranged using the quadratic formula to give

$$H = \frac{-4.308\ell_c^2 + \sqrt{4.308\ell_c^2} \sqrt{4.308\ell_c^2 + 4R}}{2R}. \quad (5.14)$$

Figure 5.5 (b) shows Equation 5.14 plotted for $\ell_c = 2.7$ mm, along with the shape pa-

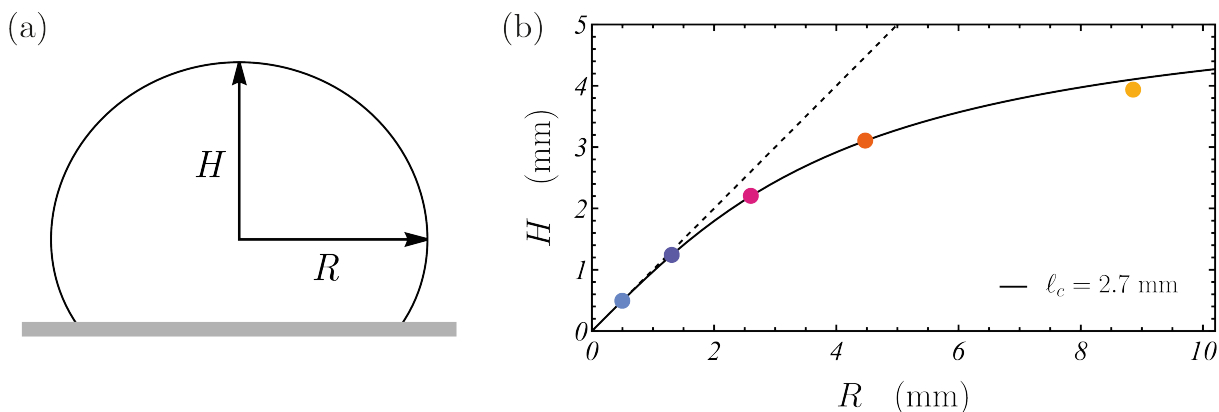


Figure 5.5: (a) Shape parameters in Equation 5.13 for a sessile drop with a contact angle greater than or equal to 90° . (b) Shape parameters H and R plotted for solid drop profiles in Figure 5.3. Equation 5.14 is plotted with $\ell_c = 2.7$ mm as a solid line, the dashed line is defined by $H = R$, which is satisfied for non-deformed spherical caps.

rameters for the equilibrium water in air drop profiles presented in Figure 5.3. Notice that the largest, most deformed drop, is more deformed than predicted by Equation 5.14, indicative of the limitations of this relationship. For mildly deformed sessile drops, fitting of Equation 5.14 to shape parameters R and H permits an evaluation of the capillary length. Feric *et al* have used this technique to measure the capillary length and surface tension of fluorescently tagged nucleoli [11].

5.3 Methods

5.3.1 Sample preparation & imaging

As the reliability of ADSA capillary length measurements scales with condensate size, it is desirable to image larger Ddx4^{N^*} condensates than those generated through nucleation and growth followed by sedimentation (typically 1-5 μm tall after 1 h). To do this, samples were prepared by following the protocol detailed in Section 2.8 to step 6, modified to combine 20 μL protein with 20 μL buffer. The heated solution was then removed from the heating block and allowed to cool to room temperature for 10 min, triggering phase separation. The solution was then centrifuged for 2 min at 20 000 rcf to drive coalescence

of condensates. The sedimented condensate was resuspended by vortexing for a few seconds, before pipetting 9.1 μL onto the coverslip carrying the adhesive spacer. The sample was then sealed with a second coverslip and allowed to equilibrate for at least 1 h before imaging.

3D confocal z -stacks of sessile condensates were collected as detailed in Section 4.3.2, using 2.5 μM Alexa-488 fluorescent dye partitioned into condensates as a contrast agent. Condensate profiles were then extracted and fit to the Young-Laplace equation for sessile drops as detailed in Sections 4.3.3 and 4.3.4.

5.4 Results & discussion

5.4.1 Scheutjens-Fleer calculations

Scheutjens-Fleer theory was implemented using the SFbox software package, which minimizes Equation 5.5 and calculates interfacial energies for a given homopolymer. Briefly, the software considers that the equilibrium ϕ_p at each lattice site is given by its Boltzmann probability. Conversely, the ϕ_p dependent free energy of each lattice site returns the Boltzmann weighting. When the occupation of each lattice site matches its Boltzmann probability, and the total free energy of the lattice is at a minimum, the optimum profile $\phi_p(z)$ has been found. The procedure is to guess a profile and minimize the objective function, which is computed as the difference between the Boltzmann weights and the occupancy. This amounts to a self-consistent field scheme, where the energies associated with each site are consistent with their occupancy ϕ_p [151].

Calculations of Ddx4^{N^*} liquid-liquid interfacial tensions was performed using a 1D lattice of 200 sites, with mirrored boundary conditions. The coordination number for the lattice was set to 6, as is standard for lattice simulations of three dimensional fluids [227]. The protein is input as a polymer consisting of r monomers, where r is the amino acid

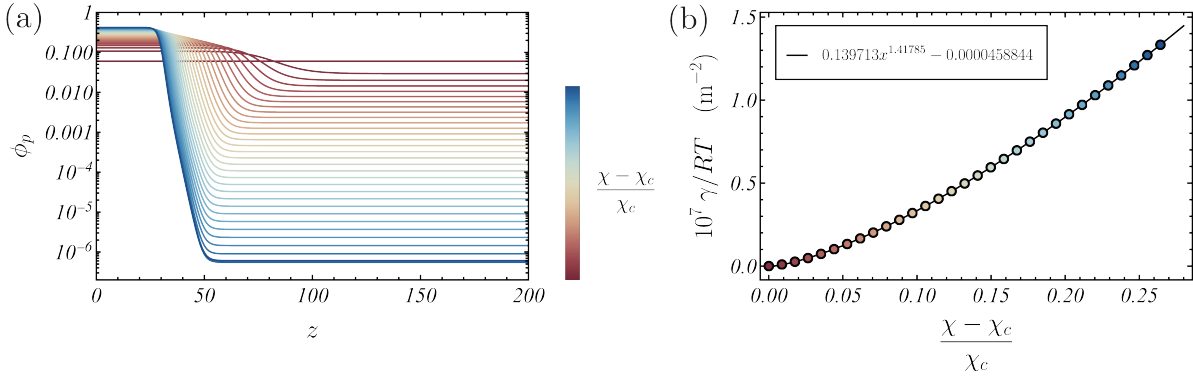


Figure 5.6: (a) Scheutjens-Fleer interfacial profiles for Ddx4^{N*} 1-229, modelled as a homopolymer of length 234, as a function of the reduced interaction parameter. (b) Fitting calculated fluid-fluid interfacial tensions γ to a power law allows extraction of γ at desired values of the interaction parameter χ

count of the construct. The lattice was populated with 12 protein ‘molecules’, giving a total ϕ_p of 0.06. The first calculation was run with $\chi = \chi_c - 0.15$, with the protein monomers constrained to the first 30 sites. This ensured only one phase separation event on the lattice. Subsequent calculations, with unconstrained protein monomers, increased χ by 0.005 up to $\chi = \chi_c$, taking the $\phi_p(z)$ profile of the previous calculation as the initial configuration. As 0.06 is very close to the ϕ_c given by 3.30 for the Ddx4^{N*} constructs studied ($r = 234, 236, 239, 241$), phase separation was observed in all calculated profiles, except when $\chi = \chi_c$. The series of calculations generated profiles and interfacial tensions, shown in Figure 5.6 for Ddx4^{N*} 1-229. Interfacial tensions at desired values of χ were obtained by using the best fit power law, shown in Figure 5.6 (b) for Ddx4^{N*} 1-229. This permitted a propagation of uncertainty in χ to uncertainty in γ .

The mean-field interfacial tensions for the Ddx4^{N*} constructs at 22 °C are displayed in Table 5.2, being of order 10^{-4}J m^{-2} . The mean-field prediction is fluid-fluid interfacial tension depends only on the strength, not the nature, of intermolecular interactions, as condensates formed by constructs with larger values of χ display greater interfacial tensions in Table 5.2. Using the phase diagrams from 3.4, we can calculate a density difference $\Delta\rho$ and therefore a mean-field capillary length, also shown in Table 5.2, looking forward to a comparison with experimentally measured values of ℓ_c .

Table 5.2: Fluid-fluid interfacial tensions calculated using SFbox, with the associated interaction parameter and predicted density difference and capillary length at 22 °C. Uncertainties are calculated from the upper and lower bounds on χ , based on uncertainties in ΔU and ΔS (see Section 3.4).

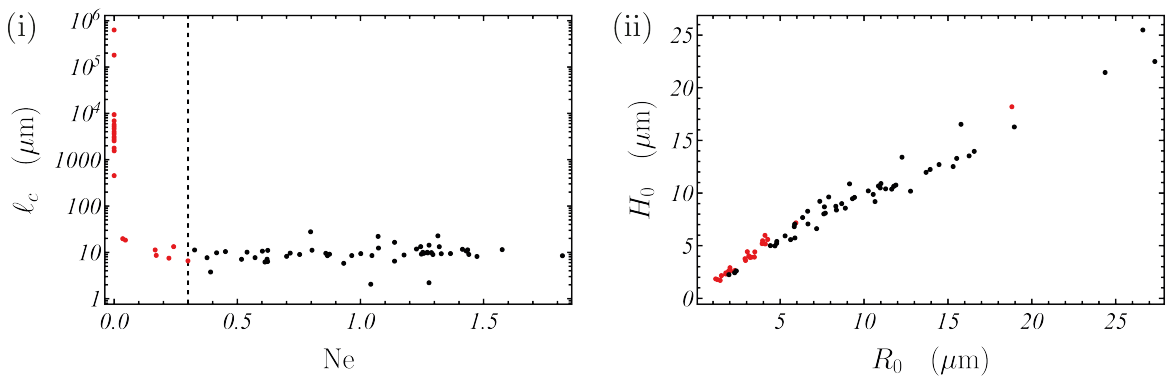
Ddx4 ^{N*} construct	γ (10^{-6}J m^{-2})	χ	$\Delta\rho$ (kg m^{-3})	ℓ_c μm
1-229	94.7 $\pm$ 0.8	0.6303 $\pm$ 0.0004	87.8 $\pm$ 1.2	331.6 $\pm$ 2.7
1-231	83.76 $\pm$ 0.35	0.6251 $\pm$ 0.0002	84.3 $\pm$ 0.6	318.3 $\pm$ 1.3
1-234	71 $\pm$ 0.6	0.6182 $\pm$ 0.0003	79.5 $\pm$ 1.1	301.8 $\pm$ 2.5
1-236	57.5 $\pm$ 0.04	0.61088 $\pm$ 0.00002	74.04 $\pm$ 0.08	281.36 $\pm$ 0.17
1-236 F $\rightarrow$ Y	90.71 $\pm$ 0.10	0.62757 $\pm$ 0.0005	89.07 $\pm$ 0.16	322.21 $\pm$ 0.34

5.4.2 Measurement of capillary length

The mean-field prediction of capillary length for Ddx4^{N*} 1-236 was 281 μm at 22 °C, the smallest for the Ddx4^{N*} constructs investigated in this thesis. Verification of this value would require imaging condensates with $\sqrt{R_0 H_0} > 153 \mu\text{m}$ to achieve $\text{Ne} > 0.3$. The thickness of the sample chambers used in this work (120 μm) prevents the observation of condensates this tall. However, many reported values of IDP biomolecular condensate interfacial tensions are much lower than our mean-field predictions, taking values as small as 10^{-7}J m^{-2} . For the same density differences as the Ddx4^{N*} condensates taken from the phase diagrams in Section 3.4, roughly 80 kg m^{-3} , an interfacial tension this low would give $\ell_c \approx 10 \mu\text{m}$, readily measurable using our experimental setup.

Larger Ddx4^{N*} 1-236 condensates, prepared by centrifugation, were imaged on siliconized glass and borosilicate glass coverslips. As the capillary length is a property of only the fluid-fluid interfacial tension and condensate density, measured values should be independent of the supporting solid surface. Figures 5.7 (a) (i) and (b) (i) shows their measured capillary lengths and Neumann numbers, both obtained from ADSA, divided into reliable and unreliable measurements by a dashed line at $\text{Ne} = 0.3$. The mean capillary length of condensates with $\text{Ne} > 0.3$ is 10.12 μm on siliconized glass and 17.7 μm on borosilicate glass, indicating an interfacial tension around 10^{-7}J m^{-2} . To investigate the reliability of these measurements, we check to see if these values are consistent with the condensate size distributions.

(a) siliconized glass



(b) borosilicate glass

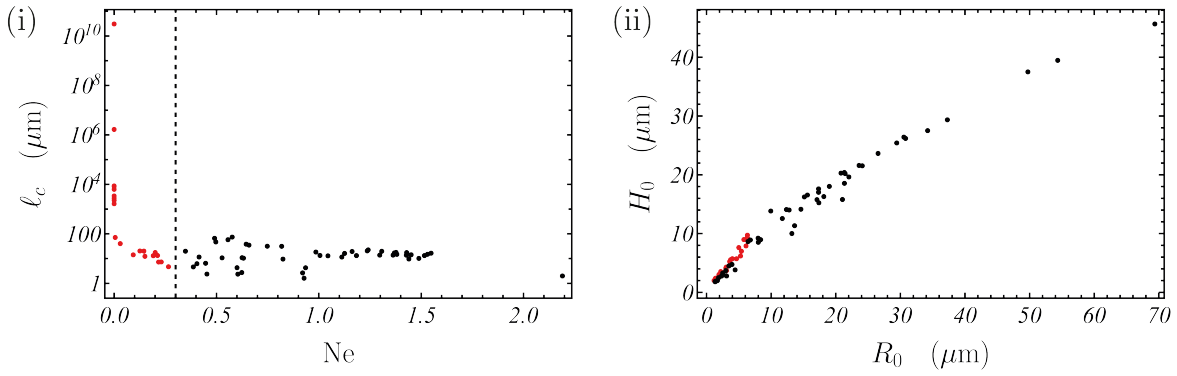


Figure 5.7: (i) Capillary length ℓ_c emann number Ne and (ii) shape parameters for sessile $\text{Ddx4}^{\text{N}*}$ 1-236 condensates prepared on (a) siliconized and (b) borosilicate glass coverslips. Red dots indicate condensates with $Ne < 0.3$.

Partitioning data by $Ne > 0.3$ is expected to divide the condensate population based on their size, with smaller condensates not exhibiting enough gravitational distortion to permit capillary length measurement [136, 222, 223]. To test this, in Figures 5.7 (a) (ii) and (b) (ii) we plot the condensate heights against their radius of curvature at their apex, with condensates where $Ne < 0.3$ plotted with red markers. The condensates where $Ne < 0.3$ are generally the smallest in the population, indicated by low values of H_0 and R_0 , as expected. However, we do observe condensates with heights exceeding $20.24\text{ }\mu\text{m}$ and $35.4\text{ }\mu\text{m}$, twice the mean ℓ_c values for siliconized and borosilicate glass respectively. $2\ell_c$ represents the upper limit of Equation 5.12, If the mean values of ℓ_c measured from condensates with $Ne > 0.3$ are representative of the true value of ℓ_c , then we expect these large droplets to be heavily distorted towards a puddle shape, with a contact angle approaching 180° . In Figure 5.8 (a) we plot capillary length against condensate height examining two xz images of condensates overlaid with their ADSA fit in (i) and (ii). The larger condensate with a contact angle of 78.9° , Figure 5.8 (a) (i), does not appear as a puddle and has a measured ℓ_c of $74.1\text{ }\mu\text{m}$.

It may be the case that the landscape explored by the minimization algorithm during condensate profile fitting is relatively flat with regards to ℓ_c , as would be expected if the true ℓ_c is much larger than the condensate. This would result in the minimizer being sensitive to small deviations in the experimentally obtained (r, z) profile coordinates, resulting in an artificial ℓ_c . To explore this, the profile coordinates from the condensates in Figures 5.8 (a) (i) and (ii) were refit to Equation 4.14, with fixed values of ℓ_c , optimizing only R_0 and Δz . Figure 5.8 (b) shows how the value of the root mean squared error $\sqrt{\lambda}$, defined by Equation 4.22, varies with fixed values of ℓ_c . For both condensates, (i) and (ii), $\sqrt{\lambda}$ decreases sharply for very small values of ℓ_c , before reaching a minima (the values plotted in Figure 5.8 (a)) and then slowly increasing and plateauing at a stable value, where the resultant profile forms part of an undeformed spherical cap by the time ℓ_c reaches its mean-field value (see Section 5.4.1). These $\sqrt{\lambda}$ landscapes are not relatively flat, indicating proper minimization of ℓ_c .

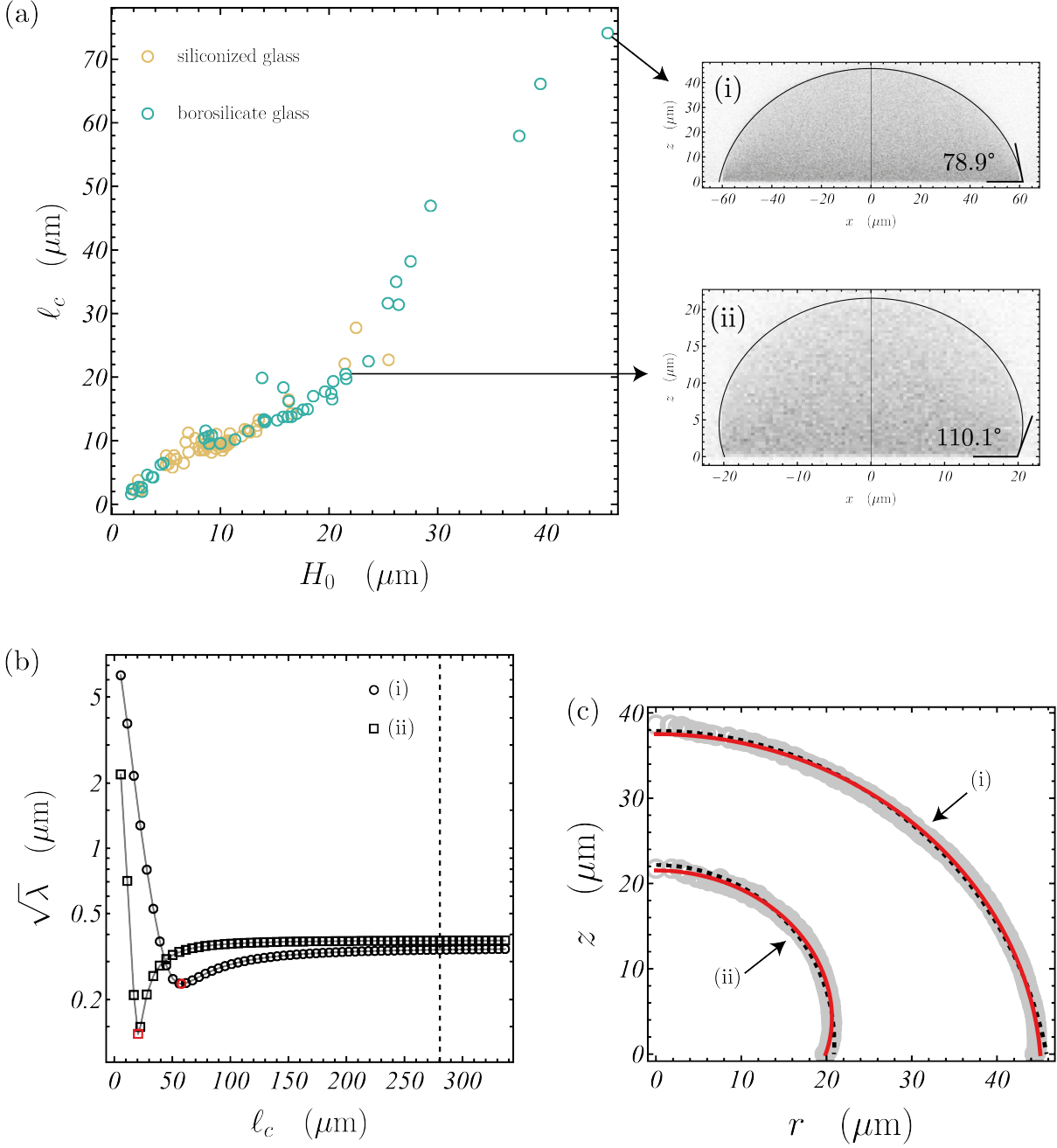


Figure 5.8: (a) Condensate heights H_0 plotted against their measured capillary lengths ℓ_c for Ddx4^{N*} 1-236 condensates resting on siliconized and borosilicate glass coverslips. (i) and (ii) show xz slices, taken from the centre of condensates, overlaid with their ADSA profiles, with their ADSA contact angles inset. (b) Root mean squared error (Equation 4.22) for ADSA profile fits with fixed ℓ_c values for condensates (i) and (ii) from (a). Red points indicate the optimum fits, the dashed line indicates $\ell_c = 280.6 \mu\text{m}$, the predicted mean-field value of ℓ_c . (c) Profile coordinates and ADSA optimum fits (red) and ADSA fits setting $\ell_c = 280.6 \mu\text{m}$ (black, dashed) for condensates (i) and (ii).

In Figure 5.8 (c) we show the profile coordinates, along with their best fit profile (red) and the profile obtained assuming $\ell_c = 280.6 \mu\text{m}$ (black, dashed). Artificially fixing the capillary length to the mean-field value results in a profile fit that does undulates around the optimum fit, increasing the mean squared error from $0.02 \mu\text{m}$ to $0.34 \mu\text{m}$ for condensate (i) and from $0.02 \mu\text{m}$ to $0.37 \mu\text{m}$ for condensate (ii). Taken together with Figure 5.8 (b), these data show the sensitivity of the minimization to small changes in experimental drop profile coordinates.

According to the work of Hell *et al*, we should observe a decrease in fluorescence intensity as our focal plane extends deeper into the sample [201]. This may have an adverse affect on identification of condensates through binarization, especially near the apex of large condensates, reducing their apparent height and capillary length. In Figure 5.9 (a) we illustrate a vertical line, through the xyz centroid of the condensate shown in Figure 5.8 (a) (i). In Figure 5.9 (b) we plot the pixel intensity, measured along this corresponding line, in the Gaussian filtered 3D confocal image containing the condensate, generated as detailed in Section 4.3.3. There is a general decrease in intensity through the droplet, as expected. I_T marks the binarization threshold identified using Otsu's algorithm [204] as detailed in Section 4.3.3. The threshold intersects the intensity trend at the apex of the droplet, as opposed to slicing the cap off. This indicates minimal impact of this aberration on the identification of the droplet apex.

To further examine the capillary length of Ddx4^{N*} 1-236, we consider Equation 5.14, which provides an alternative route to ℓ_c through fitting the function to H and R for condensates with contact angles greater than 90° . In Figures 5.10 (a) and (b) we see Equation 5.14 fit to condensate shape parameters for Ddx4^{N*} 1-236 on siliconized and borosilicate glass coverslips respectively. On both surfaces, the small condensates lie on the dashed line, indicating they are well described as undeformed spherical caps. For larger values of R and H , condensates deviate from the spherical cap geometry, but in a manner poorly described by Equation 5.14. For a constant value of ℓ_c , R is expected to increase faster than H , until H plateaus. The data in Figures 5.10 (a) and (b) deviates

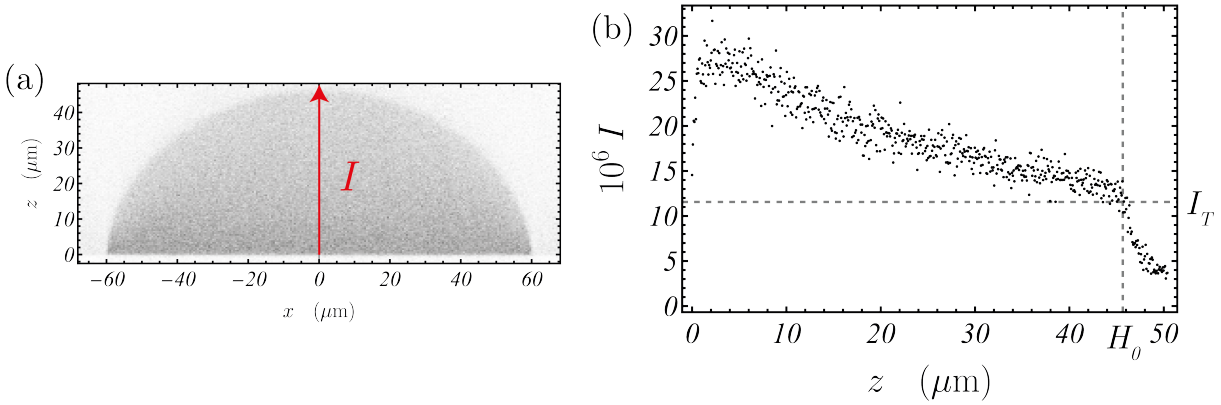


Figure 5.9: (a) Maximum diameter xz slice of a Ddx4^{N*} 1-236 condensate. (b) Intensity along I from (a) acquired after applying the Gaussian filter as detailed in Section 4.3.3. I_T represents the binarization threshold obtained using Otsu's algorithm as detailed in Section 4.3.3 and H_0 is the position of the apex of the condensate according to this binarization threshold.

from spherical cap geometry at roughly $8 \mu\text{m}$ in both cases, before transitioning to another linear regime. Again, this is indicative of an apparent increase in ℓ_c with condensate size.

To test whether the positive relationship between ℓ_c and condensate size is unique to Ddx4^{N*} 1-236, we proceeded to perform ADSA on Ddx4^{N*} 1-229 condensates of siliconized glass and borosilicate glass coverslips, also prepared through centrifugation. In Figures 5.11 (a) (i) and (b) (i) the ADSA capillary lengths are plotted against Neumann numbers. Again, we see populations of condensates with $Ne > 0.3$, with $\ell_c \sim 10 \mu\text{m}$. However, Figures 5.11 (a) (ii) and (b) (ii) show no clear relationship between $Ne < 0.3$ and H_0 or R_0 , unlike the data presented in Figures 5.7 (a) (ii) and (b) (ii). In Figure 5.11 (a) we plot the Ddx4^{N*} 1-229 condensate heights against their capillary lengths, for those with $Ne > 0.3$. A similar trend is observed as in Figure 5.11 (c), whereby larger condensates exhibit larger capillary lengths. The two xz slices overlaid with ADSA profile fits show condensates with similar contact angles, indicating this effect is not a result of contact angle differences, such as those seen in Figure 5.8 (a).

To further examine the ubiquity of size dependent values of ℓ_c , we performed ADSA on Ddx4^{N*} 1-231, 1-234 and 1-236 F $\rightarrow$ Y condensates on borosilicate and siliconized glass. Figure 5.12 (a) shows significant populations of condensates with $Ne > 0.3$ for all Ddx4^{N*} constructs. All Ddx4^{N*} constructs display an increasing ℓ_c with condensate height, sug-

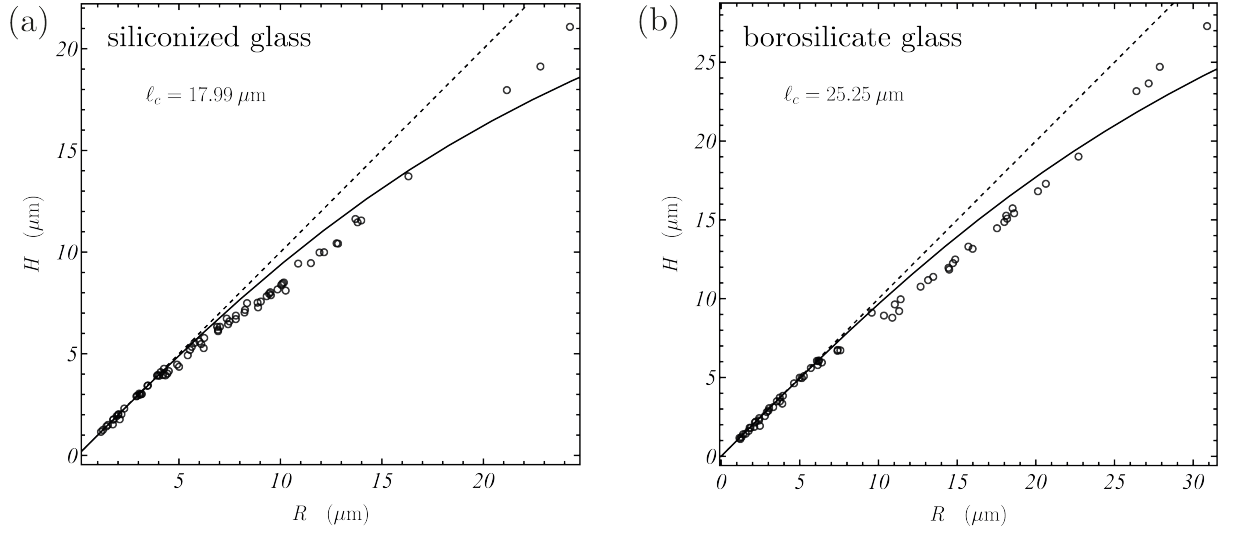
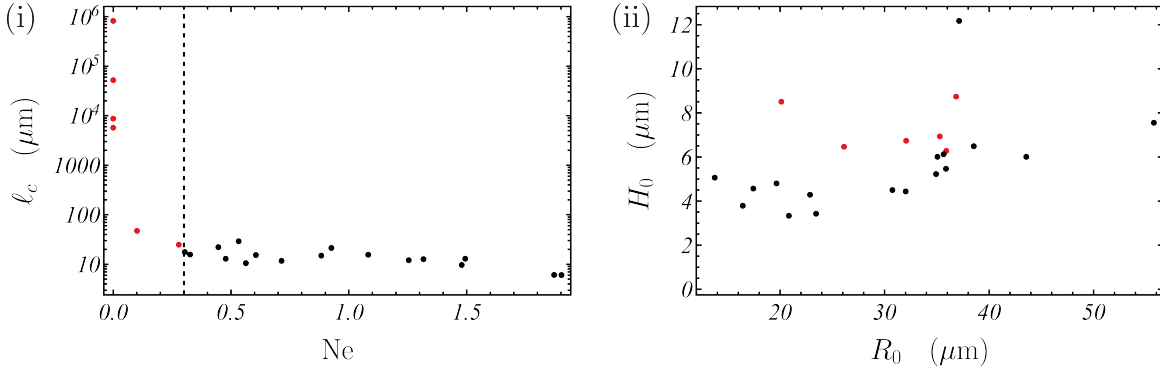


Figure 5.10: Equation 5.14 fit to shape parameters H and R for Ddx4^{N*} 1-236 condensates on (a) siliconized glass and (b) borosilicate glass coverslips. The dashed line at $H = R$ indicates the relationship expected for a spherical cap.

gesting the general effect is indifferent to the mutations introduced in Chapter 3. Taken together, these data do not allow the experimental evaluation of a single capillary length for each Ddx4^{N*} construct. They also do not permit a rejection of the predicted capillary lengths presented in Table 5.1, as there remains the possibility that much larger condensates would return these values.

(a) siliconized glass



(b) borosilicate glass

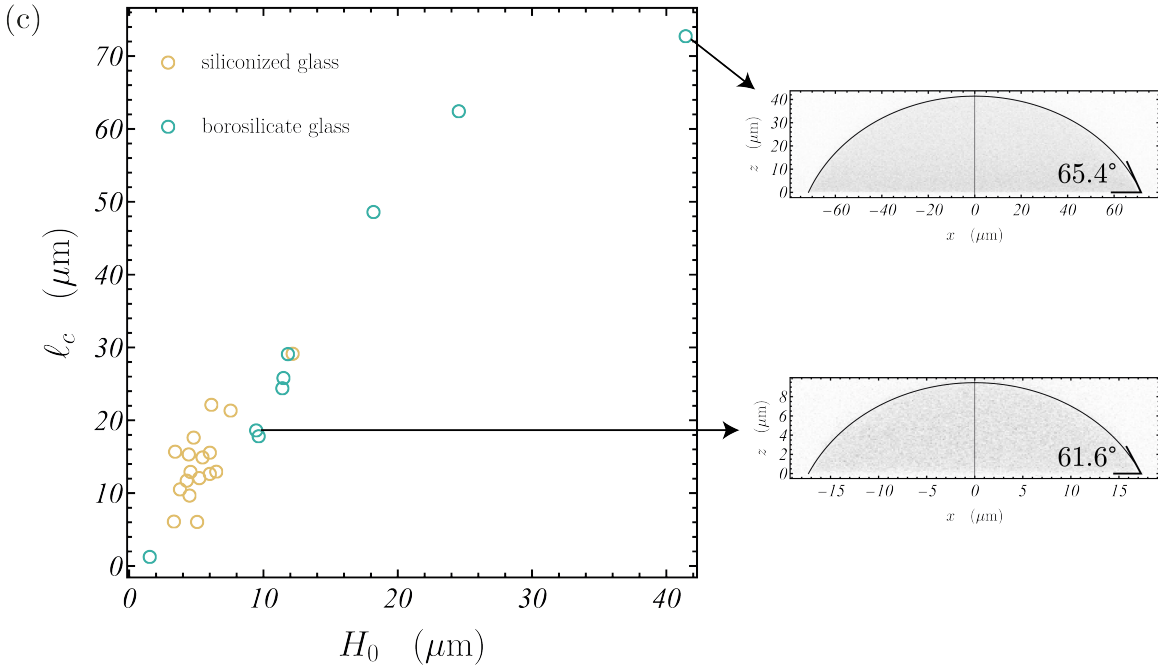
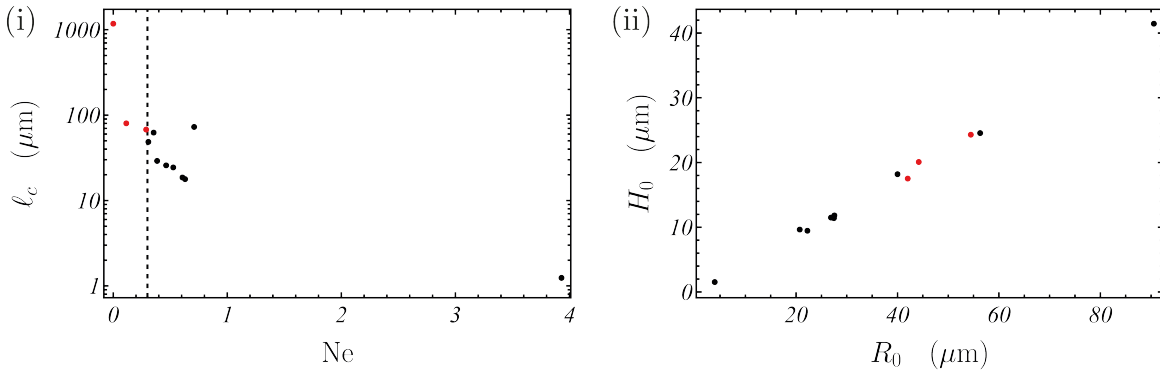


Figure 5.11: (i) Capillary length ℓ_c plotted against Neumann number Ne and (ii) shape parameters for sessile Ddx4^{N^*} 1-229 condensates prepared on (a) siliconized and (b) borosilicate glass coverslips. Red dots indicate condensates with $Ne < 0.3$. (c) Condensate heights H_0 plotted against their measured capillary lengths ℓ_c for Ddx4^{N^*} 1-229 condensates resting on siliconized and borosilicate glass coverslips. Arrows point to condensate xz images overlaid with ADSA profile fits with their ADSA contact angles.

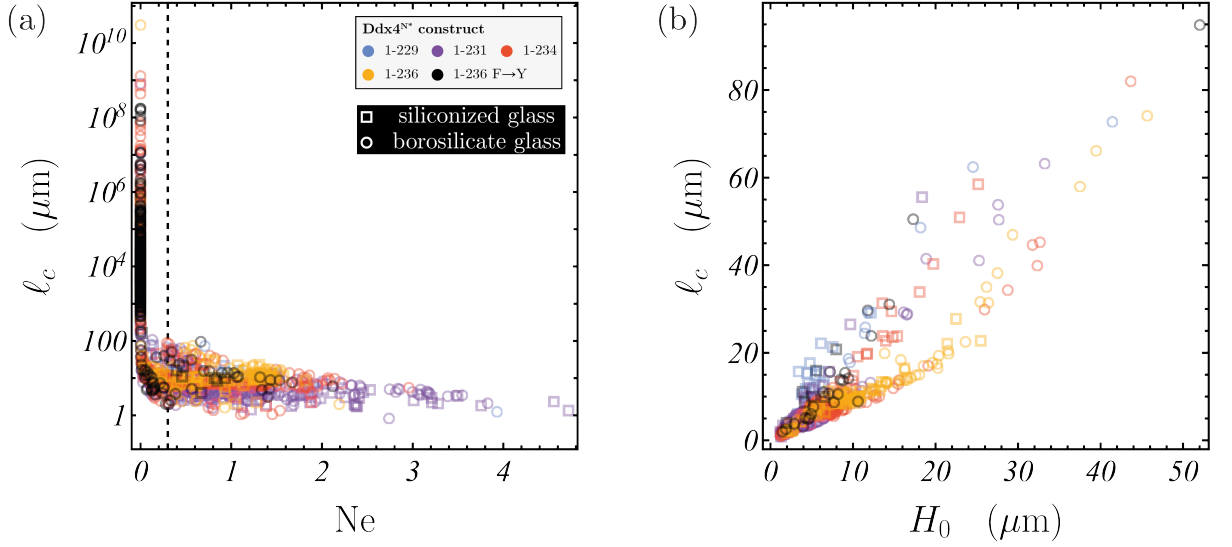


Figure 5.12: (a) Capillary length ℓ_c plotted against Neumann number Ne for all Ddx4^{N*} condensates on borosilicate and siliconized glass coverslips. 14 points lie outside the plot range; 13 with $Ne < 10^{-30}$ and $\ell_c > 10^{11}\mu\text{m}$, one with $(Ne, \ell_c) = (14.05, 8.3\mu\text{m})$. (b) ℓ_c as a function of condensate height H_0 for all Ddx4^{N*} condensates where $Ne > 0.3$ on borosilicate and siliconized glass coverslips.

Discussion

The presented measurements of ℓ_c using both axisymmetric drop shape analysis and the semi-empirical relationship given by Equation 5.13, are suggestive of a value of a size-dependent capillary length. We have shown this is unlikely to be caused by the image analysis and subsequent shape fitting and is also independent of condensate contact angle. What has not been explored, is an alternative imaging set up, allowing an investigation of inherent, unaccounted for, aberrations in our confocal microscopy system. In Section 4.3.3, the change in refractive index between the glass surface and the sample solution was seen to introduce a blur in the image, making it difficult to identify the solid-liquid interface using the fluorescence channel. The condensate phase is expected to have an increased refractive index, relative to the surrounding protein poor phase (~ 1.36 vs 1.33), [202]. It is possible this introduces a similar aberration at the apex of condensates, where the change is most stark. As this is an issue with the imaging set-up, any error will be carried through to profile fitting.

To consider a physical explanation of condensate size-dependent capillary lengths, we recall

$$\ell_c = \sqrt{\frac{\gamma}{g\Delta\rho}}. \quad (5.15)$$

Therefore, we are concerned with an increase in the ratio of the interfacial tension to density difference between the condensate and its surrounding protein-poor phase. Fluid-fluid interfacial tensions are known to be dependent on the local curvature of the interface R , and therefore condensate size, as described by the Tolman length δ , which augments γ by a factor $1 + \frac{2\delta}{R}$ [228]. Typically, δ is on the order of the molecular lengthscale. Increased ℓ_c with size suggests an increasing γ , therefore a negative Tolman length. In this case, small condensates would be predicted to have very low fluid-fluid interfacial tensions, with decreasing capillary lengths. We do observe this behaviour, but not in the form $\ell_c \sim 1/\sqrt{R}$, in accordance with a Tolman length effect. Raising the value of γ as condensate size increases would be expected to have a knock-on effect on contact angles, driving large condensates towards a value of 90° . This is not consistent with the data subsequently presented in Chapter 6, therefore we find a size dependent fluid-fluid interfacial tension unlikely.

Alternatively, an increasing ℓ_c may be explained by a decreasing $\Delta\rho$ with condensate size. The contact angle is also dependent on the interfacial tension of the condensate with solid surface below, inherently governed by the composition of the condensate. As $\Delta\rho$ parameterises the composition of the condensate, we may again, expect to see a size dependent contact angle. In Chapter 6 we do present size dependent contact angles, but with values that stabilise for larger condensates, as opposed to the unstable values of ℓ_c reported in this section, providing evidence to reject an effect on $\Delta\rho$. To investigate size dependent values of $\Delta\rho$, future work could focus on quantifying the condensates interior Ddx4^{N*} concentration, using a carefully fluorescently labelled protein enabling concentration measurement through fluorescence microscopy. The work of McCall *et*

al [202] provides an alternative, label free measurement of protein concentration through quantitative phase microscopy, allowing us to address this issue.

5.5 Conclusions & outlook

We have demonstrated the numerical calculation of fluid-fluid interfacial tension for Ddx4^{N*} condensates utilising the Scheutjens-Fleer framework. These calculations take as input the Flory-Huggins mean-field interaction parameters obtained by the fitting of phase diagrams presented in Chapter 3. Numerical calculation of fluid-fluid interfacial tension also permitted the prediction of capillary lengths. To verify the applicability of Scheutjens-Fleer theory to biomolecular condensates, we obtained experimental values of condensate capillary lengths using axisymmetric drop shape analysis. In doing so, we observed an unexpected size dependency, wherein the capillary length was seen to increase with condensate heights.

To investigate this observation, we closely examined the drop shape analysis procedure. The positive relationship between capillary length and condensate size was not explained by poor shape fitting, or by aberrations. We also found a similar trend when using an alternative semi-empirical relationship, entirely independent of condensate contact angles, to analyse the capillary length of a population of condensates. We considered a possible a Tolman length effect, as well as potential variance of composition between condensates, but found these to be inconsistent with the data presented in this chapter, and subsequently in Chapter 6.

As a whole, we find it speculative that the Ddx4^{N*} condensate systems presented do indeed exhibit a size-dependent capillary length. Further experiments are required to confirm this and to enable a confirmation or rejection of our mean-field calculations of capillary length. In 2021, Ijavi *et al* [136] measured biomolecular condensate interfacial tensions and densities using a combination of ADSA and sedimentation velocity mea-

surements. They utilised bright field microscopy to image sessile condensates $\sim 500\text{ }\mu\text{m}$ in height, capturing images from a side-on perspective to obtain capillary lengths. This circumvents the axial aberrations associated with the confocal microscopy set-up used in this chapter. The implementation of a similar experimental set up would allow the verification of the predicted Ddx4^{N^*} capillary lengths. We also note that they did not mention any size dependent $\Delta\rho$ or γ during their experiments on condensates composed of intrinsically disordered protein FUS₂₆₇. Future experiments should also focus on alternative measures of capillary lengths, for example through characterisation of the menisci formed by condensed protein phases in the vicinity of vertical walls [219]. This removes any effect of size from the experiment, as the form of the menisci is dependent only on the capillary length and contact angle.

Acknowledgments

We thank Prof Mark Vis and Prof Remco Tuinier (Eindhoven University of Technology) for demonstrating and gifting us the SFBox software package.

Chapter 6

Molecular Determinants of Condensate Wetting

ABSTRACT

Here, we are concerned with the organization of biomolecular condensates at surfaces through wetting. We motivate this through an overview of intracellular wetting phenomena, in particular, the observation that non-specific membrane wetting by biomolecular condensates is yet to be reported. Through applying axisymmetric drop shape analysis, we perform precise measurements of Ddx4^{N} condensate contact angles on a model membrane, comprising a supported lipid bilayer, revealing an intrinsic repulsion. Carrying forwards the Ddx4^{N*} mutants introduced in Chapter 3, their contact angles are also measured on hydrophilic and hydrophobic glass surfaces, allowing an investigation into the hydrophobic forces dictating Ddx4^{N*} condensate wetting. We also reveal the effect of line tension in governing wetting behaviour, measuring a line tension length relevant for intracellular condensates.*

6.1 Introduction

The wetting behaviour of a surface describes its tendency to be wet by a given liquid, and is characterised by the contact angle θ [225]. Figure 6.1 shows three distinct cases, whereby the surface exhibits total wetting, partial wetting and total drying - an absence of wetting. The particular contact angle is determined by a minimization of the total interfacial energy, which is dependent on the compositions of the three phases in contact and the interaction of molecules at their surfaces. Wetting phenomena are familiar in nature; the structure of duck feathers and plant leaves, with high water-in-air contact angles, keep the animal dry and funnel water towards the roots respectively [229, 230]. With the advent of the study of functional, liquid-like intracellular biomolecular condensates [4, 231], there is growing interest in the contribution of wetting phenomena to the interior organization of the cell.

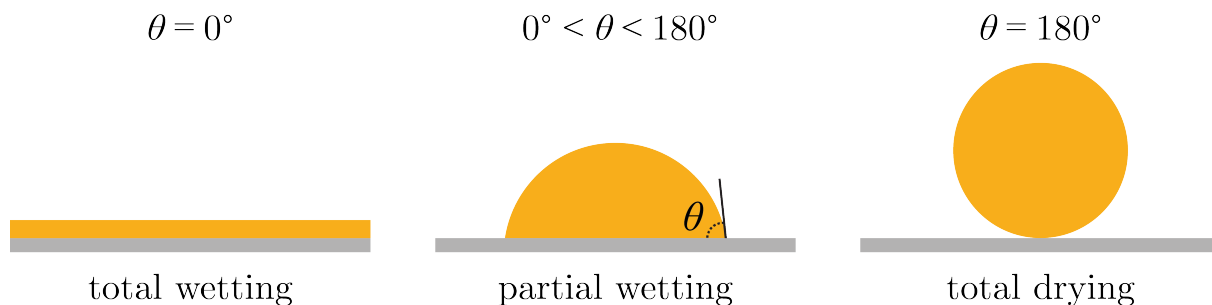


Figure 6.1: The wetting behaviour of liquids at surfaces falls into three distinct categories, and is characterised by the contact angle θ .

Recent evidence shows the formation of intracellular condensates from a well-mixed cytoplasm/nucleoplasm, by liquid-liquid phase separation [110, 177, 195]. Heterogenous nucleation theory predicts condensates, forming from a well mixed solution, should nucleate (and grow) primarily on surfaces which they preferentially wet [232–234]. The localisation of condensates to intracellular surfaces is indeed observed, with some intracellular condensates partially wetting the plasma membrane [27, 29, 115–119, 121–125], while others wet membrane bound organelles [119] or cytoskeletal elements [113, 114, 235].

In all reported cases of membrane wetting condensates, the phenomenon is driven by specific protein-protein interactions between condensate components and membrane bound proteins. It is also apparent that the majority of intracellular condensates, both endogenous and exogenous, form and remain suspended in the protoplasm, with no obvious interaction with membrane surfaces.

We hypothesise there is an innate repulsion between intracellular condensates and membrane surfaces, encoded in the sequences of their dominant (scaffold) proteinacious components. To investigate this, we use axisymmetric drop shape analysis to measure the contact angle of model Ddx4^{N*} condensates on a model membrane, composed of a 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayer resting on glass. We then proceed to measure contact angles on hydrophilic and hydrophobic glass surfaces, allowing elucidation of the contribution of hydrophobic forces to condensate wetting. In this conjunction, we discover the impact of line tension in determining the size-dependent contact angle of Ddx4^{N*} condensates.

This chapter commences with theoretical background on the interfacial forces contributing to contact angles, with reference to theoretical models for solid-liquid interfacial tensions and line tension, concluding with a discussion of line tensions in molecular systems. The preparation of POPC supported lipid bilayers is then detailed, along with the procedure used to clean borosilicate glass coverslips. The results & discussion begins with evidence supporting a line tension effect, manifest as size-dependency in measured contact angles. Contact angle measurements of the Ddx4^{N*} constructs introduced in Section 3.1.1 is then performed on the POPC bilayer, siliconized (hydrophobic) and untreated (hydrophilic) borosilicate glass surfaces. The chapter concludes with a contextual discussion of the presented results.

6.2 Theoretical background

6.2.1 Wetting & interfacial tension

Figure 6.2 shows the forces governing wetting, illustrated for a droplet of phase α , with fluid-fluid interfacial tension γ , resting on a smooth solid surface s , surrounded by its coexisting fluid phase β . γ_{sj} represents the interfacial tension between the solid and phase i , τ represents the line tension at the three phase contact line and r represents the radius of the contact line. At equilibrium, the net force on the droplet interface is zero. For a large drop or small line tension, where $\tau/r \rightarrow 0$, resolving the horizontal forces in Figure 6.2 gives

$$\cos \theta = \frac{\gamma_{s\beta} - \gamma_{s\alpha}}{\gamma}. \quad (6.1)$$

This is Young's equation, dating back to 1805 [197], showing that contact angles, in the absence of line tension effects, are determined by the relative interfacial tensions of the three surfaces in contact. As the interaction between phases α and s becomes more favourable, the contact angle decreases, increasing the area of the $s\alpha$ interface and decreasing the area of the $s\beta$ interface. Equation 6.1 has two limiting cases. For total wetting, where $\theta = 0^\circ$,

$$\gamma_{s\beta} = \gamma_{s\alpha} + \gamma. \quad (6.2)$$

At a glance this seems like a rare case. With α and β in equilibrium, $\gamma_{s\beta}$ will never be greater than $\gamma_{s\alpha} + \gamma$, as a microscopically thin film of α is always able to form between β and s , if it serves to decrease $\gamma_{s\beta}$ [102]. This is Antonow's rule [236], and applies equally well to the converse case of total drying, where $\theta = 180^\circ$;

$$\gamma_{s\alpha} = \gamma_{s\beta} + \gamma. \quad (6.3)$$

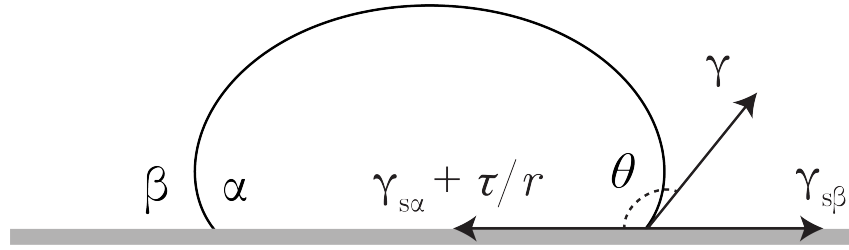


Figure 6.2: The contact angle, θ , of a sessile drop is determined by balancing forces. These are the fluid-fluid interfacial tension γ between α and β , the solid-fluid interfacial tensions $\gamma_{s\alpha}$ and $\gamma_{s\beta}$, and the line tension τ . r is the contact radius at the base of the drop.

From Antonow's rule, we see that if a contact between α and s through phase β would result in an overall increase in interfacial energy, we instead observe no contact and total drying of s by α .

Cahn, Nakanishi and Fisher [111, 237] have extended the theory of van der Waals (see Section 5.2.1) to give a description of wetting in two component systems, where the interfacial tensions at solid walls are found by minimizing the following with respect the composition profile $\phi(z)$:

$$\gamma_{s\alpha} = \Phi(\phi(z=0)) + \int_0^\infty \left[W(\phi(z)) + m \left(\frac{d\phi(z)}{dz} \right)^2 \right] dz, \quad (6.4)$$

Here, the wall is considered to be perpendicular to z and positioned at $z = 0$, and gravitational effects are ignored. The term Φ describes the free energy per unit area at the solid surface, while the first term in the integral describes the cost of deviations in ϕ from its bulk value, and the second term accounts for gradients in ϕ . The excess free energy density $W(\phi)$, and square gradient coefficient m , are discussed in Sections 3.2.5 and 5.2.1 respectively. Solid-liquid interfacial tensions are therefore determined by both the direct interactions of the constituent molecules of the liquid with the solid, and the penalty associated with disturbing these molecules from their bulk equilibrium arrangement.

6.2.2 Line tension

Considering the forces in Figure 6.2 for sessile droplets where line tension is not insignificant, gives the modified Young's equation [238, 239]

$$\cos \theta = \frac{\gamma_{s\beta} - \gamma_{s\alpha}}{\gamma} - \frac{\tau}{\gamma r}. \quad (6.5)$$

Line tension may be positive or negative and may drastically increase or decrease contact angles of sessile drops. The ratio τ/γ defines a lengthscale, the line tension length, below which line tension effects on θ are visible. Just as interfacial tension arises from the deviations from bulk equilibrium compositions necessitated by the formation of interfaces, line tension is rooted in the extra structure associated with the formation of the three phase contact line [102]. Its value may be modelled using an interface displacement model, which we proceed to outline [240].

Consider the arrangement of molecules in the vicinity of a solid substrate for the sessile drop shown in Figure 6.3 (a). As the drop interior is approached from the exterior, along vector x , the arrangement of molecules in the vicinity of the solid substrate changes continuously from one which gives rise to $\gamma_{s\beta}$, to an arrangement which gives rise to $\gamma_{s\alpha}$. The excess free energy associated with this region is the line tension, which has units J/m. The free energy potential V (per unit area) along x is plotted in Figure 6.3 (b) for positive and negative line tensions.

Substitution of Equation 6.1 into Equation 6.5 gives

$$\cos \theta = \cos \theta_0 - \frac{\tau}{\gamma r}, \quad (6.6)$$

where we consider θ_0 to be Young's angle, defined by Equation 6.1, the asymptotic limit of Equation 6.5 as $r \rightarrow \infty$ [145, 241]. We now see that positive line tensions are associated with contact angles greater than Young's angle for droplets with contact radii of order

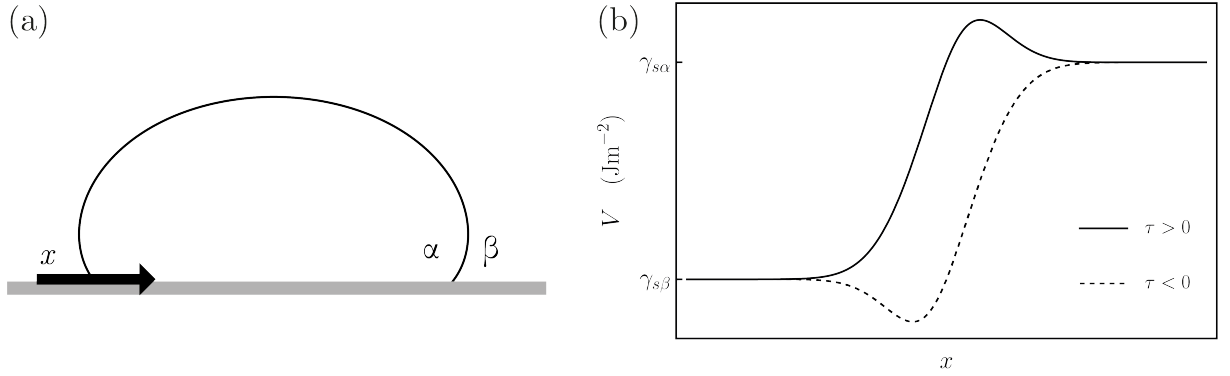


Figure 6.3: (a) Sessile drop resting on a solid surface, vector x captures molecules in the vicinity of the solid surface, at the solid-fluid interfaces. (b) Free energy per unit area V along vector x for positive (filled line) and negative (dashed line) line tensions.

τ/γ and smaller, vice versa for positive line tensions. Equation 6.6 is linear in $1/r$ and can be readily fitted to contact angle measurements, allowing an extraction of Young's angle and the line tension length [146].

Reported values of line tensions are well reviewed by Amirfazli and Nuemann [242], and more recently by Law *et al* [243]. Theoretical and computationally calculated line tensions for model systems including: colloidal particles at liquid interfaces [244, 245], electrolyte solutions on charged surfaces [246] and soap films at liquid interfaces [102, 247] give values of magnitude $10^{-12} - 10^{-10} \text{J m}^{-1}$ [248–255]. Experimental measurements of line tension are generally restricted to water-air or water-oil systems in contact with inorganic substrates, with magnitudes scattered in the range $10^{-12} - 10^{-5} \text{J m}^{-1}$ [145, 256–260]. Theoretical, computationally calculated and experimentally measured values of line tension may be positive or negative. To the authors knowledge, there have been no investigations into line tension effects in biomolecular condensate systems.

6.3 Methods

6.3.1 Supported lipid bilayer preparation by vesicle fusion

To observe the interaction of biomolecular condensates with cellular membranes in a controlled environment, a model membrane in the form of a supported lipid bilayer (SLB) was prepared. The model membrane is composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), whose skeletal formula is given in Figure 6.4 (a). Its hydrophilic headgroup and hydrophobic tail drives the spontaneous formation of bilayers, manifest in solution as vesicles, which are often multilamellar. Vesicle fusion is a standard technique for preparing SLBs [261], where vesicles fuse and rupture atop a solid, hydrophilic support, creating a lipid bilayer [262].

The following protocol is adapted from references [263,264] and uses POPC supplied as 100 mg powder in sealed ampules from Avanti[®]. First, the ampule is aliquoted and prepared as lipid films, then lipid films are resuspended and processed to form small unilamellar vesicles (SUVs). Finally, SUVs are deposited on borosilicate glass to generate a SLB.

Preparation of lipid films

20 × 10 mL and a single 10 mL glass phial(s) were washed with isopropanol, dried with nitrogen gas and desiccated under vacuum for 1 h. To a 100 mg ampule of POPC, 5 mL chloroform was added using a glass syringe previously washed with chloroform, instantly dissolving the lipids to form a clear solution. The POPC solution was then added to the 20 mL phial, the air in the phial replaced with nitrogen gas and the solution topped up to 20 mL with chloroform and bath sonicated for 5 min. After sonication, 1 mL POPC solution was added to a clean 10 mL glass phial. The chloroform was evaporated under a moderate flow of nitrogen gas, rotating the phial at a tilt to create a lipid film on the

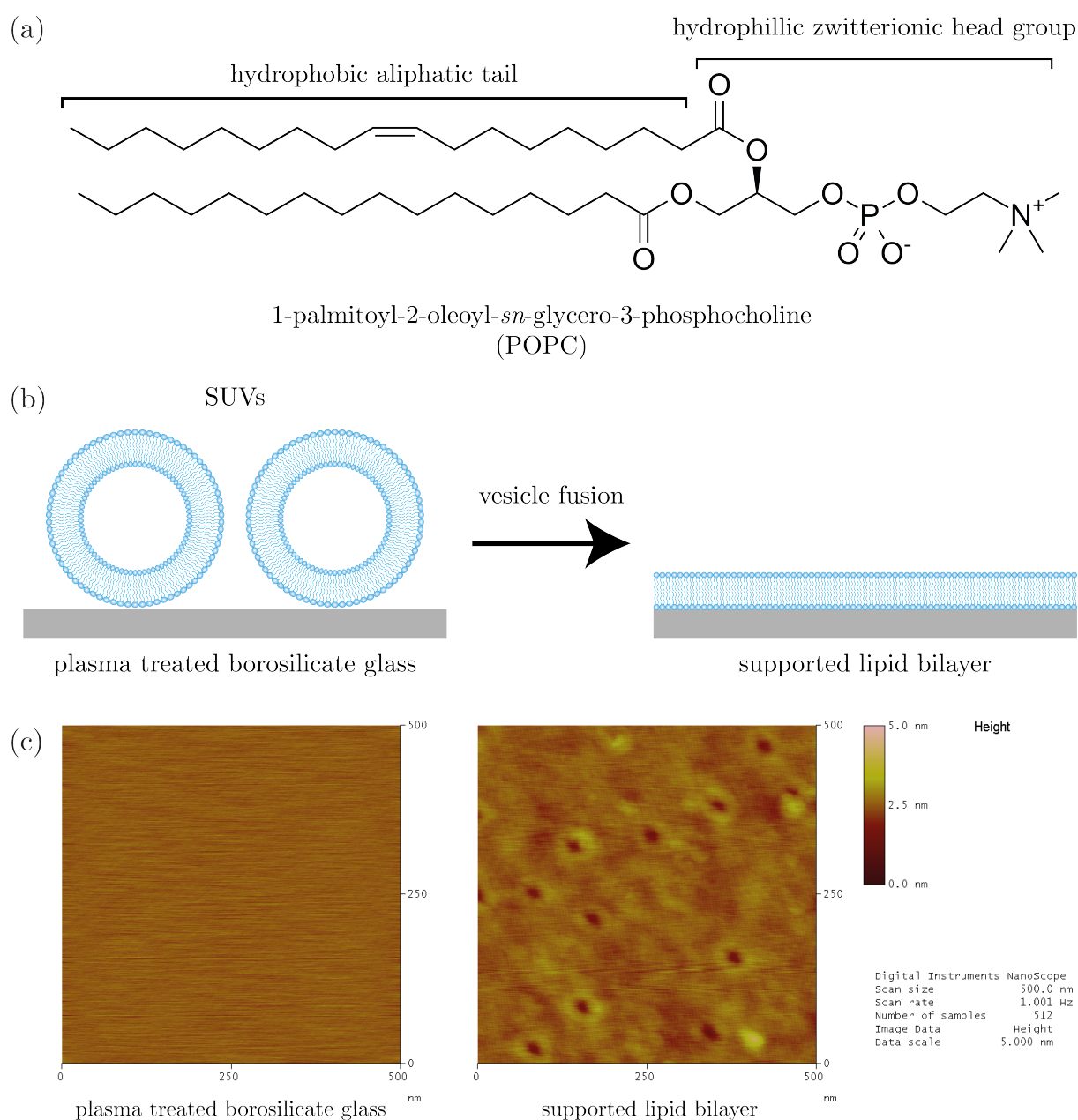


Figure 6.4: (a) Skeletal formula of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine phospholipid. (b) Illustration of vesicle fusion leading to the formation of a supported lipid bilayer. (c) Atomic force microscopy micrographs of plasma treated borosilicate glass and a supported lipid bilayer (see text).

walls of the phial. This was repeated until 20 aliquots containing 5 mg POPC had been prepared. Aliquots were stored under vacuum overnight with a loose lid to remove any moisture, then at -20°C for longer term storage with the lid screwed tight.

Preparation of SUVs

5 mg POPC was dissolved in 5 mL SUV buffer and vortexed until no lipid film was visible on the inner surface of the phial. The lipid solution was freeze-thawed 5 $\times$ by holding the phial in liquid nitrogen for 1 min and then at 50°C in a water bath for 7 min to rupture large multilamellar vesicles. The solution was transferred to a clean, plastic 250 mL beaker, before 45 mL SUV buffer was added. The beaker was placed on ice during probe sonication, performed with a Fisherbrand[®] Model 705 Sonic Dismembrator with process settings: amplitude 55, process time 15 min, pulse-on time 3 s, pulse-off time 10 s. The sonicate was centrifuged in a 50 mL Greiner Bio-One[™] Polypropylene Conical Bottom Test Tube, the supernatant was then transferred to a fresh 50 mL tube. The air in the tube was replaced with nitrogen gas before storage at 4°C . SUVs were used within 3 days of preparation.

Lipid coating of borosilicate glass

10 borosilicate glass coverslips (Agar Scientific Ltd. 22 mm No. 1.5) were cleaned with nitrogen gas, isopropanol, nitrogen gas again and wiped with lens paper (Fisherbrand[™]). Each surface of the coverslips was treated with an oxygen plasma for 5 min before submersion in 2 $\times$ petri dishes containing 25 mL POPC SUV solution (5 coverslips in each dish). The dishes were left on a rocker for 60 min to allow the supported lipid bilayer to form by vesicle fusion (see Figure 6.4 (b)). The solution in the petri dish was exchanged for fresh SUV buffer twice, with 30 min on the rocker between washes, to remove excess SUVs. Coated coverslips were dried with nitrogen gas and placed in a fresh petri dish before storage under vacuum. SLB coated coverslips were used within 3 days of preparation.

Quality control

Atomic force microscopy was performed by Dr Robert Jacobs (Oxford surface forces analysis lab) on a coverslip coated with POPC, and another coverslip cleaned with isopropanol, plasma treated and submerged in SUV buffer without any POPC and left to dry overnight. Figure 6.4 (c) shows distinct patterning on the POPC treated glass, in contrast to the untreated surface, indicating successful adhesion of a lipid bilayer.

6.3.2 Sample preparation, imaging & analysis

Sessile condensates were prepared on POPC bilayer coated coverslips, borosilicate glass coverslips and siliconized glass coverslips, roughly following the protocol detailed in Section 2.8, with modifications affording the generation of multiple sizes of condensates. Following the protocol to step 10 and allowing the sample to cool to room temperature generated condensates with maximum dimension 10 μm . Condensates with maximum dimension 20 μm were prepared by following the protocol to step 6, then allowing the tube containing the phase separating solution to cool to room temperature for 10 mins. The rest of the protocol was then followed, keeping the glass coverslips and sample at room temperature. The largest condensates were generated by centrifugation, as detailed in Section 5.3.1. Note that for samples prepared on supported lipid bilayers, the POPC coated coverslips always kept at room temperature.

3D confocal z -stacks of sessile condensates were collected at room temperature ($\approx 22^\circ\text{C}$) as detailed in Section 4.3.2, using 2.5 μM Alexa-488 fluorescent dye (unless stated otherwise) partitioned into condensates as a contrast agent. Condensate profiles were then extracted and fit to the Young-Laplace equation for sessile drops as detailed in Sections 4.3.3 and 4.3.4, permitting an evaluation of contact angle on a per-condensate basis.

6.4 Results & discussion

6.4.1 Ddx4^{N*} condensates exhibit a positive line tension

It is crucial for contact angle interpretation to establish whether line tension plays a role, resulting in size-dependent contact angles, as is illustrated by Equation 6.5. Considering the predicted fluid-fluid interfacial tensions of the Ddx4^{N*} condensates studied in this thesis are of order 10^{-5} J m^{-2} , while general values of line tension are in the range $10^{-12} - 10^{-5}$, we may expect a line tension length in the broad range $10^{-7} - 10^{-1} \text{ m}$. We now present and investigate the size dependency of Ddx4^{N*} 1-229 condensates on siliconised glass.

Preparation of sessile condensates by centrifugation generated condensates with contact radii ranging from 3 to 35 μm (see Figure 6.5 (a)). To control for any effects of high g-forces on condensates, another sample was prepared whereby the phase separating solution was left on the bench for 18 h. This generates large condensates through coalescence through gravity induced sedimentation, avoiding the use of high g-forces. Sedimented condensates were resuspended by lightly flicking the sample tube, before being loaded onto a siliconized glass coverslip, before sealing using adhesive spacers and a second siliconized glass coverslip as in Section 2.8. As usual, sessile condensates were left to equilibrate for 1 h.

Figure 6.5 (a) shows the profile coordinates, with their best fit profiles from ADSA for both sample preparation techniques. The profiles of condensates prepared by centrifugation overlap with those prepared by sedimentation, indicating the ‘harsher’ method of inducing coalescence through centrifugation does not alter the shapes of sessile condensates, suggesting both preparation methods result in equilibrium liquid drops. Figure 6.5 (b) shows xz slices of condensates taken from the centrifuged samples, with ADSA profiles reflected about $r = 0$ and overlaid, with the measured contact angle inset. The condensate

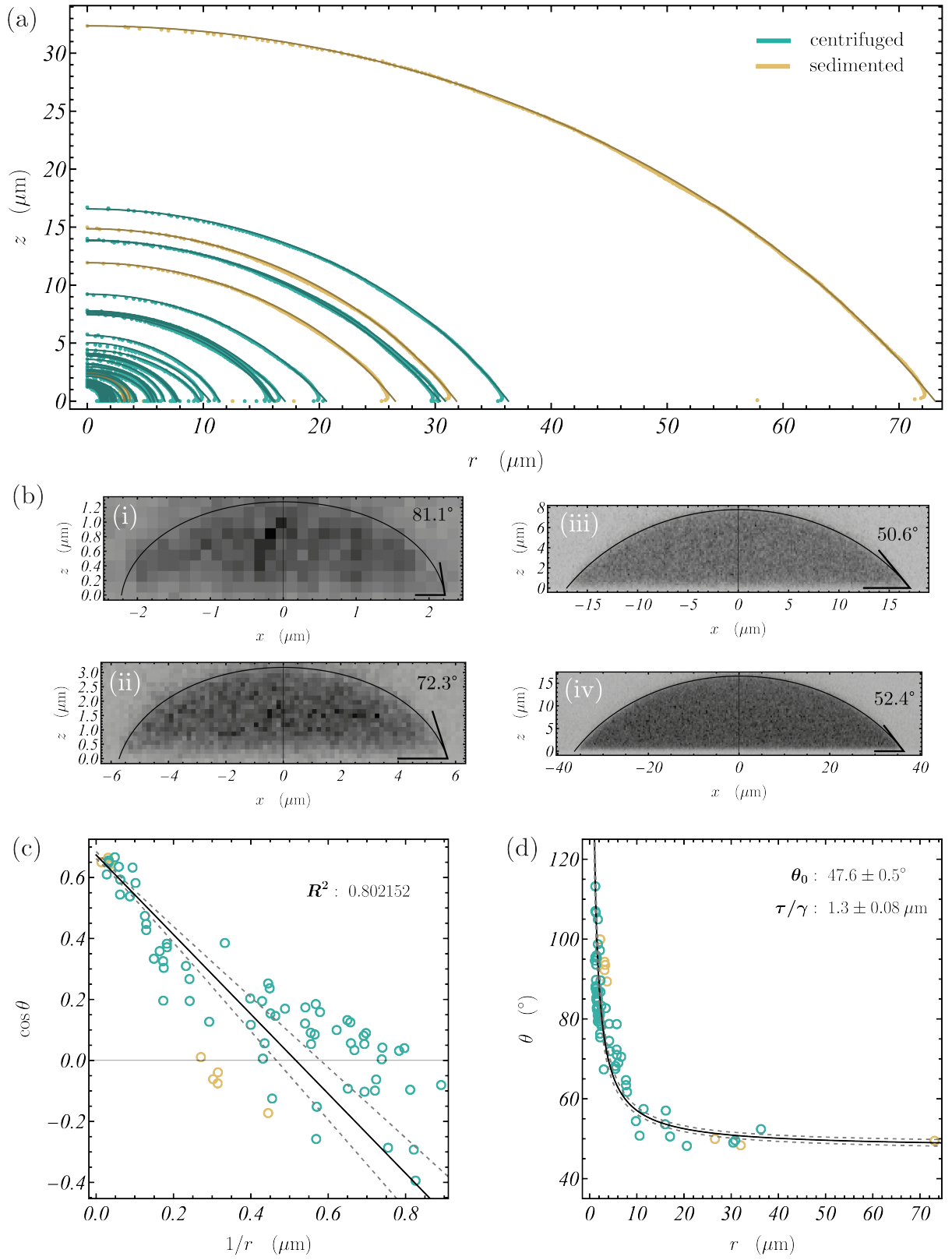


Figure 6.5: Caption on next page.

Figure 6.5: Sessile Ddx4^{N*} 1-229 condensates resting on siliconised glass, imaged with 5 μm Cy3B. (a) All profile coordinates and their fits, shown for condensates prepared by centrifugation in triplicate with 18, 40 and 8 condensates from each sample, and for condensates prepared by sedimentation in duplicate, with 6 and 2 condensates from each sample. (b) ADSA profiles overlaid on xz slices of condensates, taken at their diameter, for condensates ranging from 2.2 μm (i) to 36.6 μm (iv). The contact angles from ADSA fitting are inset. (c) Contact angles and contact radii from condensates in (a), fit to Equation 6.6 (solid line), weighting the independent variable $1/r$ as r^2 . The dashed lines indicate the 95% confidence bands on the line of best fit, with the goodness of fit shown by the R^2 value. (d) The data and best fit lines from (c) transformed into the (r, θ) plane. The best fit Young's angle and line tension length are inset with their uncertainties.

shown in (i) is at the limit of acceptable size for ADSA, and has a contact angle larger than (ii), which is larger than (iii) and (iv), a clear dependency of contact angle on condensate size. Equation 6.6 was fit in Figure 6.5 (c) with strong correlation, using linear regression, to the contact angle and contact radii extracted from the fits in Figure 6.5 (a). In the fitting, contact radii were weighted as r^2 , as the solid surface area sampled by each condensate scales as r^2 . In Figure 6.5 (d) we map the data from Figure 6.5 (c) onto the (r, θ) domain. A size dependent contact angle is clearly visible at lengthscales far beyond the axial and lateral resolution limits of the optical setup (see Section 4.3.2). The best fit Young's angle and line tension length for this system are $47.6 \pm 0.5^\circ$ and $1.3 \pm 0.08 \mu\text{m}$ respectively.

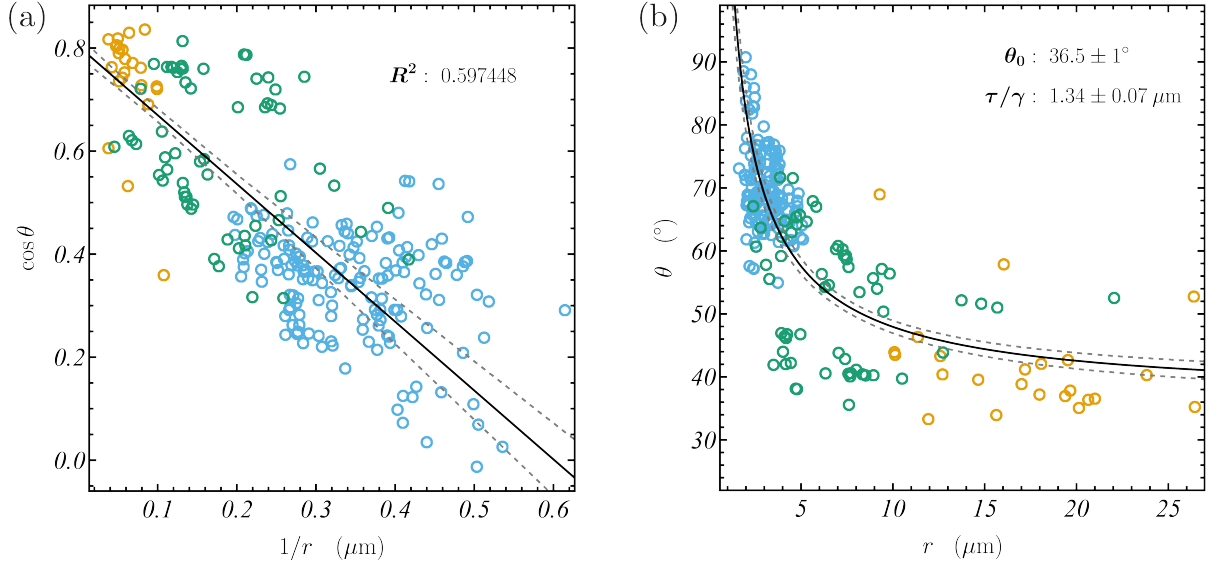


Figure 6.6: Sessile Ddx4^{N*} 1-229 condensates resting on siliconized glass, imaged with 2.5 μM Alexa-488. The 23 orange datapoints correspond to condensates prepared by centrifugation, the 61 green datapoints correspond to condensates prepared by allowing condensates to phase separate in the sample tube and allowing them to sediment and coalesce for 10 min before imaging. Blue datapoints correspond to condensates prepared as described in Section 4.3.1. (a) Linear fit of Equation 6.6 to condensate contact angles and contact radii, dashed lines show the 95% confidence bands. (b) Datapoints and fit from (a) transformed into the (r, θ) plane.

The data presented in Figure 6.5 were collected using 5 μM Cy3B as the fluorescence contrast agent. To test whether the observed line tension effect was dependent on the identity of the fluorescent dye in the sample, an analogous experiment was set up with 2.5 μM Alexa-488 dye. These data were fit to Equation 6.6, as shown in Figures 6.6 (a) and (b). The value of Young's angle has decreased, from $47.6 \pm 0.5^\circ$ to $36.5 \pm 1^\circ$, upon swapping Cy3B for Alexa-488, indicating a degree of dye-dependent wetting. However, the line tension length, $1.34 \pm 0.07 \mu\text{m}$ is the same (within error) as that measured with Cy3B, suggesting the effect is an intrinsic property of the condensate. Subsequently, we present contact angle measurements of the Ddx4^{N*} 1-229, 1-231, 1-234, 1-236 and 1-236 F $\rightarrow$ Y condensates on a supported lipid bilayer, siliconized glass and borosilicate glass in Figures 6.8 and 6.10. As the data presented in Figures 6.5 and 6.6 is suggestive of a line tension effect, we have fit Equation 6.6 to all these data. The measured line tension lengths are summarised in Table 6.1.

In the cases where a strong line tension was not observed (Ddx4^{N*} 1-236 on siliconized

Ddx4 ^{N*} construct	line tension length (μm)		
	SLB	borosilicate glass	siliconized glass
1-229	-	1.04 ± 0.03	1.34 ± 0.07
1-231	-	1.19 ± 0.05	1.67 ± 0.06
1-234	-	1.23 ± 0.05	1.26 ± 0.05
1-236	0.7 ± 0.04	-	-
1-236 F $\rightarrow$ Y	0.45 ± 0.03	1.7 ± 0.06	1.39 ± 0.08

Table 6.1: Line tension lengths measured for Ddx4^{N*} condensates on supported lipid bilayers (SLB), borosilicate glass and siliconized glass surfaces. Uncertainties are reported as the 95% confidence interval in the fit to Equation 6.6. Entries marked with a dash are state points where Equation 6.6 was poorly fit, quantified by a $R^2 < 0.4$.

glass and borosilicate glass, Ddx4^{N*} 1-229, 1-231, 1-234 on the supported lipid bilayer) we may expect the contact angle data to be too noisy to fit, or the true line tension length to be too small ($< 0.1 \mu\text{m}$) to have an effect on our condensates. Further experiments are needed to confirm this. Where Equation 6.6 is well fit, the line tension is positive, with a line tension lengths clustered around $1 \mu\text{m}$. A positive line tension length of this magnitude, is expected to have a deleterious impact on intracellular condensate wetting, as this is the relevant lengthscale describing the size of many endogenous condensates [4, 265]. The data in Table 6.1 imply greatly inhibited nucleation of Ddx4^{N*} 1-229, 1-231, 1-234 and 1-236 F $\rightarrow$ Y condensates on borosilicate glass and siliconized glass surfaces, and Ddx4^{N*} 1-236 and 1-236 F $\rightarrow$ Y on the SLB [266]. These data could therefore be validated by setting up carefully controlled nucleation experiments, providing a basis for future work.

The fluid-fluid surface tension in our system is likely of order 10^{-5}J m^{-2} (see Section 5.4.1), giving a line tension of $\sim 10^{-11} \text{J m}^{-1}$. This is on the lower range of reported experimental values, similar to theoretical values calculated for fluids considering only van der Waals interactions [243, 245]. Larger experimental values of τ , of order 10^{-6}J m^{-1} , are attributed to gravitational contributions. Clearly, we may rule this out, as this would require condensate fluid-fluid interfacial tension of order 10^{-1}J m^{-2} , greater than the interfacial tension at the water-air interface.

6.4.2 Ddx4^{N*} condensates are repelled by lipid bilayers

The lack of intracellular condensate wetting on intracellular membranes, without facilitation by direct interactions with membrane proteins, suggests an intrinsic repulsion between condensates and membrane surfaces. To study this in a controlled environment, we introduce a POPC model membrane, prepared on borosilicate glass as a supported lipid bilayer (SLB) as detailed in Section 6.3.1. POPC is a common reagent used to prepare biomimetic SLBs, with its phosphatidyl head group found on over 50% of the eukaryotic membrane phospholipids [267]. In the sample set up used in this thesis, gravity serves to bring condensates into contact with the SLB, allowing a measurement of their contact angles. By comparing the wetting behaviour of the Ddx4^{N*} constructs introduced in Section 3.1.1 on the model membrane, we aim to infer how the interaction between condensates and membranes is affected by protein-protein electrostatic and cation- π interactions.

In Section 6.3.1, atomic force microscopy was used to examine the surface of the SLB coated glass, showing a distinct structure compared to uncoated glass (see Figure 6.4 (c)). To further confirm the presence of a SLB, Ddx4^{N*} 1-229 condensates were prepared on both coverslips treated with POPC and coverslips equivalently treated with isopropanol, plasma and SUV buffer, without any POPC to serve as a control. Figure 6.7 we plot the measured contact angles as a function of contact radius. We see contact angles in the control experiment are well separated from those measured on the supported lipid bilayer, indicating a successful coating of coverslips.

Confident in the generation of a supported lipid (POPC) bilayers, Ddx4^{N*} condensates were prepared on SLBs and imaged 1-4 h after preparation. Figure 6.8 (a) shows all Ddx4^{N*} constructs showed partial wetting behaviour, with contact angles almost unanimously greater than 90°. As we expect a line tension effect, Young's angles were measured by fitting Equation 6.6. In the case of a poor fit, as for constructs 1-229, 1-231 and 1-234, we defined Young's angle as the mean contact angle $\bar{\theta}$, weighted by r^2 . In Figure

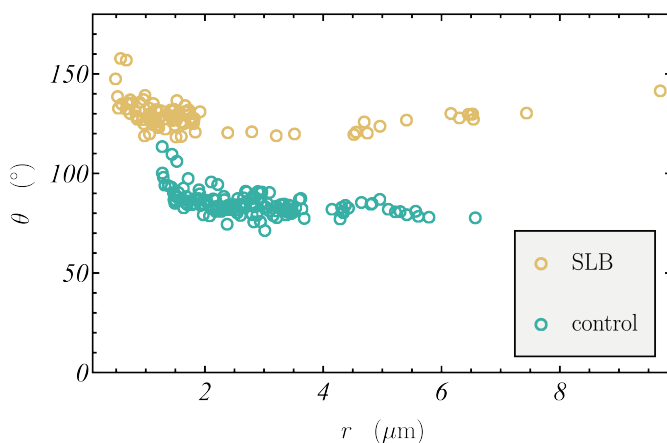


Figure 6.7: Contact angles and radii plotted for sessile Ddx4^{N*} 1-229 condensates resting on a supported lipid bilayer and a surface prepared equivalently, in the absence of POPC (see text).

6.8 (b) we plot the measured Young's angles against the absolute net charge per residue (NCPR) for each Ddx4^{N*} construct. All constructs show a Young's angle greater than 90°, therefore a higher interfacial tension between the condensates and the bilayer than between the bilayer and the protein-poor phases. This indicates the bilayer prefers to be wet by the protein-poor phase, due to a relative repulsive interaction between the bilayer and condensates. These data show no obvious dependency of θ_0 on NCPR. Equally, the contact angle is unaffected by the F→Y substitution. Taken together, these data show condensate-POPC bilayer interactions are unlikely to be electrostatic in nature, or involve the cation- π interaction.

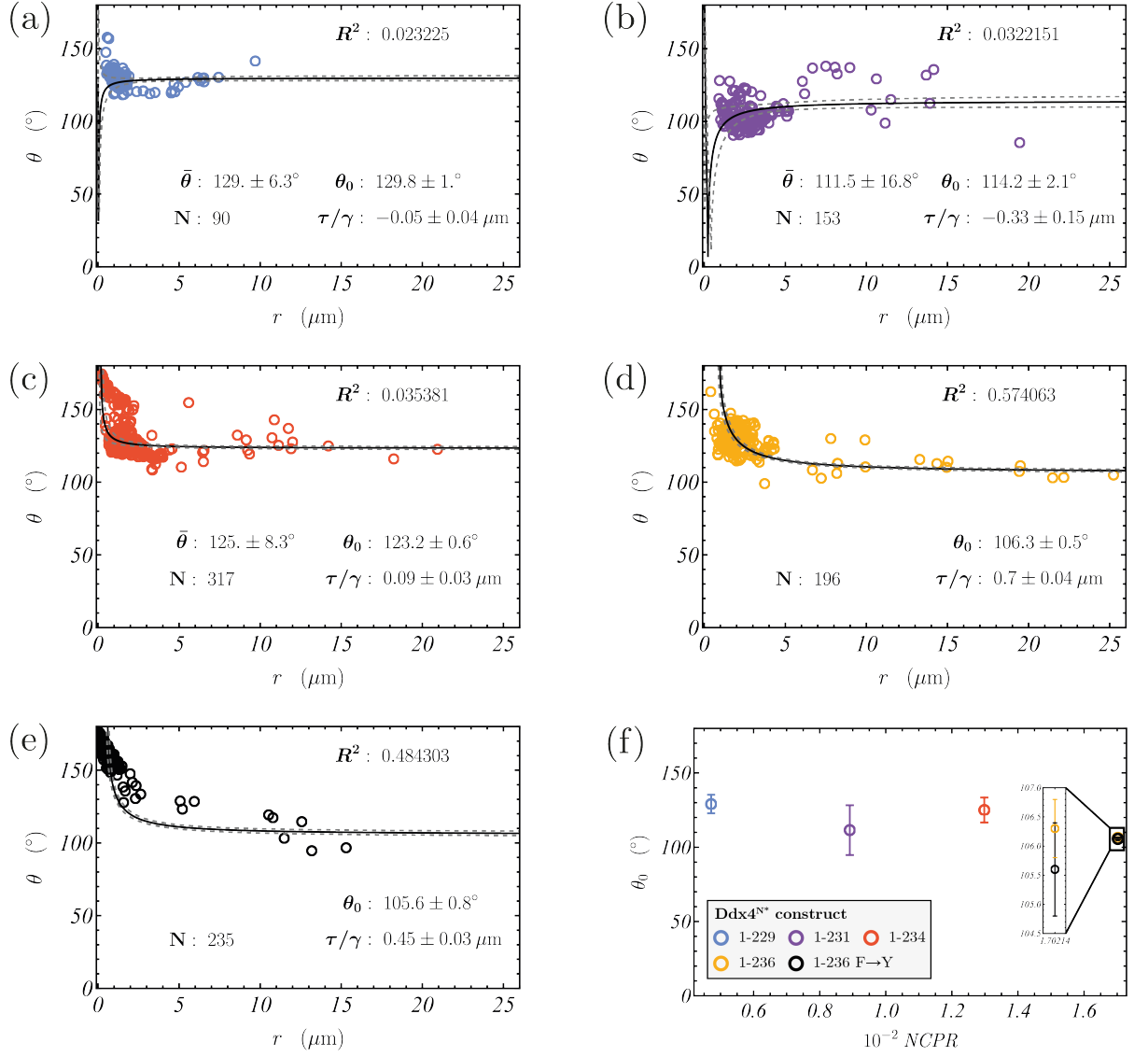


Figure 6.8: (a)-(e) Contact angles and radii plotted for sessile Ddx4^{N*} condensates resting on a POPC supported lipid bilayer. $\bar{\theta}$ is calculated as the mean contact angle, weighted by the contact area, with the uncertainty reported as the equivalently weighted standard deviation. All data were collected between 1 and 3 hours after phase separation. We did not see any time-dependent phenomena. (f) Young's angles (see text) for Ddx4^{N*} condensates on a POPC supported lipid bilayer plotted against the absolute net (negative) charge per residue of the Ddx4^{N*} chain.

6.4.3 Hydrophobic determinants of condensate wetting

In Section 6.4.2 we saw the relative repulsion between Ddx4^{N*} condensates and the POPC bilayer is not electrostatic in nature, or affected by cation- π interactions. In Section 3.4 we identified hydrophobic interactions between hydrophobic residues as a key driver of Ddx4^{N*} phase separation. We now characterise the effect of hydrophobic interactions on condensate wetting, by introducing two more surfaces: siliconized glass and borosilicate glass. To characterise these surfaces, we measure the in-air contact angle of the aqueous experimental buffer (150 mM NaCl 20 mM Tris pH 8.0), accounting for 99.9% (v/v) of the protein-poor phase coexisting with Ddx4^{N*} condensates. In Figure 6.9 we see increased hydrophobicity (decreased hydrophilicity) in the order: SLB, borosilicate glass, siliconized glass.

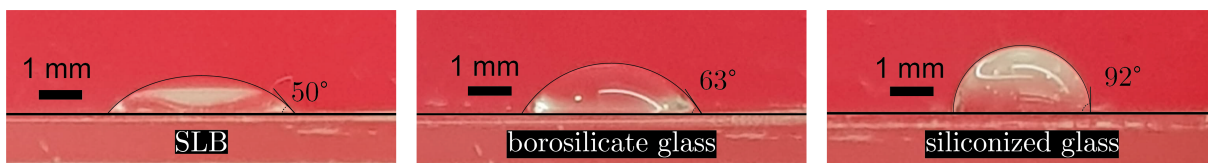


Figure 6.9: Images of 10 μ L sessile drops of 150 mM NaCl 20 mM Tris pH 8.0 buffer resting on a POPC bilayer (SLB), borosilicate glass and siliconized glass. Images were captured using a Samsung Galaxy S8. Contact angles were measured by fitting Equation 4.14 to hand drawn drop profiles.

Next we measured the contact angles of sessile Ddx4^{N*} condensates on siliconized and borosilicate glass surfaces. Figure 6.10 shows these contact angles as a function of contact radius for the five Ddx4^{N*} constructs, along with their fits to Equation 6.6. All constructs, except Ddx4^{N*} 1-236 (Figures 6.10 (d) and (i)), are well fit by Equation 6.6, with a positive line tension. As θ_0 for Ddx4^{N*} 1-236 is poorly fit, we define Young's angle as the mean contact angle, $\bar{\theta} \pm SD$, weighted as r^2 . Generally, we see decreased wetting on borosilicate glass, compared to siliconized glass, for all Ddx4^{N*} condensates.

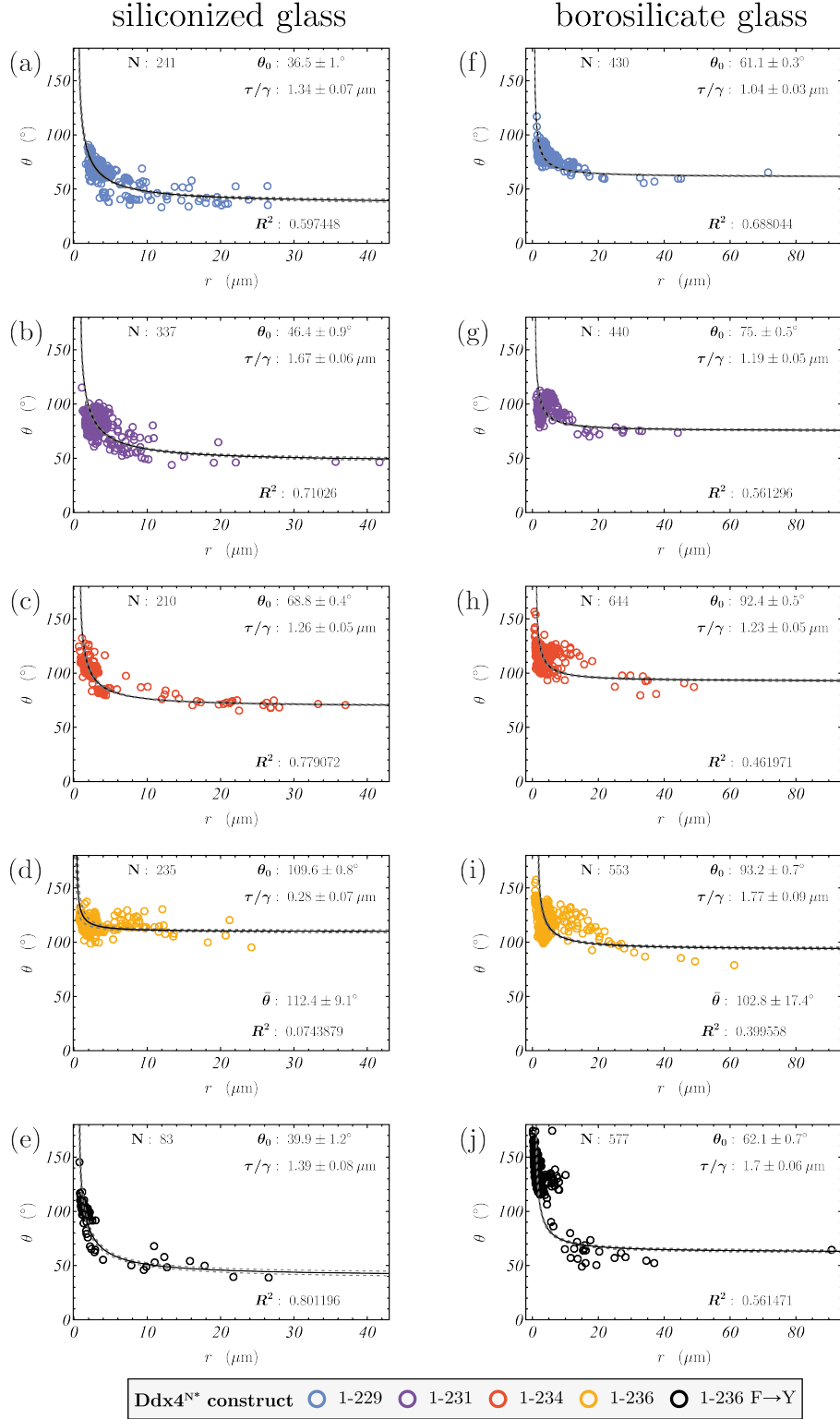


Figure 6.10: Contact angles and radii plotted for sessile Ddx4^{N*} condensates resting on siliconized glass (a)-(e) and borosilicate glass (f)-(j). Solid lines represent the best fit Equation 6.6, dashed lines show the corresponding 95% confidence bands with the goodness of fit indicated by R^2 , inset are the corresponding fitting parameters τ/r and θ_0 . $\bar{\theta}$ is calculated as the mean contact angle, weighted by the contact area, with the uncertainty reported as the equivalently weighted standard deviation. All data were collected between 1 and 3 hours after phase separation - we did not see any time dependency in contact angle values.

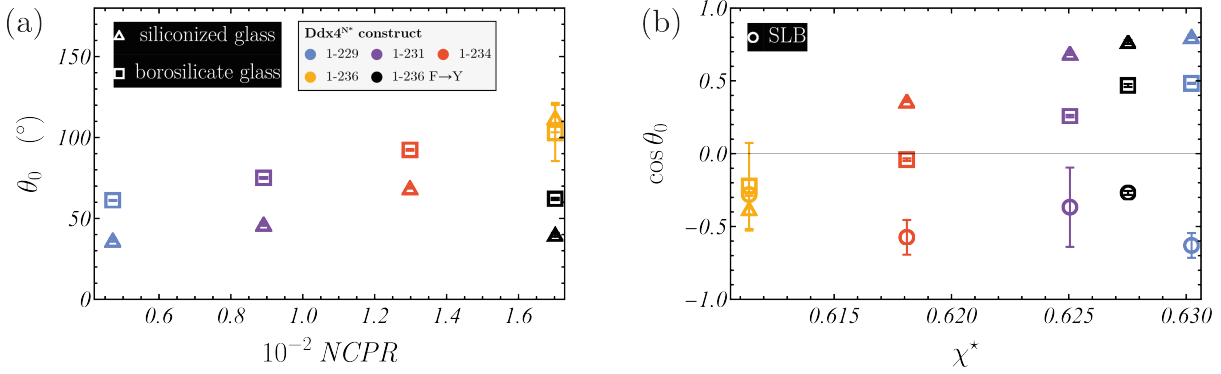


Figure 6.11: (a) Young's angles of Ddx4^{N*} condensates on siliconized glass and borosilicate glass surfaces plotted against the absolute net (negative) charge per residue of the Ddx4^{N*} chain. (b) The cosine of Young's angles of Ddx4^{N*} condensates on siliconized glass, borosilicate glass and POPC supported lipid bilayer surfaces plotted against their Flory-Huggins interaction parameter at 22 °C (measured in Section 3.4).

In Figure 6.11 (a) we plot the measured Young's angles against the NCPR of each Ddx4^{N*} construct. We see the contact angle on both surfaces is decreased by reducing NCPR, and also by substituting all phenylalanine residues on Ddx4^{N*} 1-236 for tyrosine residues. In Section 3.4 we saw these mutations increased the phase separation propensity of Ddx4^{N*}, characterised by an increased Flory-Huggins interaction parameter χ at ambient temperatures. In Figure 6.11 (b) we plot χ at 22 °C (χ^*) against $\cos \theta_0$, where increased $\cos \theta_0$ corresponds to increased wetting and decreased θ_0 . We see a clear relationship between χ^* and $\cos \theta_0$ on both siliconized glass, and borosilicate glass surfaces, with wetting increasing with χ^* , and consistently greater wetting on siliconized glass. We now recall the definition of Young's angle,

$$\cos \theta_0 = \frac{\gamma_{s\beta} - \gamma_{s\alpha}}{\gamma}, \quad (6.7)$$

where in our system α represents the condensate phase and β represents the protein poor phase. In Section 5.4.1 we saw a positive relationship between χ^* and γ is expected. Taking this to be true, we see the positive relationship between χ^* and $\cos \theta_0$ on siliconized glass and borosilicate glass is directly due to an increased value of $\gamma_{s\beta} - \gamma_{s\alpha}$. For $\cos \theta_0 > 0$ this corresponds to preferential adsorption of protein, over solvent, at the solid surface

(vice-versa for $\cos \theta_0 < 0$). We now consider the origin of preferential protein adsorption, and how this may explain the data in Figure 6.11 (b).

As χ^* increases, so does the relative protein-protein interaction strength, and the amount of Ddx4^{N*} in condensates. Because Ddx4^{N*} 1-236 F→Y falls on the trend remarkably well, between Ddx4^{N*} 1-231 and 1-229, we suggest $\gamma_{s\beta} - \gamma_{s\alpha}$ depends on an interaction mode unaltered between all Ddx4^{N*} constructs. As the wetting is consistently decreased on (hydrophilic) borosilicate glass, relative to (hydrophobic) siliconized glass, and the trend between Ddx4^{N*} constructs is the same, this interaction mode is identified as hydrophobic in nature, and expected to be strongest between Ddx4^{N*} and siliconized glass. This framework implies the direct, single-molecule interaction between the glass surfaces and protein is unaffected by the mutational series investigated. The density of hydrophobic contacts in Ddx4^{N*} condensates naturally increases with the amount of Ddx4^{N*} in condensates, and therefore with χ^* . Adsorption of protein at the solid surface is associated with a free energy penalty, as the phase is disturbed from its equilibrium composition (see Equation 6.4). As the amount of Ddx4^{N*} inside condensates increases, we propose greater adsorption of protein to the solid interface, as less deformation of the structure of the condensate in the vicinity of the surface is required to minimize $\gamma_{s\alpha}$, decreasing $\gamma_{s\alpha}$, and θ_0 .

We also recognize in Figure 6.11 (b) Ddx4^{N*} 1-236 shows $\cos \theta_0 < 0$ on both glass surfaces, as does Ddx4^{N*} 1-234 on borosilicate glass. While we suggest there is an attractive force between both surfaces and Ddx4^{N*}, driving adsorption, this suggests there is also a repulsive interaction, which manifests at lower values of χ^* . Ddx4^{N*} is an intrinsically disordered protein, successfully modelled as a flexible polymer in solution [62]. There exists an intrinsic repulsive entropic force between polymers and walls, the depletion force, where the (large) number of available polymer conformations is necessarily reduced [163]. The depletion interaction is expected to increase with protein concentration, and therefore with χ^* . As the data in Figure 6.11 (b) shows increased wetting with χ^* , we suggest reduced scaling of the effect of the depletion force with protein concentration on $\gamma_{s\alpha}$, com-

pared to the attractive (hydrophobic) forces between Ddx4^{N*} and the two glass surfaces. Therefore we propose the $\cos \theta_0 < 0$ regime on borosilicate glass and siliconized glass surfaces in Figure 6.11 (b) shows the regime where the repulsive depletion force outweighs the attractive hydrophobic forces.

With this model of Ddx4^{N*} adsorption driven by hydrophobic interactions, we now consider the repulsive interaction between Ddx4^{N*} condensates and the POPC supported lipid bilayer. Figure 6.8 (f) showed Young's angles greater than 90° for all Ddx4^{N*} condensates, and Figure 6.11 (b) shows the protein poor phase shows the protein poor phase preferentially wets the bilayer to the greatest extent for all Ddx4^{N*} condensates, except Ddx4^{N*} 1-236 where all values of θ_0 are within error. We have also seen how the aqueous buffer wets the POPC bilayer to the greatest extent, with an in-air contact angle of 50°.

Previously, Schwierz *et al* [268] have shown solid surfaces become resistant to adsorption by hydrophilic and hydrophobic peptides when their water-in-air contact angle decreases below 50-60°, due to preferential solvation of these hydrophilic surfaces by water. Furthermore, they showed that adsorption of proteins on hydrophobic surfaces is driven by entropy, explained by the hydrophobic effect. This cross-over value of the water-in-air contact angle is in close agreement with the established Berg limit [269], below which the water entropy at the solid interface is greater than in the bulk, due to greater numbers of polar and hydrogen bonding interactions available at the surface [270]. In this regime, the release of water associated with a hydrophobic interaction between a solute and the solid surface is entropically unfavourable. This is consistent with our observation that Ddx4^{N*} condensate wetting decreases on surfaces with decreased water-in-air contact angles, and our suggestion that this is driven by hydrophobic interactions. Furthermore, as the POPC bilayer is below the Berg limit, there is no hydrophobic attraction with Ddx4^{N*}, as the protein is unable to outcompete water for the surface of the bilayer. On a molecular level, the POPC bilayer headgroups are especially suited to solvation by water over proteins, as the small size of water allows its close approach with the positive and negative charge densities on the headgroups [271]. The same cannot likely be achieved by proteins, due

to the relative bulkiness of their chemical structure.

Extending this picture, we hypothesize that the necessarily hydrophilic nature of the surfaces of intracellular membranes places them below the Berg limit, implying an intrinsic repulsion between proteins and the surface of membranes, mediated by water. This is consistent with the observation that specific membrane-binding domains are required to traffick proteins to membrane surfaces [272]. Furthermore, this is in line with our observation that all reported cases of condensates wetting intracellular membranes is mediated through specific interactions between membrane-bound proteins.

6.5 Conclusions & outlook

The axisymmetric drop shape analysis protocol presented in Chapter 4 has enabled the precise measurement of contact angles for thousands of Ddx4^{N*} condensates, spanning a range of sizes. Through analysis of the size dependence of these contact angles, we saw a positive line tension effect. This effect showed a characteristic lengthscale of $\sim 1\mu\text{m}$, relevant for intracellular biomolecular condensates, expected to greatly inhibit their wetting of intracellular surfaces. Our measurements of line tension also emphasise the importance of measuring contact angles for a wide range of condensates. Future work in this area should focus on validation of the line tension effect through nucleation experiments [266].

Motivated by the absence of reports of non-specific adhesion of endogenous biomolecular condensates to intracellular membranes, we used axisymmetric drop shape analysis to precisely measure the contact angles of model Ddx4^{N*} condensates on a supported lipid bilayer. We saw a repulsive interaction for all variants of Ddx4^{N*}, characterised by contact angles above 90° , regardless of their size. By further measuring contact angles of Ddx4^{N*} mutants on hydrophobic and hydrophilic glass surfaces, we were able to ascertain the contribution of hydrophobic interactions to condensate wetting. Combining our observations with reported characterisations of proteins at hydrophilic surfaces, we suggested that the

repulsion between biomolecular condensates is an intrinsic property of their constituent proteins, and is modulated by their content of hydrophobic residues. Our data also showed cross over from partial condensate wetting to partial drying, on both glass surfaces, as the Ddx4^{N^*} Flory-Huggins interaction parameter decreased. This lead us to hypothesize the presence of a repulsive depletion interaction, mediated by the high conformational entropy of Ddx4^{N^*} chains.

The immediate next steps of this work concern application of Cahn-Nakanishi-Fisher wetting formalism [111,237] to our contact angle data, allowing a quantitative inference of the interactions between the protein and the surface. Further modulation of the Ddx4^{N^*} sequence would allow further testing of the interaction mode of Ddx4^{N^*} condensates with the surfaces presented. Increasing the its hydrophobic content would be predicted to have an increased contact angle on siliconized glass, in a manner that does not lie on the trends shown in Figure 6.11 (b), with increased wetting on siliconized glass and borosilicate glass, and decreased wetting on the POPC bilayer, at a given value of χ^* .

The proposed replusive depletion force between Ddx4^{N^*} and solid surfaces is expected to scale with the length of the protein chain, although at a rate much slower than the range (234-241) of lengths investigated [163]. Carefully varying the length of the Ddx4^{N^*} construct, by 100-200 amino acids, retaining sequence features essential for phase separation, should allow an inference of the contribution of this force to wetting phenomenon. The depletion force may also be measured in a more direct manner, through observing its effect on colliodal particles [273,274]. The current experimental system allows easy introduction of bilayers with alternative compositions, for example introducing charged lipids to investigate the role of charge in condensate bilayer interactions, or measuring contact angles on bilayers functionalised with membrane proteins. Expanding our experiments to alternative biomolecular condensate systems, will allow a test of the ubiquity of our observations.

Acknowledgments

We thank Dr Luke Clifton (Rutherford Appleton laboratory) for advising us on the preparation of supported lipid bilayers.

Bibliography

- [1] Isabella Ellinger and Adolf Ellinger. Smallest unit of life: Cell biology. In *Comparative Medicine: Anatomy and Physiology*, pages 19–33. Springer-Verlag Wien, dec 2014.
- [2] Edmund B Wilson. The structure of protoplasm. *Science*, 10(237):33—45, 1899.
- [3] Anthony A Hyman, Christoph A Weber, and Frank Jülicher. Liquid-Liquid Phase Separation in Biology. *Annu. Rev. Cell Dev. Biol.*, 30:39–58, 2014.
- [4] Salman F. Banani, Hyun O. Lee, Anthony A. Hyman, and Michael K. Rosen. Biomolecular condensates: Organizers of cellular biochemistry. *Nature Reviews Molecular Cell Biology*, 18(5):285–298, may 2017.
- [5] Catherine Rabouille and Simon Alberti. Cell adaptation upon stress: the emerging role of membrane-less compartments. *Current Opinion in Cell Biology*, 47:34–42, aug 2017.
- [6] Robert D. Phair and Tom Misteli. High mobility of proteins in the mammalian cell nucleus. *Nature 2000 404:6778*, 404(6778):604–609, apr 2000.
- [7] Miroslav Dundr, Michael D. Hebert, Tatiana S. Karpova, David Stanek, Hongzi Xu, Karl B. Shpargel, U. Thomas Meier, Karla M. Neugebauer, A. Gregory Matera, and Tom Misteli. In vivo kinetics of Cajal body components. *Journal of Cell Biology*, 164(6):831–842, mar 2004.
- [8] Stefanie Weidtkamp-Peters, Thorsten Lenser, Dmitri Negorev, Norman Gerstner, Thomas G. Hofmann, Georg Schwanitz, Christian Hoischen, Gerd Maul, Peter Dittich, and Peter Hemmerich. Dynamics of component exchange at PML nuclear bodies. *Journal of Cell Science*, 121(16):2731–2743, aug 2008.
- [9] Clifford P Brangwynne, Christian R Eckmann, David S Courson, Agata Rybarska, Carsten Hoege, Jöbin Gharakhani, Frank Jülicher, and Anthony A Hyman. Germline P granules are liquid droplets that localize by controlled dissolution/condensation. *Science (New York, N.Y.)*, 324(5935):1729–32, jun 2009.

- [10] Sonja Kroschwald, Shovamayee Maharana, Daniel Mateju, Liliana Malinovska, Elisabeth Nüske, Ina Poser, Doris Richter, and Simon Alberti. Promiscuous interactions and protein disaggregases determine the material state of stress-inducible RNP granules. *eLife*, 4(AUGUST2015), aug 2015.
- [11] Marina Feric, Nilesch Vaidya, Tyler S Harmon, Richard W Kriwacki, Rohit V Pappu, Clifford P Brangwynne Correspondence, Diana M Mitrea, Lian Zhu, Tiffany M Richardson, and Clifford P Brangwynne. Coexisting Liquid Phases Underlie Nucleolar Subcompartments Article Coexisting Liquid Phases Underlie Nucleolar Subcompartments. *Cell*, 165:1686–1697, 2016.
- [12] Saumya Jain, Joshua R. Wheeler, Robert W. Walters, Anurag Agrawal, Anthony Barsic, and Roy Parker. ATPase-Modulated Stress Granules Contain a Diverse Proteome and Substructure. *Cell*, 164(3):487–498, jan 2016.
- [13] Ekaterina Voronina, Geraldine Seydoux, Paolo Sassone-Corsi, and Ippei Nagamori. RNA Granules in Germ Cells. *Cold Spring Harbor Perspectives in Biology*, 3(12):a002774, dec 2011.
- [14] Carolyn J. Decker and Roy Parker. P-Bodies and Stress Granules: Possible Roles in the Control of Translation and mRNA Degradation. *Cold Spring Harbor Perspectives in Biology*, 4(9):a012286, sep 2012.
- [15] Yang Luo, Zhenkun Na, and Sarah A. Slavoff. P-Bodies: Composition, Properties, and Functions. *Biochemistry*, 57(17):2424–2431, may 2018.
- [16] Jennifer T. Wang and Geraldine Seydoux. P granules. *Current biology : CB*, 24(14):R637, jul 2014.
- [17] Jarrett Smith, Deepika Calidas, Helen Schmidt, Tu Lu, Dominique Rasoloson, and Geraldine Seydoux. Spatial patterning of P granules by RNA-induced phase separation of the intrinsically-disordered protein MEG-3. *eLife*, 5(DECEMBER2016), dec 2016.
- [18] Jieyan Pan, Mary Goodheart, Shinichiro Chuma, Norio Nakatsuji, David C. Page, and P. Jeremy Wang. RNF17, a component of the mammalian germ cell nuage, is essential for spermiogenesis. *Development*, 132(18):4029–4039, sep 2005.
- [19] Y. Fujiwara, T. Komiya, H. Kawabata, M. Sato, H. Fujimoto, M. Furusawa, and T. Noce. Isolation of a DEAD-family protein gene that encodes a murine homolog of *Drosophila vasa* and its specific expression in germ cell lineage. *PNAS*, 91(25):12258–12262, feb 2006.

- [20] Mikhail V. Kibanov, Vladimir A. Gvozdev, and Ludmila V. Olenina. Germ granules in spermatogenesis of *Drosophila*. *Communicative & Integrative Biology*, 5(2):130–133, mar 2012.
- [21] Sadaki Yokota. Nuage proteins: Their localization in subcellular structures of spermatogenic cells as revealed by immunoelectron microscopy. *Histochemistry and Cell Biology*, 138(1):1–11, jul 2012.
- [22] Vidya Balagopal and Roy Parker. Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs. *Current Opinion in Cell Biology*, 21(3):403–408, jun 2009.
- [23] Paul Anderson and Nancy Kedersha. Stress granules. *Current Biology*, 19(10):R397–R398, may 2009.
- [24] J. G. Gall. Cajal Bodies: The First 100 Years. *Annual Review of Cell and Developmental Biology*, 16:273–300, nov 2000.
- [25] Beáta E. Jány, Patricia Richard, Edouard Bertrand, and Tamás Kiss. Cell Cycle-dependent Recruitment of Telomerase RNA and Cajal Bodies to Human Telomeres. *Molecular Biology of the Cell*, 17(2):944, feb 2006.
- [26] Martin Machyna, Patricia Heyn, and Karla M. Neugebauer. Cajal bodies: where form meets function. *Wiley Interdisciplinary Reviews: RNA*, 4(1):17–34, jan 2013.
- [27] Menglong Zeng, Yuan Shang, Yoichi Araki, Tingfeng Guo, Richard L. Huganir, and Mingjie Zhang. Phase Transition in Postsynaptic Densities Underlies Formation of Synaptic Complexes and Synaptic Plasticity. *Cell*, 166(5):1163–1175.e12, aug 2016.
- [28] Austin A. Coley and Wen Jun Gao. PSD95: A synaptic protein implicated in schizophrenia or autism? *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 82:187–194, mar 2018.
- [29] Menglong Zeng, Xudong Chen, Dongshi Guan, Jia Xu, Haowei Wu, Penger Tong, and Mingjie Zhang. Reconstituted Postsynaptic Density as a Molecular Platform for Understanding Synapse Formation and Plasticity. *Cell*, 174(5):1172–1187.e16, aug 2018.
- [30] LARS EHRENBERG. INFLUENCE OF TEMPERATURE ON THE NUCLEOLUS AND ITS COACERVATE NATURE. *Hereditas*, 32(3-4):407–418, may 1946.
- [31] François Michel Boisvert, Silvana Van Koningsbruggen, Joaquín Navascués, and Angus I. Lamond. The multifunctional nucleolus. *Nature Reviews Molecular Cell Biology* 2007, 8(7):574–585, jul 2007.

- [32] Denis L.J. Lafontaine, Joshua A. Riback, Rümeyza Bascetin, and Clifford P. Brangwynne. The nucleolus as a multiphase liquid condensate, sep 2020.
- [33] D. L. Spector, X. D. Fu, and T. Maniatis. Associations between distinct pre-mRNA splicing components and the cell nucleus. *The EMBO Journal*, 10(11):3467–3481, nov 1991.
- [34] Tom Misteli, Javier F. Cáceres, and David L. Spector. The dynamics of a pre-mRNA splicing factor in living cells. *Nature* 1997 387:6632, 387(6632):523–527, may 1997.
- [35] Jiah Kim, Kyu Young Han, Nimish Khanna, Taekjip Ha, and Andrew S. Belmont. Nuclear speckle fusion via long-range directional motion regulates speckle morphology after transcriptional inhibition. *Journal of Cell Science*, 132(8), apr 2019.
- [36] Susan E. Liao and Oded Regev. Splicing at the phase-separated nuclear speckle interface: a model. *Nucleic Acids Research*, 49(2):636–645, jan 2021.
- [37] Anireddy S.N. Reddy, Irene S. Day, Janett Göhring, and Andrea Barta. Localization and dynamics of nuclear speckles in plants. *Plant physiology*, 158(1):67–77, 2012.
- [38] Elizabeth S. Freeman Rosenzweig, Bin Xu, Luis Kuhn Cuellar, Antonio Martinez-Sanchez, Mirosława Schaffer, Mike Strauss, Heather N. Cartwright, Pierre Ronceray, Jürgen M. Plitzko, Friedrich Förster, Ned S. Wingreen, Benjamin D. Engel, Luke C.M. Mackinder, and Martin C. Jonikas. The Eukaryotic CO₂-Concentrating Organelle Is Liquid-like and Exhibits Dynamic Reorganization. *Cell*, 2017.
- [39] Tobias Wunder, Steven Le Hung Cheng, Soak Kuan Lai, Hoi Yeung Li, and Oliver Mueller-Cajar. The phase separation underlying the pyrenoid-based microalgal Rubisco supercharger. *Nature Communications* 2018 9:1, 9(1):1–10, nov 2018.
- [40] James Barrett, Philipp Girr, and Luke C.M. Mackinder. Pyrenoids: CO₂-fixing phase separated liquid organelles. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1868(5):118949, apr 2021.
- [41] Elise K. van Buskirk, Peter V. Decker, and Meng Chen. Photobodies in Light Signaling. *Plant Physiology*, 158(1):52–60, jan 2012.
- [42] Eirini Kaiserli, Katalin Páldi, Liz O’Donnell, Olga Batalov, Ullas V. Pedmale, Dmitri A. Nusinow, Steve A. Kay, and Joanne Chory. Integration of Light and Photoperiodic Signaling in Transcriptional Nuclear Foci. *Developmental Cell*, 35(3):311–321, nov 2015.
- [43] Samantha K. Powers, Alex S. Holehouse, David A. Korasick, Katherine H. Schreiber, Natalie M. Clark, Hongwei Jing, Ryan Emenecker, Soeun Han, Eric Tycksen, Ildoo

- Hwang, Rosangela Sozzani, Joseph M. Jez, Rohit V. Pappu, and Lucia C. Strader. Nucleo-cytoplasmic Partitioning of ARF Proteins Controls Auxin Responses in *Arabidopsis thaliana*. *Molecular Cell*, 76(1):177–190.e5, oct 2019.
- [44] Yanniv Dorone, Steven Boeynaems, Eduardo Flores, Benjamin Jin, Shannon Hateley, Flavia Bossi, Elena Lazarus, Janice G. Pennington, Emiel Michiels, Mathias De Decker, Katlijn Vints, Pieter Baatsen, George W. Bassel, Marisa S. Otegui, Alex S. Holehouse, Moises Exposito-Alonso, Shahar Sukenik, Aaron D. Gitler, and Seung Y. Rhee. A prion-like protein regulator of seed germination undergoes hydration-dependent phase separation. *Cell*, 184(16):4284–4298.e27, aug 2021.
- [45] Bálint Mészáros, Gábor Erdos, Beáta Szabó, Éva Schád, Ágnes Tantos, Rawan Abukhairan, Tamás Horváth, Nikoletta Murvai, Orsolya P. Kovács, Márton Kovács, Silvio C.E. Tosatto, Péter Tompa, Zsuzsanna Dosztányi, and Rita Pancsa. PhaSe-Pro: the database of proteins driving liquid–liquid phase separation. *Nucleic Acids Research*, 48(D1):D360–D367, jan 2020.
- [46] Ryan J. Emenecker, Alex S. Holehouse, and Lucia C. Strader. Biological Phase Separation and Biomolecular Condensates in Plants. *Annual Review of Plant Biology*, 72:17–46, jun 2021.
- [47] Rudolph Wagner. Einige bemerkungen und fragen über das keimbläschen (vesicular germinativa). *Müller's Archiv Anat Physiol Wissenschaft Med*, 268:373—7, 1835.
- [48] Gabriel Valentin. *Repertorium für anatomie und physiologie*. Berlin, 1837.
- [49] Steven Boeynaems, Simon Alberti, Nicolas L. Fawzi, Tanja Mittag, Magdalini Polymeridou, Frederic Rousseau, Joost Schymkowitz, James Shorter, Benjamin Wolozin, Ludo Van Den Bosch, Peter Tompa, and Monika Fuxreiter. Protein Phase Separation: A New Phase in Cell Biology. *Trends in Cell Biology*, 28(6):420–435, jun 2018.
- [50] Christopher D. Reinkemeier, Gemma Estrada Girona, and Edward A. Lemke. Designer membraneless organelles enable codon reassignment of selected mRNAs in eukaryotes. *Science*, 363(6434), mar 2019.
- [51] Christopher D. Reinkemeier and Edward A. Lemke. Synthetic biomolecular condensates to engineer eukaryotic cells, oct 2021.
- [52] Darwin Charles. *The origin of species*. PF Collier & son New York, 1909.
- [53] Alan Fersht. *Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding*. Macmillan, 1999.

- [54] Prakash Kulkarni and Vladimir N. Uversky. Intrinsically Disordered Proteins: The Dark Horse of the Dark Proteome. *PROTEOMICS*, 18(21-22):1800061, nov 2018.
- [55] Robin Van Der Lee, Marija Buljan, Benjamin Lang, Robert J. Weatheritt, Gary W. Daughdrill, A. Keith Dunker, Monika Fuxreiter, Julian Gough, Joerg Gsponer, David T. Jones, Philip M. Kim, Richard W. Kriwacki, Christopher J. Oldfield, Rohit V. Pappu, Peter Tompa, Vladimir N. Uversky, Peter E. Wright, and M. Madan Babu. Classification of intrinsically disordered regions and proteins. *Chemical Reviews*, 114(13):6589–6631, jul 2014.
- [56] Masato Kato, Tina W. Han, Shanhai Xie, Kevin Shi, Xinlin Du, Leeju C. Wu, Hamid Mirzaei, Elizabeth J. Goldsmith, Jamie Longgood, Jimin Pei, Nick V. Grishin, Douglas E. Frantz, Jay W. Schneider, She Chen, Lin Li, Michael R. Sawaya, David Eisenberg, Robert Tycko, and Steven L. McKnight. Cell-free formation of RNA granules: Low complexity sequence domains form dynamic fibers within hydrogels. *Cell*, 149(4):753–767, may 2012.
- [57] Shana Elbaum-Garfinkle, Younghoon Kim, Krzysztof Szczepaniak, Carlos Chih-Hsiung Chen, Christian R Eckmann, Sua Myong, Clifford P Brangwynne, and Ronald D Vale. The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics. *PNAS*, 2015.
- [58] Amandine Molliex, Jamshid Temirov, Jihun Lee, Maura Coughlin, Anderson P. Kanagaraj, Hong Joo Kim, Tanja Mittag, and J. Paul Taylor. Phase Separation by Low Complexity Domains Promotes Stress Granule Assembly and Drives Pathological Fibrillization. *Cell*, 163(1):123–133, sep 2015.
- [59] Timothy J. Nott, Evangelia Petsalaki, Patrick Farber, Dylan Jervis, Eden Fussner, Anne Plochowitz, Timothy D. Craggs, David P. Bazett-Jones, Tony Pawson, Julie D. Forman-Kay, and Andrew J. Baldwin. Phase Transition of a Disordered Nuage Protein Generates Environmentally Responsive Membraneless Organelles. *Molecular Cell*, 2015.
- [60] Huaiying Zhang, Shana Elbaum-Garfinkle, Erin M. Langdon, Nicole Taylor, Patricia Occhipinti, Andrew A. Bridges, Clifford P. Brangwynne, and Amy S. Gladfelter. RNA Controls PolyQ Protein Phase Transitions. *Molecular Cell*, 60(2):220–230, oct 2015.
- [61] E Huckel and P Debye. Zur theorie der elektrolyte. i. gefrierpunktserniedrigung und verwandte erscheinungen. *Phys. Z.*, 24:185—206, 1923.
- [62] Jacob P Brady, Patrick J Farber, Ashok Sekhar, Yi-Hsuan Lin, Rui Huang, Alaji Bah, Timothy J Nott, Hue Sun Chan, Andrew J Baldwin, Julie D Forman-Kay, and

- Lewis E Kay. Structural and hydrodynamic properties of an intrinsically disordered region of a germ cell-specific protein on phase separation. *PNAS*, 2017.
- [63] Benjamin S. Schuster, Gregory L. Dignon, Wai Shing Tang, Fleurie M. Kelley, Aishwarya Kanchi Ranganath, Craig N. Jahnke, Alison G. Simpkins, Roshan Mammen Regy, Daniel A. Hammer, Matthew C. Good, and Jeetain Mittal. Identifying sequence perturbations to an intrinsically disordered protein that determine its phase-separation behavior. *Proceedings of the National Academy of Sciences of the United States of America*, 117(21):11421–11431, may 2020.
- [64] Chi W. Pak, Martyna Kosno, Alex S. Holehouse, Shae B. Padrick, Anuradha Mittal, Rustam Ali, Ali A. Yunus, David R. Liu, Rohit V. Pappu, and Michael K. Rosen. Sequence Determinants of Intracellular Phase Separation by Complex Coacervation of a Disordered Protein. *Molecular Cell*, 63(1):72–85, jul 2016.
- [65] Michael Dzuricky, Bradley A. Rogers, Abdulla Shahid, Paul S. Cremer, and Ashutosh Chilkoti. De novo engineering of intracellular condensates using artificial disordered proteins. *Nature Chemistry*, 12(9):814–825, sep 2020.
- [66] Yi Hsuan Lin and Hue Sun Chan. Phase Separation and Single-Chain Compactness of Charged Disordered Proteins Are Strongly Correlated. *Biophysical Journal*, 112(10):2043–2046, may 2017.
- [67] Jason J. Madinya, Li-Wei Chang, Sarah L. Perry, and Charles E. Sing. Sequence-dependent self-coacervation in high charge-density polyampholytes. *Molecular Systems Design & Engineering*, 5(3):632–644, mar 2020.
- [68] Clifford P. Brangwynne, Peter Tompa, and Rohit V. Pappu. Polymer physics of intracellular phase transitions. *Nature Physics*, 2015.
- [69] Yuan Lin, Simon L. Currie, and Michael K. Rosen. Intrinsically disordered sequences enable modulation of protein phase separation through distributed tyrosine motifs. *Journal of Biological Chemistry*, 292(46):19110–19120, nov 2017.
- [70] Jie Wang, Jeong-Mo Choi, Alex S. Holehouse, Hyun O. Lee, Xiaojie Zhang, Marcus Jahnke, Shovamayee Maharana, Régis Lemaitre, Andrei Pozniakovsky, David Drechsel, Ina Poser, Rohit V. Pappu, Simon Alberti, and Anthony A. Hyman. A Molecular Grammar Governing the Driving Forces for Phase Separation of Prion-like RNA Binding Proteins. *Cell*, 174(3):688–699.e16, jul 2018.
- [71] Erik W. Martin, Alex S. Holehouse, Ivan Peran, Mina Farag, J. Jeremias Incicco, Anne Bremer, Christy R. Grace, Andrea Soranno, Rohit V. Pappu, and Tanja Mittag. Valence and patterning of aromatic residues determine the phase behavior of prion-like domains. *Science*, 367(6478):694–699, feb 2020.

- [72] Seema Qamar, Guo Zhen Wang, Suzanne J. Randle, Francesco Simone Ruggeri, Juan A. Varela, Julie Qiaojin Lin, Emma C. Phillips, Akinori Miyashita, Declan Williams, Florian Ströhl, William Meadows, Rodylyn Ferry, Victoria J. Dardov, Gian G. Tartaglia, Lindsay A. Farrer, Gabriele S. Kaminski Schierle, Clemens F. Kaminski, Christine E. Holt, Paul E. Fraser, Gerold Schmitt-Ulms, David Klennerman, Tuomas Knowles, Michele Vendruscolo, and Peter St George-Hyslop. FUS Phase Separation Is Modulated by a Molecular Chaperone and Methylation of Arginine Cation- π Interactions. *Cell*, 173(3):720–734.e15, apr 2018.
- [73] Anne Bremer, Mina Farag, Wade M. Borchers, Ivan Peran, Erik W. Martin, Rohit V. Pappu, and Tanja Mittag. Deciphering how naturally occurring sequence features impact the phase behaviours of disordered prion-like domains. *Nature Chemistry*, 14(2):196–207, dec 2022.
- [74] Matteo Paloni, Rémy Bailly, Luca Ciandrini, and Alessandro Barducci. Unraveling Molecular Interactions in Liquid-Liquid Phase Separation of Disordered Proteins by Atomistic Simulations. *Journal of Physical Chemistry B*, 124(41):9009–9016, oct 2020.
- [75] Priyanka Dogra, Ashish Joshi, Anupa Majumdar, and Samrat Mukhopadhyay. Intermolecular Charge-Transfer Modulates Liquid-Liquid Phase Separation and Liquid-to-Solid Maturation of an Intrinsically Disordered pH-Responsive Domain. *Journal of the American Chemical Society*, 141(51):20380–20389, dec 2019.
- [76] Titus M Franzmann, Marcus Jahnel, Andrei Pozniakovsky, Julia Mahamid, Alex S Holehouse, Elisabeth Nüske, Doris Richter, Wolfgang Baumeister, Stephan W Grill, Rohit V Pappu, Anthony A Hyman, and Simon Alberti. Phase separation of a yeast prion protein promotes cellular fitness. *Science (New York, N.Y.)*, 359(6371):eaao5654, jan 2018.
- [77] Leo E. Wong, Tae Hun Kim, D. Ranjith Muhandiram, Julie D. Forman-Kay, and Lewis E. Kay. NMR Experiments for Studies of Dilute and Condensed Protein Phases: Application to the Phase-Separating Protein CAPRIN1. *Journal of the American Chemical Society*, 142(5):2471–2489, feb 2020.
- [78] Christine M. Clemson, John N. Hutchinson, Sergio A. Sara, Alexander W. Ensminger, Archa H. Fox, Andrew Chess, and Jeanne B. Lawrence. An Architectural Role for a Nuclear Noncoding RNA: NEAT1 RNA Is Essential for the Structure of Paraspeckles. *Molecular Cell*, 33(6):717–726, mar 2009.
- [79] Momoyo Hanazawa, Masafumi Yonetani, and Asako Sugimoto. PGL proteins self associate and bind RNPs to mediate germ granule assembly in *C. elegans*. *Journal of Cell Biology*, 192(6):929–937, mar 2011.

- [80] Alexander M. Ishov, Alexey G. Sotnikov, Dmitri Negorev, Olga V. Vladimirova, Norma Neff, Tetsu Kamitani, Edward T.H. Yeh, Jerome F. Strauss, and Gerd G. Maul. Pml Is Critical for Nd10 Formation and Recruits the Pml-Interacting Protein Daxx to This Nuclear Structure When Modified by Sumo-1. *Journal of Cell Biology*, 147(2):221–234, oct 1999.
- [81] Daniela Teixeira and Roy Parker. Analysis of P-body assembly in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell*, 18(6):2274–2287, jun 2007.
- [82] Bhalchandra S. Rao and Roy Parker. Numerous interactions act redundantly to assemble a tunable size of P bodies in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America*, 114(45):E9569–E9578, nov 2017.
- [83] Salman F. Banani, Allyson M. Rice, William B. Peeples, Yuan Lin, Saumya Jain, Roy Parker, and Michael K. Rosen. Compositional Control of Phase-Separated Cellular Bodies. *Cell*, 166(3):651–663, jul 2016.
- [84] Ammon E. Posey, Alex S. Holehouse, and Rohit V. Pappu. Phase Separation of Intrinsically Disordered Proteins. In *Methods in Enzymology*, volume 611, pages 1–30. Academic Press, jan 2018.
- [85] Jorge R. Espinosa, Jerelle A. Joseph, Ignacio Sanchez-Burgos, Adiran Garaizar, Daan Frenkel, and Rosana Collepardo-Guevara. Liquid network connectivity regulates the stability and composition of biomolecular condensates with many components. *Proceedings of the National Academy of Sciences of the United States of America*, 117(24):13238–13247, jun 2020.
- [86] Diana M. Mitrea and Richard W. Kriwacki. Phase separation in biology; Functional organization of a higher order Short linear motifs - The unexplored frontier of the eukaryotic proteome, jan 2016.
- [87] Richard J Wheeler and Anthony A Hyman. Controlling compartmentalization by non-membrane-bound organelles. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 373(1747), 2018.
- [88] Carla J. Beckham and Roy Parker. P Bodies, Stress Granules, and Viral Life Cycles, apr 2008.
- [89] Terunao Takahara and Tatsuya Maeda. Transient Sequestration of TORC1 into Stress Granules during Heat Stress. *Molecular Cell*, 47(2):242–252, jul 2012.
- [90] Frank Wippich, Bernd Bodenmiller, Maria Gustafsson Trajkovska, Stefanie Wanka, Ruedi Aebersold, and Lucas Pelkmans. Dual Specificity Kinase DYRK3 Couples

- Stress Granule Condensation/Dissolution to mTORC1 Signaling. *Cell*, 152(4):791–805, feb 2013.
- [91] Timothy J. Nott, Timothy D. Craggs, and Andrew J. Baldwin. Membraneless organelles can melt nucleic acid duplexes and act as biomolecular filters. *Nature Chemistry*, 8(6):569–575, 2016.
- [92] Clifford P. Brangwynne, Timothy J. Mitchison, and Anthony A. Hyman. Active liquid-like behavior of nucleoli determines their size and shape in *Xenopus laevis* oocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 108(11):4334–4339, mar 2011.
- [93] Ming-Tzo Wei, Shana Elbaum-Garfinkle, Alex S Holehouse, Carlos Chih-Hsiung Chen, Marina Feric, Craig B Arnold, Rodney D Priestley, Rohit V Pappu, and Clifford P Brangwynne. Phase behaviour of disordered proteins underlying low density and high permeability of liquid organelles. *Nature Chemistry*, 9, 2017.
- [94] Ibraheem Alshareedah, George M. Thurston, and Priya R. Banerjee. Quantifying Viscosity and Surface Tension of Multi-Component Protein-Nucleic Acid Condensates. *Biophysical Journal*, jan 2021.
- [95] Christina M. Caragine, Shannon C. Haley, and Alexandra Zidovska. Surface Fluctuations and Coalescence of Nucleolar Droplets in the Human Cell Nucleus. *Physical Review Letters*, 121(14):148101, oct 2018.
- [96] Sonja Kroschwald, Matthias C. Munder, Shovamayee Maharana, Titus M. Franzmann, Doris Richter, Martine Ruer, Anthony A. Hyman, and Simon Alberti. Different Material States of Pub1 Condensates Define Distinct Modes of Stress Adaptation and Recovery. *Cell Reports*, 23(11):3327–3339, jun 2018.
- [97] Yi Shen, Francesco Simone Ruggeri, Daniele Vigolo, Ayaka Kamada, Seema Qamar, Aviad Levin, Christiane Iserman, Simon Alberti, Peter St George-Hyslop, and Tuomas P.J. Knowles. Biomolecular condensates undergo a generic shear-mediated liquid-to-solid transition. *Nature Nanotechnology*, 15(10):841–847, jul 2020.
- [98] Louise M. Jawerth, Mahdiye Ijavi, Martine Ruer, Shambaditya Saha, Marcus Jahnel, Anthony A. Hyman, Frank Jülicher, and Elisabeth Fischer-Friedrich. Salt-Dependent Rheology and Surface Tension of Protein Condensates Using Optical Traps. *Physical Review Letters*, 121(25), dec 2018.
- [99] Louise Jawerth, Elisabeth Fischer-Friedrich, Suropriya Saha, Jie Wang, Titus Franzmann, Xiaojie Zhang, Jenny Sachweh, Martine Ruer, Mahdiye Ijavi, Shambaditya Saha, Julia Mahamid, Anthony A. Hyman, and Frank Jülicher. Protein condensates as aging Maxwell fluids. *Science*, 370(6522):1317–1323, dec 2020.

-
- [100] Avinash Patel, Hyun O. Lee, Louise Jawerth, Shovamayee Maharana, Marcus Janel, Marco Y. Hein, Stoyno Stoyanov, Julia Mahamid, Shambaditya Saha, Titus M. Franzmann, Andrej Pozniakovski, Ina Poser, Nicola Maghelli, Loic A. Royer, Martin Weigert, Eugene W. Myers, Stephan Grill, David Drechsel, Anthony A. Hyman, and Simon Alberti. A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation. *Cell*, 162(5):1066–1077, aug 2015.
- [101] Felipe Garcia Quiroz, Vincent F. Fiore, John Levorse, Lisa Polak, Ellen Wong, H. Amalia Pasolli, and Elaine Fuchs. Liquid-liquid phase separation drives skin barrier formation. *Science (New York, N.Y.)*, 367(6483), mar 2020.
- [102] John Shipley Rowlinson and Benjamin Widom. *Molecular theory of capillarity*. Courier Corporation, 2013.
- [103] Monika Zelazowska, Wincenty Kilarski, Szczepan M. Bilinski, Daniel D. Podder, and Malgorzata Kloc. Balbiani cytoplasm in oocytes of a primitive fish, the sturgeon *Acipenser gueldenstaedtii*, and its potential homology to the Balbiani body (mitochondrial cloud) of *Xenopus laevis* oocytes. *Cell and Tissue Research*, 329(1):137–145, jul 2007.
- [104] Alison B. Singer and Joseph G. Gall. An inducible nuclear body in the *Drosophila* germinal vesicle. *Nucleus*, 2(5):403, 2011.
- [105] Joel Berry, Stephanie C. Weber, Nilesch Vaidya, Mikko Haataja, Clifford P. Brangwynne, and David A. Weitz. RNA transcription modulates phase transition-driven nuclear body assembly. *Proceedings of the National Academy of Sciences of the United States of America*, 112(38):E5237–E5245, sep 2015.
- [106] Daniel S.W. Lee, Ned S. Wingreen, and Clifford P. Brangwynne. Chromatin mechanics dictates subdiffusion and coarsening dynamics of embedded condensates. *Nature Physics 2021 17:4*, 17(4):531–538, jan 2021.
- [107] Archishman Ghosh, Divya Kota, and Huan-Xiang Zhou. Shear relaxation governs fusion dynamics of biomolecular condensates. *Nature Communications 2021 12:1*, 12(1):1–10, oct 2021.
- [108] Andrew W. Folkmann, Andrea Putnam, Chiu Fan Lee, and Geraldine Seydoux. Regulation of biomolecular condensates by interfacial protein clusters. *Science*, 373(6560), sep 2021.
- [109] Hanieh Falahati, Bobbie Pelham-Webb, Shelby Blythe, and Eric Wieschaus. Nucleation by rRNA dictates the precision of nucleolus assembly. *Current Biology*, 26(3):277–285, feb 2016.

-
- [110] Shunsuke F. Shimobayashi, Pierre Ronceray, David W. Sanders, Mikko P. Haataja, and Clifford P. Brangwynne. Nucleation landscape of biomolecular condensates. *Nature*, 599(7885):503–506, sep 2021.
- [111] John W. Cahn. Critical point wetting. *The Journal of Chemical Physics*, 66(8):3667–3672, apr 1977.
- [112] Jose A. Morin, Sina Wittmann, Sandeep Choubey, Adam Klosin, Stefan Golfier, Anthony A. Hyman, Frank Jülicher, and Stephan W. Grill. Sequence-dependent surface condensation of a pioneer transcription factor on DNA. *Nature Physics*, 18(3):271–276, feb 2022.
- [113] Matthew R. King and Sabine Petry. Phase separation of TPX2 enhances and spatially coordinates microtubule nucleation. *Nature Communications* 2020 11:1, 11(1):1–13, jan 2020.
- [114] Sagar U. Setru, Bernardo Gouveia, Raymundo Alfaro-Aco, Joshua W. Shaevitz, Howard A. Stone, and Sabine Petry. A hydrodynamic instability drives protein droplet formation on microtubules to nucleate branches. *Nature Physics*, 17(4):493–498, jan 2021.
- [115] Yan G. Zhao and Hong Zhang. Phase Separation in Membrane Biology: The Interplay between Membrane-Bound Organelles and Membraneless Condensates, oct 2020.
- [116] Piong Li, Sudeep Banjade, Hui-Chun Chun Cheng, Soyeon Kim, Baoyu Chen, Liang Guo, Marc Llaguno, Javoris V. Hollingsworth, David S. King, Salman F. Banani, Paul S. Russo, Qiu-Xing Xing Jiang, B. Tracy Nixon, and Michael K. Rosen. Phase transitions in the assembly of multivalent signalling proteins. *Nature*, 483(7389):336–340, mar 2012.
- [117] Sudeep Banjade and Michael K Rosen. Phase transitions of multivalent proteins can promote clustering of membrane receptors. *Banjade and Rosen. eLife*, 3:4123, 2014.
- [118] Zelin Shan, Yuting Tu, Ying Yang, Ziheng Liu, Menglong Zeng, Huisha Xu, Jiafu Long, Mingjie Zhang, Yu Cai, and Wenyu Wen. Basal condensation of Numb and Pon complex via phase transition during *Drosophila* neuroblast asymmetric division. *Nature Communications* 2018 9:1, 9(1):1–16, feb 2018.
- [119] Weirui Ma and Christine Mayr. A Membraneless Organelle Associated with the Endoplasmic Reticulum Enables 3UTR-Mediated Protein-Protein Interactions. *Cell*, 175(6):1492–1506.e19, nov 2018.

- [120] Oliver Beutel, Riccardo Maraschini, Karina Pombo-García, Cécilie Martin-Lemaitre, and Alf Honigmann. Phase Separation of Zonula Occludens Proteins Drives Formation of Tight Junctions. *Cell*, 179(4):923–936.e11, oct 2019.
- [121] Marta Alenquer, Sílvia Vale-Costa, Temitope Akhigbe Etibor, Filipe Ferreira, Ana Laura Sousa, and Maria João Amorim. Influenza A virus ribonucleoproteins form liquid organelles at endoplasmic reticulum exit sites. *Nature Communications* 2019 10:1, 10(1):1–19, apr 2019.
- [122] Xiandeng Wu, Qixu Cai, Zeyu Shen, Xudong Chen, Menglong Zeng, Shengwang Du, and Mingjie Zhang. RIM and RIM-BP Form Presynaptic Active-Zone-like Condensates via Phase Separation. *Molecular Cell*, 73(5):971–984.e5, mar 2019.
- [123] Yuko Fujioka, Jahangir Md Alam, Daisuke Noshiro, Kazunari Mouri, Toshio Ando, Yasushi Okada, Alexander I. May, Roland L. Knorr, Kuninori Suzuki, Yoshinori Ohsumi, and Nobuo N. Noda. Phase separation organizes the site of autophagosome formation. *Nature*, 578(7794):301–305, feb 2020.
- [124] Jaime Agudo-Canalejo, Sebastian W. Schultz, Haruka Chino, Simona M. Migliano, Chieko Saito, Ikuko Koyama-Honda, Harald Stenmark, Andreas Brech, Alexander I. May, Noboru Mizushima, and Roland L. Knorr. Wetting regulates autophagy of phase-separated compartments and the cytosol. *Nature*, 591(7848):142–146, mar 2021.
- [125] Wilton T. Snead, Ameya P. Jalihal, Therese M. Gerbich, Ian Seim, Zhongxiu Hu, and Amy S. Gladfelter. Membrane surfaces regulate assembly of ribonucleoprotein condensates. *Nature Cell Biology* 2022, pages 1–10, apr 2022.
- [126] Kevin L. Shaw, Gerald R. Grimsley, Gennady I. Yakovlev, Alexander A. Makarov, and C. Nick Pace. The effect of net charge on the solubility, activity, and stability of ribonuclease Sa. *Protein Science*, 10(6):1206–1215, jun 2001.
- [127] Karolina L. Zapadka, Frederik J. Becher, A. L. Gomes dos Santos, and Sophie E. Jackson. Factors affecting the physical stability (aggregation) of peptide therapeutics, dec 2017.
- [128] Albert H Mao, Scott L Crick, Andreas Vitalis, Caitlin L Chicoine, and Rohit V Pappu. Net charge per residue modulates conformational ensembles of intrinsically disordered proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 107(18):8183–8188, may 2010.
- [129] Joseph R. Simon, Nick J. Carroll, Michael Rubinstein, Ashutosh Chilkoti, and Gabriel P. López. Programming molecular self-assembly of intrinsically disordered

- proteins containing sequences of low complexity. *Nature Chemistry*, 9(6):509–515, jun 2017.
- [130] Robert M. Vernon and Julie D. Forman-Kay. First-generation predictors of biological protein phase separation. *Current Opinion in Structural Biology*, 58:88–96, oct 2019.
- [131] Thomas Dannenhoffer-Lafage and Robert B. Best. A Data-Driven Hydrophobicity Scale for Predicting Liquid-Liquid Phase Separation of Proteins. *Journal of Physical Chemistry B*, 125(16):4046–4056, apr 2021.
- [132] Xiaoquan Chu, Tanlin Sun, Qian Li, Youjun Xu, Zhuqing Zhang, Luhua Lai, and Jianfeng Pei. Prediction of liquid-liquid phase separating proteins using machine learning. *BMC Bioinformatics*, 23(1):1–13, dec 2022.
- [133] Wenwei Zheng, Gregory L Dignon, Nina Jovic, Xichen Xu, Roshan M Regy, Nicolas L Fawzi, Young C Kim, Robert B Best, and Jeetain Mittal. Molecular Details of Protein Condensates Probed by Microsecond Long Atomistic Simulations. *J. Phys. Chem*, 2020.
- [134] Jeong Mo Choi, Furqan Dar, and Rohit V. Pappu. LASSI: A lattice model for simulating phase transitions of multivalent proteins. *PLoS Computational Biology*, 15(10):e1007028, 2019.
- [135] Huan Wang, Fleurie M. Kelley, Dragomir Milovanovic, Benjamin S. Schuster, and Zheng Shi. Surface tension and viscosity of protein condensates quantified by micropipette aspiration. *Biophysical Reports*, 1(1):100011, sep 2021.
- [136] Mahdiye Ijavi, Robert W. Style, Leonidas Emmanouilidis, Anil Kumar, Sandro M. Meier, Alexandre L. Torzynski, Frédéric H.T. Allain, Yves Barral, Michel O. Steinmetz, and Eric R. Dufresne. Surface tensiometry of phase separated protein and polymer droplets by the sessile drop method. *Soft Matter*, 17(6):1655–1662, feb 2021.
- [137] Ronald Lovett, Patrick W. Dehaven, James J. Viecelli, and Frank P. Buff. Generalized van der Waals theories for surface tension and interfacial width. *The Journal of Chemical Physics*, 58(5):1880, aug 1973.
- [138] D. G.A.L. Aarts, R. P.A. Dullens, H. N.W. Lekkerkerker, D. Bonn, and R. Van Roij. Interfacial tension and wetting in colloid-polymer mixtures. *Journal of Chemical Physics*, 120(4):1973–1980, jan 2004.
- [139] Souheng Wu. Calculation of interfacial tension in polymer systems. *Journal of Polymer Science Part C: Polymer Symposia*, 34(1):19–30, mar 2007.

- [140] R. Tuinier and G. J.P. Krooshof. Interfacial tension between benzene and water in the presence of caprolactam. *Journal of Colloid and Interface Science*, 382(1):105–109, sep 2012.
- [141] Robert A. Riggleman, Rajeev Kumar, and Glenn H. Fredrickson. Investigation of the interfacial tension of complex coacervates using field-theoretic simulations. *Journal of Chemical Physics*, 136(2):024903, jan 2012.
- [142] D. N. Staicopolus. The computation of surface tension and of contact angle by the sessile-drop method. *Journal of Colloid Science*, 17(5):439–447, jun 1962.
- [143] J. Bachmann, R. Horton, R. R. van der Ploeg, and S. Woche. Modified sessile drop method for assessing initial soil-water contact angle of sandy soil. *Soil Science Society of America Journal*, 64(2):564–567, mar 2000.
- [144] Y. Rotenberg, L. Boruvka, and A. W. Neumann. Determination of surface tension and contact angle from the shapes of axisymmetric fluid interfaces. *Journal of Colloid And Interface Science*, 93(1):169–183, may 1983.
- [145] J. Gaydos and A. W. Neumann. The dependence of contact angles on drop size and line tension. *Journal of Colloid And Interface Science*, 120(1):76–86, nov 1987.
- [146] D. Li and A. W. Neumann. Determination of line tension from the drop size dependence of contact angles. *Colloids and Surfaces*, 43(2):195–206, jan 1990.
- [147] O. I. Del Río and A. W. Neumann. Axisymmetric drop shape analysis: Computational methods for the measurement of interfacial properties from the shape and dimensions of pendant and sessile drops. *Journal of Colloid and Interface Science*, 196(2):136–147, dec 1997.
- [148] D. Y. Kwok and A. W. Neumann. Contact angle measurement and contact angle interpretation. *Advances in Colloid and Interface Science*, 81(3):167–249, sep 1999.
- [149] Laura R. Ganser and Sua Myong. Methods to Study Phase-Separated Condensates and the Underlying Molecular Interactions, nov 2020.
- [150] JMHM Scheutjens and GJ Fler. Statistical theory of the adsorption of interacting chain molecules. 1. Partition function, segment density distribution, and adsorption isotherms. *Journal of physical chemistry*, 83(12):1619–1635, 1979.
- [151] Gerard Fler, MA Cohen Stuart, Jan MHM Scheutjens, T Cosgrove, and B Vincent. *Polymers at interfaces*. Springer Science & Business Media, 1003.
- [152] D F Swinehart, Eugene The, and Beer-Lambert Law. The Beer-Lambert Law. *Journal of Chemical Education*, 39(7):333–335, 1962.

- [153] Douglas Hanahan. Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology*, 166(4):557–580, jun 1983.
- [154] F. William Studier and Barbara A. Moffatt. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology*, 189(1):113–130, may 1986.
- [155] Je Nie Phue, Santosh B. Noronha, Ritabrata Hattacharyya, Alan J. Wolfe, and Joseph Shiloach. Glucose metabolism at high density growth of *E. coli* B and *E. coli* K: Differences in metabolic pathways are responsible for efficient glucose utilization in *E. coli* B as determined by microarrays and Northern blot analyses. *Biotechnology and Bioengineering*, 90(7):805–820, jun 2005.
- [156] A. M. Maxam and W. Gilbert. A new method for sequencing DNA. *Proceedings of the National Academy of Sciences of the United States of America*, 74(2):560–564, 1977.
- [157] Allan M. Maxam and Walter Gilbert. [57] Sequencing End-Labeled DNA with Base-Specific Chemical Cleavages. *Methods in Enzymology*, 65(C):499–560, jan 1980.
- [158] Stanley Tabor. Expression Using the T7 RNA Polymerase/Promoter System. *Current Protocols in Molecular Biology*, 11(1):16.2.1–16.2.11, jul 1990.
- [159] F. William Studier. Protein production by auto-induction in high-density shaking cultures. *Protein Expression and Purification*, 41(1):207–234, may 2005.
- [160] Jay A Glasel and Murray P Deutscher. *Introduction to biophysical methods for protein and nucleic acid research*. Academic Press, 1995.
- [161] Kathleen A. Burke, Abigail M. Janke, Christy L. Rhine, and Nicolas L. Fawzi. Residue-by-Residue View of In Vitro FUS Granules that Bind the C-Terminal Domain of RNA Polymerase II. *Molecular Cell*, 60(2):231–241, oct 2015.
- [162] Michael D. Crabtree, Jack Holland, Purnima Kompella, Leon Babl, Noah Turner, Andrew J. Baldwin, and Timothy J. Nott. Repulsive electrostatic interactions modulate dense and dilute phase properties of biomolecular condensates. *bioRxiv*, page 2020.10.29.357863, feb 2021.
- [163] Pierre-Gilles De Gennes. *Scaling concepts in polymer physics*. Cornell university press, 1979.
- [164] Håkan Wennerström, Eloy Vallina Estrada, Jens Danielsson, and Mikael Oliveberg. Colloidal stability of the living cell, may 2020.

- [165] Kevin L. Shaw, Gerald R. Grimsley, Gennady I. Yakovlev, Alexander A. Makarov, and C. Nick Pace. The effect of net charge on the solubility, activity, and stability of ribonuclease Sa. *Protein science : a publication of the Protein Society*, 10(6):1206–1215, jun 2001.
- [166] Rahul K. Das and Rohit V. Pappu. Conformations of intrinsically disordered proteins are influenced by linear sequence distributions of oppositely charged residues. *Proceedings of the National Academy of Sciences of the United States of America*, 110(33):13392–13397, aug 2013.
- [167] BY J Lawrence Henderson. CONCERNING THE RELATIONSHIP BETWEEN THE STRENGTH OF ACIDS AND THEIR CAPACITY TO PRESERVE NEUTRALITY. *American Journal of Physiology*, 1908.
- [168] Henry N. Po and N. M. Senozan. The Henderson-Hasselbalch Equation: Its History and Limitations. *Journal of Chemical Education*, 78(11):1499–1503, 2001.
- [169] Gerald R. Grimsley, J. Martin Scholtz, and C. Nick Pace. A summary of the measured pK values of the ionizable groups in folded proteins. *Protein Science*, 18(1):247–251, jan 2009.
- [170] Alex S. Holehouse, Rahul K. Das, James N. Ahad, Mary O.G. Richardson, and Rohit V. Pappu. CIDER: Resources to Analyze Sequence-Ensemble Relationships of Intrinsically Disordered Proteins. *Biophysical Journal*, 112(1):16–21, jan 2017.
- [171] Paul J. Flory. Thermodynamics of High Polymer Solutions. *The Journal of Chemical Physics*, 10(1):51, dec 2004.
- [172] Maurice L. Huggins. THERMODYNAMIC PROPERTIES OF SOLUTIONS OF LONG-CHAIN COMPOUNDS. *Annals of the New York Academy of Sciences*, 43(1):1–32, mar 1942.
- [173] Ken A. Dill, Sarina Bromberg, and Dirk Stigter. *Molecular Driving Forces*. Garland Science, oct 2010.
- [174] John W Cahn. Phase separation by spinodal decomposition in isotropic systems. *The Journal of Chemical Physics*, 42(1):93–99, 1965.
- [175] M. Adams, Z. Dogic, S. L. Keller, and S. Fraden. Entropically driven microphase transitions in mixtures of colloidal rods and spheres. *Nature*, 393(6683):349–352, may 1998.
- [176] Michael Rubinstein and Ralph H Colby. *Polymer Physics*. Oxford university press New York, 2003.

- [177] Anatol W. Fritsch, Andres F. Diaz-Delgadillo, Omar Adame-Arana, Carsten Hoege, Matthaus Mittasch, Moritz Kreysing, Mark Leaver, Anthony A. Hyman, Frank Julicher, and Christoph A. Weber. Local thermodynamics govern formation and dissolution of *Caenorhabditis elegans* P granule condensates. *Proceedings of the National Academy of Sciences of the United States of America*, 118(37), sep 2021.
- [178] Yi-Hsuan Lin, Jianhui Song, Julie D. Forman-Kay, and Hue Sun Chan. Random-phase-approximation theory for sequence-dependent, biologically functional liquid-liquid phase separation of intrinsically disordered proteins. *Journal of Molecular Liquids*, 228:176–193, feb 2017.
- [179] H S Chan and K A Dill. Polymer Principles in Protein Structure and Stability. *Annual Review of Biophysics and Biophysical Chemistry*, 20(1):447–490, jun 1991.
- [180] Julio Atkins, Peter W and De Paula. *Atkins' Physical Chemistry*. Oxford university press, 2006.
- [181] R. D. Allen, G. B. David, and G. Nomarski. The zeiss-Nomarski differential interference equipment for transmitted-light microscopy. *Zeitschrift fur Wissenschaftliche Mikroskopie und Mikroskopische Technik*, 69(4):193–221, nov 1969.
- [182] Zheng Wang, Gangming Zhang, and Hong Zhang. Protocol for analyzing protein liquid–liquid phase separation. *Biophysics Reports 2018 5:1*, 5(1):1–9, dec 2018.
- [183] Wolfram Research Inc. Mathematica, 2021.
- [184] Hua Zhao. Viscosity B-coefficients and standard partial molar volumes of amino acids, and their roles in interpreting the protein (enzyme) stabilization. *Biophysical Chemistry*, 122(3):157–183, aug 2006.
- [185] Caroline A. Schneider, Wayne S. Rasband, and Kevin W. Eliceiri. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods 2012 9:7*, 9(7):671–675, jun 2012.
- [186] Sara S. Ribeiro, Nirnay Samanta, Simon Ebbinghaus, and João C. Marcos. The synergic effect of water and biomolecules in intracellular phase separation. *Nature Reviews Chemistry*, 3(9):552–561, jul 2019.
- [187] Georg Krainer, Timothy J. Welsh, Jerelle A. Joseph, Jorge R. Espinosa, Sina Wittmann, Ella de Csilléry, Akshay Sridhar, Zenon Toprakcioglu, Giedre Gudīškytė, Magdalena A. Czekalska, William E. Arter, Jordina Guillén-Boixet, Titus M. Franzmann, Seema Qamar, Peter St George-Hyslop, Anthony A. Hyman, Rosana Collepardo-Guevara, Simon Alberti, and Tuomas P.J. Knowles. Reentrant liquid

- condensate phase of proteins is stabilized by hydrophobic and non-ionic interactions. *Nature Communications*, 12(1):1–14, feb 2021.
- [188] Noel T. Southall, Ken A. Dill, and A. D.J. Haymet. A View of the Hydrophobic Effect. *Journal of Physical Chemistry B*, 106(3):521–533, jan 2001.
- [189] Rungsima Chollakup, John B. Beck, Klaus Dirnberger, Matthew Tirrell, and Claus D. Eisenbach. Polyelectrolyte Molecular Weight and Salt Effects on the Phase Behavior and Coacervation of Aqueous Solutions of Poly(acrylic acid) Sodium Salt and Poly(allylamine) Hydrochloride. *Macromolecules*, 46(6):2376–2390, mar 2013.
- [190] Charles E. Sing. Development of the modern theory of polymeric complex coacervation, jan 2017.
- [191] Jacob N Israelachvili. *Intermolecular and surface forces*. Academic Press, 2011.
- [192] Wenwei Zheng, Gregory L. Dignon, Nina Jovic, Xichen Xu, Roshan M. Regy, Nicolas L. Fawzi, Young C. Kim, Robert B. Best, and Jeetain Mittal. Molecular details of protein condensates probed by microsecond long atomistic simulations. *Journal of Physical Chemistry B*, 124(51):11671–11679, dec 2020.
- [193] Manzar Abbas, Wojciech P. Lipiński, Karina K. Nakashima, Wilhelm T.S. Huck, and Evan Spruijt. A short peptide synthon for liquid–liquid phase separation. *Nature Chemistry*, 13(11):1046–1054, oct 2021.
- [194] Jonas Wessén, Tanmoy Pal, Suman Das, Yi Hsuan Lin, and Hue Sun Chan. A simple explicit-solvent model of polyampholyte phase behaviors and its ramifications for dielectric effects in biomolecular condensates. *Journal of Physical Chemistry B*, 125(17):4337–4358, may 2021.
- [195] Joshua A. Riback, Lian Zhu, Mylene C. Ferrolino, Michele Tolbert, Diana M. Mitrea, David W. Sanders, Ming Tzo Wei, Richard W. Kriwacki, and Clifford P. Brangwynne. Composition-dependent thermodynamics of intracellular phase separation. *Nature*, 581(7807):209–214, may 2020.
- [196] Lev Davidovich Landau and Evgenii Mikhailovich Lifshitz. *Fluid Mechanics: Landau and Lifshitz: Course of Theoretical Physics, Volume 6*. Elsevier, 2013.
- [197] By Thomas Young and M D For Sec. III. An essay on the cohesion of fluids. *Philosophical Transactions of the Royal Society of London*, 95:65–87, dec 1805.
- [198] Pierre Simon marquis de Laplace. Supplément au dixième livre du Traité de Mécanique Céleste. In *Traité de mécanique céleste, Volume 4*, pages 1–79. Courcier, 1808.

- [199] M. Minsky. Memoir on inventing the confocal scanning microscope. *Scanning*, 10(4):128–138, jan 1988.
- [200] David J. Griffiths. *Introduction to Electrodynamics*. Prentice Hall, 1999.
- [201] S. HELL, G. REINER, C. CREMER, and E. H.K. STELZER. Aberrations in confocal fluorescence microscopy induced by mismatches in refractive index. *Journal of Microscopy*, 169(3):391–405, mar 1993.
- [202] Patrick M. McCall, Kyoo Hyun Kim, Anatol W. Fritsch, J.M. Iglesias-Artola, L.M. Jawerth, Jie Wang, M. Ruer, J. Peychl, Andrey Poznyakovskiy, Jochen Guck, Simon Alberti, Anthony A. Hyman, and Jan Brugués. Quantitative phase microscopy enables precise and efficient determination of biomolecular condensate composition. *bioRxiv*, oct 2020.
- [203] James B. Pawley. *Handbook of biological confocal microscopy: Third edition*. Springer US, 2006.
- [204] Nobuyuki Otsu. THRESHOLD SELECTION METHOD FROM GRAY-LEVEL HISTOGRAMS. *IEEE Trans Syst Man Cybern*, SMC-9(1):62–66, 1979.
- [205] J. A. Nelder and R. Mead. A Simplex Method for Function Minimization. *The Computer Journal*, 7(4):308–313, jan 1965.
- [206] Johannes Schindelin, Ignacio Arganda-Carreras, Erwin Frise, Verena Kaynig, Mark Longair, Tobias Pietzsch, Stephan Preibisch, Curtis Rueden, Stephan Saalfeld, Benjamin Schmid, Jean Yves Tinevez, Daniel James White, Volker Hartenstein, Kevin Eliceiri, Pavel Tomancak, and Albert Cardona. Fiji: an open-source platform for biological-image analysis. *Nature Methods*, 9(7):676–682, jun 2012.
- [207] J Willard Gibbs. *The collected works of J. Willard Gibbs*. Yale Univ. Press, 1948.
- [208] Arthur W Adamson and Alice Petry Gast. *Physical chemistry of surfaces*. Interscience publishers New York, 1967.
- [209] Els H.A. De Hoog and Henk N.W. Lekkerkerker. Breakup of an Elongated Droplet in a Centrifugal Field†. *Journal of Physical Chemistry B*, 105(47):11636–11640, nov 2001.
- [210] Els H.A. De Hoog and Henk N.W. Lekkerkerker. Measurement of the Interfacial Tension of a Phase-Separated ColloidPolymer Suspension. *Journal of Physical Chemistry B*, 103(25):5274–5279, jun 1999.
- [211] B-H Chen, B Payandeh, and M Robert. Order parameter and interfacial tension of a colloid-polymer system. *Physical Review E*, 62:2369, 2000.

- [212] D G A L Aarts, J H Van Der Wiel, and H N W Lekkerkerker. Interfacial dynamics and the static profile near a single wall in a model colloid–polymer mixture. *Journal of Physics: Condensed Matter*, 15(1):S245, dec 2002.
- [213] Diana M. Mitrea, Jaclyn A. Cika, Christopher B. Stanley, Amanda Nourse, Paulo L. Onuchic, Priya R. Banerjee, Aaron H. Phillips, Cheon Gil Park, Ashok A. Deniz, and Richard W. Kriwacki. Self-interaction of NPM1 modulates multiple mechanisms of liquid–liquid phase separation. *Nature Communications* 2018 9:1, 9(1):1–13, feb 2018.
- [214] Nicole Taylor, Shana Elbaum-Garfinkle, Nilesh Vaidya, Huaiying Zhang, Howard A. Stone, and Clifford P. Brangwynne. Biophysical characterization of organelle-based RNA/protein liquid phases using microfluidics. *Soft Matter*, 12(45):9142–9150, nov 2016.
- [215] Han Yi Chou and Aleksei Aksimentiev. Single-Protein Collapse Determines Phase Equilibria of a Biological Condensate. *Journal of Physical Chemistry Letters*, 11(12):4923–4929, jun 2020.
- [216] Evan Spruijt, Joris Sprakel, Martien A. Cohen Stuart, and Jasper Van Der Gucht. Interfacial tension between a complex coacervate phase and its coexisting aqueous phase. *Soft Matter*, 6(1):172–178, dec 2009.
- [217] J. T. OVERBEEK and M. J. VOORN. Phase separation in polyelectrolyte solutions. Theory of complex coacervation. *Journal of Cellular and Comparative Physiology*, 49(S1):7–26, may 1957.
- [218] D. G. A. L. Aarts, Matthias Schmidt, and Henk N W Lekkerkerker. Direct Visual Observation of Thermal Capillary Waves. *Science*, 304(5672):847–850, may 2004.
- [219] D. G. A. L. Aarts. Capillary Length in a FluidFluid Demixed ColloidPolymer Mixture. *The Journal of Physical Chemistry B*, 109(15):7407–7411, apr 2005.
- [220] J. D. van der Waals. The thermodynamic theory of capillarity under the hypothesis of a continuous variation of density. *Journal of Statistical Physics*, 20(2):200–244, feb 1979.
- [221] Jean-Louis Barrat and Jean-Pierre Hansen. *Basic concepts for simple and complex liquids*. Cambridge University Press, 2003.
- [222] Jinlong Yang, Kyle Yu, and Yi Y. Zuo. Accuracy of Axisymmetric Drop Shape Analysis in Determining Surface and Interfacial Tensions. *Langmuir*, 33(36):8914–8923, sep 2017.

- [223] Guangle Li, Gabriel Robles Del Hierro, Jimmy Z. Di, and Yi Y. Zuo. Compound Drop Shape Analysis with the Neumann Number. *Langmuir*, 36(26):7619–7626, jul 2020.
- [224] WN Bond and Dorothy A Newton. LXXXII. Bubbles, drops, and Stokes’ law. (Paper 2). *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science*, 5(30):794–800, apr 1928.
- [225] Pierre-Gilles de Gennes, Françoise Brochard-Wyart, and David Quéré. *Capillarity and Wetting Phenomena*. Springer New York, 2004.
- [226] F. K. Hansen. Surface tension by image analysis: Fast and automatic measurements of pendant and sessile drops and bubbles. *Journal of Colloid And Interface Science*, 160(1):209–217, oct 1993.
- [227] Takeo Matsubara and Hirotugu Matsuda. A Lattice Model of Liquid Helium, I. *Progress of Theoretical Physics*, 16(6):569–582, dec 1956.
- [228] Richard C. Tolman. The Effect of Droplet Size on Surface Tension. *The Journal of Chemical Physics*, 17(3):333, dec 1949.
- [229] A. B.D. Cassie and S. Baxter. Large Contact Angles of Plant and Animal Surfaces. *Nature*, 155(3923):21–22, 1945.
- [230] A Szent-Gyorgyi and V B Orth. Quantitative studies on the wetting of leaves by water. *Proceedings of the Royal Society of London. Series B - Biological Sciences*, 134(877):503–522, sep 1947.
- [231] Anthony A. Hyman, Christoph A. Weber, and Frank Jülicher. Liquid-liquid phase separation in biology. *Annual review of cell and developmental biology*, 30(1):39–58, oct 2014.
- [232] D. Turnbull. Kinetics of heterogeneous nucleation. *The Journal of Chemical Physics*, 18(2):198–203, dec 1950.
- [233] N. H. Fletcher. Size effect in heterogeneous nucleation. *The Journal of Chemical Physics*, 29(3):572–576, sep 1958.
- [234] Richard P. Sear. Nucleation: theory and applications to protein solutions and colloidal suspensions. *Journal of Physics: Condensed Matter*, 19(3):033101, jan 2007.
- [235] Ruensern Tan, Aileen J. Lam, Tracy Tan, Jisoo Han, Dan W. Nowakowski, Michael Vershinin, Sergi Simó, Kassandra M. Ori-McKenney, and Richard J. McKenney.

- Microtubules gate tau condensation to spatially regulate microtubule functions. *Nature Cell Biology* 2019 21:9, 21(9):1078–1085, sep 2019.
- [236] G. N. Antonow. Sur la tension superficielle à la limite de deux couches. *Journal de Chimie Physique*, 5:372–385, 1907.
- [237] Hisao Nakanishi and Michael E. Fisher. Multicriticality of Wetting, Prewetting, and Surface Transitions. *Physical Review Letters*, 49(21):1565, nov 1982.
- [238] L. Boruvka and A. W. Neumann. Generalization of the classical theory of capillarity. *The Journal of Chemical Physics*, 66(12):5464, aug 1977.
- [239] Elizabeth Vitt and Kenneth R Shull. Equilibrium Contact Angle for Polymer/Polymer Interfaces. *Macromolecules*, 28(18):6349–6353, 1995.
- [240] J. O. Indekeu. Line tension near the wetting transition: results from an interface displacement model. *Physica A: Statistical Mechanics and its Applications*, 183(4):439–461, may 1992.
- [241] B Widom. Line Tension and the Shape of a Sessile Drop. *J. Phys. Chem*, 99:2803–2806, 1995.
- [242] A. Amirfazli and A. W. Neumann. Status of the three-phase line tension: A review. *Advances in Colloid and Interface Science*, 110(3):121–141, aug 2004.
- [243] Bruce M. Law, Sean P. McBride, Jiang Yong Wang, Haeng Sub Wi, Govind Paneru, Santiago Betelu, Baku Ushijima, Youichi Takata, Bret Flanders, Fernando Bresme, Hiroki Matsubara, Takanori Takiue, and Makoto Aratono. Line tension and its influence on droplets and particles at surfaces. *Progress in Surface Science*, 92(1):1–39, feb 2017.
- [244] Fernando Bresme and Nicholas Quirke. Computer simulation study of the wetting behavior and line tensions of nanometer size particulates at a liquid-vapor interface. *Physical Review Letters*, 80(17):3791–3794, apr 1998.
- [245] Fernando Bresme and Nicholas Quirke. Nanoparticulates at liquid/liquid interfaces. *Physical Chemistry Chemical Physics*, 1(9):2149–2155, jan 1999.
- [246] Ingrid Ibagon, Markus Bier, and S. Dietrich. Three-phase contact line and line tension of electrolyte solutions in contact with charged substrates. *Journal of Physics: Condensed Matter*, 28(24):244015, apr 2016.
- [247] J. A. De Feijter and A. Vrij. I. Transition regions, line tensions and contact angles in soap films. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, 37(1):9–22, jun 1972.

- [248] N. V. Churaev, V. M. Starov, and B. V. Derjaguin. The shape of the transition zone between a thin film and bulk liquid and the line tension. *Journal of Colloid And Interface Science*, 89(1):16–24, sep 1982.
- [249] C. Varea and A. Robledo. Evidence for the divergence of the line tension at the wetting transition. *Physical Review A*, 45(4):2645, feb 1992.
- [250] M. V. Toshev and M. Z. Avramov. Van der Waals attraction forces and line tension. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 100(C):203–205, jul 1995.
- [251] W. Koch, S. Dietrich, and M. Napiórkowski. Morphology and line tension of liquid films adsorbed on chemically structured substrates. *Physical Review E*, 51(4):3300, apr 1995.
- [252] T. Getta and S. Dietrich. Line tension between fluid phases and a substrate. *Physical Review E*, 57(1):655, jan 1998.
- [253] Harvey Dobbs. Line Tension of n-Alkanes on Water from a Cahn-Type Theory. *Langmuir*, 15(7):2586–2591, mar 1999.
- [254] B. V. Toshev, D. Platikanov, and A. Scheludko. Line tension in three-phase equilibrium systems. *Langmuir*, 4(3):489–499, mar 2002.
- [255] Haeng Sub Wi, Sreeram Cingarapu, Ken J. Klabunde, and Bruce M. Law. Nanoparticle adsorption at liquid-vapor surfaces: Influence of nanoparticle thermodynamics, wettability, and line tension. *Langmuir*, 27(16):9979–9984, aug 2011.
- [256] Robert J. Good and M. N. Koo. The effect of drop size on contact angle. *Journal of Colloid and Interface Science*, 71(2):283–292, sep 1979.
- [257] Jaroslaw Drelich, Jan D. Miller, and Jan Hupka. The Effect of Drop Size on Contact Angle over a Wide Range of Drop Volumes. *Journal of Colloid and Interface Science*, 155(2):379–385, feb 1993.
- [258] Robert Aveyard and John H. Clint. New method for the measurement of line tensions at the solid/liquid/air three-phase contact line. *Journal of the Chemical Society, Faraday Transactions*, 91(1):175–176, jan 1995.
- [259] Jinbo Wu, Mengying Zhang, Xiang Wang, Shunbo Li, and Weijia Wen. A simple approach for local contact angle determination on a heterogeneous surface. *Langmuir*, 27(10):5705–5708, jun 2011.

- [260] P. Chen, S. S. Susnar, A. Amirfazli, C. Mak, and A. W. Neumann. Line Tension Measurements: An Application of the Quadrilateral Relation to a Liquid Lens System. *Langmuir*, 13(11):3035–3042, may 1997.
- [261] Gregory J. Hardy, Rahul Nayak, and Stefan Zauscher. Model cell membranes: Techniques to form complex biomimetic supported lipid bilayers via vesicle fusion. *Current Opinion in Colloid & Interface Science*, 18(5):448–458, oct 2013.
- [262] † Ralf P. Richter, *, , Rémi Bérat, and Alain R. Brisson. Formation of Solid-Supported Lipid Bilayers: An Integrated View. *Langmuir*, 22(8):3497–3505, apr 2006.
- [263] Ralf Richter, Anneke Mukhopadhyay, and Alain Brisson. Pathways of Lipid Vesicle Deposition on Solid Surfaces: A Combined QCM-D and AFM Study. *Biophysical Journal*, 85(5):3035, 2003.
- [264] Luke A. Clifton, Stephen C. L. Hall, Najet Mahmoudi, Timothy J. Knowles, Frank Heinrich, and Jeremy H. Lakey. Structural Investigations of Protein–Lipid Complexes Using Neutron Scattering. *Methods in Molecular Biology*, 2003:201–251, 2019.
- [265] Julie D. Forman-Kay, Jonathon A. Ditlev, Michael L. Nosella, and Hyun O. Lee. What are the distinguishing features and size requirements of biomolecular condensates and their implications for RNA-containing condensates? *RNA*, 28(1):36–47, jan 2022.
- [266] G. Navascués and P. Tarazona. Line tension effects in heterogeneous nucleation theory. *The Journal of Chemical Physics*, 75(5):2441–2446, aug 1981.
- [267] Gerrit Van Meer, Dennis R. Voelker, and Gerald W. Feigenson. Membrane lipids: where they are and how they behave. *Nature reviews. Molecular cell biology*, 9(2):112, feb 2008.
- [268] Nadine Schwierz, Dominik Horinek, Susanne Liese, Tobias Pirzer, Bizan N. Balzer, Thorsten Hugel, and Roland R. Netz. On the relationship between peptide adsorption resistance and surface contact angle: A combined experimental and simulation single-molecule study. *Journal of the American Chemical Society*, 134(48):19628–19638, dec 2012.
- [269] Johan M Berg, L G Tomas Eriksson, M Claesson, Kari Grete, and Nordli Borvet. Three-Component Langmuir-Blodgett Films with a Controllable Degree of Polarity. *Langmuir*, 10:1225–1234, 1994.
- [270] Erwin A. Vogler. Structure and reactivity of water at biomaterial surfaces. *Advances in Colloid and Interface Science*, 74(1-3):69–117, feb 1998.

-
- [271] Ken Åman, Erik Lindahl, Olle Edholm, Pär Håkansson, and Per Olof Westlund. Structure and dynamics of interfacial water in an $L\alpha$ phase lipid bilayer from molecular dynamics simulations. *Biophysical Journal*, 84(1):102–115, jan 2003.
- [272] Mark A. Lemmon. Membrane recognition by phospholipid-binding domains. *Nature Reviews Molecular Cell Biology* 2008 9:2, 9(2):99–111, feb 2008.
- [273] X. Ye, T. Narayanan, P. Tong, J. S. Huang, M. Y. Lin, B. L. Carvalho, and L. J. Fetters. Depletion interactions in colloid-polymer mixtures. *Physical Review E - Statistical Physics, Plasmas, Fluids, and Related Interdisciplinary Topics*, 54(6):6500–6510, dec 1996.
- [274] Andrew Milling and Simon Biggs. Direct measurement of the depletion force using an atomic force microscope. *Journal of Colloid and Interface Science*, 170(2):604–606, mar 1995.

Acknowledgements

"If I have seen further it is by standing on the shoulders of giants" - Isaac Newton.

I've been **extremely** fortunate to lead a life surrounded by giants. Here I thank these big friendly giants for fostering my dreams. The first giant I thank is my mother, Joy - I am forever indebted to you, for raising me, for nurturing my curiosity, and most of all for your everpresent, unconditional love. This message also goes out to the rest of the Holland family; Scott, Elaine, Chris - you are amazing, and I hope you realise that. Secondly, I thank Tim and Dirk for your supervision over the last four years. It has been an incredible journey, I have learned so much from you and am proud to have been your student. An extra special thanks goes to Michael. I think you are well aware of how much you taught me, your patience knows (almost) no bounds, and I've taken great joy in conversing with you about (mainly) science, politics and football over the last four years. Thanks also goes to Nott and Aarts lab members, past and present, for providing a stimulating and friendly working environment throughout my DPhil. I also thank Chris Lagerholm - thanks for the support at the WIMM, it is a great shame that portion of my DPhil work was thwarted by the pandemic.

The people I surround myself with outside of the lab are of utmost importance to me, it is through them that I live a fulfilling life full of joy. To Sabrina, my loving partner (howdy) - you are a real inspiration to me, improving my life to no end and I look forward to many more adventures together. To Jeff, Boon, Sophie, Arthur, Joe DW - you guys are incredible, here is to many many more good times! Joe H and Miranda, thanks for pub Friday. Special thanks also go to Tony, Meghan, Ben, Laura, Jenny, the Teletubbies and the wider Corpus Christi crowd.

None of this would have happened if not for the teachers I've been privileged enough to spend hours with throughout my life. At primary school I was taught the four keys to success are 'confidence', 'persistence', 'getting along' and 'organization'. If I managed the final one maybe I'd be a success! There are four teachers, Mr Yapp, Dr Beard, Mrs Brackenridge & Mr Phillips, who I thank especially, for their endless encouragement throughout my A-levels. Without you, the subsequent 9 years of my life would have been very different (worse) indeed. During my undergraduate degree, it was the time spent in tutorials with Mark, Peter and Rachel which totally revolutionised my mind. It was you who gave me the ability to climb giants and see further.

During 2020, the government enforced coronavirus lockdown resulted in me upping sticks, and moving in with my mother and her partner Mark for four months. This was a difficult time for everyone involved. A special place here is reserved for Mark and my Mom (again) for their support during this time. Tim, you also get a special recognition here, for keeping the Nott lab active and engaged with science when it seemed like the world was falling apart.

I sign off by reiterating a phrase often used by myself throughout the last four years.

This took longer than I expected.