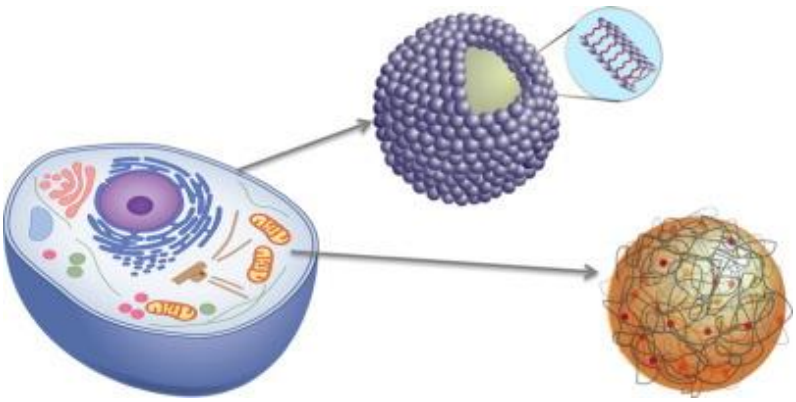

Function of membraneless organelles in cells

ABSTRACT
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Summary

In this thesis the concept of membraneless organelles and their function in cells will be discussed. First, we will emphasize on explaining how the formation of membraneless organelles occur. There are two main factors that drive the formation of these organelles: IDRs and cation-pi or pi-pi interactions. IDRs consist of long repetitive motifs and sequences with low complexity. They contain mostly charged residues and some hydrophobic residues. These charged residues are responsible for electrostatic repulsion and attraction within and between the proteins that form the coacervates. The formation of MLOs is regulated by post translational modifications. Secondly, the role of MLOs in signal transduction will be discussed. In neurons, MLOs form in the cell body upon synaptic activation. They contain mRNAs, phosphatases and kinases and travel to the axonal and dendritic parts, where these RNAs are released and further translated. The mRNAs are thought to be protected from decapping and exonuclease activities by either the existence of different compartments within a MLO where the mRNAs are shielded or the switching off of the molecules responsible for this by post translational modifications. Furthermore, we will study how MLOs can be involved in gene expression. In some pathways MLOs are involved in gene suppression and in others in gene overexpression. In certain viruses like Epstein-Barr virus phase separation is also used for the enhancement of gene expression. Finally, it is explained how dysfunctional MLOs can form and cause neurodegenerative and infectious diseases and cancer. These dysfunctional coacervates are formed due to changes in the mechanism of formation, due to changes in the molecules that regulate the phase separation or due to changes in the physicochemical condition of the cell. In certain neurodegenerative diseases these forms of dysfunctional membraneless organelles are found and it has been shown that this can cause different dynamic, composition and size of the granules. The MLOs could also transition from liquid-like to solid-like and form aggregates due to mutations. In certain types of cancers dysfunctional MLOs can alter signalling pathways and affect gene expression. In addition to that, viruses use MLOs to replicate their genome and protect them from the immune system of the host.

Table of content

Introduction

Coacervates are formed by liquid-liquid phase separation (LLPS) and are dispersed droplets of a dense phase in a dilute aqueous phase. This dense phase is rich in macromolecules like nucleic acids and proteins. The process is called liquid-liquid phase separation because both the dilute phase and the droplets of the dense phase have liquid-like properties. This process is divided in two parts, simple coacervation and complex coacervation. In simple coacervation only one macromolecule is involved and in complex coacervation multiple macromolecules, mostly polyelectrolytes with opposite charges, are involved [1]. LLPS has been a topic of interest for many years now in science. One of the first articles reporting about this phenomenon dates back to the 1930s [2]. It was thought that the phenomenon of phase separation which exists in macromolecules, was the first step in the origin of life. This was important in the development of protocells, which are proposed to be crucial for the origin of life. In 2009 Brangwynne et al. suggested that the formation of coacervates by liquid-liquid phase separation plays a role in the development of membraneless organelles. Formation of coacervates by LLPS was first observed in Cajal bodies and P granules [3]. Nowadays, it is widely believed that coacervates play an important role in many biological processes. In marine biology for example, extracellular formation of coacervates in mussels is key for creating strong adhesion in order to withstand the rough sea and the tides. Intracellularly, coacervates play an important role in compartmentalisation, vesicle formation and self-assembly [4]. Finally, coacervation is also used in biophysics, biomaterials and the food industry [5].

Phase separation and formation of coacervates encompass many different aspects. In this thesis we will focus on one form of coacervates: membraneless organelles (MLOs). MLOs often consist of proteins and nucleic acids and can be found in both cytoplasmic and nucleoplasmic environments. They can be classified as biomolecular condensates, which means their composition is not dependent on a bounding membrane. Classic membrane-bound organelles are separated from their surroundings by lipid bilayers, while MLOs are not and are held together mostly by weak intermolecular interactions [6]. To fully understand MLOs we will try to answer the following research question: What are the functions of membraneless organelles in cells? For this purpose, we will examine four different aspects of MLOs. Firstly, we will discuss the formation of MLOs and the underlying mechanism. This includes the components that are required in order to successfully form MLOs and the conditions in which the mechanism is activated. In addition, it has already been shown that MLOs play a role in the signal transduction of neurons and therefore we will examine next the role of MLOs in signal transduction [7]. Another important function of MLOs is their function in gene expression. It has been shown that MLOs can amplify as well as suppress gene expression [8,9]. Therefore we will discuss the procedure of these mechanisms, in which situations certain genes are amplified and in which certain genes are suppressed. MLOs are also linked to some diseases when they do not function as expected due to different causes [10]. Therefore, in the last part we will discuss what causes this dysfunction of MLOs and additional consequences. By treating all these different aspects we hope to provide a comprehensive review on some interesting aspects of MLOS.

Formation of MLOs

Over the past couple of years different membraneless organelles have been found in the cell such as nucleoli, germ granules, pericentriolar material, stress granules and P bodies. Besides the fact that these MLOs are not surrounded by a lipid bilayer, they are also able to respond rapidly to environmental changes. The reason for this is the liquid-like properties that MLOs possess. In 2009 Brangwynne et al. showed that MLOs behave like liquid droplets instead of granules when interacting with the nuclear envelope [3]. A decade later other researchers tried to find the factors that drive these MLOs to phase separate.

One of these factors is the interactions of intrinsically disordered regions IDRs with other components of MLOs [11]. IDRs are protein domains that lack stable conformational structures and consist of repetitive motifs and sequences with low complexity [12]. Research has shown that about 44% of the human genes that code for proteins contain IDRs longer than 30 amino acids [13]. Brady et al. found that IDRs have no stable structure because they usually contain many charged and polar residues and some bulky hydrophobic residues [14]. The charged residues can be both positively and negatively charged, which is usually evenly distributed in an IDR. The electrostatic repulsion within an IDR, that is between its residues, causes the disordered structure. These ever changing conformations of IDRs appear to be an ideal microenvironment with solvent properties for very specific substrates like proteins and RNAs. Within an IDR charged residues cause electrostatic repulsion, but the charged residues can also undergo electrostatic interactions with oppositely charged residues from other IDRs or nucleic acids, these intermolecular interactions promote phase separation. It was also found that a lot of Arg-Phe pairs are formed between IDRs which are involved in phase separation. This indicates that another form of intermolecular interactions is also involved in phase separation, namely pi-pi and cation-pi interactions. These two forms of molecular interactions are thought to be the main driving forces of phase separation. Studies have shown that in vitro phase separation of IDRs is more rapid than in vivo. In addition, besides phase separation to liquid-like droplets, IDRs are also responsible for phase separation of droplets with properties ranging from hydrogels to crystals and amyloid fibers [15].

To regulate the formation of MLOs and prevent unnecessary formation, post translational modifications are used. Three of the most common post translational modifications are phosphorylation, acetylation and methylation and are found to be used as a mechanism for the tight regulation of MLOs. Kim et al. studied two proteins, CAPRIN1 and FMR, which both contain an IDR and are involved in the formation of membraneless organelles [16]. They found that phosphorylation affected this process. When both proteins or neither protein was phosphorylated, phase separation was lower. But when one of the proteins was phosphorylated, it was found that phase separation was increased. The IDRs of both proteins are rich in positively charged Arg residues and it is thought that this causes repulsion, but this repulsion disappears when the Arg residues are phosphorylated. Also in this paper it was shown that pi-interactions were important for phase separation. When the Tyr and Phe residues of the CAPRIN1 protein were phosphorylated, an increase in phase separation was observed when CAPRIN1 was paired with unphosphorylated FMRP. A decrease in phase separation was

observed when the protein was paired with phosphorylated FMRP. Acetylation and deacetylation is also important in the formation of stress granules [17]. They are formed when a cell is under environmental pressure and contains mRNA molecules that are stalled before initiation of their translation. Saito et al. found that acetylation of Lys rich regions in the IDRs of proteins involved in LLPS, impaired the LLPS. It was suggested that this was due to the neutralization of the positively charged residue which results in the loss of the intermolecular interaction between the cationic Lys and the anionic residues or the residues with aromatic pi-interaction. Formation of stress granules was observed when adding a deacetylase.

The role of MLOs in signal transduction

MLOs respond to environmental changes. This is done by activating or regulating signalling pathways. One of the first discoveries that connected MLOs to signal transduction was the release of mRNA by MLOs [18]. These mRNAs usually contain codes for signalling molecules and are stored in MLOs, like granules, in a state in which they are ready or almost ready for translation. Responding to environmental fluctuations sometimes requires a rather rapid process and one particular type of cell in which rapid signal transduction is required and that makes use of MLOs are neuronal cells. Formicola et al. found that synaptic activation and extracellular stimuli induced signaling events involving MLOs in the axonal and dendritic parts [7]. It was shown that in response to these activations and stimuli neuronal ribonucleoproteins (RNPs), granules were formed. These RNPs were formed by RNA-RNA, protein-RNA and RNA-protein intermolecular interactions as is depicted in figure 1. Also it was found that IDRs of proteins were involved in the formation of the granules. It is thought that these RNPs are assembled in the cell bodies of neurons since there the concentration of granule components and RNA is high. Then, the granules travel to the axonal and dendritic parts of the neurons. Depending on the specific synaptic activation or extracellular stimuli the RNPs contain different mRNAs for different compartments of the cell. Upon arriving at their destination they release the mRNAs, which are then locally translated and induce the necessary changes in the cell.

What is still unknown about the released mRNAs is how they are protected from decapping and exonuclease activities when stored in the granules. Namely, it has been shown that molecules possessing these characteristics are present in these RNPs. There is still more research needed to understand this mechanism and its possible effect on signal transduction. At the moment there are two hypotheses which have the most evidence based support [19]. In the first one, it is thought that granules have different areas which can be separated so the enzymes will not react with each other. When certain signalling pathways need to be activated or deactivated these areas can become more permeable upon stimulation of other molecules which can interact with each other. The second hypothesis states that these enzymes in the granules can be regulated by phosphorylation and dephosphorylation by posttranslational modifications. In certain instances the released mRNAs are also involved in suppressing or enhancing gene transcription, which will be discussed in detail in the next section.

Zhang et al. describe that besides mRNA, MLOs can also contain protein kinases and phosphatases [20,21]. These signalling molecules were observed in P-bodies and different types of granules and are often involved in cell survival and growth. It is believed that in MLOs these molecules are involved in the cells response to stress via signal transduction. In stress granules, mentioned in the previous section, different signal molecules were observed. One of these molecules is RACK1, a scaffolding protein that plays a role in the activation of MTK1, a MAP kinase [22]. MTK1 regulates the activation of two downstream mitogen kinases. When a cell is under a certain type of stress, MTK1 reduces the level of apoptosis. By Thedieck et al. it was found that by recrutement of another protein, Raptor, programmed cell death can be avoided [23]. Namely, this protein is a key component of a signalling complex. When this Raptor is recruited by the granules, completion of this complex is not possible and programmed cell death is stopped. Many protein kinases that have been observed in P-bodies, are involved in many different processes of the cell and when it comes to MLOs, they are also involved in several steps of the meiotic process [24]. When meiosis starts the protein kinases localized to the P-bodies to protect them from degradation. If this localization fails, proteasomes will degrade the protein and the cell will not be able to fully complete its meiosis. Besides a role in signal transduction, MLOs also have a role in gene expression and this will be discussed in the next section.

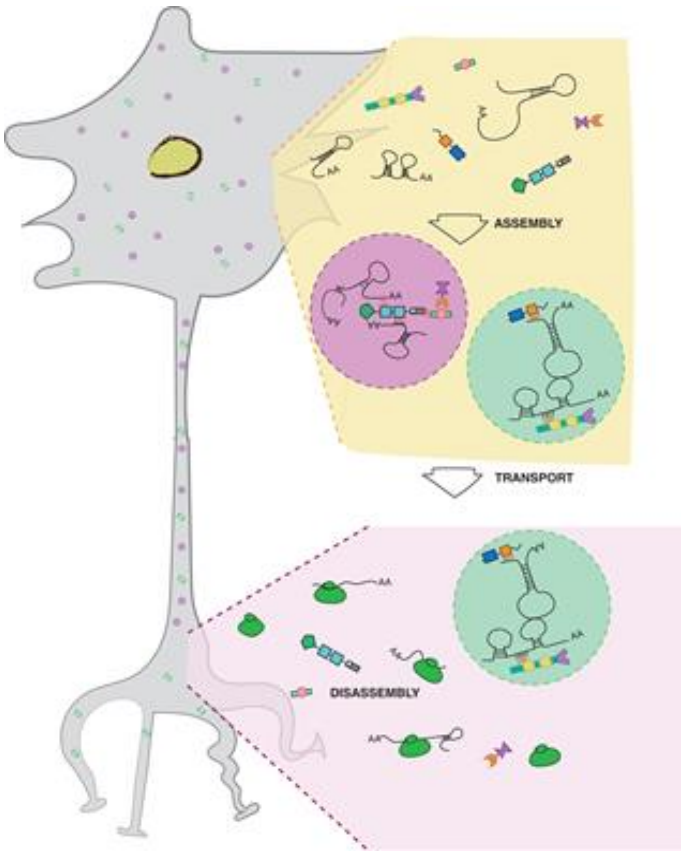

Figure 1. Assembly of the neural RNPs driven by protein-protein, RNA-protein and RNA-RNA molecular interactions. The RNPs originate in the cell body and transport to other parts of the cell to disassemble and release mRNAs. The coloured rectangles are protein domains, multiple rectangles together form proteins that interact with RNA molecules to form MLOs. The green ovals are ribomsomes that translate the releases mRNA.

The role of MLOs in gene expression

MLOs are involved in the amplification and suppression of different genes. Although further research is required, this could be important in genetically related diseases. In this section we will discuss a few of these mechanisms involved in gene suppression and amplification. The first is a mechanism concerning gene silencing by piRNA in *Caenorhabditis elegans* described by Suen et al. [8]. It was shown in previous studies that small non-coding RNAs are found to have sites on MLOs for gene silencing [25,26]. piRNA is such a small non-coding RNA molecule involved in a specific pathway called after the molecule. piRNAs are usually involved in the repression of transposable elements (TE). For this repression interaction with proteins from the Argonaute family are necessary [27]. In *C. elegans* piRNA interacts with a protein of that family and targets mRNAs [28]. Suen et al, tried to discover the relation between this complex formed by these two proteins and MLOs. Their experiments showed that the Argonaute protein has a binding site for a certain protein. This protein was involved in the formation of P granules. When the binding site on the Argonaute protein was mutated, less P granules were formed and gene silencing by piRNA was decreased significantly. These experiments show that MLOs might play a vital role in the silencing of certain genes.

The next mechanism that plays a role in the amplification of genes is the mechanism of nucleated transcriptional MLOs in gene amplification, which was investigated by Wei et al. [29]. These nucleated transcriptional MLOs, also referred to as nucleated transcriptional condensates, have been shown to contain proteins with IDRs and proteins with binding sites for nucleic acids and in that way promote phase separation [30,31]. They are also thought to be involved in gene expression by forming these MLOs at certain parts of the genome and with that influence bimolecular interactions. In their research, Wei et al. examined three transcriptional regulators which are hypothesised to phase separate and enhance gene expression. One of the three proteins that was tested for phase separation in nucleoplasm was three times lower than in cytoplasm, meaning phase separation of that protein took place more in the nucleus. This was due to the charge distribution of the protein within the IDR. This charge distribution strengthened the interaction of the molecule with polymerase II (Pol II). The molecule interacting with Pol II showed a higher nucleation rate and thus faster formation of the MLO when Pol II was not present. Finally, a significantly larger gene expression was observed in cells with this polymerase-complex compared to cells in which it was missing, indicating amplification of gene expression.

The third mechanism is related to amplified gene expression in cells, but in this case is caused by a virus. Peng et al. studied this for the Epstein-Barr virus (EBV) [32]. EBV infection has been linked with different types of cancer like lymphoma and gastric cancers [33,34]. Moreover, two transcription factors encoded by EBV are used to enhance gene expression of the genes which are related to cell survival and growth in infected cells [35]. These two transcription factors are co-expressed and seem to participate in LLPS. Both proteins contain IDRs which promote phase separation due to the formation of electrostatic interactions. A second factor that drives phase separation of these molecules has to do with the large number

of prolines they contain. It was observed that when these prolines were replaced by arganines, phase separation was almost completely absent. Proto-oncogenes and genes coding for transcription factors are targets of these two transcription factors. The complex of the two transcription factors was shown to recruit host co-activators and transcription factors, form coacervates with them at superenhancer sites of the genes and increase their expression. This process is schematically depicted in figure 2. Superenhancer sites are sites where multiple transcriptional enhancers are clustered together and form coacervates together with co-activators and can be amplified significantly. In this last example we see that functional coacervates can cause diseases, but we will discuss the role of dysfunctional coacervates in diseases in the next section.

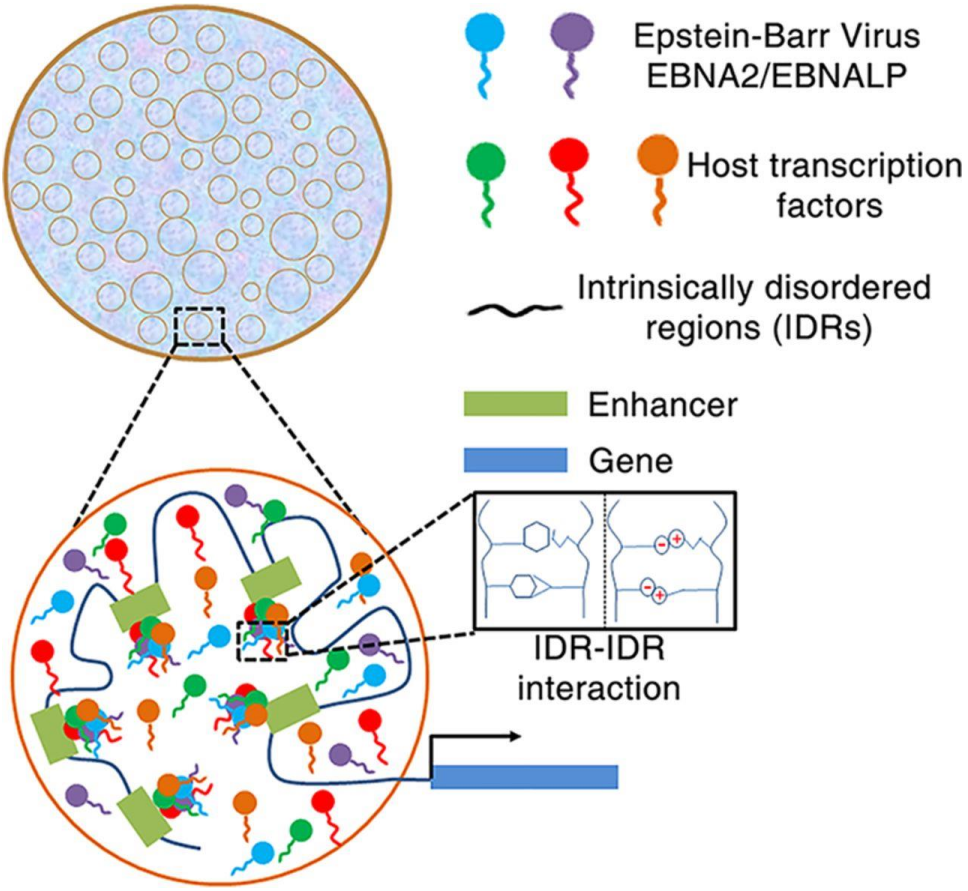

Figure 2. Schematic model of the formation and the components of MLOs. The two transcription factors of EBV recrute host transcription factors and co-activators at superenhancer sites to increase gene expression.

Dysfunctional MLOs and diseases

In the previous sections we talked about what coacervates are, how they form and what their functions are in signal transduction and gene expression. However, several dysfunctions could occur which can be harmful to humans and cause diseases. These are mostly neurodegenerative diseases like amyotrophic lateral sclerosis (ALS), Frontotemporal Dementia (FTD) and Alzheimer Disease (AD) but it has also been shown that can be infectious diseases or cancers

[36]. Deviated MLOs that lose their function or gain unwanted functions have been linked to these diseases [37,38]. Alberti et al., propose three different ways on how these dysfunctional MLOs can form [37].

First, it is hypothesised that changes in the mechanism that is used to form MLOs can result in dysfunctional MLOs. Genetic mutations could change the composition of proteins involved in the formation of MLOs. The electrostatic properties of these molecules can be changed. This will affect the intermolecular interactions within the coacervates that could affect its morphology, size or number. In addition, mutations can influence the solubility or localization of a MLO. As described in the previous section, some MLOs have a function in a specific part of the cell and some MLOs have different solubility in different parts. The second hypothesis is that an alteration in molecules that regulate phase separation is the cause. An example is the overexpression or silencing of a kinase, which is important in the formation of MLOs by post translational modifications. Missexpression of molecules that are part of the coacervate and are key to the nucleation of these MLOs can also have severe consequences and result in premature or modified MLOs with different material properties. Thirdly, it is hypothesised that a shift in the physicochemical conditions of the cell can cause disease related phenotypes. Parameters such as pH, osmotic regulation, salt concentration and energy metabolism are all important for the formation of MLOs [39]. So changes in these conditions can have fatal consequences for the formation, state and properties. These three hypotheses have also been depicted in figure 3.

In ALS and FTD some of these hypotheses of dysfunctional MLOs have been proven to be present. Mutations in RNA-binding proteins like TDP-43 and FUS cause accumulation of these molecules in stress granules [40,41]. This results in different dynamic, composition, size and of the granules. In addition to that, it has been suggested that these deviated stress granules are precursors of protein aggregates, the main cause of neurodegenerative diseases, which was found in post-mortem brains of patients suffering from ALS and FTD [42]. Mutations in the IDRs of FUS have also been proven to cause over time a shift from coacervate droplets with liquid-like properties to solid-like aggregates. The nuclear localisation signal of FUS can also be mutated, this causes the protein to have less binding affinity to nuclear import receptors. This results in accumulation and phase separation of the protein in the cytoplasm instead of the nucleoplasm and in favors the liquid-like droplets to transform to solid-like aggregates. Finally, PTMs can also be a cause for ALS and FTD [43]. In AD a protein that binds microtubules, is often mutated or wrongly post translationally modified. Under normal conditions this nucleates microtubules locally so they can undergo phase separation but with these mutations and PTMs they are shown to form aggregates.

As discussed in a previous section, MLOs are important for signalling pathways. Aberrant MLOs have been proposed to play an important role in the alteration of some signalling pathways that are related to cancer. One example is demonstrated in a paper by Bouchard et al., where it is shown that a tumor suppressor involved in the ubiquitination and degradation of oncogenic proteins, is mutated in certain types cancers [44]. Due to these mutations it fails to phase separate, localize to the nucleoplasm and the interactions between the substrates are disrupted. As was seen for EBV, certain cancers are also caused by phase separated coacervates

comprising different transcriptional factors located at superenhancer sites [45]. Besides enhancing gene transcription, in viruses MLOs are also thought to be used for the replication of the genome and other components of the virus and for evading the host's immune system. In Rabies for example, the viral coacervates are formed in the cytoplasm of the infected cells and this phase separation is promoted by the IDR of the P protein of the virus [46]. In these MLOs it has been shown that replication of the genome of the virus takes place.

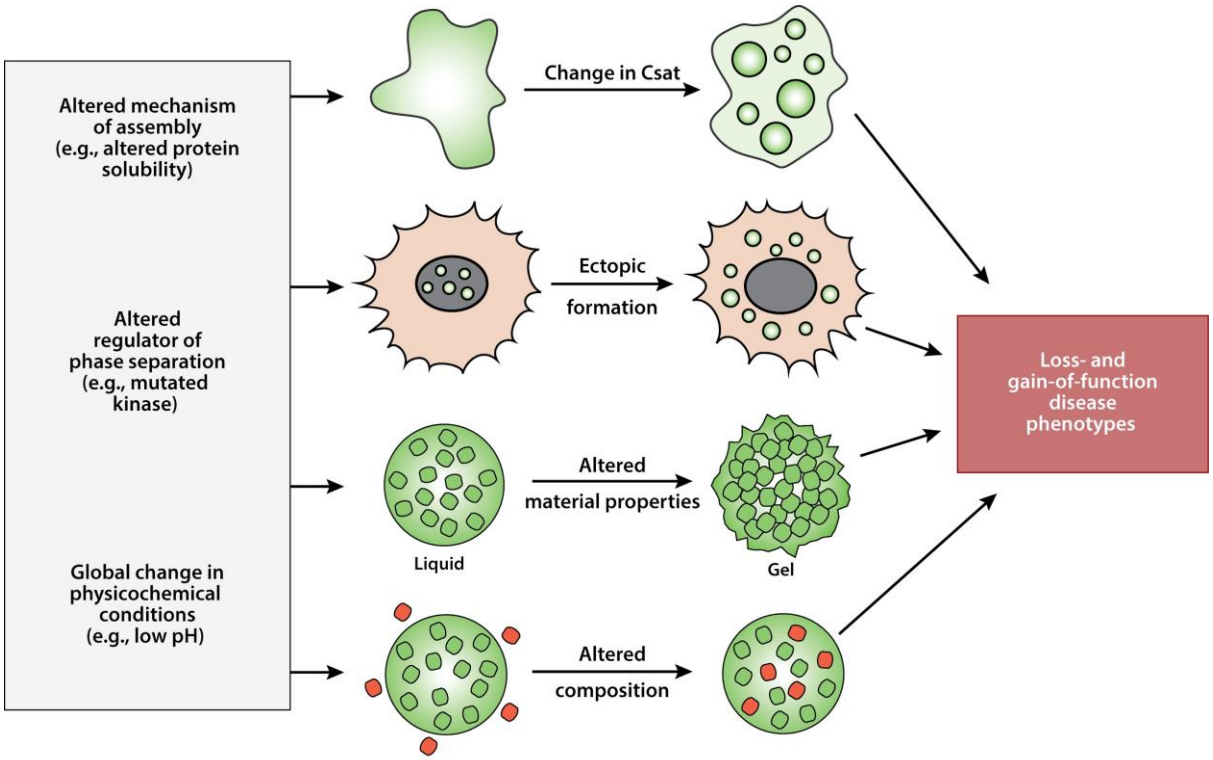

Figure 3. Possible causes of dysfunctional MLOs and the emergence of related diseases. Dysfunctional coacervates are formed due to changes in the mechanism of formation, changes in the molecules that regulate the phase separation or changes in the physicochemical condition of the cell.

Discussion

In the first section we described the formation of MLOs. Studies seem to agree that this formation is driven by two different factors: interactions of IDRs and cation-pi or pi-pi interactions. These forces are the base of the underlying protein-protein, protein-RNA and RNA-RNA molecular interactions. IDRs consist of long repetitive motifs and sequences with low complexity. They contain mostly charged residues and some hydrophobic residues. These charged residues are positively and negatively charged and in that way are responsible for electrostatic repulsion and attraction within and between the proteins that formed the coacervates. Considering their low complexity and repetitiveness it is unclear how sensitive are these molecular interactions between IDRs and pi-interaction. According to Brady et al. more research is needed on the mechanism that regulates the specificity of the different MLOs [14]. Marnik & Updike and Saito et al. found that formation of MLOs is regulated by post translational modifications like phosphorylation, acetylation and methylation [11,17]. Brady et al. suggest that this mechanism should be more elaborated or another mechanism is involved. However, it is clear that more research on the current driving forces and their specificity is needed. A very likely possibility could be that more forces drive phase separation.

Regarding the signal transduction, it has been shown by Formicola et al. that in neurons, MLOs form in the cell body upon synaptic activation [19]. They contain mRNAs and travel to the axonal and dendritic parts, where these RNAs are released and further translated. Often these mRNAs code for signal molecules and depending on the kind of activation the MLOs contain different types of mRNAs. In this study, further research is required on the link between the synaptic activation, changes in the environment and the reaction of the granules to that. To achieve an even better image research should be done very locally on both the long-term and short-term responses to different synaptic activation. The different mRNAs that are translated in different parts of the neurons upon the different activations should also be measured. Beside mRNAs, MLOs can also contain phosphatases and kinases that are involved in signalling pathways related to cell growth and survival. This should also be taken into consideration during the experiments and data collection. It is thought by Zhang & Herman that the mRNAs are protected from decapping and exonuclease activities by either the existence of different compartments within a MLO where the mRNAs are shielded or the switching off of the molecules responsible for this by post translational modifications [20]. These hypotheses need further research which is necessary for the confirmation of the hypotheses and future research based on these assumptions.

Moreover, we investigated the involvement of MLOs in gene expression. In some pathways MLOs are involved in gene suppression and in other pathways they are involved in gene overexpression. Suen et al. showed that in the piRNA pathway the formation of MLOs is vital for the silencing of certain genes [8]. Small non-coding RNA, like piRNA, are thought to be a promising new factor in the formation of coacervates. Since this is one of the first studies on the involvement of small non-coding RNA, more research is needed to confirm the results and elaborate the knowledge on this topic. Examining the mechanism of a certain nucleated

transcriptional MLO, Wei et al. showed that this organelle was formed in the nucleoplasm by binding RNA and was located at specific parts of the genome to enhance gene expression. In this research though, an optogenetically induced version of the protein was used which most likely makes the scale of the MLOs much larger when compared to the MLOs in normal cells. To check to what extent this influenced the result, this process could be further studied in vivo or on a scale comparable to the one in cells. In a study by Peng et al. it was observed that in EBV-infected cells, two viral transcription factors recruit host co-activators and form MLOs at superenhancer sites of certain genes to increase expression of these genes [33]. For treatment against this virus, research on how to prevent these two viral factors from phase separating can be very useful.

Later, we studied the formation of dysfunctionals MLOs which cause neurodegenerative diseases, infectious diseases and cancer. It was proposed by Alberti & Dormann that these dysfunctional coacervates are formed due to changes in the formation mechanism or in the molecules that regulate the phase separation or in the physicochemical condition of the cell [37]. In ALS en FTD it has been shown that mutation in molecules involved in the phase separation can cause different dynamic, composition and size of the granules. The MLOs could also transition from liquid-like to solid-like and form aggregates because of mutations. Wrongly executed PTMs have been found to cause dysfunctional MLOs in ALS, FTD and AD. In certain types of cancers, dysfunctional MLOs can alter signalling pathways and affect gene expression. Viruses use MLOs to replicate their genome and protect them from the immune system of the host. From these studies it becomes clear that for understanding these diseases and their mechanisms, focussing on one single molecule is not sufficient. Research on phase separation and related diseases shows that in order to find new effective therapeutics we need to look at the interactions between multiple molecules. Depending on the situation, there needs to be screening for MLO promoting or MLO destructive drugs.

In conclusion, this thesis shows that, although the research on phase separation is only at the beginning, there is great potential for future appliances.

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