Effect of additives on liquid droplets and aggregates of proteins

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Abstract

This review briefly summarizes the effect of additives on the formation of liquid droplets and aggregates of proteins. Proteins have the property of forming liquid droplets and aggregates both in vivo and in vitro. The liquid droplets of proteins are mainly stabilized by electrostatic and cation– π interactions, whereas the amorphous aggregates are mainly stabilized by hydrophobic interactions. Crowders usually stabilize liquid droplets, whereas ions and hexandriols destabilize the droplets. Additives such as kosmotropes, sugars, osmolytes, and crowders promote the formation of amorphous aggregates, whereas additives such as arginine and chaotropes can prevent the formation of amorphous aggregates. Further, amyloid has a different mechanism for its formation from amorphous aggregates because it is primarily stabilized by a cross- β structure. These systematic analyses of additives will provide clues to controlling protein aggregations and will aid the true understanding of the transition of proteins from liquid droplets and aggregates.

Keywords Liquid droplets · Dispersion · Aggregation · Amyloid · Additive

Introduction

Proteins comprise 20 naturally occurring amino acids, which have the property to form assemblies, typically liquid droplets and amorphous aggregates in vitro (Iwashita et al. 2018b). For example, when an antibody is mixed with a poly (amino acid) at a neutral pH, the protein solution becomes cloudy (Mimura et al. 2019). This cloudiness disappears with the addition of hundreds of mM of salt. The cloudiness is stabilized by the electrostatic interaction between the antibody and the poly (amino acid) and hence dissociated by the electrostatic shielding effect by increasing ionic strength (Matsuda et al. 2018). Similarly, hen egg-white lysozyme is mixed with ovalbumin (Iwashita et al. 2018a) or ovotransferrin (Iwashita et al. 2019), and cloudiness is observed in vitro. These data showed that proteins are prone to form liquid droplets and/or aggregates in vitro. In this review, we briefly summarize the stabilization factors of liquid droplets, amorphous aggregates, and amyloid fibrils. After that, we overview the solution additives that control these protein assemblies. This review will

provide important information to understanding on liquid–liquid phase separation of proteins in living cells.

Stabilization of liquid droplets and aggregates

Protein solutions can be classified into four types according to their intermolecular interactions and fluidity (Fig. 1). The solution states are mainly influenced by the hydrophobicity that changes the fluidity of the protein assembly. (1) A dispersed solution of protein is an ideal state without intermolecular interactions. It should be noted that many researches on protein structure and function have been performed under the dispersed state. (2) The liquid droplet state of protein is a state stabilized by intermolecular interactions, although the molecules have fluidity inside the liquid droplets, which contain many water molecules. Hydrogels of proteins are more stable than droplets owing to mature intermolecular interactions (Kato et al. 2012). (3) Amorphous aggregates of proteins are solid precipitates without fluidity due to hydrophobic interactions between protein molecules. (4) The amyloid state is a typical aggregated state of proteins stabilized by cross- β structures.

The main factors that stabilize liquid droplets are electrostatic and cation– π interactions. In addition, π – π interactions

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Fig. 1 Representative solution states of proteins. Proteins has typically four states in aqueous solutions that stabilize different interactions

	Disperse	Liquid Droplet	Amorphous Aggregate	Amyloid
Interaction	(None)	Electrostatic Cation- π Cross- β	Hydrophobic	Cross- β
Fluidity	Yes	Yes	No	No

and multivalent interactions are effective in stabilizing the liquid droplet state of proteins. In contrast, the main factor that stabilizes aggregates is hydrophobic interaction. Cross- β interactions stabilize both liquid droplets and amyloid aggregates, and the former is mainly stabilized by short cross- β structures (Murray et al. 2017). These stabilizing factors are briefly summarized as follows.

Electrostatic interactions are generated by an electric attractive force or a repulsive force between charged groups of protein molecules. Electrostatic interaction is the most important factor in the stabilization of liquid droplets because it is the only long-ranged interaction among others. Additionally, electrostatic interactions are affected by post-translational modifications. For example, the C-terminal domain of RNA polymerase II has many sites that are phosphorylated, but phosphorylation allows it to form stable droplets with other histidine-rich proteins (Lu et al. 2018). The RNA-binding proteins Ddx4 (Nott et al. 2015) and hnRNP A2 (Ryan et al. 2018) become soluble at low temperatures when lysine and arginine are methylated. Similarly, two research groups have reported that the liquid droplet of fused-in sarcoma (FUS) protein is tightly controlled by arginine methylation in the disordered region (Qamar et al. 2018; Hofweber et al. 2018).

Cation- π interactions are interactions between the π electron and a cation. Compared with lysine, arginine contributes to a stronger cation- π interaction because arginine has a planar positive charge on the side chain that has the cation- π interaction alongside the aromatic ring. In fact, there are many RGG sequences in which arginine (R) and glycine (G) are aligned in the low complexity region that causes phase separation of RNA-binding proteins (Chong et al. 2018). It is thought that the small amino acid of glycine is present to facilitate the interaction between the large side chain of arginine and the target aromatic amino acid on proteins.

π - π interactions are those in which the side chains of aromatic amino acids are arranged. The π - π interaction stabilizes a dispersion force between the delocalized π electrons on the

aromatic ring and is one of the stabilizing factors of liquid droplets; hence, tyrosine (Tyr) and phenylalanine (Phe) compose the low complexity region of proteins for phase separation (Boeynaems et al. 2018). Note that Trp, which is the most hydrophobic amino acid, is not found in the low complexity region. This may be because Trp is not favorable for the formation of liquid droplet.

Hydrophobic interactions are interactions between the non-polar regions of macromolecules in aqueous solution. Generally, hydrophobic interactions are the most important factors that stabilize amorphous aggregates, as well as the tertiary structure of proteins (Chiti et al. 2002). To more clearly see the difference, the amorphous aggregates of proteins are mainly stabilized by hydrophobic interactions, whereas the liquid droplets of proteins are mainly stabilized by non-hydrophobic interactions (Iwashita et al. 2018b).

Cross- β structures are intermolecular interactions of hydrogen bonds between the main chains of proteins. The cross- β structure was originally known as a structure that stabilizes amyloid aggregates (Nelson et al. 2005). The amyloid fibrils associated with the pathogenesis of Alzheimer's and Parkinson's diseases exhibit a common structural feature known as the cross- β , known as "dry steric zipper structures" (Vitagliano et al. 2009). On the other hand, recent studies have suggested that many RNA-binding proteins such as FUS can form functional amyloid fibrils with distinct reversibility that cannot be interpreted by the dry steric zipper structure (Kato et al. 2012). Solid-state nuclear magnetic resonance and molecular dynamics simulations have revealed that liquid droplets of FUS, which is thought to cause amyotrophic lateral sclerosis, have a cross- β structure as short as several residues (Murray et al. 2017). In addition, the cross- β structure that stabilizes liquid droplets is basically characterized by a short bend (Luo et al. 2018); thus, it is naturally thought that amyloid fibrils grow by forming liquid droplets.

Notably, liquid droplets of proteins and RNAs are determined by the respective interactions described above and by

the arrangement order and tertiary structure of the macromolecules. For example, proteins are prone to form liquid droplets when the distribution of positive and negative charges is biased (Chang et al. 2017). Additionally, RNA contained in RNA-granules is less likely to form liquid droplets when conformational change occurs by a mutation (Ditlev et al. 2018). These results indicate that the liquid droplet state of proteins and RNA is more difficult to understand than that of synthetic polymers of simple repetitive sequences.

In summary, proteins form liquid droplets, aggregates, as well as dispersed state (Fig. 1). This simple view may change the understanding of the molecular mechanisms occurring in living cells. For example, FUS protein is responsible for familial neurodegenerative diseases by point mutations (Vance et al. 2009). The mutations of FUS cause cytotoxicity because it tends to form aggregates after the formation of liquid droplets (Patel et al. 2015). Similar data were obtained from an RNA-binding protein hnRNPA1, which is thought to be involved in neurodegenerative diseases. The hnRNPA1 forms a highly fluid liquid droplet with short cross- β structures, which are highly reversible, although the formation of long cross- β structures results in insoluble amyloid (Gui et al. 2019). In another case of α -synuclein, first, the disordered region of the N-terminus forms a liquid droplet, followed by amyloid formation in the C-terminal region (Ray et al. 2019).

Effects of additives on the solution states of proteins

Here, we summarize the effects of small molecules on the stabilizing factors of liquid droplets, amorphous aggregates, and amyloids of proteins (Table 1). The additives that affect protein structure and solubility can be experimentally understood to some extent by considering the above interactions

that stabilize the liquid droplets, amorphous aggregates, and amyloids of proteins.

First, it should be noted that additives primarily affect the solubility of proteins, and their properties are divided into two types. Small-molecular-weight additives, such as amino acids and sugars, have the property of requiring high concentrations (typically 100 mM or more). This is because they change the properties of the solvent, rather than the binding to proteins. Conversely, polyamino acids and polyvalent metal ions have properties that are different from small molecules because they interact with proteins in multivalent interactions that change the solubility of the proteins. Accordingly, such polymers will work with just the same concentration of proteins (typically 1 mM or less).

Inorganic and organic ions weaken electrostatic interactions between solutes via the electrostatic shielding effect. For example, the addition of 50 mM sodium chloride completely dissociates the protein complex that is stabilized only by electrostatic interactions (Iwashita et al. 2018a). Notably, ions can be classified from kosmotropes to chaotropes depending on the strength of binding to solutes, so called Hofmeister series (Zhang and Cremer 2010). For example, sulfide and fluoride ions are typical kosmotropes, which tend to form amorphous aggregates of proteins. In contrast, iodide ions and thiocyanate ions are typical chaotropes, which tend to unfold the protein structure and suppress the formation of amorphous aggregates. Metal ions are prone to binding to protein, RNA, and DNA, which changes the ability of biomolecules to form liquid droplets, which is the different mechanism of monovalent ions. For example, magnesium and calcium ions accelerate the formation of nucleosome self-assembly liquid droplets by phase separation (Ohyama 2019).

Denaturants are substances that disrupt the tertiary structure of proteins. Denaturants, typically urea and guanidinium, have the function of weakening the hydrophobic interactions between solutes, resulting in the dispersion of hydrophobic

Table 1 Effect of typical additives on liquid droplets, amorphous aggregates, and amyloids of proteins

	Liquid droplet	Amorphous aggregate	Amyloid
Stabilization	TMAO ^a	Sugar ^g	Sugar ^g
	Crowder ^b	Crowder ^h	Crowder ^h
	Ethanol ^b	Kosmotrope ⁱ	Polyelectrolyte ^{m,n}
Destabilization	Hexanediol ^c	Polyelectrolyte ^j	Catechin ^o
Suppression	Sugar ^b	Arginine ^k	Polyphenol ^p
	Osmolyte ^d	Denaturant ^l	Aromatic compound ^q
	Ion ^e	Chaotrope ⁱ	
	ATP ^f		

^a Choi et al. 2018. ^b Mimura et al. 2019. ^c Lin et al. 2016. ^d Choi et al. 2018. ^e Iwashita et al. 2018a. ^f Patel et al. 2017. ^g Katyal and Deep 2017. ^h Kuznetsova et al. 2015. ⁱ Zhang and Cremer 2010. ^j Kurinomaru et al. 2012. ^k Hamada et al. 2009. ^l Hevehan and De Bernardez 1997. ^m Kovachev et al. 2019. ⁿ Zhang et al. 2015. ^o He et al. 2009. ^p Porat et al. 2006. ^q Levy-Sakin et al. 2009

substances into solution (Vanzi et al. 1998). Representative denaturants include guanidine hydrochloride and urea. In addition, protein aggregation is mainly driven by hydrophobic interactions, so the addition of denaturants can prevent protein aggregation (Hevehan and De Bernardes 1997). Similarly, it is naturally believed that liquid droplets are dissolved by the addition of denaturants if the liquid droplets are stabilized mainly by hydrophobic interactions.

Sugars stabilize the tertiary structure of proteins to increase the interfacial energy and promote the formation of amorphous aggregates. For example, glucose and trehalose dissolve the liquid droplets of immunoglobulin G and polyglutamic acid (Mimura et al. 2019). This may be because the addition of the sugars differs the hydration of poly (amino acids), rather than their tertiary structure, resulting in the dissolution of the liquid droplet. Trehalose accelerates the formation of amorphous aggregates, whereas it suppresses the formation of amyloid aggregates of prion domain (Katyal and Deep 2017) and amyloid- β 40 and 42 (Liu et al. 2005).

Osmolyte is a molecule found in the cells of marine organisms that are resistant to osmotic pressure (Yancey et al. 1982). For example, osmolytes such as betaine, taurine, and trimethylammonium N-oxide (TMAO) stabilize the three-dimensional structure of proteins. In cases where the structure of the natural denatured region plays an important role as a stabilizing factor for liquid droplets, the addition of osmolytes results in the dissolution of the liquid droplets in a similar way to sugars. TMAO stabilizes the liquid droplets of an intrinsically disordered protein of TDP-43, which prevents amyloid fibrilization (Choi et al. 2018). By contrast, TMAO promotes the amyloid formation of tau protein (Scaramozzino et al. 2006). Therefore, the effect of osmolytes remains unclear.

Arginine is a naturally occurring amino acid with a basic side chain. Arginine is the most versatile additive for various applications, such as suppressing aggregation due to heating, dissolving aromatic compounds, and improving refolding yield (Arakawa and Kita 2014). Because guanidinium in the side chain of arginine has a planar structure, arginine forms a stable cation- π interaction with the aromatic ring on the surface of the protein, resulting in an increase in the repulsion between protein molecules (Miyatake et al. 2016). Arginine dissolves the opalescence of high-concentration solutions of gamma globulin (Oki et al. 2018). Notably, alantoin and hydantoin are new aggregation suppressors for proteins that may complement arginine's function (Nishinami et al. 2018).

Amine compounds, such as spermine, putrescine, and glycnamide, have the function of preventing aggregation and inactivation of proteins caused by heat treatment (Shiraki et al. 2015). A similar effect can be seen for ammonium ions (Hirano et al. 2007). The mechanism by which amine compounds prevent thermal stress mainly protects the amine on the side chain of asparagine (Tomita and Shiraki 2011). It is important to note that such chemical reactions

affect the formation of liquid droplets and amorphous aggregates.

Alcohol solutions have a lower dielectric constant than water. Solutions with low dielectric constants enhance the electrostatic interactions and hydrogen bonds between the protein molecules; hence, alcohols tend to form α -helix-rich protein structures (Shiraki et al. 1995). For example, liquid droplets of immunoglobulin G and polyglutamic acid are stabilized in an ethanol solution (Mimura et al. 2019). In contrast, low concentration of 1,6-hexanediol dissolves liquid droplets because alcohol weakens the weak hydrophobic interactions between protein molecules (Lin et al. 2016). Accordingly, the molecular mechanism of alcohol on liquid droplets are controversial.

Neutral macromolecules, typically polyethylene glycol and ficoll, stabilize the tertiary structure of proteins via the crowding effect (Kuznetsova et al. 2015). The crowding effect prevents the formation of liquid droplets to make the protein structure more compact; however, it stabilizes the droplets once formed (Mimura et al. 2019). Crowders accelerate the formation of amyloids (Munishkina et al. 2004). The crowding effect plays an important role in understanding the inside of living cells.

Polyelectrolytes affect the formation of liquid droplets, amorphous aggregates, and amyloid. Polyelectrolytes change the solvent properties of proteins by complex formation. This is different from the above-described property of changing the solvent properties by the addition of small molecules in solution. For example, poly (lysine) and poly (glutamic acid) precipitate various types of proteins and peptides (Kurinomaru et al. 2014) because the complex of polyelectrolytes and proteins becomes electrostatically neutral; thus, it easily precipitates (Matsuda et al. 2018). On the other hand, a polyelectrolyte that binds strongly to proteins unfolds proteins to stabilize aggregates (Kurinomaru et al. 2012). One of the polyelectrolytes of RNA suppresses the formation of amyloids, such as mammalian prion (Kovachev et al. 2019) and poly Q (Zhang et al. 2015).

In conclusion, this review has summarized the fact that proteins easily assemble into liquid droplets and amorphous aggregates. The liquid droplets and aggregates of proteins have different stabilizing factors, typically hydrophobic, electrostatic, cation- π , and cross- β interactions. As a result, the effects of additives are systematically classified into liquid droplets and aggregates. This information will provide important clues for understanding how liquid-liquid phase separation occurs in living cells and will be useful in various biotechnological applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing.

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