Oil and water: The basics of biomolecular condensates and Alzheimer's disease

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T00644569

BIOL4480
Dr. Don Nelson
April 2023

What are condensates and why are they important?

Our everyday experiences can illustrate complex biological topics more eloquently than we ever could, but we often do not notice. Understanding these concepts could advance a broad range of medical research, involving diseases such as Alzheimer's. For example, take the seemingly mundane mixing of water and oil; everyone knows that the two will separate. Does everyone know, however, that the droplets of oil that form in the water perfectly demonstrate liquid-liquid phase separation (LLPS)? At the cellular level, phase separation is a process by which proteins and other biological macromolecules self-organize within the cytoplasm or nucleus, like the droplets of oil within the water (Boeynaems et al., 2018; Leslie, 2021). Like the droplets of oil, which demonstrate phase separation by self-organizing to form a distinct phase on top of the water, molecules within our cells also self-organize and condense to form distinct phases, called condensates.

Cell biologists hypothesize that condensates, membraneless organelles within the cytoplasm and nucleus, can trigger or carry out important biological processes, ranging from regulating gene expression to forming stress granules (SGs) (Boeynaems et al., 2018). They are hopeful that the phenomenon of phase separation could answer a fundamental biological question: how do cells arrange their constituents so that the molecules necessary for specific biological functions are in the right places at the right times (Leslie, 2021)? If the answer to this question is that cells use phase separation to form condensates, it could pave the way for new and exciting discoveries in molecular cell biology. In addition to wanting to know how cells arrange their constituents so that

they're in the right places at the right times, I also want to know what happens when cells arrange their constituents to be in the wrong places at the wrong times. Could understanding how condensates form at the wrong place and time also pave the way for new and exciting discoveries?

Cell biologists currently speculate that the misregulation of biomolecular condensate formation, composition maintenance, and clearance could provide an avenue for pathological aggregation (Boeynaems et al., 2018). In terms of water and oil, we can liken aggregation to hot oil being poured down a cold sink. The oil congeals and clogs the sink's pipes in the same way a condensate might aggregate and inhibit a biological function if improperly formed, maintained, or cleared. This has major implications for protein aggregation diseases—especially those strongly correlated with ageing and neurodegeneration, like Alzheimer's. To understand how the misregulation of condensates may cause aggregation, however, we must first understand how they are formed, maintained, and cleared.

How are condensates formed, maintained, and cleared?

There are many factors that effectuate the assembly of proteins to form a condensate. But intrinsically disordered structural regions of proteins may be the most important one. These regions of intrinsic disorder, also known as IDRs, are as termed as such because they allow for intraprotein and interprotein interactions that result in various structural rearrangements of a specific peptide sequence (Tompa, 2012). That is to say, proteins with IDRs do not have one, single, native functional structure (Tompa,

2012). This dynamism is contrary to the long-standing structure-function paradigm of proteins, which states that the amino acid sequence of a protein determines its structure, and its structure determines its function (Tompa, 2012). Thus, tertiary structure proteins with IDRs may be described as *super*tertiary structure proteins, a hybrid of tertiary and quaternary structure proteins (Tompa, 2012). It should be noted that within a supertertiary structure protein, domains with clear tertiary structure may be well-established, but there is not a clear overall tertiary structure (Tompa, 2012). This is a result of the multiplicity of supertertiary protein structures, based on a variety of conformational states due to motifs and domain arrangements within IDRs (Tompa, 2012).

Motifs are local, secondary structure elements, e.g., alpha-helices and beta-sheets, found in characteristic combinations that confer structural function; this indicates supersecondary structure, a hybrid of secondary and tertiary structure proteins (Stollar & Smith, 2020). Moreover, domains are tertiary structure elements with potential functional importance, and they are independently stable (Stollar & Smith, 2020). The motifs and domains of IDRs may include features of domain or motif organization, domain-domain interactions, domain-motif interactions, and/or residual disorder, giving rise to dynamism (Tompa, 2012). Domain or motif organization designates which interactions may occur, based on where the domains and motifs are located within a protein sequence's IDRs (Tompa, 2012). Folding transitions due to organization are a direct result of which motifs and domains can be incorporated during induced folds, leading to various structures at different times (Tompa, 2012).

Interactions that occur between domains include stable and transient intramolecular interactions between separated domains, inducing regulation or further disordered states (Tompa, 2012). This is similar to interactions that occur between domains and motifs, which also include stable and transient intramolecular interactions that can induce regulation or further disordered states (Tompa, 2012). Finally, residual disorder is a state in which a part of the protein retains disorder while the rest of its structure is stable (Tompa, 2012).

Due to their dynamic nature, it is believed that condensates incorporate hundreds to thousands of supertertiary proteins with IDR features (Alberti & Hyman, 2021). Formed by LLPS, a condensate's liquid-like state is dependent on the physical and chemical conditions of the cytoplasm it forms in, as well as its constituents. It is also largely dependent on the continuous rearrangements of intramolecular and intermolecular interactions between its constituents (Alberti & Hyman, 2021). These continuous interactions are governed by multivalency—the ability of a molecule to form multiple, reversible, chemical interactions at once—and heterotypic interactions—interactions between dissimilar molecules (Alberti & Hyman, 2021). Multivalency and heterotypic interactions are both strongly associated with IDRs, especially those that are organized to have domains and motifs separated, with ordered spacer regions in between (Tompa, 2012; Alberti & Hyman, 2021). When condensates form, it is hypothesized that some supertertiary structure proteins with high numbers of valences act as scaffolds. These scaffold proteins are thought to recruit client molecules with lower numbers of valences, acting as drivers of phase separation, and

thus condensate assembly, by promoting assembly through the linkage of IDRs (Alberti & Hyman, 2021).

So how are the interactions between scaffold proteins and client molecules controlled and maintained within the cytoplasm and nucleus? Aside from the physical and chemical properties of the cytoplasm or nucleus the condensate forms in, as well as its constituents, cell biologists believe there may be a variety of different mechanisms. For example, post-translational modifications (PTMs) may regulate scaffold protein and client molecule valency, chemical properties, and interactions dictating what can bind when (Ditlev et al., 2018). In addition, protein quality control (PQC) machinery, such as molecular chaperones and protein degradation systems, could be responsible for preventing the accumulation of defective ribosomal products that could cause aggregation (Alberti & Hyman, 2021). There is also competition between cellular processes and factors for essential components that may control which condensates can be formed and maintained at any given time (Alberti & Hyman, 2021). These components include ribosomal binding proteins (RBPs), which strongly interact with other proteins, and whose concentrations dramatically alter phase behaviours through their binding capabilities (Ditlev et al., 2018; Alberti & Hyman, 2021). Overall, there is a multitude of mechanisms that may be used to maintain the reactions of a condensate's constituents.

What may be even more incredible than a condensate maintaining its interactions between scaffold proteins and client molecules in the cytoplasm or the

nucleus, however, is a condensate maintaining its interactions between scaffold proteins and client molecules within another condensate (Boeynaems et al., 2018; Ditlev et al., 2018). These droplet within a droplet, inception-esque condensates are referred to as multi-phase droplets (Boeynaems et al., 2018). Recent work suggests that multiphase systems, such as multi-phase droplets, maintain specificity through different surface tensions, which could also serve as the basis of assembly and control in other membraneless organelles, such as the nucleolus (Boeynaems et al., 2018; Ditlev et al., 2018). Like singular condensates, these systems are also dependent on controlled and maintained scaffold protein and client molecule interactions, as well as the physical and chemical conditions of the cytoplasm or the nucleus and the condensate's constituents (Boeynaems et al., 2018; Alberti & Hyman, 2021).

Eventually, condensates must undergo dissolution or degradation. This is critical to the prevention of aggregation. Similar to the processes of condensate formation and maintenance, the physical and chemical conditions of the cytoplasm or the nucleus a condensate is formed and maintained within, as well as its constituents, have a considerable effect on the process of dissolution (Boeynaems et al., 2018; Alberti & Hyman, 2021). This is because these conditions dictate the saturation concentration—the concentration of protein at which LLPS will occur in a solution, forming a condensate (Alberti & Hyman, 2021). When a condensate has completed its biological function and it is time to undergo dissolution, a mechanism or process that changes the solution's saturation concentration must occur. This could be a charge inversion that solubilizes the condensate by "salting it in" (Alberti & Hyman, 2021). Or it

could be PTMs, altering the properties or availability of condensate-forming proteins and causing condensate dissolution (Ditlev et al., 2018; Alberti & Hyman, 2021). Alternatively, phosphorylations that decrease IDR interactions could be used to promote dissolution (Boeynaems et al., 2018).

In contrast, when a condensate becomes aberrant during formation or maintenance, it must also undergo dissolution or degradation to prevent aggregation. In a situation like this, the aberrant condensate may recruit PCQ factors that promote dissolution or degradation (Alberti & Hyman, 2021). Some of these factors may be autophagy-inducing factors, like p62, that can be incorporated into a condensate to induce autophagocytosis (Alberti & Hyman, 2021). In cases where autophagy-inducing factors are incorporated during aberration, the autophagy machinery of the innate immune system will selectively degrade the condensate (Alberti & Hyman, 2021). Interestingly, autophagy-inducing factors may also induce condensate formation, binding to ubiquitylated proteins to form an assembly (Alberti & Hyman, 2021). This indicates that condensates may be used to remove damaged, or misfolded proteins within our cells, preventing aggregation through inconceivably calculated foresight (Alberti & Hyman, 2021).

How are condensates studied?

Knowing about condensate formation, maintenance, and clearance, one might wonder how these processes, and condensates as a whole, are studied. There are

multiple approaches to examining the processes, properties, and interactions of condensates.

In a 2018 article, Mitrea et al. highlighted a variety of microscopy, spectroscopy, and -omics techniques, as well as a rheology technique for the characterization of phase-separated bodies and membraneless organelles. For morphological and quantitative characterizations of condensates, Mitrea et al. reviewed microscopy techniques ranging from light to fluorescence, including phase contrast imaging, fluorescence recovery after photobleaching (FRAP), and fluorescence loss in photobleaching (FLIP) (2018). Alternatively, for structural characterization of condensates, Mitrea et al. reviewed spectroscopy techniques ranging from NMR to a variety of fluorescence methods (2018). For compositional characterization of condensates, Mitrea et al. included -omics techniques in their review, like proteomics and transcriptomics (2018). Finally, Mitrea et al. reviewed rheology for the characterization of the material properties of condensates (2018).

In a different review, Ganser and Myong highlighted phase separation assays, FRAP, microrheology, droplet fusion, and surface tension techniques. They assert that phase separation assays, using microscopy, are capable of tracking droplets over time and assessing the effects of a variety of conditions, such as concentration, temperature, and pH, on droplet formation, droplet dissolution, and droplet miscibility (Ganser & Myong, 2019). In addition, they postulate that FRAP and microrheology uncover how molecules diffuse through droplets (Ganser & Myong, 2019). Finally, they also state

that droplet fusions assays, surface wetting and right-angle imaging, can aid in the determination of droplet surface tension (Ganser & Myong, 2019). It is clear that there is not a single, all-encompassing method to study condensates; chosen methodology must reflect the information one aspires to ascertain. It should also be noted that the majority of methods covered in the reviews by Mitrea et al in 2018, and Ganser and Myong in 2019, are only appropriate *in vitro* or in select *in vivo* environments.

A recent Focus Article in *Nature Methods*, however, describes four "Methods to Watch" for the study of condensates in living cells: 1, a microfluidic device that examines the intrinsic effects of protein sequence, and the extrinsic effects of protein concentration, salt concentration, and temperature on LLPS in a microenvironment; 2, an optogenetic platform that provides spatiotemporal control of intracellular mesoscopic phase transitions, using optoDroplets; 3, ultrafast-scanning fluorescence correlation spectroscopy, that determines molecular interactions and droplet viscosity; 4, a DNA assay that uses HP1α to induce LLPS in the nucleus (Tang, 2019).

The first method, generated by Simon et al., presents design rules for proteins with low complexity IDRs, which utilizes a microfluidic device to examine a condensate under changing internal and external conditions, allowing for programming and synthesis of self-assembly condensate models (2017). On top of this, method two includes an optogenetic platform, introduced by Shin et al. in 2017, which uses light to activate IDR-mediated phase separation, resulting in spatiotemporal control of condensates, as well as the ability to elucidate both physiological phase transitions and

their links to pathological aggregates. Furthermore, the third method, established by Wei et al., ultrafast-scanning fluorescence correlation spectroscopy, is a novel technique that can be employed to measure molecular interactions and protein concentrations within condensates (2017). Lastly, in 2017, two research groups identified HP1α as a protein that can be modified to induce LLPS in the nucleus, allowing for the study of chromatin domains and how they regulate nuclear functions (Larson et al., 2017; Strom et al., 2017).

What are the biological implications of condensates?

As prior mentioned, condensates can trigger or carry out important biological processes, ranging from regulating gene expression to forming SGs (Boeynaems et al., 2018). In addition, biomolecular condensates are presumed to be involved in convening resources for transcription, transducing chemical or physical signals, polymerizing actin, and many more cellular processes (Boeynaems et al., 2018; Alberti & Hyman, 2021; Leslie, 2021). But how?

Let's first explore how condensates are involved with heritable information molecules. Occurring in the nucleus, the regulation of gene expression, and transcription, are both essential to development (Sabari, 2020). Evidence suggests that the condensation of biomolecules fundamental to these processes may govern which genes are expressed, and when (Shin et al., 2019; Sabari, 2020). In 2019, Shin et al. used CasDrop optogenetic technology to demonstrate that proteins with IDRs phase separate in low-density, euchromatic regions, acting as mechano-active chromatin

filters. This indicates that nuclear condensates may be responsible for dynamic restructuring, including the formation of HP1-rich, silent foci (Shin et al., 2019). Interestingly, these same condensates often include gene-control machinery, including RNA polymerase II, pointing to a role in transcribing the active parts of the genome (Shin et al., 2019; Sabari, 2020). Evidence supports compartmentalization, or condensates, as a mode to ensure the hundreds of proteins and RNAs responsible for gene activation and expression gather in the right place, at the right time (Sabari, 2020).

Condensates are also involved with signal transduction, and actin polymerization (Su et al., 2016; Banani et al., 2017). In 2016, Su et al. used an *in vitro* system to monitor the clustered signaling molecules within T cell receptors. This research group found that linker protein for activation of T cells (LAT) clusters form through phosphorylation-dependent associations, promoting signaling output and increasing the activity of enzymes specific to actin polymerization (Su et al., 2016). The phosphorylation-dependent associations occur between the phosphotyrosines of three specific LATs; their clustering during association promotes signaling and increases enzyme activity by activating several downstream modules (Su et al., 2016). This is corroborated by the progressive, decreased clustering *in vitro* that was observed when one, two, or all three tyrosines were mutated experimentally (Su et al., 2016). Further, Banani et al. found that *in vitro* actin polymerization rates could be accelerated by concentrating the Arp2/3 complex and N-WASP into droplets on model membranes (2017).

Additionally, condensates are involved in the formation of SGs (Wheeler et al., 2016; Hofmann et al., 2021). SGs form upon sensing environmental stressors, incorporating proteins, and mRNAs stalled in translation in response to stress-induced ribosome and polysome disassembly (Hofmann et al., 2021). The molecular mechanism behind the formation of SGs is not well understood, however, it is thought to be primarily driven by LLPS—this is because LLPS droplets formed *in vitro* demonstrate fusion, shearing, and dynamicity properties, similar to those of SGs (Wheeler et al., 2016; Hofmann et al., 2021). This is a logical conclusion, given the IDRs within the large amounts of protein and mRNA that are contained within SGs (Hofmann et al., 2021). It should be noted that although SGs are presumed to aid the cell in its response to a stressor by binding large amounts of protein and mRNA, aberrant formation or disassembly resulting in pathological aggregation may occur as a direct result (Wheeler et al., 2016; Hofmann et al., 2021).

What are the biological implications of aberrant condensates?

Unfortunately, in addition to triggering or carrying out important biological processes and cellular functions, condensates may become aberrant, inducing negative effects. As previously insinuated, aberration is a mechanism by which aggregation may ensue. It often occurs through the misregulation of condensate formation, composition maintenance, and clearance. Aberration may arise via changes in physical or chemical conditions like PTMs, protein mutations and concentrations, and condensate ageing.

As previously noted, changes in physical or chemical conditions dictate the saturation concentration and may come about through charge inversions or phosphorylations (Alberti & Hyman, 2021). These changes alter the concentration at which LLPS will occur or be maintained in a solution, with direct implications for condensates (Boeynaems et al., 2018; Ditlev et al., 2018; Alberti & Hyman, 2021). Through misregulation, these same physical and chemical alterations may cause aberrant condensate formation, composition maintenance, or clearance (Boeynaems et al., 2018; Alberti & Hyman, 2021). This is because when the solubility limit of a saturation concentration is reached at an inopportune time, proteins aggregate and fall out of solution resulting in dense aggregates with fibril and amyloid-like morphology (Boeynaems et al., 2018; Alberti & Hyman, 2021). Moreover, given PTMs' importance in LLPS, and their role in changing physical and chemical conditions, it is also important to consider how they may contribute to condensate aggregation (Ambadipudi et al., 2017). It has been demonstrated that several protein aggregates display PTM signatures. For instance, tau phosphorylation is a strong indicator of pathology in Alzheimer's disease and is a PTM that has been demonstrated to promote aggregation *in vitro* (Ambadipudi et al., 2017).

Another way by which aggregation may arise is through genetic mutations or changes in protein concentrations. For example, some fused in sarcoma (FUS) or RNA binding protein (RBP) mutations may increase aggregation propensity (Alberti & Hyman, 2016). Some of these FUS and RBP mutations seemingly affect β-zippers in IDRs—pairs of β-sheets, with the side chains of the two facing sheets

interlinked—making them prone to fold into amyloid-like fibrils, reminiscent of β-amyloid plaques and tau tangles in Alzheimer's patients (Sawaya et al., 2007; Patel et al., 2015; Alberti & Hyman, 2016). Additionally, some mutations may lead to increases in RBP concentrations (Molliex et al., 2015). Recent experimental evidence suggests that if excess RBPs are accumulated during condensate formation, the additional attractive interactions will promote aggregation within the condensate through protein misfolds (Molliex et al., 2015).

Finally, condensate ageing may also promote aggregation through aberration. With ageing, high protein concentrations in tandem with the absence of their physiological binding partners, as well as low water content, are seen; it is plausible that post-translation modifications and molecular chaperones may not be capable of continuously maintaining condensates in these conditions (Boeynaems et al., 2018; Alberti & Hyman, 2021). Contrarily, because condensate ageing into a gel or glass-like state can also act as a "kinetic trap" for aggregated proteins, preventing the ageing of condensates could allow further aggregation to take place (Alberti & Hyman, 2021). Clearly, all of the above processes are delicate and may cause condensate aggregation when misregulated. This aggregation may lead to many other issues, including a variety of neurodegenerative diseases, such as Alzheimer's.

Condensates and Alzheimer's Disease

As indicated above, Alzheimer's is a progressive neurodegenerative disease that affects cognitive function, such as thinking and behaviour, and memory (CDC, 2021). It

is the most common cause of dementia, a term used in the medical field to describe a decline in cognitive abilities that affects a person's daily life (CDC, 2021). The etiology of Alzheimer's is unknown, but it is known that each case is a unique combination of genetic, environmental, and lifestyle factors (CDC, 2021). And although there are treatments available to help manage the symptoms and slow down the progression of the disease, there is no cure for Alzheimer's disease.

As we don't know the exact cause of Alzheimer's, the disease as a whole is difficult to characterize. Broadly speaking, however, it has some hallmarks:

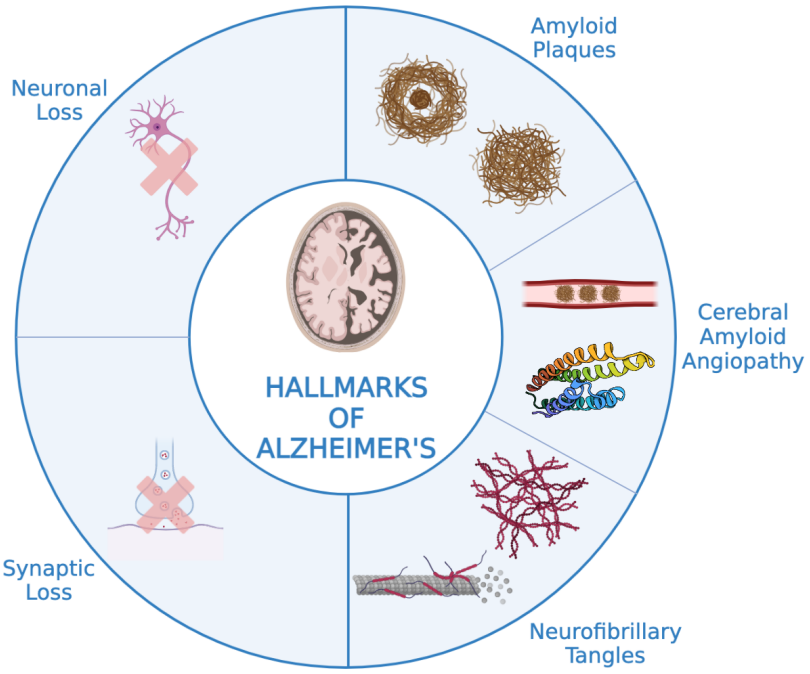

Figure 1. The hallmarks of Alzheimer's.

In a pathological sense, these hallmarks are negative lesions, and positive lesions (Serrano-Pozo et al., 2011). The term "negative lesions" refers to regions of

neuronal or synaptic loss, whereas the term “positive lesions” (Figure 2) refers to abnormal results that would show up on a PET scan, such as amyloid plaques, cerebral amyloid angiopathy, and neurofibrillary (or tau) tangles (Serrano-Pozo et al., 2011). These lesions all contribute to memory loss, difficulty communicating, behaviour changes, and eventually an inability to care for oneself (Serrano-Pozo et al., 2011). As the presence of positive lesions indicates either the presence or imminent presence of negative lesions, this paper will focus on positive lesions (Serrano-Pozo et al., 2011). Both amyloid plaques and tau tangles, from their amyloid-beta and tau derivatives, respectively, have demonstrated properties aligned with condensates, especially through their fibrillization processes.

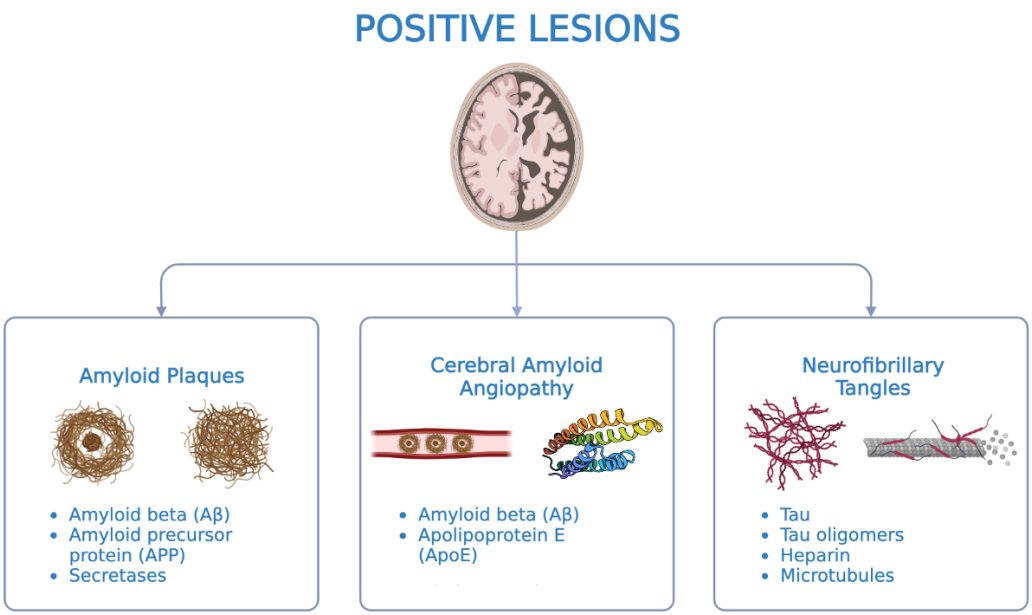

Figure 2. Positive lesions and their derivatives.

How do fibrils form?

Given that the fibrillization processes of both amyloid-beta and tau demonstrate condensate-like tendencies, it is pertinent to understand how fibrils form. Figure 3 exemplifies the fibrillization process of α-synuclein, which is a protein strongly associated with Parkinson's rather than Alzheimer's but will still allow for an understanding of the steric zipper motif. On the left side of Figure 3, in A, there are thousands of copies of a short segment, stacking to form a pair of beta sheets (Sawaya et al., 2021). B demonstrates a view that reveals the tight fit between the sidechains, and C shows that the sidechains have a zipper-like mating pattern which extends along the entire length of the fibril (Sawaya et al., 2021). D demonstrates the parallel and in-register stacking of the protofibrils via hydrogen bonds (dotted lines), and in E and F, it is apparent that this steric zipper formation liberates protein-bound water molecules, which contributes to fibril stability with a hydrophobic effect (Sawaya et al., 2021). From G-L, however, the extended protofibrils are stacked in sheets, with a slight twist; H demonstrates the same interaction as B but with longer chains (Sawaya et al., 2021). I and J are analogous to C and D, whereas K and L demonstrate the formation of seven steric zippers, not just one, creating an incredibly stable amyloid fibril (Sawaya et al., 2021).

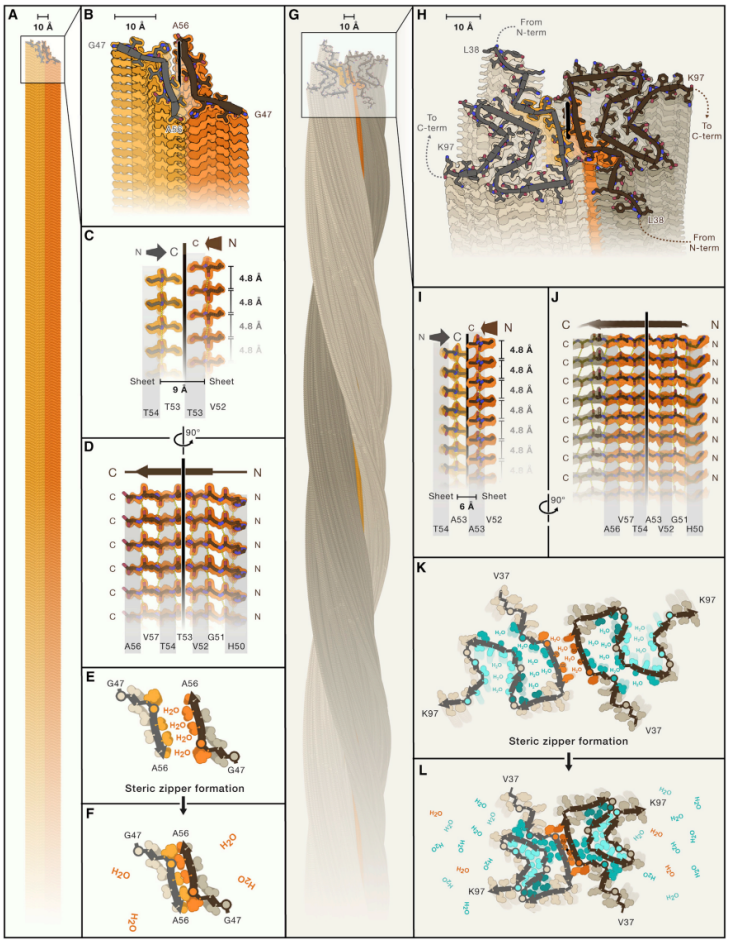

Figure 3. Fibril formation of α-synuclein (Sawaya et al., 2021).

This complex process involves both primary and secondary nucleation, where primary nucleation begins with the formation of small aggregates (metastable oligomers) from monomers and is continued with growth by monomer addition, and secondary nucleation is a reaction in which the formation of nuclei is catalyzed by existing seeds (pre-formed aggregates) composed of the same monomers (Sawaya et al., 2021).

Amyloid beta as a condensate

Amyloid beta protein (Aβ) is a 40–42 amino acid peptide (Chen et al., 2017). Naturally present in the brain, Aβ has garnered significant attention from the scientific community for its potential link to Alzheimer's. It is produced through the cleavage of amyloid precursor protein (APP), a cell surface receptor and transmembrane precursor protein, which is proteolytically cleaved by the enzymes β-secretase and γ-secretase (Chen et al., 2017; Calabrese et al., 2022). Normally, APP is cleaved by β-secretase after residue 671, generating sAPPβ, a long amino-terminal soluble fragment for extracellular release, and a corresponding cell-associated carboxy-terminal fragment (β-CTF) (Chen et al., 2017; Calabrese et al., 2022). β-CTF is then processed by γ-secretase, which catalyzes its intramembrane cleavage, yielding Aβ for secretion as well as APP intracellular C-terminal domain (AICD) (Chen et al., 2017; Calabrese et al., 2022). In the presence of some disease-associated mutations, however, APP cleavage into Aβ fragments increases (Calabrese et al., 2022). This is concerning because Aβ is highly hydrophobic with a multitude of IDRs and self-assembles via both primary and secondary nucleation (Thacker et al., 2020). And, because Aβ elongates via nucleation, it has a distinct propensity to oligomerize and deposit, forming amyloid fibrils, and ultimately insoluble amyloid plaques (Thacker et al., 2020).

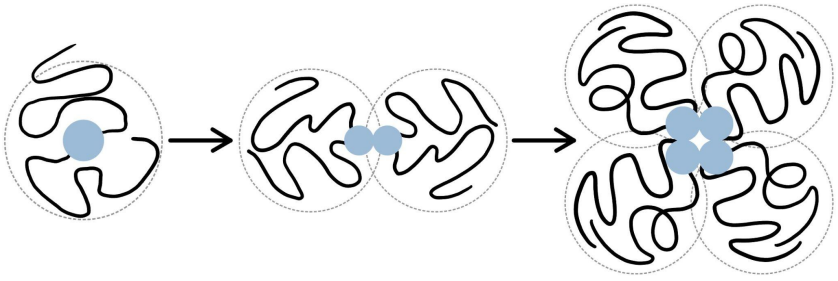

Figure 4. An illustration of Aβ nucleation.

Since Aβ is inclined to aggregate, the acquisition of pure samples for structural analyses has also proved difficult (Chen et al., 2017). Furthermore, its tendency to bind to lipids and other proteins makes it challenging to determine its precise interactions within the body (Chen et al., 2017). Despite these difficulties, however, recent advances have provided insights into Aβ's properties and role in Alzheimer's, revealing that Aβ adopts varying conformations depending on its surrounding environment (Connor et al., 2022). As prior stated, when Aβ aggregates, it leads to the formation of insoluble amyloid fibrils. These fibrils are the primary component of amyloid plaques, which accumulate between nerve cells in the brains of Alzheimer's patients, causing inflammation, which interferes with neuron function and triggers cell death, playing a key role in Alzheimer's disease's pathogenesis (Serrano-Pozo et al., 2011; Chen et al., 2017; Connor et al., 2022).

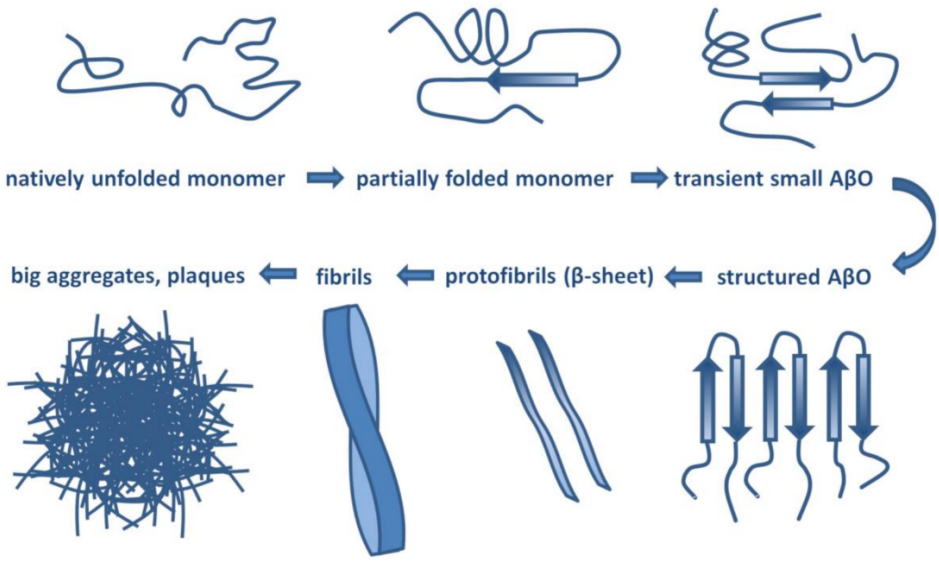

Figure 5. An Aβ aggregation pathway (Penke et al. 2020).

Figure 5 demonstrates the transition of Aβ from a monomer to a partially folded monomer, to a transient, small AβO, to a structured AβO, to protofibrils, and to fibrils (Penke et al., 2020). It is hypothesized that this formation is enabled by Aβ IDRs, lacking a stable 3D structure—these IDRs associate with other molecules and the structural "evolution" of Aβ peptides results in assemblies of growing size (Penke et al., 2020). This hypothesis takes volume interactions and hydrophobic interactions of Aβ's IDRs into account, forming a client/scaffold model for Aβ oligomerization through liquid-liquid phase-separation (LLPS) into condensates (Penke et al., 2020; Connor et al., 2022). This LLPS facilitates the further nucleation of Aβ. It has been found that different fractions of Aβ, including Aβ with varying N-terminal amino acid sequences, have different LLPS critical concentrations, once again demonstrating Alzheimer's uniqueness across individuals (Penke et al., 2020).

It should be noted that Aβ aggregation also contributes to cerebral amyloid angiopathy (CAA), as a result of amyloid-beta plaque buildup in the cardiovascular system (Morrone et al., 2020). In physiological conditions, soluble Aβ is cleared from the brain into vessels along the perivascular spaces and lumen of arteries and veins by Apolipoprotein E (ApoE) (Morrone et al., 2020). 191 amino acids in length and containing multiple amphipathic alpha helices, ApoE enhances the proteolytic breakdown of Aβ both within and between cells (Morrone et al., 2020). The ApoE-ε4 allele, however, is known to be associated with CAA, in particular, as the isoform is not as effective at promoting Aβ proteolytic reactions (Morrone et al., 2020). This genetic variation results in increased vulnerability to CAA, as in pathological arteries, Aβ is

deposited along the smooth muscle cells and endothelial cells, forming a 'double-barrel' morphology that causes arterial smooth muscle and endothelial cell loss (Morrone et al., 2020). Additionally, in pathological veins, Aβ deposition forms globular deposits in perivascular spaces, contributing to extensive venous collagenosis along venular walls, significant enlargement of the perivascular space, and endothelial cell loss (Morrone et al., 2020). Overall, Aβ buildup, in blood vessels especially, can lead to CAA, playing a key role in Alzheimer's disease's pathogenesis.

Tau as a condensate

In addition to the major contribution of Aβ in AD, the tau protein is another key peptide that has sparked scientific inquiry. Tau protein is a component of the central nervous system, which serves as a crucial contributor to the maintenance of neuronal structure and function (Mandelkow & Mandelkow, 2012). Encoded by MAPT on chromosome 17, tau protein is expressed predominantly in neurons, where axons are its principal location of accumulation (Boeve & Hutton, 2008). This is because tau is a microtubule-associated protein, responsible for stabilizing microtubules in neurons, which facilitate the transportation of neurotransmitters along axons (Mandelkow & Mandelkow, 2012). Recent studies also suggest that tau protein may have functions outside of microtubules, such as synaptic plasticity (Biundo et al., 2018). To function properly, tau undergoes a plethora of PTMs, including but not limited to, phosphorylation, acetylation, glycosylation, and ubiquitination (Calabrese et al., 2022). These modifications significantly impact the protein's function, stability, and interaction with other proteins.

Sometimes, however, PTMs are negatively impactful and cause tauopathies, neurodegenerative diseases characterized by the accumulation of abnormal forms of tau; in Alzheimer's, hyperphosphorylation leads to tau protein detachment from microtubules, oligomerization, and ultimately the formation of neurofibrillary tangles (NFTs) (Calabrese et al., 2022). As tau protein aggregates into NFTs in several neurodegenerative diseases, it remains an area of active research.

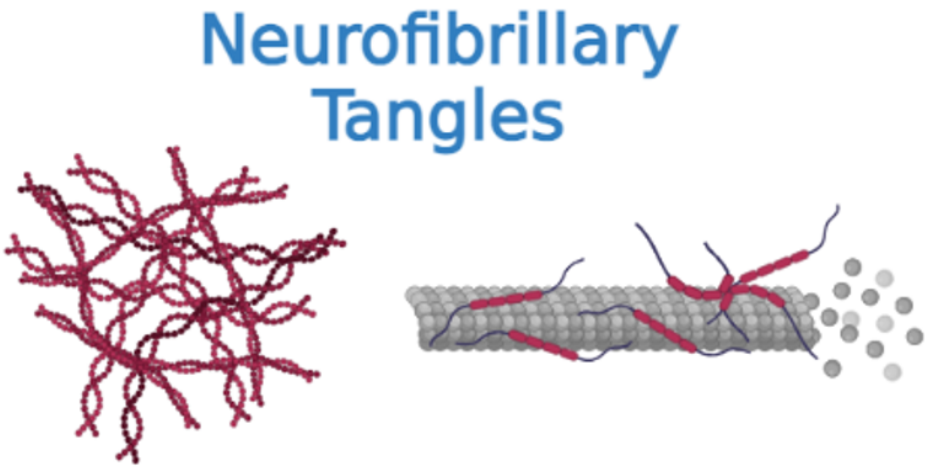

Figure 6. Tau tangles.

NFTs or tau tangles are oligomers of tau protein that accumulate within nerve cells (Brion et al., 2001). They are thought to be toxic and interfere with the brain's normal functioning. In Alzheimer's patients, aggregation of tau results in paired helical fragments (PHFs), and these PHFs can further accumulate into intracellular NTFs (Calabrese et al., 2022). Even though the exact mechanism of tau aggregation is still unclear, its accumulation is considered to play another major role in Alzheimer's pathogenesis. It is believed that tau fibril formation occurs under an array of

physicochemical conditions, which can be mediated by heparin (Ambadipudi et al., 2017).

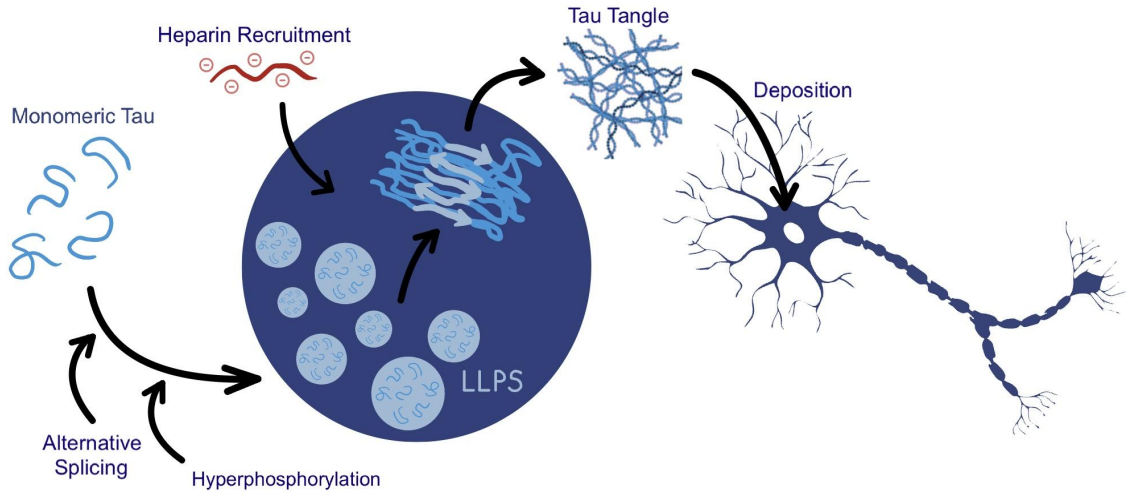

Figure 7. The process of tau fibrillization to tau tangle deposition.

Similar to Aβ, tau also has IDRs and ultimately becomes fibrillated through LLPS (Ambadipudi et al., 2017). However, this process is unsurprisingly convoluted. As such, it can be visualized in Figure 7. It is hypothesized that this process begins when tau is alternatively spliced, and then hyperphosphorylated (Ambadipudi et al., 2017). As we know, phosphorylation events can modulate protein-protein interactions, and thus the aggregation propensity of tau, in a complex manner. For instance, phosphorylation involving glycogen synthase kinase-3 (GSK3), casein kinase 1/2 (CK1/2), leucine-rich repeat kinase 2 (LRRK2) and Fyn have been shown to promote protein-protein interactions, propagating LLPS events in tau by lowering its critical concentration (Calabrese et al., 2022). It appears that LLPS is a driving factor, used to increase the local concentration of tau to supersaturation (Ambadipudi et al., 2017). At

supersaturation, these tau droplets recruit negatively charged heparin, via their many lysine-rich microtubule binding domains, which in turn promotes fibrillization (Ambadipudi et al., 2017). In addition to the polyanion heparin, a laser capture microdissection found 72 proteins within NFTs, that may influence tau accumulation (Ambadipudi et al., 2017).

Condensate-based Alzheimer's treatments

So, can we treat Alzheimer's with a condensate-based methodology? Many researchers are attempting to do so. One promising condensate-based treatment involves the use of miniprotein inhibitors as a mechanism of seeding prevention (Murray et al., 2022). Recent advances have enabled amyloid fibril structures to be determined with an atomic-level resolution via cryoelectron microscopy, microelectron diffraction, and solid-state NMR, allowing for the possibility of structure-based inhibitor design (Murray et al., 2022). Through de novo protein design using a computational method, researchers have developed a library of miniprotein inhibitors of 35 to 48 residues that target the amyloid structures of tau, and Aβ (Murray et al., 2022). These miniproteins bind to the ends of amyloid fibrils, "capping" them and preventing further growth (Murray et al., 2022). Furthermore, biophysical characterization of in silico-designed inhibitors shows that "they form stable folds, have no sequence similarity to naturally occurring proteins, and specifically prevent the aggregation of their targeted amyloid-prone proteins in vitro" (Murray et al., 2022).

In vitro, this methodology has exhibited excellent results. The inhibitors halt protein aggregation into fibrils and halt the ability of fibrils to induce or “seed” fibril growth in other cells (Murray et al., 2022). Inhibition of primary nucleation was demonstrated through the use of ThT kinetics assays, and secondary nucleation in cell assays (Murray et al., 2022). In vivo evaluation has also revealed their ability to reduce aggregation and decrease motor deficits in *C. elegans* models of Alzheimer’s disease (Murray et al., 2022). Already considered a promising eventual therapeutic, an additional advantage of miniproteins is that they could be genetically encoded and delivered to diseased brains by viral vectors or brain-penetrating nanoparticles (Murray et al., 2022). Miniprotein inhibitors’ stability, size, and ease of expression are preferred over established antibody therapeutics, and their adaptability and designability further distinguish them from current therapeutics (Murray et al., 2022). Further improvements to the inhibitor designs presented thus far could improve binding affinity, as well as delivery method (Murray et al., 2022).

Another potential treatment comes from a paper that describes 16 rounds of laboratory evolution, which led to the generation of a bacterial transpeptidase sortase A (SrtA) that can mediate the covalent modification of Aβ peptides (Podracky et al., 2021). SrtA is an epitope-specific enzyme, which provides the ability to induce site-specific protein modifications of Aβ (Podracky et al., 2021). Naturally, Aβ monomers contain a five-amino-acid sequence (LMVGG) that shares features with SrtA’s native recognition site, and this is what allows SrtA to recognize and modify endogenous amyloid-β (Aβ) protein. Using a yeast display selection, the lab group evolved a SrtA variant that

prefers LMVGG substrates with a >1,400-fold change in substrate preference from a starting enzyme that preferred LPESG substrates (Podracky et al., 2021). This was done by conjugating the yeast display sortase variants to triglycine peptides with N termini free for sortase-catalyzed reactions, incubating the library with an N-terminally biotinylated target substrate and non-biotinylated off-target substrates, isolating the yeast cells with biotinylated surfaces and staining them with fluorophore-linked streptavidin before growing them up and subjecting them to enrichment by fluorescence-activated cell sorting (FACS) before mutating them further (Podracky et al., 2021). The overall evolved SrtA can be used to label endogenous Aβ in human CSF, enabling the sensitive detection of Aβ (13-56%) at a level rivalling other commercial assays (Podracky et al., 2021).

Furthermore, it was shown that the evolved SrtA is capable of conjugating a hydrophilic peptide to Aβ, impeding its ability to aggregate into higher-order structures (Podracky et al., 2021). Purified Aβ was treated with SrtAβ and 200μM of GGGRR, replacing the last five residues of Aβ (GVVIA) with GGGRR, yielding a more hydrophilic protein (Podracky et al., 2021). The new Aβ with GGGRR, as confirmed by mass spectroscopy and high-performance liquid chromatography, had a lower aggregation propensity when assayed with continuous thioflavin T (ThT) binding assay; SrtAβ-modified peptides were found to take much longer to nucleate into aggregates, taking approximately 40-fold longer to reach half-maximal aggregation (Podracky et al., 2021). These results demonstrate potential future applications of sortase-mediated labelling of Aβ peptides, which could include but are not limited to, generating new

Aβ-conjugates for vaccine development, conjugating peptides to endogenous Aβ in human CSF, attaching fluorophores to Aβ for imaging, or tagging Aβ to inhibit aggregation or mark them for degradation (Podracky et al., 2021).

Finally, as tau hyperphosphorylation is a key feature of Alzheimer's disease pathology, leading to the formation of neurofibrillary tangles in the brain, several kinases have been identified as potential targets for therapeutic intervention to prevent or reduce tau aggregation (Calabrese et al., 2022). One suggested approach is to target the kinases that promote tau phosphorylation. For example, inhibiting glycogen synthase kinase-3 (GSK3) has been shown to reduce tau phosphorylation and aggregation in animal models of Alzheimer's (Toral-Rios et al., 2020; Calabrese et al., 2022). In addition, inhibition of Fyn kinase has also been proposed as a potential therapeutic target for AD (Briner et al., 2020). A different approach is to target the protein interactions that contribute to tau aggregation, such as interactions promoted by cyclin-dependent kinase 5 (CDK5) (Seo et al., 2017). Inhibiting CDK5 has been shown to restore long-term potentiation in mice, indicating that it may mitigate the pathological development of Alzheimer's disease (Seo et al., 2017). Unfortunately, finding effective compounds that can specifically target the molecules of interest while minimizing unintentional effects remains a challenge.

Conclusion

To summarize, biomolecular condensates have emerged as an exciting area of research that has broad implications for our understanding of cellular processes and

disease. As discussed in this review, condensates are dynamic, membraneless organelles that concentrate proteins and other biomolecules through liquid-liquid phase separation to play critical roles in diverse cellular processes, from regulating gene expression to forming SGs. While our understanding of condensates is still evolving, we have made significant strides in understanding how they are formed, maintained, and cleared. Studies have identified specific proteins and domains that promote or inhibit phase separation, and there is growing evidence that post-translational modifications, such as phosphorylation, can also regulate condensate formation. Furthermore, novel imaging and biochemical techniques have allowed researchers to study these structures in unprecedented detail, providing new insights into their functions, properties, and responses to different stimuli, while proteomic approaches have provided a wealth of information about their composition and organization.

While condensates play critical roles in normal cellular processes, aberrant condensates have been implicated in a range of human diseases, including neurodegenerative disorders such as Alzheimer's disease. Studies have shown that Aβ and tau, proteins that aggregate pathologically in Alzheimer's disease, form as condensates. This has led to the development of novel therapeutic approaches aimed at disrupting Alzheimer’s-related condensates to prevent or reduce fibril formation. Overall, biomolecular condensates represent a fascinating frontier in our understanding of cellular processes and disease. By studying these structures, we may gain new insights into the molecular mechanisms that underlie health and disease, and ultimately develop new treatments for a range of human illnesses. As of right now, we are a long

way off, but the field is expanding exponentially. More and more scientists are working to understand biomolecular condensates every day, but I think we need to bring it back to the basics first, and that all starts with a droplet of oil in water.

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