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Molecular Mechanisms Involved in the Ageing of Protein Condensates

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Abstract

Protein condensates, formed through liquid-liquid phase separation, continuously cement their importance in cellular organisation and regulation. However, their long-term stability is challenged by ageing, a process in which condensates transition from liquid-like to gel-like or solid-like states with deep functional and pathological implications. This thesis reviews current research on condensate ageing, starting with the fundamentals of phase separation and the roles of protein condensates in cells. It then assesses the scope of experimental and computational techniques used to explain their behaviour. Furthermore, biological systems, environmental factors and intrinsic properties are taken into consideration. Subsequently, the thesis discusses the findings which shape our understanding of protein condensate behaviour and outlines future outlooks for research.

Keywords Condensate Ageing, Liquid-Liquid Phase Separation, Neurodegenerative Diseases, Protein Condensates, Environmental and Intrinsic Factors

Table of Contents

Symbols and Abbreviations

Symbols

G	Gibbs free energy
H	enthalpy
S	entropy
T	absolute temperature

Operators

ΔG	change in G
ΔH	change in H
ΔS	change in S

Abbreviations

ALS	amyotrophic lateral sclerosis
BAC	bacterial artificial chromosome
C	circular dichroism
CG	coarse-grained
DNA	deoxyribonucleic acid
EM	electron microscopy
FCS	fluorescence correlation spectroscopy
FRAP	fluorescence recovery after photobleaching
FUS	fused in sarcoma
GFP	green fluorescent protein
hnRNPA1	heterogeneous nuclear ribonucleoprotein A1
IDP	intrinsically disordered protein
IDR	intrinsically disordered region
LLPS	liquid-liquid phase separation
MD	molecular dynamics
NMR	nuclear magnetic resonance
RNA	ribonucleic acid
TAR	transactive response
TDP-43	TAR DNA-binding protein 43

1 Introduction

In recent years, protein condensates have emerged as an intriguing research topic, as new encouraging discoveries have been generated regarding their diverse states and crucial roles in both physiological and pathological mechanisms [1]. This subgroup of biomolecular condensates is a product of liquid-liquid phase separation (LLPS), a process in which two unique liquid phases emerge after a spontaneous separation of a homogenous solution of liquids [2]. However, these protein condensates have been shown to be thermodynamically unstable, because of a development known as ageing or maturation [3]. This is a time-dependent process by which formerly liquid-like protein condensates progressively undergo internal structural rearrangements and physicochemical changes that decrease molecular mobility and dynamic exchange with the environment [3]. The importance of understanding this transition into a solid-like state expands due to several reasons: its association with disease research, where neurodegenerative diseases are entangled with the ageing of condensates [4]; a possibility of controlling ageing and then utilising it regarding biomaterials; and even insights on cellular compartmentalisation and its regulation. Although new insights have emerged into the study of condensates, there have only been a small number of connections on the in-between shifts of the different states and other molecular mechanisms involved in the ageing of protein condensates. Thus, the aim of this thesis is to determine the extent of current research on the ageing of the protein condensates by conducting a state-of-the-art literature review. The rest of the thesis is divided into five sections. Section 2 describes the general knowledge on LLPS, protein condensates, and the concept of ageing. Section 3 presents different experimental and computational models. Section 4 explores the molecular mechanisms of ageing and the various factors that influence them. Finally, Section 5 draws conclusions from the other sections and poses possible outlooks for the future.

2 LLPS and Protein Condensates

This section first introduces the fundamentals of LLPS and summarises the main principles, which include the thermodynamic and molecular drivers, and also environmental factors which regulate this mechanism. Next, it will depict the functional roles and biophysical properties of protein condensates, which analyses their part in cellular organisation and molecular regulation, as well as their specific biophysical properties and dynamic behaviours. Finally, the section will conclude with an exploration of the concept of ageing, that provides a foundational understanding.

2.1 Fundamentals of Liquid-liquid Phase Separation

LLPS or liquid-liquid demixing is a process in which two distinct liquid phases are spontaneously formed from one single-phase mixture. The two phases are separated into a usually dense, protein-rich concentrated phase and a coexisting dilute phase, whose composition and properties depend on the concentration of the protein [2]. This results in the formation of condensates also known as membraneless compartments, which usually lack a surrounding lipid bilayer membrane as opposed to membrane-bound organelles. However, LLPS does not only initiate the formation of condensates but also contributes to cellular organisation by enabling the spatial compartmentalisation of biomolecules [5]. In this way, condensates represent specifically organised assemblies rather than random collections of components.
To acknowledge the organisation and dynamic quality of these compartments, it is important to analyse the driving forces behind them. As explained by Banani *et al.* [5], LLPS is a spontaneous thermodynamically driven reaction. This implies that it is a result of a set of required conditions being met without any continuous input of energy, which can be described by Gibbs free energy:

$$\Delta G = \Delta H - T\Delta S$$

where ΔG is the change in Gibbs free energy, ΔH the change in enthalpy, T is absolute temperature, and ΔS is the change in entropy. For a reaction to be spontaneous, there needs to be a negative change in Gibbs free energy ($\Delta G < 0$), corresponding here to the transition from a single homogenous mixture to two phase-separated liquid [5]. This thermodynamic principle can be visualised using free energy and chemical potential diagrams (Fig. 1), which illustrate how phase separation corresponds to a transition from a higher to a lower energy state.

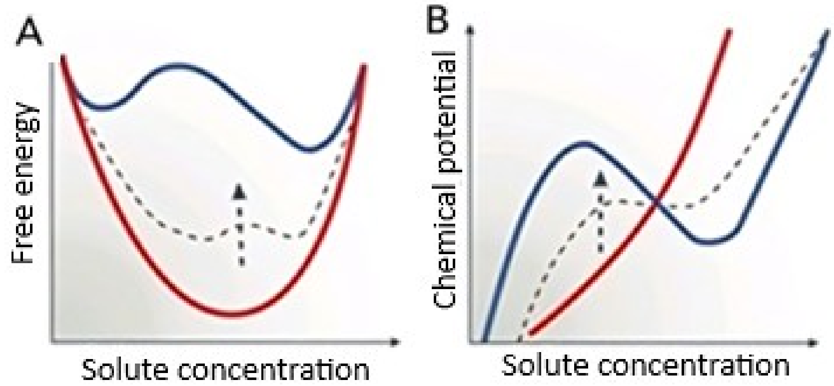

Fig. 1. Thermodynamic basis of phase separation. (A) Free energy of solution as a function of solute concentration. For non-interacting molecules, the free energy curve is unimodal (red line). When attractive interactions are present, the free energy curve becomes multimodal (blue line), and the system lowers its free energy by separating into two phases of different solute concentrations (arrow). (B) Corresponding chemical potential curves. For non-interacting molecules, the chemical potential increases monotonically with concentration (red line). With interactions, the curve becomes non-monotonic (blue line), allowing two concentrations to coexist at equal chemical potential. Together, these panels illustrate that phase separation occurs when intermolecular interactions stabilise coexistence of a dilute and a condensed phase. Adapted from [5].

This crucial shift to low free energy is usually a consequence of low enthalpy in the concentrated dense phase, in which plentiful weak macromolecule-macromolecule interactions are formed (e.g., hydrophobic effects, electrostatic interactions, hydrogen bonding, or pi-stacking) and high entropy in the surrounding phase [5 - 7].
While the thermodynamic forces do drive LLPS, Banani *et al.* [5] stress that the principles are realised through the molecular characteristics and interactions of the biomolecules involved [5]. Multivalency, i.e., the ability of a molecule to form multiple simultaneous interactions, is one such feature which strongly promotes phase separation. The authors note the importance of this, as multivalent proteins or other macromolecules readily assemble into larger oligomeric or polymeric networks when mixed. This network formation promotes phase separation, because as multivalent molecules assemble into a dense structure, their overall solubility decreases. This decrease is driven by entropy, which favours the coexistence of a protein-rich compartment and a more dilute solution. The authors further note that the network is ordinarily formed by individually weak and temporary interactions. However, the source of sufficient binding energy necessary for the stabilisation of the

condensed phase is the cumulation of the effects of these many weak reversible interactions. This allows molecules in the condensate to be continually associating and dissociating, which in return causes the formation of a liquid-like network instead of a static aggregate. It should be noted, however, that multivalency is not the only route to phase separation. Even simple spherical particles with attractive potentials can undergo LLPS. Nevertheless, in biological systems, multivalency is a key determinant of condensate behaviour.
To understand how biomolecules organise into dynamic networks, it is important to consider several features. Banani *et al.* [5] point to the importance of modular interaction domains (i.e., well-folded domains that bind specific motifs and enable multivalency). Having multiple such domains on one protein makes it multivalent, which enables formation of interconnected networks. The authors further note that proteins may also contain intrinsically disordered regions (IDRs). Unlike structured domains, IDRs lack a fixed three-dimensional fold. Instead, they provide flexibility and multiple short motifs that can engage in many weak interactions, which collectively stabilise condensates. As illustrated in Fig. 2, proteins can therefore range from well-folded domains to highly disordered regions, or combine both within the same molecule. Together, these features give proteins the multivalency and reversibility required for condensate formation and regulation.

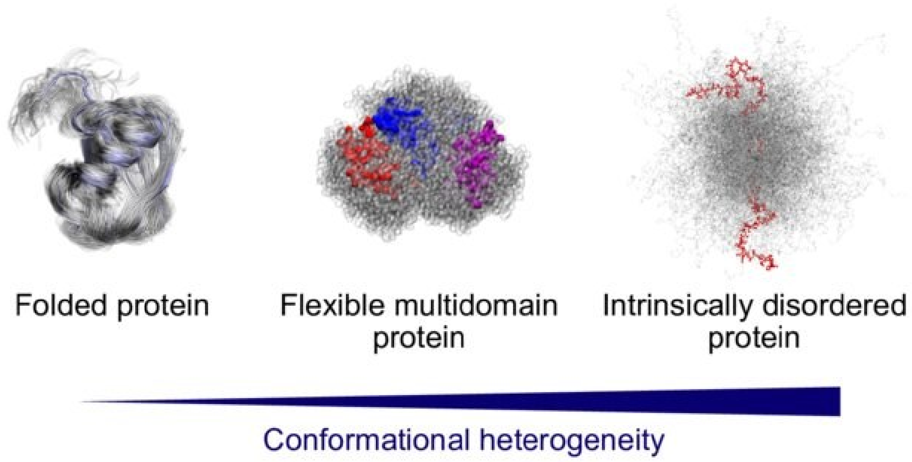

Fig. 2. Conformational heterogeneity of proteins. Left: folded protein with a well-defined three-dimensional structure. Middle: flexible multidomain protein in which structured domains (coloured regions) are connected by flexible linkers, allowing dynamic rearrangements. Right: intrinsically disordered protein (IDP) lacking stable tertiary structure, shown as ensemble of conformations. Coloured segments highlight short linear motifs that can mediate weak, transient interactions. Adapted from [8].

Other than the protein-centred aspects, a third molecular component is often crucial for the formation and regulation of biomolecular condensates. Among these, ribonucleic acid (RNA) plays a particularly important role. Banani *et al.* [5] describe RNA as inherently multivalent. It also grants binding sites through its varied structures and sequences. This facilitates its function as a scaffold, i.e., recruits many proteins to assemble or strengthen a condensate. Furthermore, the study notes that RNA can also participate in the separate interactions by base-pairing with other nucleic acids or through electrostatic interactions with proteins. Moreover, RNA may act as a modulator, where its concentration, length and structure impact condensate size, dynamics and overall composition.

Although molecular properties provide the fundamental mechanisms of LLPS, Alberti and Hyman [3] underline that the surrounding cellular environment is the leading factor which determines the specific characteristics of biomolecular condensates and their formation or dissolution. For example, the study notes that the local concentration of phase separating components is key, since condensates form only when a certain saturation threshold is exceeded. Temperature also plays a vital role as even minimal changes as close as to 1 °C can lead to a drastic phase transition, such as condensation or dissolution of droplets. Additionally, Banani *et al.* [5] stress that pH and ionic strength (i.e., salt concentration) also have a crucial role, since variations in salt concentration or pH modulate the electrostatic interactions that drive phase separation. Furthermore, the authors note that promotion of condensation through volume effects can be achieved by increasing local concentration of macromolecule. This means that crowded intracellular environment substantially influences LLPS. Finally, the study explains how post-translational modifications on proteins (e.g., phosphorylation or methylation) can function as important cellular switches. These modifications quickly alter protein charge, hydrophobity, or valency, and therefore directly regulate assembly and disassembly of condensate.

Table I. Summary of Factors Influencing LLPS

Factor/ Feature	**Influence on LLPS**	**Example/ Mechanism**
Thermodynamic drive ($\Delta G < 0$)	Phase separation occurs when Gibbs free energy decreases, favouring coexistence of two phases	Transition from homogenous mixture to dilute and concentrated phases
Enthalpy (ΔH)	Favourable interactions lower enthalpy in the condensed phase	Hydrophobic effects, electrostatic interactions, hydrogen bonding, π-stacking
Entropy (ΔS)	Entropy of solvent molecules contributes to stabilisation of two-phase system	Molecular disorder outside condensate increases
Multivalency	Promotes formation of two-phase system, reduces solubility, drives phase separation	Modular interaction domains, repeated binding motifs
IDRs	Provide flexibility and multiple weak interaction sites, stabilising dynamic condensates	Short linear motifs forming transient contacts
Additional molecular components	Third components can scaffold or modulate condensates	RNA recruits proteins

Environmental conditions	Regulate stability and transitions of condensates	Temperature, pH, ionic strength
Post-translational modifications	Rapid switches controlling condensate assembly or disassembly	Phosphorylation, methylation

2.2 Functional Roles and Biophysical Properties of Protein Condensates

Protein condensates, formed through LLPS, are dynamic assemblies that contribute to the spatial and functional organisation of biomolecules within both cytoplasm and nucleus of eukaryotic cells. Their changing character of swift assembly and disassembly results in efficiently regulated biochemical processes, due to the suitability of the compartmentalised cells [10]. These suitable compartmentalised organelles are represented by their exclusive and varied molecular composition. Protein condensates are dynamic assemblies of various proteins, RNA, and other biomolecules that are organised in a functional network, in contrast to amorphous, nonfunctional aggregates. Their exact function and character are determined directly by the precise combination of these components [5].

A leading function is spatial organisation. Shin and Brangwynne [1] show that condensates operate as molecular centres, which concentrate proteins, nucleic acids, and other molecules within enclosed spaces. This high concentration promotes efficiency of biochemical reactions, whereas their segregation from competing processes happens synchronously. The authors note that a simple example of this would be the formation of stress granules in cellular stress environment to seclude mRNAs and promote cell survival by transiently stopping unnecessary translation.

Beyond sheer cell compartmentalisation, protein condensates actively regulate molecular activity solely by their formation [5]. They can enhance reaction kinetics, due to their ability to concentrate specific enzymes, regulatory proteins or signalling components. The study notes, contrarily, that the sequestration of molecules could generally be also possible, which would result in the inhibition of their actions outside of the complex and would prevent undesirable interactions. This capability of controlling various interactions proves to be a swift and reversible switch for different cellular pathways.

Protein condensates also engage in the cells as storage sites, as a result of their ability to reversibly absorb excessive concentrations of certain molecules [5]. This is crucial in the crowded cellular environment for prevention of their wide-ranging interactions. This immensely influences the maintenance of cellular homeostasis and supports the fast release of accumulated components if necessary. These diverse functions are illustrated in Fig. 3, which summarises how condensates contribute to cellular organisation, regulation and homeostasis.

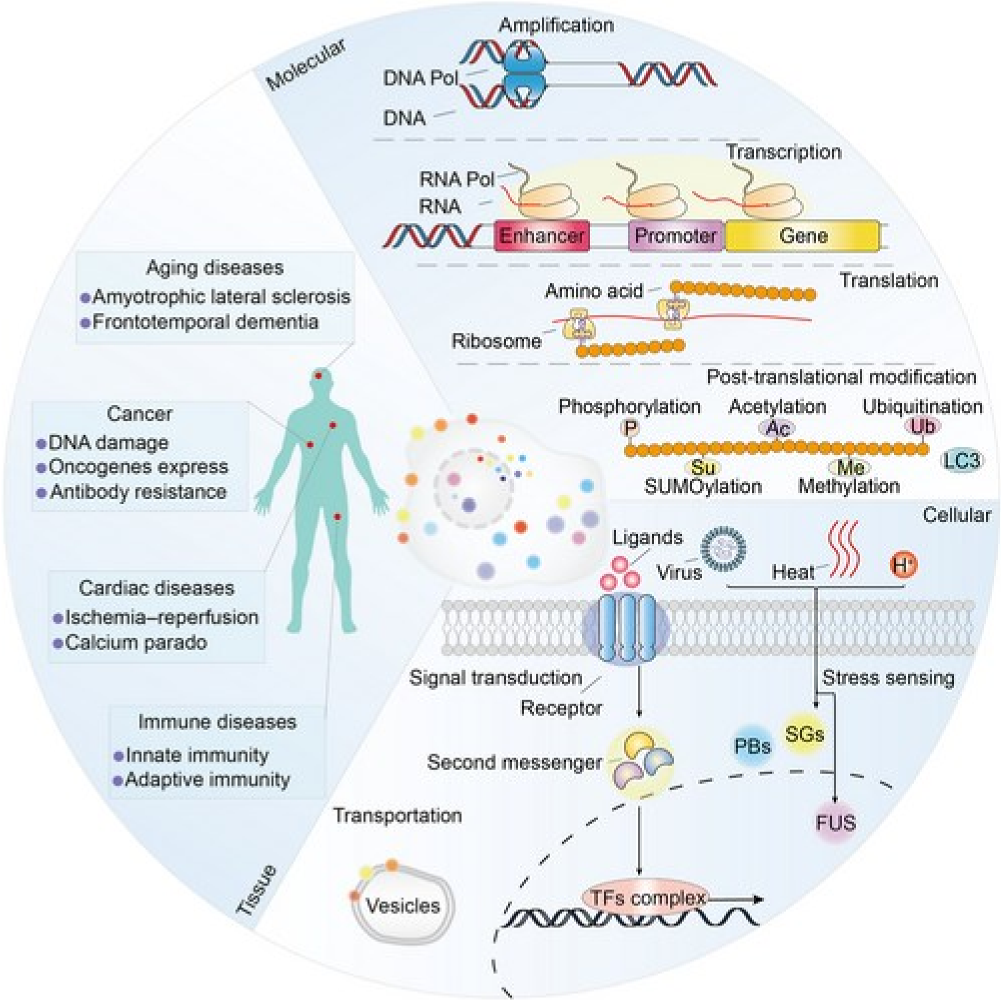

Fig. 3. Functional roles of condensates in cells. Adapted from [9].

The main contributors to these definite characteristics are IDRs, scaffolding and modulating features of RNA, and the dynamic regulation provided by post-translational modifications, all of which have been discussed in the previous section. The importance of these building blocks is then established by the purposeful design of synthetic protein condensates with desired properties and functions [10]. This highlights that the structure and function of protein condensates is determined by their built-in molecular features. Surfacing from this detailed molecular architecture and its control over the precise composition are the distinct biophysical properties and dynamic behaviours, which are the essence of the definition of protein condensates. These properties are significant for their roles inside the cell. They impact their ability to exchange within and around the condensate as well as regulate the responses of the compartments to cellular cues [10].

The material properties of protein condensates are best described as a spectrum, which usually operate in the form of liquid-like droplets, that have the capability undergo fusion and intramolecular rearrangements [11]. Defined by higher viscosity in comparison to the bulk cytoplasm, but still substantially more fluid than a solid, this liquid-like quality allows free diffusion of various components within the protein condensate, and also rapid exchange with the surroundings. However, this does not mean that material properties of protein condensates are static. On the contrary, they can transition to a gel-like state which is characterised by enhanced elasticity and viscoelastic behaviour, or further into solid-like arrangements which are more rigid, all as a possible response to specific conditions [11].
The capacity of condensates to change states from fluid to more rigid ones is not just a physical property but also performs important functional roles. While irreversible liquid to solid transitions are necessary for specific cellular processes, they also prove to have negative effects, as they advance formation of pathological aggregates, which are linked to neurodegenerative diseases, as their conversion into stable aggregates disrupts normal cellular function [1].
Apart from the possible differences in fluidity, protein condensates exhibit liquid-like behaviours such as wetting and dripping, which are evident in their ability to fuse upon contact and to flow when mechanical stress is applied [11]. What further accentuates this behaviour is presence of surface tension, i.e., mechanical tension occurring at the interface in-between the condensate and the cellular environment that surrounds it [7]. This directly leads to their characteristic spherical shape, as this shape resembles the minimum area of the interface, which is caused by continuous reduction of this area due to the tendencies of mechanical tension. This shape also has a role in the maintenance of confines of condensates from their surroundings.

2.3 Concept and Consequences of Ageing

Over time, protein condensates undergo a process known as ageing or maturation. During this process, their material properties shift from a fluid, liquid-like state to a more rigid gel-like or solid-like forms [12]. This transition is functionally significant, as it can impair condensate dynamics and has been implicated in several pathological conditions, including neurodegenerative diseases [5, 7].
The shift of protein condensates during ageing can be visualised as spectrum of material states. This means that they do not suddenly change from a liquid to a solid. Instead, their properties continuously change along a continued scope, which includes several transitional stages, neither purely liquid nor solid [14]. When formed, protein condensates usually display liquid-like

characteristics. These are expressed by high internal molecular flexibility, supporting diffusion of components in the condensate, and also rapid exchange with the cytoplasmic environment that surrounds it [5]. The authors also indicate that this fluidity assists dynamic mechanisms such as enzymatic reactions, swift responses to cellular cues, and reversible molecular sequestration. As ageing advances, protein condensates can transition into a gel-like state. This intermediate phase is described by increased viscosity and viscoelastic properties, which results in restrictions of internal diffusion and molecular exchange with the bulk phase [15, 16]. Gel-like condensates present reduced dynamic behaviour and efficiency of their functions, but they still retain slight deformability. The final stage of protein condensate ageing, can, in some cases, conclude with a solid-like state. In this stage, the structures are characterised by their remarkable rigidity, low to no internal molecular mobility, are generally irreversible, and usually conclude with the loss of their typical spherical shape [12, 17].
The evolution from dynamic liquid-like state to a rigid, stiff arrangement does not result in a mere loss of shape but also implies deep functional consequences for the cell. As ageing progresses, condensates become more viscous and display stronger viscoelastic behaviour, which reduces the efficiency of molecular exchange and diffusion across boundaries [15]. As an outcome, catalytic effectiveness of the enzymes gathered within the compartments is substantially weakened, since substrates are slow in connecting with active sites, and products struggle to be released and are essentially trapped [18] (Fig. 4)

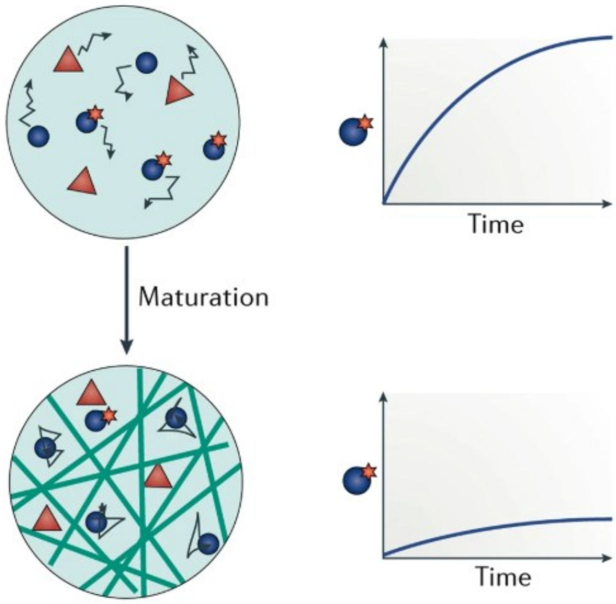

Fig. 4. Effect of condensate maturation on reaction kinetics. Left: in the liquid-like state (top), molecules within the condensate are mobile and can diffuse or interact, facilitating biochemical reactions. After maturation into a

gel-like or solid-like state (bottom), a dense network forms that restricts mobility of molecules. Right: corresponding reaction kinetics are shown by substrate-product conversion over time. In liquid-like condensate (top graph), rapid diffusion supports faster reaction rates, while in matured condensates (bottom graph), diffusion is inhibited, which results in slower reaction kinetics. Adapted from [5].

Furthermore, hindrance or complete prevention of transient protein-protein or protein-RNA interactions, that are important for multitude of dynamic cellular processes, is unavoidable in a rigid environment [13].
In addition, protein condensates that turn into a solid-like state lose their fundamental ability to adapt and respond to cellular cues. As opposed to their fluid complements, which can assemble, disassemble, or reconstruct as a response to changes in metabolic requirements or stress signals, rigidified protein condensates are static entities [16]. This means that due to the loss of dynamic regulation they can no longer suitably respond to the network of cellular signalling pathways. This negatively impacts timely cellular regulation, possibly contributing to a state of chronic cellular dysfunction, due to their inability to dissolve when no longer needed, or to rearrange to execute new functions [19].
Loss of dynamic regulation also hints at a serious risk of irreversible sequestration of vital biomolecules. With growing rigidity of protein condensates, essential proteins, RNA, and other cellular components can become physically captured within these structures, causing them to be functionally unusable for their roles in the cytoplasm [4]. This unrestrained sequestration expends the cellular collection of these resources, inducing an imbalance in cellular homeostasis and possible impairment of a broad number of metabolic pathways, gene expression, or signalling cascades which depend on the availability of these components [20].
Finally, the utmost detrimental outcome of protein condensate ageing is its linking to numerous pathological conditions. The irreversible shift to a highly stable, usually insoluble aggregates such as amyloid-like structures is a hallmark of condensate ageing [21]. While these rigid assemblies could be solely described as static entities, they can actively become cytotoxic by disorganising cellular architecture, overrunning the cultured protein quality control systems in the cell, preventing integral organelle functions, or causing stress responses which induce damage [22]. This emphasises that reduced or accelerated condensate ageing is vital pathological mechanism, as incorporations of protein in such a way are widely acknowledged as trademark of diseases like Amyotrophic lateral Sclerosis (ALS), Alzheimer's disease and Parkinson's disease [3].

3 Experimental Approaches and Theoretical Models

Researchers apply a varied array of advanced methodologies to effectively unravel the complex behaviour and transformations of protein condensates, especially their ageing process. These methods are largely categorised into experimental techniques, that allow direct observation and description of condensates, and theoretical and computational models, which supply a framework to understand the fundamental physical principles and predict intricate molecular dynamics. This section will offer an examination of these methodologies, depicting their particular contributions to clarify the mechanisms of liquid-liquid phase separation and the following material ageing of protein condensates. Comprehending these various tools is necessary to analyse the problems conferred in the prior sections and inform future research courses.

3.1 Experimental Techniques

Understanding the ageing of protein condensates requires a diverse set of experimental approaches, each capturing different aspects of their behaviour. No single method can fully describe this process, since condensates undergo changes in dynamics, mechanics, and molecular organisation over time. Broadly, experiments can be carried out *in vitro* (i.e., under controlled laboratory conditions, where variables such as protein concentration, ionic strength, or pH can be controlled), or *in vivo* (i.e., within living cells where the full complexity of the cellular environment is maintained) [2, 3, 16].
The following subsections review three major categories of techniques that have become central to condensate ageing research.

3.1.1 Microscopy Techniques

Microscopy is a fundamental category of techniques in protein condensate study. It provides direct visualisation of their formation, morphology, and dynamics. Both *in vitro* and *in vivo* applications of microscopy have been instrumental in attempts to reveal the processes underlying condensate ageing, although the insights they provide differ considerably in scope and limitations.
A primary tool in this category is fluorescence microscopy, which enables real-time surveillance of condensates [1]. Among fluorescence-based methods, fluorescence recovery after photobleaching (FRAP) has become the most widely applied. FRAP quantifies mobility of molecules by bleaching a small region inside of a droplet which is fluorescently marked and measures the recovery of fluorescence over time [17]. The authors note that a fluid-like

behaviour is implied by a high recovery rate of fluorescence, whereas hampered or even not present recovery signifies a conversion with more viscosity or solidification, which are principal signs of ageing (Fig. 5).

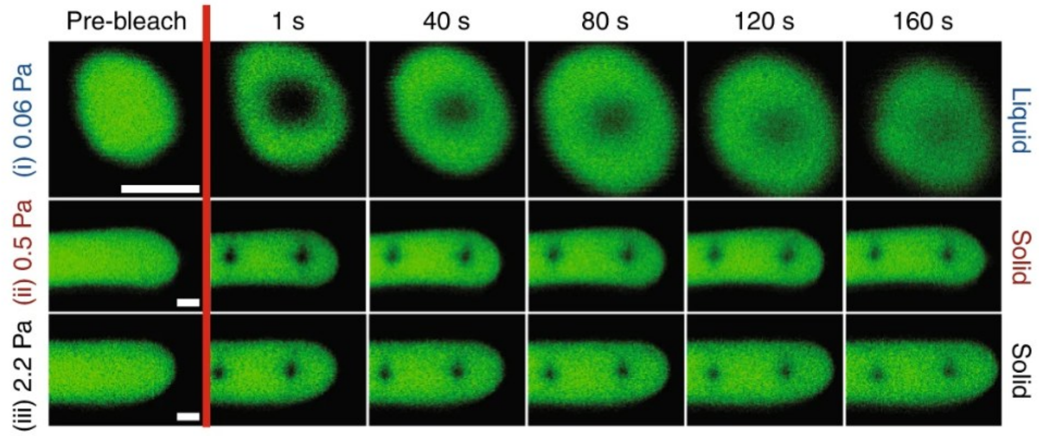

Fig. 5. FRAP of protein condensates at different material states. Rows show recovery at increasing pressures (i) 0.06 Pa (liquid-like), (ii) 0.5 Pa (solid-like), and (iii) 2.2 Pa (solid-like). Pre0bleach images confirm uniform fluorescence. After bleaching (vertical red bar), the liquid-like condensate (top row) shows high fluorescence recovery within seconds, which indicates high molecular mobility. In contrast, the solid-like condensates (middle and bottom rows) show little to no recovery, which reflects restricted diffusion and rigid internal structures. Adapted from [17].

FRAP has been widely employed in research of condensates, which includes those assembled by proteins such as fused in sarcoma (FUS) and heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), proteins whose mutations and aggregations are heavily associated with neurodegenerative diseases [13, 21]. In *in vitro* studies, FRAP offers the advantage of a simplified system, as protein concentration, pH, ionic strength, or mutations can be systematically varied to examine their effects on mobility. This makes FRAP a powerful tool for uncovering intrinsic properties of condensates. However, such reductions of conditions lack the regulatory complexity of a cell. This means that ageing transitions are often accelerated or exaggerated. In contrast, *in vivo* FRAP integrates the influence of molecular crowding, chaperone activity (i.e., the process by which specialised proteins, known as molecular chaperones, assist in the proper folding or unfolding of other proteins), and other cellular processes. For example, disease associated FUS mutations promote rapid liquid-to-solid transitions in vitro, but in vivo these transitions may be substantially delayed by cellular quality-control machinery [21]. Therefore, FRAP is an example of both strengths and shortcomings of microscopy: it quantifies dynamics with high precision, but its interpretation must account for differences between isolated systems and living cells.

To complement these dynamic measurements, electron microscopy (EM) presents high-resolution information about structure. It facilitates visualisation of internal composition and surface morphology of protein condensates. For instance, Emmanouilidis *et al.* [4] applied EM to image FUS droplets that have gone through the ageing process, uncovering surface densification and morphological differences coherent with structural ordering during ageing. Its strength lies in resolving nanoscale features which fluorescence techniques are unable to access. Yet, EM typically requires fixation or cryopreservation, which lats dynamic processes and risks introducing artifacts, i.e., artificial features or structural distortions that are not present in the living cell but are introduced during sample preparation. Consequently, EM excels at providing high-resolution snapshots, but it must be paired with live-cell imaging to reconstruct the temporal dimension of condensate behaviour.
Live-cell microscopy provides that temporal perspective by allowing condensates to be tracked directly, within their native environment. Advanced fluorescence imaging has revealed condensate formation, fusion, fission, and dissolution in response to cellular stressors [1, 5]. A particularly powerful application is fluorescent protein tagging, in which proteins that form condensates are genetically fused to markers such as green fluorescent protein (GFP). This has been applied to proteins such as FUS, where bacterial artificial chromosome (BAC) transgeneOmics approaches enabled expression of GFP-tagged variants from their original location [21]. These experiments showed that in unstressed cells, FUS localises to the nucleus, consistent with roles in transcription and splicing [23, 24], but that this organisation becomes compromised during ageing. Such tagging provides a direct window into localisation and dynamics in living cells, although potential artifacts must be considered. This includes the possibility that the tag itself alters molecular interactions or biases phase behaviour.
Beyond basic observations, microscopy has also been critical for linking condensate ageing to disease. *In vivo* imaging studies have shown that chronic stress or proteostasis decline can convert stress granules into pathological inclusions containing FUS or TAR DNA-binding protein 43 (TDP-43) [3, 22]. These transitions which are often not observable in simplified *in vitro* systems, demonstrate the importance of cellular quality-control networks such as chaperones and autophagy (i.e., a cellular process that breaks down and recycles damaged or unneeded components, which include misfolded proteins and entire organelles). Comparisons across model systems, which include transgenic mouse studies, further confirm that condensate ageing can progress differently *in vivo*, as mutations linked to diseases accelerate transitions toward irreversible aggregates [21].
Taken together, methods based on microscopy offer complementary strengths. FRAP quantifies mobility with high temporal resolution but lacks ultrastructural detail. EM provides nanometre-scale snapshots but sacrifices dynamics. Live-cell imaging and fluorescent tagging restore physiological

relevance but risk protein behaviour irritation. No single approach is sufficient to capture the full complexity of condensate ageing, Instead, the strongest insights emerge from comparative use of multiple microscopy techniques. This balances the clarity of *in vitro* simplifications with the realism of *in vivo* environments. In this way, microscopy remains vital in ageing research, while simultaneously reminding us of the need for critical interpretation.

3.1.2 Rheological Techniques

While microscopy provides insight into the visual appearance and dynamics of condensates, it does not quantify their mechanical properties. For this, rheological techniques are indispensable. They measure viscosity, elasticity, parameters that change significantly as condensates mature from liquid-like droplets to gel-like or solid-like states. Rheological studies therefore provide a bridge between molecular interactions and bulk material behaviour. However, similarly to microscopy, these techniques must be critically assessed for their scope, limitations, and complementarity.

One of the most commonly used approaches is microrheology. This method examines material characteristics by installing traceable microscopic particles inside of a condensate. Then it tracks its motion over time and analyses the thermal or externally driven dislocation of the particles, generating quantitative data signifying balance between fluidity and elasticity [15]. This data is helpful in catching slight changes in condensate behaviour, where weak and reversible interactions are replaced by more stable molecular networks (Fig. 6).

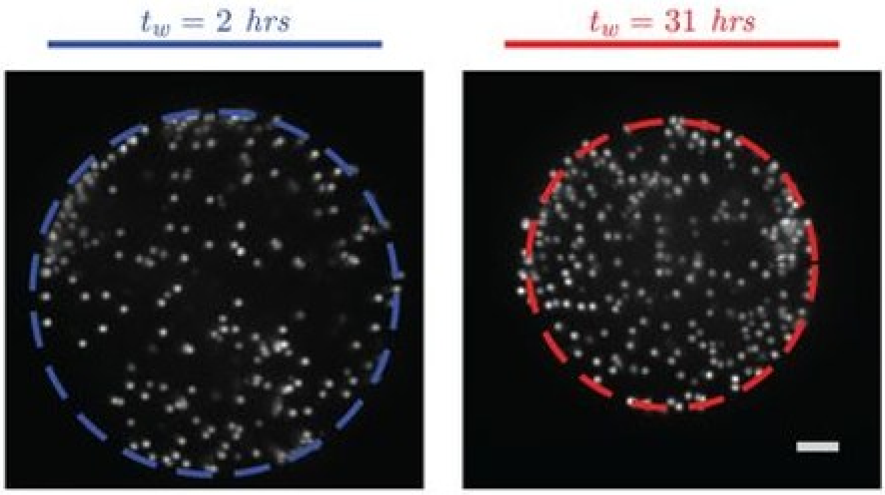

Fig. 6. Passive microrheology of protein condensates at different ageing times. Fluorescent tracer particles embedded in condensates are tracked to monitor their thermal motion. At 2 hours (left), particles display large displacements, which indicates liquid-like interior with high molecular

mobility. At 31 hours (right), particle motion is restricted, consistent with ageing into a more solid-like state. Adapted from [16].

Jawerth *et al.* [16] depicted ageing protein condensates as Maxwell fluids, displaying viscous and elastic behaviour based on the period of time of deformation. Their study points out that during the maturation of condensates, the resistance of deformation increases, resulting in growth of elastic stress and structural reinforcement as time passes. This is tied to reduced internal molecular rearrangement and typically foreshadows the formation of more rigid and irreversible aggregates. Such measurements provide critical evidence that condensate ageing is a progressive shift in material state.
The strength of microrheology lies in its ability to determine gradual changes invisible to microscopy. However, it is not without limitations. Embedding probe particles can perturb the system and interpretation of their motion requires careful modelling. Moreover, while microrheology excels in controlled *in vitro* systems, applying it *in vivo* remains challenging [16]. Tracking probe particles inside living cells is technically demanding and can alter cellular physiology, which limits the general applicability of the technique.
Beyond microrheology, alternative mechanical assays have been developed. Droplet formation experiments and optical tweezer-based force measurements probe how condensates respond to external stresses [16, 17]. These approaches can directly measure interfacial tension and mechanical resistance, properties which undergo significant changes during ageing. Their strength lies in providing direct mechanical readouts, but their applicability is again restricted, due to the previously discussed limitations of *in vitro* conditions.
Critically, rheological studies consistently show that increase in viscoelasticity correlates to a decline in molecular exchange and responsiveness. Michieletto and Marenda [15] remarked that these rheological alterations influence protein condensate function, since stiffer arrangements deter reactions of enzymes and dynamics of interactions become limited. This indicates the functional cost of ageing: as condensates become mechanically reinforced, they lose flexibility necessary for dynamic processes. This correlation also highlights a key limitation, as rheology captures bulk mechanical developments but not molecular determinants. Without complementary methods such as spectroscopy or simulations, it cannot explain why specific proteins or sequences expedite rigidification.
Another important comparative point is timescale. Diffusion-based fluorescence methods are most sensitive to fast molecular mobility, whereas rheology captures slower, collective responses to deformation. This complementarity proves to be important, as microscopy can reveal that diffusion slows, while rheology confirms that the system simultaneously develops elastic resistance.

In summary, rheological techniques are valuable for quantifying the material transitions of protein condensates. Their advantages lie in sensitivity to gradual changes and the ability to directly measure mechanical parameters. On the other hand, their limitations are in their applicability *in vivo* and reliance on artificial probe systems. Possibly the best conclusions arise when rheology is combined with other techniques, which include microscopy for visualisation, spectroscopy for molecular signatures, and computational models for mechanistic explanations. Therefore, similarly to microscopy, rheology contributes a unique but partial view.

3.1.3 Spectroscopic Techniques

Spectroscopic techniques provide a distinct perspective on condensate ageing by probing features on the molecular level. Unlike microscopy and rheology, spectroscopy is uniquely positioned to reveal how condensate material properties are rooted in changes of protein folding and molecular organisation. This focus makes spectroscopy yet another powerful complement. However, it also introduces limitations in spatial resolution and experimental complexity.

A central strength of spectroscopy lies in its ability to detect structural changes that accompany ageing. For example, circular dichroism (CD) spectroscopy and spectrofluorescence have been utilised to characterise changes in secondary and tertiary structure of proteins within condensates. In one study, serum albumin, the most abundant blood protein, showed age-related conformational modifications when exposed to alkaline conditions (i.e., exposure to high pH environment), leading to altered related α-helical content [25]. Although this work did not focus on protein condensates, such conformational changes are relevant for understanding how protein structure and environment can influence condensate stability and ageing. Such observations highlight how spectroscopic methods not only capture the physical state of condensates but also identify the structural predispositions which may influence whether condensates undergo ageing or remain liquid-like. These insights are largely inaccessible to microscopy, which cannot directly observe changes in secondary structures.

Further methods, which include Raman spectroscopy and nuclear magnetic resonance (NMR) spectroscopy, extend this molecular detail. Raman spectroscopy discerns vibrational modes of chemical bonds. This can be utilised to detect different secondary structures such as β-sheets, which signify a solid or amyloid-like state [4]. The authors applied Raman spectroscopy to unveil the assembly of β-sheet content at the surface of droplets during ageing. NMR provided complementary evidence by quantifying reductions in molecular flexibility during ageing, consistent with characteristics of maturation. A notable advantage of both Raman and NMR is their sensitivity to subtle molecular changes which precede visible solidification. This essentially provides

an early “warning” system for condensate ageing. However, both methods come with their challenges. Raman spectroscopy suffers from background noise in complex samples and NMR demands high protein concentrations and is experimentally complicated. Both are therefore more manageable in *in vitro* conditions.
To bridge this gap, fluorescence correlation spectroscopy (FCS) has emerged as a valuable tool. This technique measures molecular diffusion rates by autocorrelating the fluorescence intensity fluctuations within a confocal volume [15]. Unlike FRAP, FCS provides nanoscopic information on local viscosity and molecular exchange. This sensitivity can reveal heterogeneity within a condensate that might otherwise be averaged out in bulk rheological measurements. Nevertheless, similarly to previous spectroscopy techniques, FCS is technically demanding, requires careful calibration, and may yield values which diverge from bulk viscoelastic properties [26]. Even so, when coupled with FRAP and microrheology, FCS is a potent technique when describing the dynamics within a condensate.
Despite its strengths, spectroscopy faces limitations in contextual relevance. Many spectroscopic techniques are constrained to *in vitro* systems. Applying them *in vivo* is significantly more complex due to issues such as autofluorescence, crowding, and lower signal-to-noise ratios. This restriction contrasts with microscopy, which can directly visualise condensates in living cells. As a result, spectroscopy often provides depth without context, i.e., rich molecular detail, but in settings that may not fully replicate physiological conditions.
Taken together, spectroscopic methods excel at displaying the molecular mechanisms of condensate ageing, especially structural transitions and changes in flexibility. Furthermore, spectroscopy does not compete with neither microscopy nor rheology but complements them. While microscopy shows where and when condensates form, and rheology explains how their material properties evolve, spectroscopy reveals why these changes occur.

3.2 Theoretical and Computational Models

Theoretical and computational models serve as an important complement for understanding the principles behind liquid-liquid phase separation and ageing of protein condensates. Compared to experimental techniques, models present insight into molecular interactions, material properties, and long-term conversions which are difficult to investigate directly. They also support systematic testing of how different variables influence condensate formation, stability, and material transitions. The following subsections introduce various modelling techniques which have been utilised in regard to protein condensates.

3.2.1 Polymer Physics and Mean-Field Approaches

Polymer physics offers a convenient theoretical framework to illustrate the forces underlying the protein condensate assembly and their further progression to rigid states. These models represent proteins as polymers with multivalent interactions sites, which allows predictions of how sequence composition and molecular connectivity form condensate behaviour [11]. One of the most broadly employed mean-field approaches is the Flory-Huggins theory [27, 28]. It has been originally developed for synthetic homopolymers. The model describes the balance between mixing entropy and interaction enthalpy in polymer solutions. In the context or protein condensates, this framework has been altered by considering proteins as effective polymers, where the interaction parameter (χ) represents the average strength of intermolecular attractions. For instance, experimental phase diagrams of proteins such as FUS have been successfully interpreted through Flory-Huggins models, where changes in χ capture how sequence composition or solution conditions modulate condensate stability [29]. Higher values of χ increase the probability of phase separation. While this idealisation provides a standard description of condensate demixing, it does not factor in sequence heterogeneity, which is principal in real protein systems [11].
Continuations of these mean-field models have been established to include specific aspects of intrinsically disordered proteins. The “stickers and spacers” methodology is a notable example. In this model, amino acid residues are considered (stickers) drive cohesive interactions, while intervening sequences (spacers) modulate solubility and chain flexibility [30]. This principle is illustrated in Fig. 7.

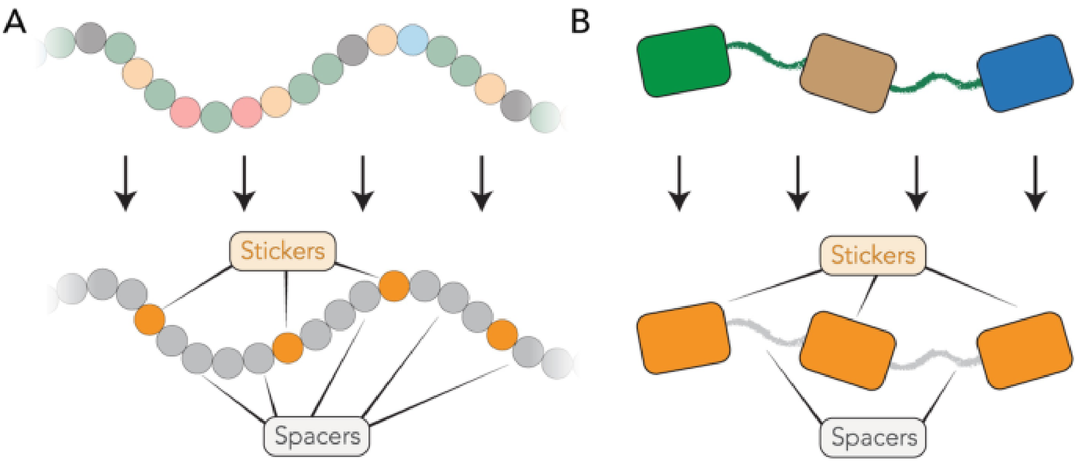

Fig. 7. Stickers-and-spacers model for multivalent macromolecules. (a) IDPs are represented as amino acid chains, where certain residues act as stickers (orange) which drive weak, reversible interactions, while intervening residues function as spacers (grey) that modulate flexibility and spacing between stickers. (b) Folded modular domains connected by flexible linkers can be

described in the same framework, where the folded domains act as stickers and the connecting segments act as spacers. Adapted from [31].

More recent work has extended this concept to ageing, which links sticker lifetime and interaction persistence to condensate rigidification [12]. The structure and density of stickers along the chain decide the likelihood of network formation. At high sticker density, proteins incline to condensation into networks which may lose fluidity and become more gel-like. This links sequence design to the material states which are fundamental to protein condensate ageing.

Adapted polymer models also present perception of the viscoelastic characteristics of protein condensates. Conjectures from sticker-spacer theories show that longer sticker lifetimes intensify transitions from liquid-like to solid-like states [12]. These theoretical results are consistent with the rheological observations which describe ageing condensates as Maxwell fluids with increasing elastic stress over time [16]. Mean-field approaches therefore provide a way to connect molecular sequence components to resulting material properties. This offers explanations for why condensates mature variously depending on protein composition.

Regardless of their usefulness, these models face challenges when applied to protein condensates. The Flory-Huggins framework oversimplifies proteins by disregarding their heteropolymeric nature. Furthermore, the sticker-spacer model simplifies protein sequences into a conceptual one-dimensional arrangement, while the resulting interactions remain inherently three-dimensional, since stickers engage in spatial contacts that drive condensate formation. These simplifications prove their value for predicting phase behaviour, but they are not able to fully explain molecular events such as β-sheet formation during ageing [4]. Another limitation is that mean-field approaches do not capture kinetic effects, which are crucial for whether condensates mature into reversible gels or irreversible aggregates.

From another perspective, the simplicity of these models is an advantage to a certain extent. They provide an intuitive framework which facilitate comparisons between different proteins and conditions. They also form a theoretical baseline for interpretation of more precise computational and experimental discoveries. Still, their predictions must be incorporated cautiously with experimental data, since of molecular details may lead to generalised conclusions.

3.2.2 Continuum and Kinetic Models

In addition to molecular descriptions, protein condensates can also be studied using continuum and kinetic models. These models treat them as mesoscale or bulk entities. They further intend to display how condensates behave during cellular timescales. This includes their growth, fusion, and maturation into rigid states. But instead of characterising each detail, they focus on the physical laws which control droplet dynamics [7].

Continuum models describe spatial and temporal evolution of condensates as droplets in a continuum. These models are specifically applied to examine developments such as Ostwald ripening, where small droplets become smaller and larger ones grow, or droplet coalescence, where fusion reduces surface tension [7]. In the context of ageing, continuum models are able to clarify the reasons for possible evolutions into multiphase structures of condensates, as shown experimentally for single-component systems [32]. Usage of such models is beneficial due to their ability to predict long-term dynamics inaccessible to atomistic or coarse-grained (CG) simulations. On the other hand, they disregard specific sequence details, i.e., they are not capable of explaining why some proteins transition into solid states, while others remain in a liquid-like state.

Kinetic and thermodynamics models allow for a different perspective. They highlight the molecular interactions rates and the energetic profile of condensates. While kinetic models specify the rates of association or dissociation with or from protein condensates, continuum thermodynamic models are fixated on the free energy changes accompanying structural transitions [7]. Utilising these capacities, the models proved to be suitable to explain large-scale behaviours such as droplet growth and coalescence. Phenomena like kinetic trapping (i.e., condensates stuck in metastable states) or nucleation barriers can also be addressed, although these are more directly captured in CG simulations which resolve molecular-scale rearrangements [12, 21]. Their employment is especially important in disease contexts, where small alterations in reaction kinetics or nucleation rates can introduce pathological inclusions.

The predictive accuracy of these models is dependent on their capacity to recreate experimentally discovered behaviours. These behaviours include formation of core-shell structures [33] and time-dependent decline of molecular mobility [16]. However, the models are greatly susceptible to input parameters, which are usually taken from experimental assessments, instead of coming from general principles. This presents an obvious issue: a model adjusted for one system may not be applicable in a different one. Moreover, they often omit the active physical processes such as active regulation through chaperones, post-translational modifications, and more [3, 19].

A notable strength of continuum and kinetic approaches is their potential to present long-scale behaviours that shorter, that mean-field models or direct experimental observations cannot reach. This makes them complementary to polymer physics models, which provide sequence-level intuition, while continuum models instead capture mesoscale behaviours such as growth, coalescence, and ageing trajectories.

3.2.3 Molecular Dynamics and Coarse-Grained Simulations

Molecular dynamics (MD) is a computational technique that simulates the physical movements of atoms and molecules over time by solving Newton's equations of motion. In these simulations, molecules are represented as collections of atoms (or coarse-grained groups of atoms), whose trajectories evolve under the influence of interatomic forces defined by a force field. This enables direct observation of how proteins associate, how weak bonds are formed and broken, and how environmental changes impact condensate stability [6]. These models are especially useful when studying intrinsically disordered regions, where structural analysis proves to be challenging due to the absence of secondary and tertiary structures [11].
Atomistic MD simulations offer high resolution and describe proteins and their solvent at atomic detail. They are helpful when it comes to inspecting interactions such as hydrogen bonding or side-chain hydrophobic clustering, which contribute to condensate stability [6]. However, atomistic simulations are limited by system size and timescales. Protein condensates undergo changes over milliseconds and seconds to hours or even days, while atomistic MD generally captures nanoseconds to microseconds. As a result, atomistic simulations are suited for comprehension of short-term interactions and are not satisfactory when studying long-term processes [14].
To overcome these restrictions, CG models are often utilised. In CG simulations, groups of atoms are reduced to beads, which simplifies the system but still preserves essential interaction patterns. This reduction not only enables simulation of larger systems but also allows the use of longer integration time steps, thereby it extends the accessible timescales compared to all-atom MD. CG simulations have been able to reproduce fusion events, coarsening of droplets, and materialisation of viscoelastic behaviour during ageing [12]. They have also been employed in tests on condensate material properties determined by sequence composition. For example, different sticker densities or charge distributions can be systematically examined to predict which proteins are more likely to shift into liquid states, and which may progress towards solid aggregates [13].
Knowledge gained from simulations is often compared with rheological and spectroscopic experiments. For instance, CG models anticipating rigidity agree with experimental research of condensates behaving as ageing Maxwell fluids [16]. Additionally, simulations which trace the arranging of chains into β-sheet structures have been associated with experimental studies showing surface β-sheet formation during FUS ageing [4]. These comparisons emphasise the effectiveness of MD approaches in linking molecular-scale mechanisms to bulk material transitions.
Nevertheless, MD simulations do have their own limitations. Atomistic simulations are strongly dependent on the accuracy of force fields. These may not properly depict the complex balance of hydrophobic, electrostatic and π-π interactions in disordered proteins [14]. On the other hand, coarse-grained

models are heavily parametrised and usually fitted to experimental data. This creates a risk, as the models may reproduce experiments well, but not because of their predictive accuracy. Instead, it is because they have been pieced to known results. Another issue is that simulations frequently neglect cellular crowding and active processes, which play a substantial role *in vivo* [3].
From a critical perspective, theoretical and computational models together form a hierarchy of approaches, each with distinct advantages and drawbacks. Mean-field and polymer physics frameworks provide intuitive, sequence-level rules and enable rapid comparison across proteins, but they oversimplify molecular heterogeneity and neglect kinetic effects. Continuum and kinetic models extend the picture to mesoscale behaviours such as droplet coalescence, ripening, or ageing trajectories, though they necessarily sacrifice sequence-level detail. MD and CG simulations fill the gap between these. They have the capability to reveal atomistic or mesoscopic mechanisms that underlie condensate transitions, but remain limited in accessible size and timescales, and often depend heavily on parameterisation. A comparison of theoretical, computational and experimental approaches is summarised in Table II.

Table II. Comparison of Theoretical, Computational and Experimental Approaches for Protein Condensates

Approach	Level of detail	Timescale	System size	Strengths	Limitations
Mean-field/polymer physics	Sequence-averaged, statistical interactions	Equilibrium	Very large	Simple, intuitive	Ignores heterogeneity, kinetics, and 3D structure
Continuum/ kinetic models	Mesoscale droplets, rate equations	Seconds to hours	Micron-sized	Captures macroscopic droplet behaviours	No sequence specificity, strongly parameter-dependent
Molecular Dynamics	Atomistic resolution	ns-µs	Tens to hundreds of nanometres	High detail	Very short timescales, computationally expensive
Coarse-Grained simulations	Groups of atoms reduced to beads	µs- ms	Hundreds of nanometres to micros	Larger systems and longer processes	Heavily parametrised, may reproduce data without predictive power

FRAP	Protein mobility via fluorescence recovery	Seconds to minutes	Micron-sized	Directly measures molecular dynamics *in vivo*	Photobleaching artifacts, limited temporal resolution
Microrheology	Probe particle motion reports viscoelastic moduli	Seconds to hours	Micron-sized	Quantifies mechanical properties	Probe perturbation, modelling assumptions, hard in vivo
Spectroscopy techniques	Protein secondary/ tertiary structure	ps-hrs	Molecules to bulk	Sensitive to conformational change	Often indirect link to phase behaviour

4 Systems, Conditions and Molecular Mechanisms of Ageing

Transitions of protein condensates from liquid-like to gel-like or possibly solid-like states are not uniform but strongly reliant on the biological system and the surrounding conditions. Different proteins exhibit different propensities for ageing, and these behaviours can vary depending on cellular contexts. At the same time, external factors and intrinsic factors regulate the pace and results of this process. Therefore, understanding protein condensate ageing necessitates considering the systems in which it occurs as well as the conditions which regulate it. Following this, the section will also examine the molecular mechanisms underlying the observed transitions.

4.1 Systems and Biological Contexts

The study of the ageing of protein condensates extends through number of systems, from minimal *in vitro* constructs to complex living organisms. Each of these systems emphasises various features of the ageing process, and their comparison presents a distinct view on how condensates behave in health and disease.

Model organisms have shown significant value in revealing how condensate ageing happens during the natural course of a life of an organism [34, 35, 21]. In yeast, stress granules formed by proteins binding RNA display ageing behaviour dependent on chaperone activity [34]. Deletion of disaggregases (i.e., molecular chaperones that dissolve protein aggregates and remodel condensates) reveals increase in granule persistence and decline of reversibility, which indicates that quality-control machinery directly regulates the transition of protein condensates in this system [34]. In *Drosophila*, disruption of the proteins FUS or TDP-43 which form condensates results in locomotor dysfunction (i.e., impaired mobility and climbing defects) and reduced lifespan [35]. This demonstrates that modified condensate behaviour is responsible for systematic decline when proteostasis is compromised. Mouse models further add evidence, as they unveil that disease-linked mutations in these proteins stimulate condensate solidification and aggravate neurodegeneration [21]. All of these examples highlight the strong influence of global state of the cell and its proteostasis network in condensate ageing.

The vulnerability to ageing of different condensates also varies across cellular compartments. Nuclear condensates (i.e., a subclass of protein condensates) often hold their liquid-like characteristics even over longer time periods [5]. Their high rate of exchange of components and input from transcriptional activity make them rather resistant to unwanted maturation [5]. Another example is tau, which forms condensates in neurons that gradually lose liquid-

like properties [13]. This specific behaviour illustrates yet another connection between condensate ageing and neurodegenerative pathology.
Overall, this knowledge underscores that condensate behaviour is highly context dependent. Comprehension of ageing therefore demands examination of condensates across systems and compartments. While biological model systems such as *Drosophila* or transgenic mice present insight into organismal outcomes, they only capture a subset of the complexity present in living cells. Minimalistic systems reduce molecular interactions, while *in vivo* models integrate proteostasis networks and cellular structure. Therefore, no single system fully depicts condensate behaviour, findings must be generalised with care. Still, bridging *in vitro* assays, *in vivo* imaging, and organismal models remains key in order to uncover further molecular mechanisms of ageing involved in both health and disease.

4.2 Environmental and Intrinsic Factors

The development of protein condensates from dynamic to rigid structures is not driven solely by time. Both environmental and intrinsic factors regulate the pace and extent of this transition [3, 5, 12]. Distinguishing between these factors generally proves to be useful, however, in practice they often perform together in ways that are difficult to split.
Environmental circumstances play a decisive role in the material states of condensates. Small changes in temperature or pH can determine whether a shift to a more rigid state occurs or if condensates remain liquid-like [2, 5, 7]. This sensitivity highlights a paradox: while condensates appear precisely tuned to their surroundings, they also are at a risk of destabilisation from slight deviations from the norm. Therefore, it is uncertain whether *in vitro* observations based on specific heat or pH stress represent processes which also occur during natural ageing. Crowded intracellular environment is another factor to consider. It adds further complexity, as macromolecular crowding supports condensation but also induces kinetically trapped states in droplets [3]. In addition, shear stress indicates that physical force may cause irreversible transitions [17]. Altogether, these remarks show that condensates are highly responsive to multiple forms of stress. However, there is still limited understanding of how these factors interact simultaneously *in vivo*.
Intrinsic properties are also necessary when trying to determine how condensates age. Instead of facilitating phase separation in general, sequence characteristics influence whether droplets remain dynamic or harden. Stickers with long lifetimes, such as aromatic clusters, advance rigidification [12]. This explains faster transitions of some proteins compared to slower transitions of different ones. A notable point is that protein features that enable reversibility of young condensates, such as IDRs, can in some cases also predispose proteins to structural rearrangements. Specifically, IDRs with

sequence tendencies towards β-sheet formation provide flexibility for condensates to transition into more rigid, aggregated states [4]. This dual behaviour presents a possibly overlooked problem, as molecular factors often considered to maintain fluidity may also trigger ageing. RNA presents a similar contradiction. It can stabilise condensates by acting as a multivalent scaffold, but changes associated with age in RNA concentration and quality control can lessen this ability. As a result, condensates may lose fluidity and shift to more solid-like states [3]. Finally, post-translational mechanics function as regulators of material state [5, 19]. Yet age-related decline in these mechanics reduces condensate adaptability, which links intrinsic features to systemic regulatory failure.

Overall, neither intrinsic nor environmental factors can explain protein condensate ageing alone. Instead, both sets interact, and their combinations determine the resulting material state of a condensate.

5 Conclusions

The aim of this thesis was to review current knowledge on the ageing of protein condensates, with a focus on the molecular mechanisms which underlie transitions across material states. The purpose was to determine how precisely current research explains this process, and how ageing relates to both cellular regulation and pathological outcomes.

This review demonstrates that condensate ageing should not be considered as a linear mechanism. Instead, it represents a spectrum of transitions that alter material properties, molecular diffusion, and biochemical activity. Environmental conditions such as pH, temperature, crowding, and mechanical stress exert strong influences on condensate dynamics. Simultaneously, intrinsic factors such as sequence patterns, disordered regions, prion-like domains, RNA interactions, and post-translational modifications significantly shape the possibility of rigidification. The combinations of these factors suggest that protein condensates possess qualities making them both resilient and adaptable, but at the same time susceptible to damage or dysfunction.

In relation to the original aim, the findings imply that neither environmental nor intrinsic factors can be considered as sufficient explanations in isolation. *In vitro* systems reveal inherent sequence-dependent risks of solidification, while *in vivo* research shows that quality control systems can reduce these risks for a significant period of an organism's lifespan. A critical issue here is that much of the literature frames ageing either as a predetermined outcome of molecular design or as a collapse of environmental regulation. Very few studies recognise that both interpretations may hold simultaneously. This therefore complicates attempts to generalise findings across systems.

Several contradictions arise from the available research. Features long considered protective, such as IDRs and RNA scaffolds, appear ambivalent. They stimulate fluidity under some conditions but enable pathologically undesirable outcomes under others. Similarly, mechanical forces, often neglected in earlier studies, can be sufficient to drive irreversible transitions. These examples indicate that the view of condensates as either "beneficial" or "detrimental" is too simple. Instead, ageing emerges from context-dependent relationships, and experimental design heavily moulds the story that is told.

The trustworthiness of these conclusions is constricted by various limitations. Much of the research focuses on a narrow set of proteins, such as FUS, TDP-43, and tau. This restricts how broadly the observed results can be applied and generalised. The field is also methodologically unbalanced. *In vitro* experiments often exaggerate ageing transitions by applying acute stress, while *in vivo* ones can mask intrinsic vulnerabilities by cellular buffering systems. Computational and theoretical models boost value but significantly depend on assumptions about molecular interactions, which often simplify biological reality. These limitations mean that the findings reviewed in this thesis should be treated as indicative rather than definitive.

Future research should expand the scope in multiple directions. First, a wider variety of protein condensates needs to be examined. This especially includes those outside neurodegenerative context, such as condensates involved in metabolism, immunity, or development. Second, experimental methods should better reflect continuous changes related to ageing, rather than acute stress conditions. This could lead to explanations of how slow decline affects condensates. Third, the role of RNA deserves deeper study. To properly understand ageing of protein condensates, we should not solely look at proteins themselves. Considering the changing role of RNA throughout the ageing process could reveal new regulatory layers. Fourth, post-translational modifications should be inspected over time to determine how age-related decline in signalling cascades impacts condensate malfunction. Finally, cross-disciplinary approaches combining rheology, spectroscopy, live-cell imaging, and advanced computational models would provide a better perspective necessary to unify current insights.

An obvious approach of harnessing protein condensate ageing presents itself in disease. Modulating condensate ageing, either by delaying rigidification or by timely dissolution, could present new strategies for neurodegenerative disease treatment. This brings forth another promising path, which is exploiting the ageing principles for biomaterials. If ageing can be controlled, condensates could be devised with adjustable lifespans or designed to sequester harmful molecules. These directions show that the questions in this field are not only fundamental but also applied.

In conclusion, this thesis has argued that condensate ageing is best understood as the intersection of intrinsic molecular characteristics and environmental factors. While the reviewed literature provides substantial evidence for both views, it remains fragmented and occasionally even contradictory. The next phase of research must therefore combine these perspectives, broaden the studied systems, and move further ahead from simplified models. Only then can condensate ageing be thoroughly explained, and its implications for biology and disease be confidently utilised.

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