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Interactions of formulation excipients with proteins in solution and in the dried state ☆

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ABSTRACT

A variety of excipients are used to stabilize proteins, suppress protein aggregation, reduce surface adsorption, or to simply provide physiological osmolality. The stabilizers encompass a wide variety of molecules including sugars, salts, polymers, surfactants, and amino acids, in particular arginine. The effects of these excipients on protein stability in solution are mainly caused by their interaction with the protein and the container surface, and most importantly with water. Some excipients stabilize proteins in solution by direct binding, while others use a number of fundamentally different mechanisms that involve indirect interactions. In the dry state, any effects that the excipients confer to proteins through their interactions with water are irrelevant, as water is no longer present. Rather, the excipients stabilize proteins through direct binding and their effects on the physical properties of the dried powder. This review will describe a number of mechanisms by which the excipients interact with proteins in solution and with various interfaces, and their effects on the physical properties of the dried protein structure, and explain how the various interaction forces are related to their observed effects on protein stability.

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1. Introduction

Many proteins are structurally unstable in solution, and are susceptible to conformational changes due to various stresses encountered during purification, processing, and storage [1–8]. These stresses include elevated temperature, exposure to extreme pH, shear strain, and surface adsorption, to name a few [5,6]. Thus, protein-based pharmaceuticals have the potential to undergo physical degradation (e.g., unfolding, aggregation, and insoluble particulate formation) by a number of mechanisms, which can negatively impact both the efficacy and safety of the therapeutic product [7,8]. The solvent environment of the protein plays a major role in determining its stability [9]. Numerous solvent additives, the so-called "osmolytes", have been shown to enhance the stability of proteins and, as a consequence, reduce the aggregation of marginally stable proteins [10–28]. In this case, protein unfolding precedes aggregation, and the structure-stabilizing co-solvents reduce aggregation by stabilizing the native structure. The lack of affinity for, or repulsive interaction with, the protein surface is the reason why these co-solvents stabilize the protein structure. Conversely, excipients such as arginine, surfactants, proteins, and polymers are often used to suppress protein aggregation without enhancing its stability [28–38]. These additives exert their effects by weakly binding to the protein surface, or by competitively binding to the surface/interface that have the potential to destabilize the protein structure. Some of these excipients are also used to stabilize proteins in the dry state. However, in the absence of water, fundamentally different mechanisms are in effect, as any mechanism that involves excipient-water interactions will not play its part. This chapter summarizes the effects of additives that are used to mitigate protein aggregation and will discuss the mechanistic basis of their effects both in solution and in the dried state. In addition, the effects of additives on protein stability during freezing will also be discussed, as freezing is an intermediate processing step involved in lyophilization. It should be noted that as water is still present, yet is gradually removed during ice crystallization, the freezing process involves an interesting physical state that is described mainly by interaction forces that are present in solution.

2. Protein stabilizers

2.1. Solution

A wide variety of protein stabilizing excipients is used for enhancing the stability of both pharmaceutical and reagent proteins and they are referred to as stabilizing co-solvents [9,22–24]. These excipients have been reported to stabilize the structure of native proteins at moderate (0.1 M) to high concentrations (1 M). In fact, these compounds played a critical role at the dawn of classical enzymology and biochemistry of cellular proteins. Many proteins are inactivated when they are isolated from their natural environment. For example, the cytoskeletal proteins, actin and tubulin, have been reported to lose the ability to polymerize as soon as they are purified, i.e., as soon as the protective components were removed. It was discovered that sugars, present at high concentrations during purification, were effective in preserving their activity [23,39–41], and thus replacing the role of the protective components that were initially present in the cellular environment. Sugars were also reported to increase the melting temperatures of various model proteins [10–12]. Thus, a correlation was drawn between the additives that stabilize proteins against thermal stress and those which stabilize the function of unstable proteins during isolation and storage. These co-solvents are also referred to as osmolytes, or compatible solutes, since they are utilized in nature to raise the osmotic pressure of the cellular environment and are compatible with the macromolecular function and cell viability [42,43]. Several examples of protein stabilization and suppression of aggregation by osmolytes are presented here, followed by a discussion of their stabilization mechanisms.

Protein-stabilizing co-solvents encompass polyols, sugars, amino acids, amines, and salting out salts. Each class of compounds has a long history of use and has been employed interchangeably. It would be difficult to find a strong reason to choose one over the others, as they all enhance the stability of proteins. The effects of salts on protein stability have been studied extensively ever since the discovery of the salting out effect of proteins by Hofmeister [44]. The main conclusion from his study was that the salting out salts increase the stability of proteins while the salting in salts either decrease or demonstrate insignificant effect on the stability of proteins. Examples of stabilizers are described below in detail.

Sugars and polyols are often used to stabilize many proteins and protect them from aggregation [45–48]. Among sugars, sucrose and trehalose have been the most frequently used. In one application, several polyols, including the two mentioned above, have been shown to be highly effective in increasing the melting temperature (T_m) of the two-domain protein, yeast hexokinase A, which resulted in significant preservation of the enzyme activity upon storage at both 4 and 25 °C [46]. Among the other effective saccharides, sorbitol has been shown to increase the T_m of human IgG and reduce its aggregation during the heating process, which is employed for viral inactivation [49]. The efficacy of glycerol in stabilizing proteins varies depending on the protein itself [50]; while glycerol conferred protection against thermal inactivation for several enzymes, it has been found to have either no effect or, at times, destabilizing effect. Sek [51] studied the effect of polyols in increasing the unfolding temperature of several antibody molecules and reported that the extent of stabilization increased with increasing polyol concentration, with larger polyols conferring greater stability. More specifically, when the data were normalized with respect to the molar concentration of alcohol groups, smaller polyols, such as glycerol and erythritol, were found to be less effective in stabilizing the antibody.

Pasteurization, normally conducted at 60 °C for 10 h or more, is a key process for virus inactivation of plasma-derived products. This process, however, can cause denaturation of proteins, often leading to aggregation. Aggregated proteins are one of the major side products of pharmaceutical protein therapeutics. Thus, it is essential to stabilize proteins against heat-induced denaturation. Caprylate and tryptophanate are the most commonly employed solvent additives for this purpose. Sorbitol and other polyols have also been demonstrated to increase the T_m of IgG solutions, thus reducing its propensity for aggregation [49,52,53].

Tetrameric hemoglobin structures readily dissociate and, as a consequence, aggregate due to thermal stress [54]. The effects of two osmolytes, sarcosine and sorbitol, were studied for their ability to stabilize hemoglobin against heat-induced dissociation followed by aggregation [55]. Hemoglobin at 1 mg/ml in 50 mM phosphate buffer, pH 7.0, was incubated at 65 °C and the amount of soluble protein was determined as a function of time. The apparent rate constant of aggregation was determined in the absence and presence of sarcosine

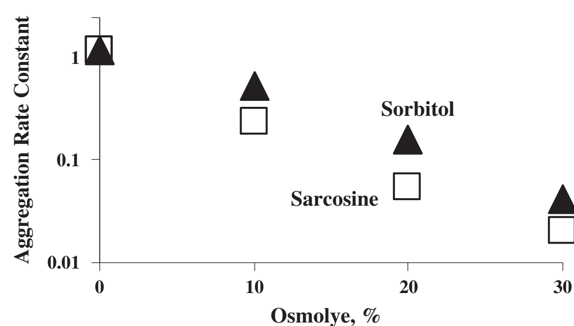

Fig. 1. The effect of osmolyte concentration on the aggregation rate constant of hemoglobin. Sorbitol and sarcosine were examined in a concentration range between 0 and 30%.
Data adapted from Domenico and Lavecchia [55].

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and sorbitol. Fig. 1 plots the log rate constant against concentration of the osmolytes. The rate constant was greatly reduced in the presence of osmolytes. Sarcosine was more effective than sorbitol, leading to over 50-fold reduction in the rate constant, when present at 30%. The stabilization effects of sorbitol are in line with its effects on many other globular proteins [10,19,56–58], as is the case with sarcosine [59,60].

Keratinocyte growth factor (KGF or FGF7) is an approved product to treat oral mucositis [61]. It is a growth factor for epithelial cells and serves as a protector from various cell toxins [62], although it may promote the growth of solid tumors [63]. KGF has a strong tendency to aggregate in solution due to its inherent instability [26,28]. KGF begins to melt at ~40 °C in 10 mM phosphate, pH 7.0, which is immediately followed by an increase in solution turbidity due to aggregation. The melting of KGF is irreversible, thus the interpretation of its stability solely from the melting data is difficult, as it depends on the rate of aggregation as well as on the thermodynamic stability of the protein. In this case, the onset temperature of thermal unfolding, T_o, may be more meaningful, as it is affected less by the aggregation process. The shelf life of KGF during storage is short, perhaps reflecting its instability and propensity for aggregation; 50% of the monomeric form of KGF disappears within 0.35 days when stored in 10 mM phosphate, pH 7.0 at 37 °C. Various protein stabilizers were tested to enhance the thermal stability of the protein in the same buffer [27]. Fig. 2 shows the effects of various stabilizing osmolytes and salts, present at 0.2 and 0.5 M, on T_o (left side panel) and the shelf life of the protein (right side panel). NaCl (gray bars) and the other osmolytes (black bar) examined in the study were only slightly effective in increasing T_o at these concentrations. However, salting out salts, including ammonium sulfate (white bar), sodium phosphate (shaded bar), and sodium citrate (dotted bar) were extremely effective, raising the temperature by over ~10 °C and ~12 °C at 0.2 and 0.5 M concentrations, respectively. Similarly to their effects on T_o, only these salting out salts were effective in increasing the shelf life of the protein. Citrate (dotted bars) in particular was extremely effective, leading to an increase in shelf life by more than 300-fold. Large differences observed between osmolytes and salts suggest the existence of specific effects of salt ions. KGF is also characterized by its ability to bind heparin and poly-anions, which results in its stabilization [64–69]. The observed stabilization of KGF by the three salts is most likely due to the binding of polyvalent anions to KGF, rather than due to their general stabilization effects of proteins [26,70,71]. Such binding is expected to be most significant for trivalent ions, such as citrate. Care must be exercised when dealing with organic acids, such as citrate, or organic bases. Organic acids and bases differ from strong electrolytes, e.g., inorganic salts, in that their ionic states depend on pH. The consequence of which is that their effects on protein stability vary and are highly dependent on their charged state.

The melting temperature appears to correlate with the increased shelf life of proteins in the examples given above. However, such correlation is not universal. This can be explained from the possible effects of co-solvents on protein stability and self-association. As depicted in Fig. 3, aggregation can occur from the association of either the unfolded or the native state of proteins. Increased melting temperature typically translates to a shift in the equilibrium constant of unfolding towards the native state, i.e., decrease in K_1. Thus, there will be a reduction in the population of unfolded protein leading to aggregation. However, the protein stabilizing excipients can enhance self-association, i.e., greater k_1, indicating that they may enhance aggregation even when there is a paucity of unfolded proteins. The stabilizing excipients can also increase the equilibrium constant, K_2, of self-association of the native state. As long as such self-association is reversible, they cause no damage to proteins, although aggregation often becomes irreversible (reflected on k_2) as the extent of self-association increases. Thus, it is clear that the effects of excipients on melting temperature may not always correlate with the storage stability of proteins.

2.2. Dry state

Lyophilization is commonly used in the manufacture of protein products that are insufficiently stable in aqueous solution [72]. In fact, pH-induced and/or temperature-induced hydrolysis and deamidation reactions have been reported to be reduced when the protein is stored in the dried state. In addition, lyophilized products are less prone to shear-induced denaturation and precipitation during transport. Freeze drying process parameters and the formulation components largely dictate the process-associated loss and consequent stability of the lyophilized product during storage [73]. Lyophilization involves two orthogonal stress vectors, freezing and drying. Both processes cause damage to the protein structure by a variety of mechanisms, and thus the selected excipients must stabilize the protein effectively against both stress vectors. In addition, the excipients must protect proteins from various stresses encountered during storage. Many structure-stabilizing co-solvents were found to be effective against freezing, but not against drying. During drying, the removal of water from the vicinity of the protein often perturbs its structure, leading to irreversible aggregation following reconstitution. The structurally altered proteins are also prone to chemical degradation [74]. There have been several reports suggesting the benefit of leaving a small amount of water in the dried structure, attesting to the detrimental consequence of over-drying. In fact, following his studies on the dehydration of calcein, Pauling [75] suggested that the protein should not be dried exhaustively, and that certain highly polar residues found on the protein surface should be maintained in the hydrated state, in order to avoid denaturation during drying. The theory that highly

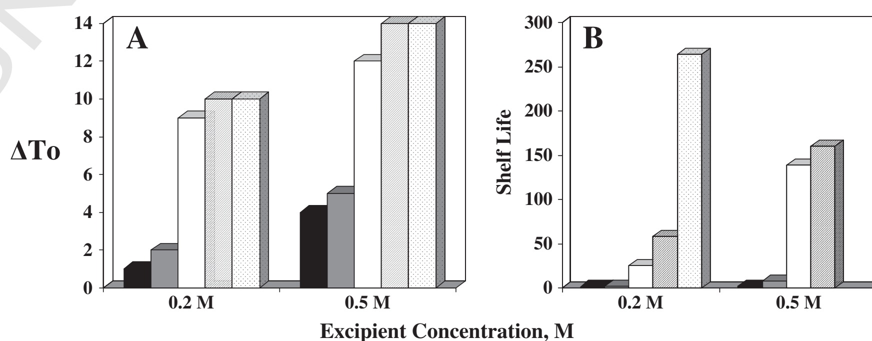

Fig. 2. The effect of various co-solvents on the (A) melting temperature (T_o) and (B) shelf-life of KGF. The effects of osmolytes, including sucrose, trehalose, glycine, proline, glycerol, mannitol, sorbitol, betaine, and sarcosine (black bars), NaCl (gray bars), ammonium sulfate (striped bars), and sodium citrate (dotted bars) are shown. ΔT_o represents the difference in onset melting temperature in the presence and absence of the excipient and the value of shelf life in (B) represent the ratio of KGF shelf life in the presence and absence of the excipient. Data adapted from Chen and Arakawa [27].

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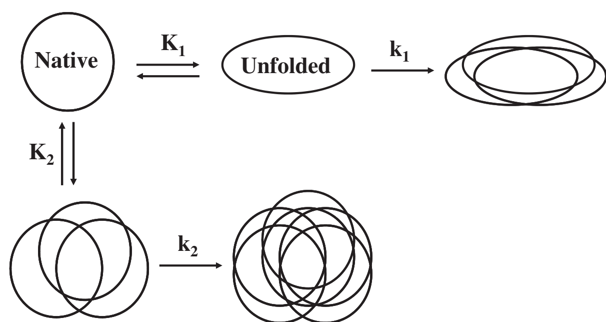

Fig. 3. The effect of excipient on the equilibrium constant of unfolding and association. K_1: Equilibrium constant for folding/unfolding, k_1: Rate constant for association of unfolded protein, K_2: Equilibrium constant for association of native structure, k_2: Rate constant for association of native protein oligomers. Aggregation can occur from the association of either the unfolded or the native state of proteins. Increased melting temperature typically translates to a shift in the equilibrium constant of unfolding towards the native state, i.e., decrease in K_1. Thus, there will be a reduction in the population of unfolded protein leading to aggregation. However, the protein stabilizing excipients can enhance self-association, i.e., greater k_1, indicating that they may enhance aggregation even when there is a paucity of unfolded proteins. The stabilizing excipients can also increase the equilibrium constant, K_2, of self-association of the native state. As long as such self-association is reversible, they cause no damage to proteins, although aggregation often becomes irreversible as the extent of self-association increases.

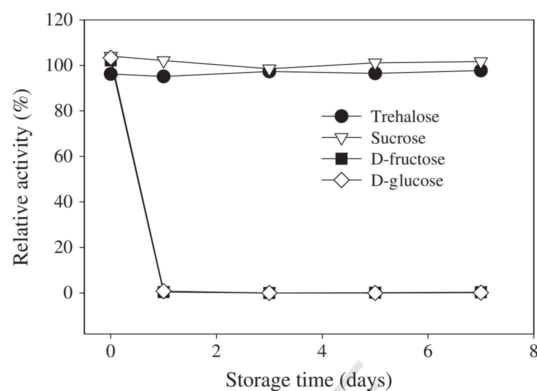

Fig. 4. The relative activity of β-galactosidase freeze dried with mono- and di-saccharides following storage at 70 °C for the indicated amount of time.
Data adapted from Izutsu et al. [80].

polar residues should be maintained in the hydrated state has also been suggested by Hsu et al. [76].

Although sugars are widely used for the preservation of protein activity following lyophilization, their amount needs to be optimized. The highest recovered activity of phosphofructokinase (PFK) at 50 μg/ml was 65% in 150 mg/ml trehalose concentration (concentration prior to lyophilization), however, the recovered activity decreased with further increases in trehalose concentration [77]. At 400 mg/ml trehalose, no PFK activity remained following freeze drying. At that level of trehalose, approximately 90% of the protein activity was recovered following freezing, thus the damage is thought to have occurred during desiccation. A similar trend was observed in the stabilization of several other lyophilized proteins in the presence of increasing concentrations of excipients, including mannitol for L-asparaginase [78], LDH [78], and β-galactosidase [79], and myo-inositol for PFK [77]. Typically, however, disaccharides have been reported to be a more effective lyoprotectant than are monosaccharides. This may be due to the higher glass transition temperature, T_g, of the former as well as their configurational flexibility. In fact, β-galactosidase freeze dried with trehalose and sucrose demonstrated no loss in activity during freeze drying and storage, whereas monosaccharides, such as glucose and fructose, were ineffective as stabilizers (Fig. 4) [80]. The simplistic view is that the higher the T_g of the amorphous sample, the greater the stability (another related parameter is the difference between T_g and storage temperature). This is because a significant change in the viscosity of the system occurs at the glass transition (lower viscosity at $T<T_g$, i.e., in the glassy state), and it is the reduction in motion that offers stability to the labile biological molecule.

In addition to preserving the activity of proteins and enzymes following lyophilization, saccharides are effective stabilizers of protein structure. Several saccharides, including sucrose, lactose, and maltose, have been shown to inhibit the random coil to β-sheet transition of poly-L-lysine [81]. Not all saccharides are effective, however, as evidenced by the ineffectiveness of mannitol and myo-inositol in preventing the conformational transition of poly-L-lysine. The authors proposed that the mechanism of protein stabilization by these additives during lyophilization is through the maintenance of its native conformation during dehydration, and the ability of each additive in interacting with the protein determines its efficacy as a stabilizer.

The preservation of protein structure does not always correlate to improved recovery of protein activity, as demonstrated by the following example. Recombinant human Factor XIII (rFXIII) freeze dried and rehydrated without additives has been reported to exhibit substantial loss of its native structure and catalytic activity [82]. Loss of the native protein appeared to be due mainly to the generation of soluble and insoluble aggregates, as was evidenced by the change in the infrared spectrum (amide I region) of the dried protein relative to that in its native state. When rFXIII was co-lyophilized with 3.5% (w/v) dextran and rehydrated, improved protection with respect to the formulation without additives was noted. However, the infrared spectrum of rFXIII dried with dextran demonstrated greater band broadening. Thus, although dextran caused increased protein unfolding, recovery of the active, native protein was improved as a result of its propensity to favor refolding over aggregation. Sucrose and trehalose, on the contrary, demonstrated greater recovery of the native structure of the protein, although their addition resulted in the formation of aggregates of decreased solubility. Thus the simplistic view of structure stability resulting in improved stability should be taken with caution.

Excipients are required not only to confer protection during processing, but also during subsequent storage as demonstrated by Chang et al. [83]. Elastase lyophilized without any excipients retained full activity immediately following freeze drying, however, it denatured upon storage at 40 °C and 75% RH, losing ~70% of the initial activity in 2 weeks (Fig. 5). The addition of sucrose or dextran 40 was effective in preventing denaturation; at up to 3 weeks of storage, residual activity was at least 80%. In another example, the effects of various saccharides, including sucrose and dextran, on the stability of a monoclonal antibody (MN12) was investigated [73]. Irrespective of the lyoprotectant used, precipitation and concomitant reduction (~10%) in the antigen-binding capacity of MN12 were observed upon reconstitution. In contrast, the additives did have a dramatic influence on antibody stability during storage. A moderate

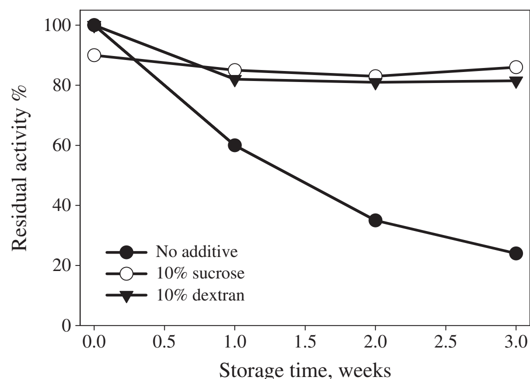

Fig. 5. Residual activity of lyophilized elastase at 40 °C and 75% RH in the presence and absence of sucrose or dextran.
Data adapted from Chang et al. [83].

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recovery of approximately 30% was obtained upon the addition of dextran. HPβCD was the most effective stabilizer examined for MN12, for which the antigen-binding recovery was approximately 70%. In the absence of lyoprotectants, insignificant amount of antibody was detected by ELISA following storage at 56 °C for 18 days.

Even within the same excipient class, the size of the molecule has been shown to have a significant effect on its capacity as a stabilizer. The stability of freeze dried bovine γ-globulin (BGG), containing dextran of varying molecular weights, was compared using size exclusion chromatography [84]. Fig. 6 shows the peak height ratio of intact BGG containing dextran of various molecular weights following 20 h of storage at 60 °C. The peak ratio represents the ratio of the peak height of the lyophilized sample to that of BGG standard solution. Dextran of smaller molecular weight (MW) demonstrated a higher degree of protein denaturation. More specifically, the formulation that contained MW 10k dextran exhibited a higher degree of denaturation than that containing MW 510k dextran. As will be described later in the chapter, changes in molecular weight for oligosaccharides and polymers have several consequences on the physical properties of the solid matrix (i.e., T_g and mobility) in addition to their mode of interaction with proteins (i.e., steric effects).

There are several reports of synergy observed between excipients, which are either ineffective on their own or only marginally effective, in conferring stability to proteins in the dried state. For example, metal ions and sugars have been reported to demonstrate such an effect in stabilizing PFK during freezing [85] and freeze drying. Carpenter et al. [86] have reported that the addition of zinc ion to enzyme-sugar mixtures significantly improved the stability of the enzyme provided by the sugars alone. It should be noted that zinc ion on its own was ineffective in conferring stability to PFK. The synergistic enhancement of enzyme stabilization by zinc was not limited to trehalose. In fact, the effect was observed with other saccharides, including maltose and sucrose. Interestingly, monosaccharides (i.e., galactose and glucose) that were ineffective on their own were converted to be a stabilizer upon the addition of $ZnSO_4$, demonstrating up to ~90% recovery of initial activity.

Besides saccharides, a number of amino acids are frequently cited as being suitable bulking agents for freeze dried formulations. Amino acids, similarly to carbohydrates, glycerol, and PEG [87], are thought to act by their preferential exclusion from the protein–water interface in solution [88]. The literature indicates that glycine crystallizes during freeze drying [89], and its behavior is dependent on pH and its salt form [90]. In fact, the protective effect in freeze dried cakes appears to correlate with the crystallinity of the excipients. Excipients which conferred protection maintained their amorphous state during processing and subsequent storage, whereas those that crystallized were ineffective in providing protection, as was the case with glycine and serine [91].

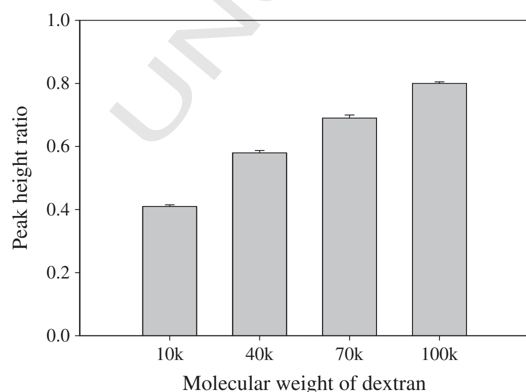

Fig. 6. Peak height ratio of bovine γ-globulin with dextran of various molecular weights, as assessed by SEC-HPLC. The lyophilized samples have been stored at 60° for 20 h. Data adapted from Yoshioka et al. [84].

Salts are present in protein formulations, typically in the form of a buffer. Salts may enhance the stability of proteins in solution by increasing the surface tension and the chemical potential of the system, however, they are not expected to confer any stability to proteins in the dried state due to their crystallization (i.e., phase separation). For example, although KCl (present at 500 mM) effectively protected LDH from thermal inactivation (at 50 °C) in solution, it failed to offer any protection during lyophilization [92]. There are notable exceptions, however. Calcium ions have been reported to stabilize lyophilized rhDNase against aggregation during storage at 40 °C [93], while Costantino et al. [94] reported that rHA co-lyophilized with NaCl prevented aggregation following prolonged incubation at 37 °C and 96% RH. In comparison, the protein without the salt lost greater than 80% solubility following 1 day of incubation under similar condition. Izutsu et al. [95] reported that several glass-forming salts (i.e., monosodium citrate) can be an effective lyoprotectant for the preservation of protein's secondary structure, including BSA and IgG. Although there are exceptions to the rule, as described above, salts are typically not employed as a protein stabilizer in the dried formulation. They are present, however, to serve a different purpose.

2.3. Mechanism

Extensive studies of the protein–solvent interaction, pioneered by Timasheff and his coworkers, resulted in enhancing our understanding of the mechanism of protein stabilization by co-solvents [96–99]. As will be described later in more detail, the co-solvents stabilize proteins by not binding to the proteins. This non-binding plays a fundamental role in cell biology and survival strategy of organisms that live in environments of high osmotic pressure, as mentioned earlier [42,43,100].

Four inter-related mechanisms, which all involve interactions with water in a different manner, have been postulated to explain the stabilization effects of co-solvents: cohesive force on water (surface tension mechanism), excluded volume effect, unfavorable interaction with peptide bonds, and preferential exclusion from the protein surface. Described below is the summary of these mechanisms:

2.3.1. Cohesive force

The protein-stabilizing co-solvents, most likely without exception, increase the surface tension of water. Namely, they exert a cohesive force on water and this was termed attraction pressure by Traube [101,102]. He correlated the attraction pressure to the effects of co-solvents, in particular the salts, on various properties of proteins. Although it was not clear in his report about how the attraction pressure relates to enhanced protein stability, such correlation provides insight into the mechanism of their effects. As will be described below in more detail, the cohesive force, and thus attraction pressure, was shown to cause the salts to be preferentially excluded from the protein surface.

2.3.2. Excluded volume effect

This mechanism has been used to explain the effect of polymers on protein stability and solubility. Any molecules that are larger than water are excluded from the vicinity of protein surface, as illustrated in Fig. 7. There is a layer of excess water (hatched area) surrounding the protein surface, from which the excipient is excluded due its hydrodynamic radius. The exclusion is thermodynamically unfavorable and repulsive, and tends to be greater, and hence more unfavorable, when the protein surface area increases. In other words, the excipient forces the protein to assume an equilibrium structure possessing the smallest solvent-exposed surface area possible. Thus, the excluded volume effect stabilizes the compact native structure of the protein.

2.3.4. Unfavorable interaction with peptide bond

Protein surface is highly heterogeneous, and as a consequence may have affinity for specific protein-stabilizing excipients. Affinity for a particular chemical structure can be examined from solubility

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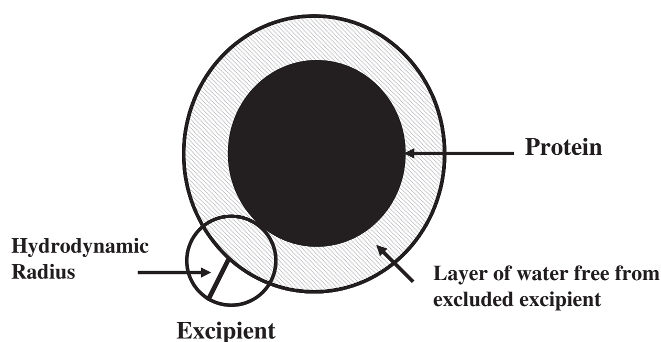

Fig. 7. Schematic illustration of the excluded volume effect. The protein is represented by the black circle and the excipient by the white circle. The striped area represents the layer of water free from the excluded excipient.

measurements. Nozaki and Tanford [103–106] pioneered such solubility experiments and reported a number of important conclusions for the mechanism of protein denaturation by urea, GdnHCl, and organic solvents. Conversely, Gekko [107–109] and Bolen [110–112] examined the interactions between protein stabilizers and amino acids. While both demonstrated the critical role of unfavorable interactions between amino acid side chains and peptide bonds, the latter concluded that the unfavorable interaction present between stabilizing excipients and peptide bonds is the primary determinant for protein stabilization. Such an unfavorable interaction may be closely related to the cohesive force, excluded volume effect, or both. In reality, both mechanisms should favor the stabilizing excipients to remain in bulk water, creating an entropically unstable condition. As all of the mechanisms cause repulsive interactions between excipients and proteins, it is generally impossible to pinpoint which mechanism plays a dominant role in stabilizing proteins.

2.3.5. Preferential interaction

Various interactions (both weak and strong) contribute to the overall interaction of the co-solvents with proteins. These interactions can be determined from equilibrium dialysis experiments and may be formally grouped into two different modes. In the first case, co-solvents (depicted by black circles) are present in excess in the vicinity of the protein surface compared to its concentration in the bulk phase, as illustrated in Fig. 8 (left panel). This case is termed "preferential interaction", indicating that the co-solvent concentration is higher at the protein surface than that in the bulk phase (arbitrarily separated by the dashed line). The opposite case is also illustrated in Fig. 8 (right panel), in which there is excess water (white circles) at the protein surface. This is called "preferential hydration" or "preferential exclusion" of the co-solvent, indicating a deficiency of co-solvent molecules in the vicinity of the protein. Osmolytes demonstrate preferential hydration of proteins; in other words, osmolytes are preferentially excluded from the protein surface [13,14]. Many sugars, polyols, and certain salts, which are known to stabilize proteins and decrease their solubility, are all preferentially excluded from the vicinity of the protein [13–15,22,23]. Furthermore, preferential exclusion is in accord with the repulsive interactions of these co-solvents with proteins, as described above.

Having introduced these concepts, the question now is, by what mechanism do these co-solvents/osmolytes increase the stability of proteins and decrease their solubility? The structure-stabilizing osmolytes are preferentially excluded from the protein surface, indicating that the interaction between osmolytes and protein is thermodynamically unfavorable. This increases the free energy of the native state of the protein, as schematically depicted in Fig. 9. Although not determined experimentally, a greater exclusion of co-solvent/osmolyte is expected from the unfolded structure, because it possesses a greater surface area compared to that of the folded, native state. The unfavorable interaction, and thus free energy, would increase even more so for the unfolded state in the presence of the co-solvent. This leads to a greater energy difference between the native and unfolded structures in the presence of stabilizing co-solvents/osmolytes, i.e., more energy is required to unfold proteins in the presence of preferentially excluded co-solvents. As preferential exclusion, and thus unfavorable interaction, increases with co-solvent/osmolyte concentration, the native structure is stabilized to a greater extent at higher co-solvent/osmolyte concentrations (Fig. 9). This concept can be extended to the situation in which there is self-association. During the process of protein self-association, the surface area per protein molecule decreases, which in turn reduces the unfavorable interaction present between the co-solvent and the protein complex or aggregates, also depicted in Fig. 9 (right side panel). Thus, the associated state is stable in the presence of stabilizing co-solvents/osmolytes; i.e., they enhance aggregation. In the examples described above, protein unfolding is the key determinant in causing aggregation, thus preferentially excluded co-solvents reduce aggregation by stabilizing the native structure.

As mentioned above, during lyophilization, both freezing and drying stresses need to be taken into account. The stresses encountered, which include cold denaturation, increased concentrations of solutes and proteins, pH shift, and dehydration, can cause protein denaturation and aggregation [113,114]. Protection during freezing is provided by a wide variety of co-solvents and is attributed to Timasheff's preferential exclusion mechanism (similar to that in solution), as free water is still present [115]. Nevertheless, as water molecules gradually crystallize, the amount of free water decreases, so stabilization by preferential exclusion mechanism may be impacted by the rate and extent of crystallization. Carpenter and Crowe [116] reported that high concentrations (>1 M) of sodium acetate, potassium phosphate, and various sulfate salts (all kosmotropes, or water structure-makers) provide significant cryoprotection of lactate dehydrogenase (LDH). In contrast, the more chaotropic salt, NaCl, yielded a much lower level of activity following freeze-thaw. During drying, the preferential interaction mechanism is no longer applicable because the bulk water, as well as the hydration shell of the protein, is removed [117–119]. It is the water molecules (not the protein) that the structure-stabilizing co-solvents influence. Furthermore, dehydration stress is different from those associated with freezing, thus many effective cryoprotectants or stabilizers in solution do not necessarily stabilize proteins during drying [83]. For many of the proteins examined by Prestrelski et al. [114], including γ-IFN, G-CSF, LDH, α-lactalbumin, bFGF, and α-casein, general disordering of the protein backbone was observed upon dehydration, as evidenced by the broadening of the individual amide I components. However, there were notable differences observed, which mainly depended on the protein itself. In general, three types of behaviors were observed during dehydration followed by rehydration. First, the protein can be resistant to conformational change during drying, thus retain its native conformation during processing. G-CSF is one example. In the second case, the protein may unfold during dehydration but refold upon rehydration, as was observed for α-lactalbumin and lysozyme. Finally, the protein may unfold during dehydration and remain unfolded during rehydration, resulting in irreversible conformational changes. For poly-L-lysine, the dehydration-induced conformational transitions appear to arise from its attempt in compensating for the lost hydrogen bonds with water. In solution, the random coil configuration has its peptide hydrogen bonding satisfied through its interaction with water molecules. Upon dehydration, these hydrogen bonding interactions are lost, and to compensate for the loss, the polypeptide forms intermolecular hydrogen bonds, resulting in β-sheet conformation. Furthermore, in the absence of water, the partial charges of the intermolecular interactions are screened to a lesser extent due to the lowered dielectric environment, thus increasing the electrostatic attraction of opposing charges between the peptides,

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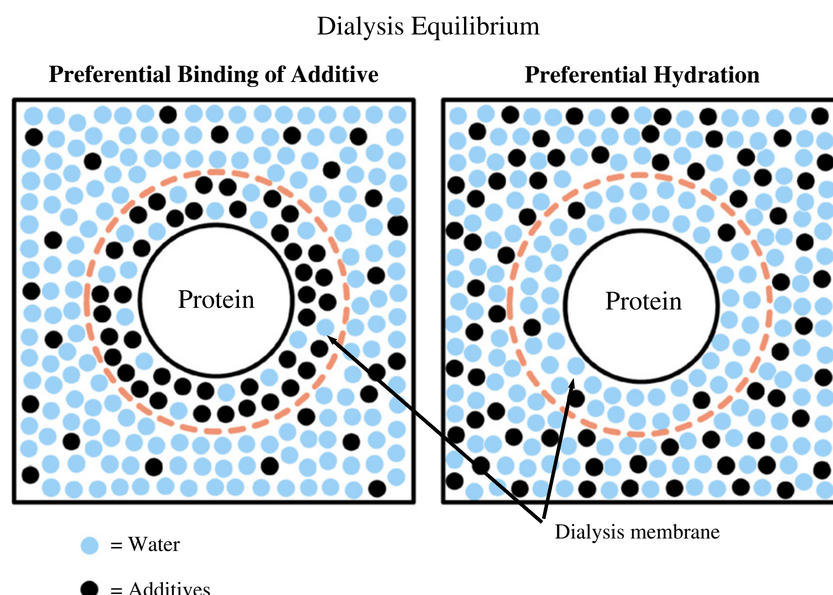

Fig. 8. Schematic presentation of preferential binding (left) and preferential exclusion (right) in a typical dialysis equilibrium experiment. The protein is represented by the white circle, water molecule by the blue circle, and the additive by the black circle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

leading to aggregation. Similarly to peptides, proteins rearrange their conformation to maximize both intra- and inter-chain hydrogen bonding to replace the lost hydrogen bonds during dehydration [114]. The above data clearly demonstrate the importance of satisfying the hydrogen bonding requirements of the polypeptide side chains and peptide bonds both in solution and upon desiccation.

Saccharides have been postulated to protect proteins through a variety of mechanisms, but two have been put forth to describe many observations, the water replacement hypothesis and vitrification. The main difference between the two proposed methods is that direct interaction is a pre-requisite for the former [77,114,120], while it is not for vitrification. For the latter mechanism, the formation of an amorphous glass (vitrification) is the only requirement for providing stability, mainly through retarding molecular motion and providing physical separation between the proteins (i.e., inhibiting aggregation) [121,122]. Although the underlying mechanisms differ, both hypotheses require the protein and the stabilizer to be in the same amorphous phase.

To support the water replacement hypothesis, many studies have confirmed the presence of hydrogen bonding in lyophilized samples between carbohydrates and proteins. Examples include lysozyme, BSA, PFK, bFGF, γ-IFN, recombinant G-CSF, bovine α-lactalbumin, and bovine α-casein, to name a few [77,81,123–125]. Spectroscopic studies of L-asparaginase freeze dried with trehalose indicated that the amide II band of the enzyme was quite similar to that observed in solution, thus suggesting that the level of hydrogen bonding for L-asparaginase was similar in the two states [116,126]. In another example, Prestrelski et al. [81] have demonstrated that the titration of sucrose with increasing amounts of protein resulted in decreased amount of residual water following lyophilization. The authors proposed that water is displaced from the dried protein through its direct interaction with the sugars. In fact, examination of the carboxylate bands in the spectrum of α-lactalbumin indicated that the addition of carbohydrate maintained these bands in the hydrogen-bonded or hydrated form after dehydration, as reported by Carpenter and Crowe [77]. Furthermore, the degree of structural protection conferred by saccharides, such as sucrose and trehalose, which are apparent in second derivative amide I infrared spectra, has been shown to correlate with the extent of hydrogen bonding between sugar and protein [125]. Direct binding (hydrogen bonding), though necessary, is insufficient to confer stability during lyophilization.

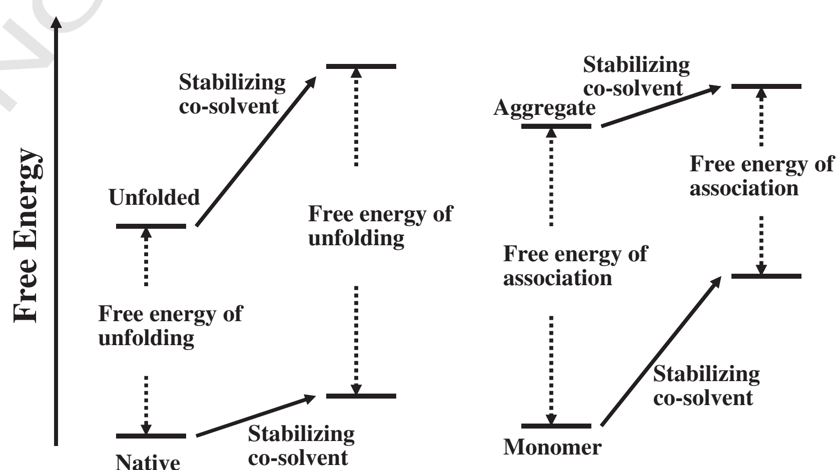

Fig. 9. Free energy diagram of protein unfolding and the effect of co-solvent interaction. The left panel illustrates protein unfolding and the right panel illustrates protein aggregation.

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For example, glucose has been shown to hydrogen bond effectively to dried proteins [127,128], however, this was insufficient to retain the native structure during freeze drying.

Vitrification hypothesis is based on the premise that the inhibition of molecular mobility, whether long-order (i.e., aggregation) or short-order (i.e., deamidation, cyclization, etc.), leads to an improvement in storage stability [129–131]. The parameter that has typically been examined for the purpose of comparing the expected stability of various formulations is the glass transition temperature (T_g). The publication of several reports illustrating the lack of direct correlation between T_g and stability has somewhat discredited the hypothesis as a stand-alone explanation for the observed stability of amorphous pharmaceuticals [132,133]. It should be noted that the occurrence of vitrification does not preclude the existence of direct interaction between the glassy matrix and the protein (i.e., water-replacement). Furthermore, the importance of reducing the molecular mobility is a common theme between the two hypotheses. Besides T_g, other parameters have been reported to correlate to stability. In one example, Yoshioka et al. [84] examined the effect of the molecular weight of dextran on the stability of freeze dried bovine γ-globulin (BGG) using ^{1}H-NMR. Changes in molecular mobility of freeze dried formulations occurring below T_g was detected and this temperature was called the molecular mobility-changing temperature (T_{mc}). T_{mc} increased as the molecular weight of dextran increased, which indicated that the molecular mobility of formulations in the microscopically liquidized state decreased as the molecular weight of dextran increased. In comparison to T_{mc}, the T_g of the freeze dried BGG formulations was determined to be higher. Thus, T_{mc} represents the temperature at which molecular mobility begins to increase in a temperature range below T_g [134], and may be a more relevant marker for stability indication.

While the amorphous or crystalline nature of excipients is clearly important in achieving optimal protein stability, the effects of the physical characteristics of the stabilizer cannot be generalized for all proteins. For example, mannitol is often used as a bulking agent in preparing lyophilized proteins due to its propensity for crystallization, thus it is phase separated from the protein, which is typically amorphous. However, the spectra of γ-IFN indicate that mannitol, and other crystallizing components such as myo-inositol, are destabilizing and induce further unfolding during dehydration [81]. This finding suggests that focusing solely on the physical properties of the excipients, while necessary, provides a limited view of the effects of the lyophilization process on protein stability. On the contrary, amorphous excipients form a part of the protein-rich glassy concentrate and behave differently from the segregated crystalline excipients. These behaviors can have important implications in regards to the stability of proteins during freezing, freeze drying, and subsequent storage. It should be emphasized that even when the physical criteria mentioned above are met (i.e., amorphous nature, T_g above storage temperature, etc.), there are cases in which a substantial loss of protein structure and activity are observed [82]. Chemical degradation, including oxidation and deamidation, could perturb the protein structure, and thus activity. These types of reactions may not be slowed sufficiently even upon the formation of the highly viscous glass, as would be for aggregation.

For optimal stability of the protein in the dried state, not only do the excipients have to replace the hydrogen bonding network lost during dehydration and remain in the amorphous phase, but they must also offer structural stabilization through direct binding. That is, the excipients must be in a specific geometrical orientation to interact favorably with the protein. This is illustrated by the example of HPβCD, which is a sugar polymer arranged in a cylindrical conformation with a hydrophilic outer surface and a hydrophobic internal cavity [135]. Reasons behind the efficacy of HPβCD include its relatively high collapse temperature (~−9 °C) and its intrinsic amorphous nature [136,137]. In addition, the hydrophilic exterior of the lyoprotectant was reported to provide the protein-HPβCD complex a higher degree of hydration and, thus, promote water structure formation. The most important requirement for the formation of a stable protein-HPβCD inclusion complex is the tight fitting, wholly or at least partially, of the protein within the cyclodextrin cavity. The hydrophobic cavity of HPβCD may enclose the amino acid side chains of mAb MN12, thus protecting them from a variety of degradation reactions. In fact, HPβCD has been reported to protect other drugs against oxidation and gastric acid degradation [135].

The effects of salts on the stability of dried proteins have also been examined, although not to the same extent as for sugars. It should be noted that the effects of salts in the dry systems are more case-specific than those in solution; in solution, stabilizing salts are universally stabilizing and destabilizing salts consistently demonstrate adverse effects. Buffer components may favorably or adversely affect the stability of proteins through direct interactions and/or through modification of its local environment (pH shift) [138–142]. Chang and Randall [127] have classified salts into 3 types based on their glass-forming tendency at a given cooling rate and subsequent thermal history: (1) crystallizing salts, (2) partially crystallizing salts, and (3) glass-forming salts. As glass-forming excipients can inhibit salt crystallization, salts can be included in the formulation when other amorphous excipients are present [143]. Interestingly, non-glass forming salts, on their own or in combination with glass-forming excipients (i.e., sugars), have been reported to demonstrate stabilizing effects on proteins following lyophilization. For example, Costantino et al. [94] reported that recombinant human albumin (rHA) co-lyophilized with NaCl did not exhibit any aggregation following prolonged incubation at 37 °C and 96% RH, while greater than 80% loss in solubility following 1 day of incubation under similar condition was noted for the protein lyophilized in the absence of the salt. As the inclusion of NaCl did not induce any significant changes to the secondary structure of lyophilized rHA, the stabilization effect of the salt was attributed to its water uptake in the vicinity of the protein, which may have facilitated protein refolding into its native and more stable conformation. Thus following this logic, the greater the affinity of salt (or excipient) for water, the greater the stabilizing effect. However, if a protein is sensitive to residual water, this stabilization mechanism is not applicable. Another stabilization mechanism proposed for salts is the prevention of protein–protein interaction and aggregation by physical dilution and separation of protein molecules. Liu et al. [144] attributed the reduced aggregation of lyophilized BSA in the presence of NaCl, along with other excipients, to the dilution effect.

In the case of glass-forming salts, the stabilization mechanism is thought to occur through a similar mechanism as that for sugars; i.e., their direct interaction with proteins to substitute for water molecules that are removed during drying. Carboxylic acid salts have been shown to provide both hydrogen bonds and electrostatic interactions with the protein, resulting in high T_g of the amorphous solid. In fact, the observed structural stabilization at specific salt-to-protein ratio indicates the presence of direct interaction. It is highly plausible that these buffer salts hydrogen bond to the protein, thereby substituting for the lost water molecules. In fact, FTIR analysis of bovine IgG in monosodium citrate buffer demonstrated the retention of intramolecular β-sheet band (1637 cm^{-1}) following lyophilization [95]. Furthermore, the lower concentration of monosodium citrate in comparison to sucrose, which was required to stabilize IgG, suggested an additional mode of interaction present between the salt and the protein (besides hydrogen bonding), which is most likely electrostatic interaction [145–147].

Salts have also been incorporated into a lyoprotectant formulation as a structure former. Chang and Randall [127] reported that one of the major stress factors that contribute to protein denaturation during a lyophilization cycle is the loss of cake structure. It was concluded that the addition of salts with eutectic melting temperatures (T_e) above −20 °C would promote rapid crystallization upon freezing and prevent the collapse of the frozen fraction during dehydration.

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However, the presence of uncrystallized salt in a freeze-concentrate usually depresses T_g', so the salt content in protein formulations should be kept to a minimum [121]. It should be noted that the occurrence of cake collapse (or lack of typical structure) does not necessarily correlate to instability, as demonstrated by several authors [148–151].

4. Polymers

4.1. Solution

Hydrophilic polymers have often been used to stabilize proteins and enhance protein assembly [152–154]. Sasahara et al. [155] demonstrated that the stability of a protein against heat treatment was increased through the incorporation of dextran. Manning et al. [156] have studied the effects of polymeric excipients on the thermally-induced aggregation of low molecular weight urokinase, and found hydroxyl ethyl (HETA) starch, PEG4000, and gelatin to all be effective in stabilizing the enzyme, which consequently suppressed aggregation [157]. In contrast, polyvinylpyrrolidone (PVP) and low molecular weight PEGs (e.g., PEG 300) were found to be ineffective, as their hydrophobic nature offset the stabilizing effects of the polymers. Unlike small molecular weight protein-stabilizers, polymers possessing a hydrophobic moiety do not always stabilize proteins. An example is shown in Fig. 10A, which plots the change in the melting temperature of β-lactoglobulin as a function of PEG concentration [158]. Both PEG200 and 1000 greatly decreased the melting temperature. Due to the smaller molecular weight of these PEGs, it appears that the excluded volume effects (i.e., stabilizing effects) are overwhelmed by their hydrophobic nature. In addition, as polymers are strong protein precipitants, they are known to enhance self-association as well as protein–protein and protein–macromolecule interactions, leading to protein aggregation [159–161]. Examples of these effects include the acceleration of α-synuclein fibril formation upon the addition of PEG, dextran, and Ficoll [162,163].

Charged polymers can stabilize proteins via electrostatic interactions through their multiple charged binding sites [164,165]. This effect is rather protein specific, as has been demonstrated for acidic fibroblast growth factor (aFGF), which has a constellation of positively charged groups on the surface [166–168]. Won and co-workers [169] found that a variety of sulfated and phosphorylated anionic polymers (heparin, dextran sulfate, pentosan sulfate, enoxaparin, phosvitin, and phytic acid) were effective at stabilizing aFGF. The only requirement for aFGF stabilization appeared to be the presence of one or more regions of high negative charge density [68]. Similarly, other negatively charged biopolymers, e.g., nucleic acids, were found to be effective. Furthermore, negatively charged dextran sulfate was found to be effective in preventing aggregation of basic ribonuclease A [170]. Andersson and Hatti-Kaul [171] examined the effect of polyethyleneimine (PEI), a cationic polymer, on the stability of lactate dehydrogenase (LDH) and found the storage stability of LDH to be improved (and prevented the aggregation) upon the addition of 0.01–1% (w/v) polymer. Unlike the protein-stabilizing excipients (e.g., sugars and salts), the addition of PEI did not increase the denaturation temperature of LDH (62 °C), although it did suppress the oxidation of free sulfhydryl groups (which are catalyzed by metal ions), thus improving the stability of the enzyme. The protective effect is attributed to the metal chelating property of PEI [172]. Furthermore, the addition of 0.1% PEI was effective in maintaining the secondary structure of the enzyme, while in its absence significant loss was observed following 2 weeks of storage. Charged DEAE-dextran polymer conferred no stabilization effect on the green fluorescent protein, suggesting the protein-specific nature of charged polymers [173].

PEG is different from the more hydrophilic polysaccharides (e.g., dextran) in that it possesses a small, non-polar moiety. PEG has been shown to decrease the surface tension of water and act as a surfactant [174]. PEG and other polymers have been used to suppress protein–protein interactions and surface adsorption through hydrophobic competitive interaction. Poloxamers, which are non-ionic co-polymers of polyoxypropylene and polyoxyethylene, have also been found to be effective in preventing aggregation induced by various stresses [175]. Poloxamers are amphiphilic, and are thus surface active. The proportion of hydrophobic and hydrophilic moieties can be modulated by the relative sizes of the polypropylene hydrophobic core and the hydrophilic polyoxyethylene moieties, and thus, a wide variety of poloxamers are commercially available. In this regard, many proteins are also amphiphilic. For example, human serum albumin (HSA) has been used as a stabilizer in pharmaceutical products [176], typically at concentrations ranging from 0.1 to 1%, as found in many patent applications and publications, e.g. [177]. Although HSA has been used as a stabilizing excipient in a number of protein therapeutics to prevent surface adsorption, recent concerns about potential infectious agents in animal-derived products have prompted regulatory agencies to restrict its usage, and non-ionic surfactants are increasingly finding use as a replacement for serum albumin [178]. As polymers and proteins are competitive inhibitors for protein adsorption, their use at low concentrations may be sufficient to cover the protein binding sites on the surface.

4.2. Dry state

Polymers have been demonstrated to be a successful additive in suppressing protein aggregation during lyophilization and to prevent the solubility decrease observed during reconstitution [179–182]. Dextran, CMC, DEAE-dextran, and PEG have all been shown to reduce the aggregation of lyophilized BSA significantly during storage at 37 °C [144]. The derivatized starch, hydroxypropyl β-cyclodextrin (HPβCD), has been used to improve the solubility and prevent the

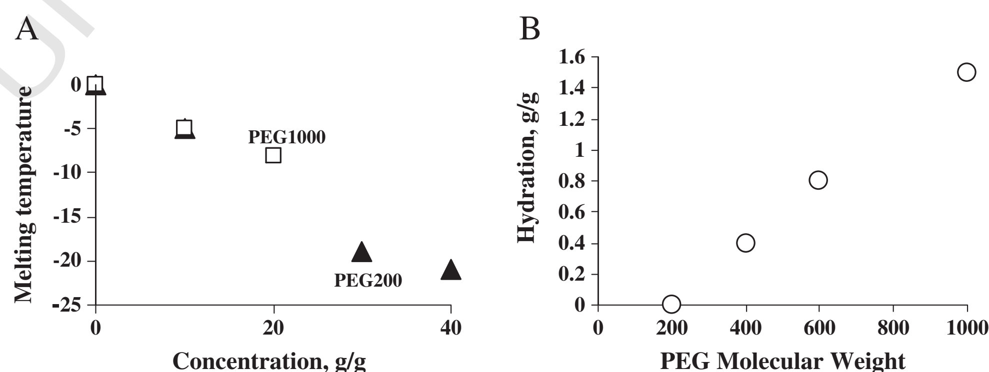

Fig. 10. Effect of PEG on (A) the melting temperature and (B) preferential hydration of β-lactoglobulin. Data adapted from Arakawa and Timasheff [158].

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lyophilization-induced insoluble aggregate formation for growth hormone, interleukin-2 (IL-2), and insulin [183]. In addition, HPβCD was found to stabilize lyophilized mouse monoclonal antibody during storage at 56 °C [73] and inhibit the dimerization of lyophilized TNF during storage at 37 °C [184]. Dextran 40 at 10% concentration increased the activity of lyophilized elastase (20 mg/ml) from 33 to 82% following storage for 2 weeks at 40 °C and 79% RH [83], while dextran (162 kD) at 3.5 and 5% (w/v) improved the storage stability of lyophilized rFXIII and *Humicola lanuginose* lipase, respectively, at 40 or 60 °C [82,185]. Several PVP's and maltodextrin were reported to stabilize lyophilized invertase during incubation at 90 °C [133,186], and 1–10% PVP or BSA have been reported to improve the recovery of LDH activity following freeze-thaw or lyophilization [187] (Fig. 11). In contrast, PEI addition failed to confer stability to LDH following freeze-thaw (Table 1). The charged polymer was, however, effective in maintaining the enzyme activity following freeze drying and the degree of protection was found to depend on the concentration of PEI used.

HSA at concentrations between 0.05 and 0.1% (w/v) has been used as a lyoprotectant in formulating hydrophobic cytokines, including interleukin-1a (IL-1a), IL-1b, IL-3, and macrophage colony stimulating factor (MCSF) [188]. Inclusion of BSA at 0.05% concentration increased the recovered activity of LDH (25 μg/ml) from approximately 30 to 80% following lyophilization [187]. LDH activity was also maintained during lyophilization in the presence of different concentrations of PEI [189] as well as with PVP (40 kD) [187]. Hydroxyethylcellulose (HEC) at 1% completely inhibited the lyophilization-induced aggregation of aFGF at 100 μg/ml in PBS containing 33 μg/ml heparin [190].

Polymers may not always stabilize proteins in the solid state, and in some cases, have adverse effects. For example, dextran of certain molecular weight may be unable to provide sufficient stability to proteins during lyophilization due to steric hindrance, which prevents efficient hydrogen bonding with proteins. Dextran (40 kD) at concentrations of up to 100 mg/ml was ineffective in inhibiting dehydration-induced unfolding of lysozyme [128], and its addition was not effective in preventing the formation of β-sheets in poly-L-lysine during dehydration [191]. Certain polymers may also cause phase separation during freezing, which can adversely affect protein stability. The presence of dextran 40 in IL-6-sucrose formulation increased protein aggregation during storage for 9 months at 30 °C [192]. Also, rehydration of PEG-containing lyophilized sample resulted in protein precipitation [114]. It is possible that the high concentration of PEG, a strong protein precipitant [158], induced precipitation of the protein during rehydration, resulting in its lower activity. PEG was also reported to be ineffective in stabilizing lysozyme, even up to concentrations of 100 mg/ml [128]. However, in combination with a smaller excipient, such as glucose, PEG was shown to be an effective lyoprotectant. In fact, as the glucose concentration was increased in lysozyme preparations

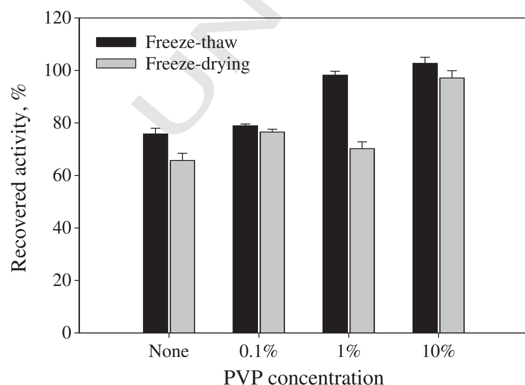

Fig. 11. Effects of PVP concentration on the recovery of LDH activity following freeze-thaw or lyophilization.
Data adapted from Anchordoquy and Carpenter [187].

Table 1
Effect of polyethyleneimine (PEI) on the stability of LDH during freeze-thaw, freeze drying, and drying. LDH concentration was 50 μg/ml in 50 mM Tris–HCl at pH 7.2. Data adapted from Andersson and Hatti-Kaul [171].

PEI	Concentration	Residual activity (%)		
		Freeze-thaw	Freeze dried	Drying
None		84 ± 2.6	27 ± 0.4	43 ± 0.3
Low MW	0.01	82 ± 1.0	43 ± 3.7	ND
	0.1	79 ± 2.8	47 ± 0.4	ND
	1	76 ± 4.3	69 ± 3.9	ND
High MW	0.01	77 ± 1.0	52 ± 5.1	77 ± 1.8
	0.1	83 ± 2.6	64 ± 0.8	79 ± 0.8
	1	83 ± 1.4	69 ± 0.7	78 ± 2.4

containing 1% PEG8000, the extent of hydrogen bonding to protein carboxylate groups increased and the lyophilized sample demonstrated structural similarity to the native lysozyme [128]. A similar positive and additive effect was observed following PEG addition to sucrose and trehalose.

4.3. Mechanism

In solution, polymers provide protein stabilization by both protein specific and non-specific mechanisms. Charged polymers mainly work in a protein-specific manner, while polar, hydrophilic polymers stabilize proteins independent of their chemical nature. Hydrophilic polymers (e.g., polyethylene glycols, polysaccharides, and inert proteins) can stabilize proteins by a multitude of mechanisms [156,158,169,170,193–196]. The dominant factor among them is the molecular crowding effect, as has been observed for protein transport in polymer gels and concentrated protein solutions [197–199]. This is schematically represented in Fig. 12, in which the aqueous solution is filled with polymers (closed circles). When a protein molecule (thick line) is in equilibrium between the native, compact structure (small dotted circle) and the unfolded, expanded structure (large dotted circle), it is thermodynamically more favorable to maintain the native structure, as it possesses a smaller radius and hence surface area for exclusion. Thus, the native protein is more favorable in the presence of a polymer, and even more so at higher polymer concentrations. As polymer exclusion increases with its size, larger polymers are generally more effective in stabilizing proteins in solution [200]. Such an effect can be replicated using a protein, e.g., HSA and other, but similar, proteins. The latter suggests that a protein is thermodynamically more stable at higher concentrations, which increases the molecular crowding effect. In Fig. 3, this corresponds to a decrease in K_1 due to high protein concentration and greater crowding effects. Of course, other factors may affect such stabilizing mechanism at high protein concentrations, e.g., reversible or irreversible self-association being one of them, as also seen in Fig. 3, in which high protein concentration can increase K_2, eventually leading to aggregation. Excluded volume effect is essentially a repulsive interaction between protein and polymer. Thermodynamic interaction measurements indicated that PEGs are preferentially excluded from the protein surface [158,201,202]. Fig. 10B depicts the effect of PEG's molecular weight on the amount of excess water in the vicinity of β-lactoglobulin as a result of PEG exclusion from the protein surface. The figure clearly illustrates that excess water increases with the molecular weight of PEG, as expected from the increasing exclusion of higher molecular weight PEGs. As is the case for protein-stabilizing co-solvents, such exclusion leads to a thermodynamically unstable state of the protein. Protein unfolding can further increase the instability and hence is suppressed in the presence of polymers.

However, polymers possessing hydrophobic character, such as PEG, can bind to proteins through hydrophobic interaction. In fact, it has been shown that the thermodynamic interaction of PEG with aromatic groups is thermodynamically favorable [Hirano et al., unpublished work]. Thus,

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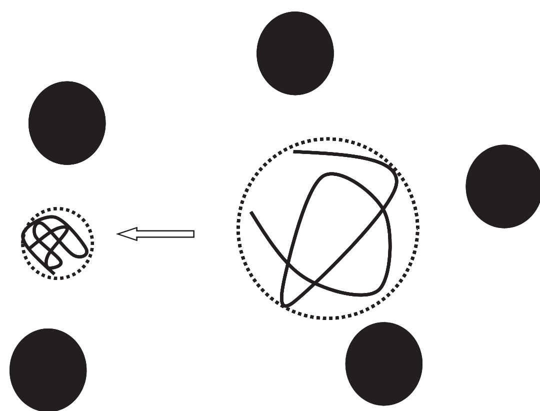

Fig. 12. Illustration of the molecular crowding effect. Protein molecules are represented by the lines and the dotted circle surrounding the lines represent the volume occupied by the folded (left) and unfolded (right) protein. Polymers are represented by the black circles.

the stabilizing effect of PEGs on proteins is a delicate balance between the two opposing effects: stabilizing effect due to steric exclusion and destabilizing effect due to hydrophobic interaction. This is schematically represented in Fig. 13. Hydrophobic sequences (thick black line) of the protein are sequestered within the native protein structure (left side), and are not expected to interact strongly with the polymer (black circle). When the protein unfolds (right side), however, the hydrophobic regions become exposed, and the polymer can bind to the unfolded structure with greater affinity, or to a greater extent, in comparison to the native state. Thus, such polymers could stabilize the unfolded structure by hydrophobic interaction and the native structure by excluded volume. The overall effect of such polymers will then be determined by the balance between the two opposing factors. In solution (and during the freezing process which will be described later), proteins tend to be adsorbed to the surface through hydrophobic (and also electrostatic) interactions. Amphiphilic polymers such as PEG, poloxamers, and HSA can compete with the protein and prevent its adsorption-mediated conformational change(s) and consequent aggregation.

A second mechanism by which polymers can stabilize proteins is via specific binding, e.g., polyanion binding to positively-charged heparin binding site of aFGF [68]. Polymers have also been shown to prevent certain types of chemical instability that can lead to aggregation, e.g., metal-ion catalyzed oxidation can be inhibited by polymers via metal ion complexation [203].

Polymers have also been shown to suppress the damage to multi-subunit proteins during freeze-thaw or lyophilization by stabilizing their quaternary structure in the frozen state via preferential exclusion mechanism [83,187]. This is because water is still present during freezing, although water activity is gradually decreased upon ice crystal formation, and as long as water is present, polymer exclusion mechanism is still in operation. Stability in the dried state has been typically attributed to hydrogen bonding between the polymer and protein [77,118], however due to steric hindrance, polymers do not readily form hydrogen bonds with proteins as is the case with sugars. The inherent stability of multimeric enzymes is known to be greater in the assembled state than as unassociated subunits [204,205]. Inhibition of subunit disassembly by polymers in solution, and its subsequent immobilization in the glassy matrix, may account for the preserved activity of the enzyme. While the formation of a glassy state, in and of itself, is insufficient for protection [118,206,207], the high viscosity in the glass could prevent the dissociation during dehydration, thus contributing to the observed protection of protein activity, as is the case for LDH. Interestingly, these polymers have also been found to inhibit freezing-induced shifts in pH, presumably by inhibiting crystallization of buffer salts, particularly in the case of phosphate buffer systems, and thus resulting in enhanced protein stability.

While non-ionic polymers stabilize proteins by being preferentially excluded from their surface [158,208], charged polymers stabilize or inactivate proteins depending on their mode of interaction. Since proteins are poly-ampholytic molecules, they can interact with polymers, or polyelectrolytes, via long-range Coulombic forces [209,210]. In case of PEI [171], it is likely that charge–charge and/or hydrophobic interactions between the protein and polymer overcome the effects of preferential exclusion, thus providing no stabilizing effect during freezing, however during dehydration, the polymer provides protection to the quaternary structure of LDH (Table 1).

Polymers, such as dextran, have also been reported to stabilize proteins by raising the T_g of the protein formulation [211,212] and by inhibiting crystallization of small stabilizing excipients, such as sucrose [213]. In fact, the T_g of dextran-formulated γ-globulin formulations increased significantly with increasing molecular weight of dextran from 10 to 510 kD [214]. Similarly, the stability of lyophilized invertase was shown to correlate to the T_g of the maltodextrin (MD) or PVP [215]. In fact, an inverse correlation was reported to exist between the remaining activity and (T–T_g) for MD and PVP of various molecular weight, suggesting that higher enzyme activity is associated with higher T_g. More specifically, PVP-360 ($T_g = 155$ °C) and PVP-40 ($T_g = 137$ °C) afforded better enzyme stabilization compared to PVP-10 ($T_g = 93$ °C), and this may be correlated with their higher T_g values (Fig. 14). However, the maintenance of the glassy state was insufficient to prevent inactivation, since PVP systems maintained at temperatures below their

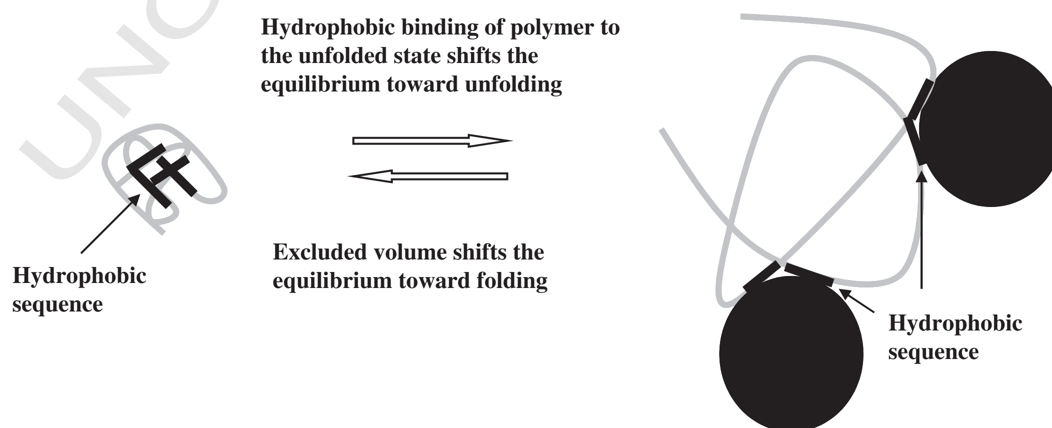

Fig. 13. Illustration demonstrating the two opposing effects of polymers (i.e., PEG) on protein structure. The hydrophobic sequence of a protein is represented by the thick black lines within the protein structure (gray lines). Hydrophobic binding of polymers (black circles) to the unfolded state shifts the equilibrium towards unfolding, while excluded volume effect shifts the equilibrium toward the folded state.

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T_g values (i.e., 90 °C) still demonstrated significant enzyme inactivation. This is most likely due to the presence of mobility at temperatures below T_g, as reported by Simatos et al. [216] among others.

Although large molecular weight carbohydrate polymers, such as dextran and HES, are effective in increasing the T_g' and the collapse temperature, allowing the freeze drying process to be conducted at higher temperatures, they are not very effective in protecting the protein during lyophilization. Unlike smaller sugars, they cannot effectively hydrogen bond to the protein as a result of steric interference. To circumvent this shortcoming, a small disaccharide can be used concurrently with the carbohydrate polymers. The efficacy of the combination stems from the independent stabilizing capabilities of the two components; polymers are an effective cryoprotectant, while sugars are effective against dehydration stress. However, care must be taken in adjusting the ratio of the two components. Allison et al. [125] suggested that in the two component system, the ability of a sugar to hydrogen bond to the protein surface may be reduced due to the partitioning of some fraction of the sugar molecules with the polymer in the dried solid. As a result, there may be less sugar available to interact with and stabilize the protein.

5. Surfactants

5.1. Solution

Surfactants are widely used to stabilize proteins, suppress aggregation and assist in protein refolding [217,218]. Polysorbate 80 (polyoxyethylene sorbitan monooleate) and polysorbate 20 (polyoxyethylene sorbitan monolaurate) are two of the widely incorporated surfactants in marketed protein pharmaceuticals [176,178,219], and are typically used in the 0.0003–0.3% range [176]. The effects of surfactants and their interaction mechanism in aqueous solution will be described in Section 8, thus this section will place more emphasis on their effects in the dry state. It has been extensively documented that surfactants suppress protein aggregation against various stresses, including heating and agitation. While the suppression of aggregation is almost universally observed in solution, the effect of a surfactant on thermal stress or denaturant-induced protein unfolding varies, depending on the protein itself and on the stress conditions. Surfactants have also been reported to be an effective stabilizer in protecting proteins against surface denaturation in non-frozen aqueous solutions [220,221].

5.2. Dry state

In the context of lyophilized formulations, surfactants have been used to prevent aggregation. For example, when 1 mg/ml solution of IL-1ra was freeze-dried with 0.1% Tween 80, less than 3% soluble aggregates was detected by size-exclusion HPLC (SE-HPLC), while in the absence of the surfactant, approximately 50% of the protein was observed to form soluble aggregates [222]. Kreilgaard et al. [82] demonstrated that Tween 20 addition (0.002% w/v) improved the recovery of native rFXIII and reduced the amount of insoluble aggregates formed. In addition, FTIR analysis of the same system revealed that the addition of 0.1% Tween 80 was sufficient in inhibiting aggregation and maintaining the secondary structure of freeze dried IL-1ra, with respect to that of the native structure in solution. Furthermore, the protective effect of surfactant was much greater than that by sucrose, for which 1% addition resulted in 8% aggregate formation.

There have been a number of examples in the literature (i.e., keratinocyte growth factor, Interleukin 2, Interleukin 1 receptor antagonist, and bovine IgG, etc.) which demonstrated the efficacy of surfactant-containing diluent in reducing aggregation following reconstitution [140,223–225]. However, this effect cannot be generalized, because it is somewhat specific to Tween/polysorbate. Other surfactants such as N-octyl glucoside and Pluronic either exhibited no effect or promoted aggregation [31,224]. Interestingly, in the case of Anti-L-Selectin, the presence of Tween in the reconstitution buffer was necessary to prevent aggregate formation, but if present during lyophilization, it increased the aggregate level in the reconstituted solution [223]. The mechanism of this protection is not well understood, as there was no evidence of Tween binding to, or stabilizing, the native state of the protein.

Spray drying subjects proteins to large air–water interfacial surface area during atomization. In a study to minimize aggregation of recombinant human growth hormone (rhGH) during spray drying, Maa et al. [226] reported that insoluble aggregate formation decreased as polysorbate concentration was increased. The aggregate level reached a plateau at a certain critical polysorbate concentration (cpc), which was independent of the protein concentration. They also demonstrated that cpc was directly proportional to the air–water interfacial area, i.e., inversely proportional to the median droplet diameter, thus demonstrating that insoluble aggregate formation was linked to denaturation of hGH at the air–water interface, and that polysorbate suppressed rhGH aggregation by competing with the protein for the air–water interface. It is interesting to note that in this study, polysorbate was unable to completely arrest soluble aggregate formation, requiring the use of Zn^{2+} as an additional excipient, thus highlighting the fact that aggregation can occur by multiple mechanisms. Effective control requires excipients that work by multiple mechanisms, e.g., competition with air–water interface, thermodynamic stabilization by preferential exclusion, or specific ligand binding.

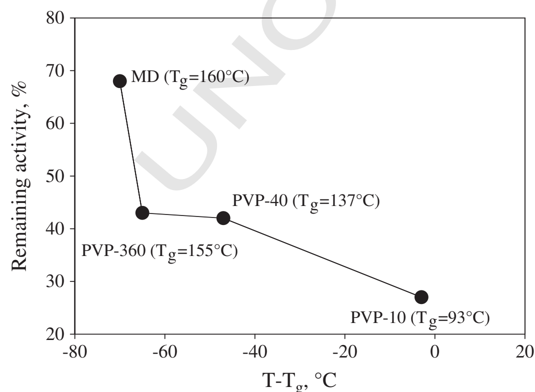

Fig. 14. Effect of polymer glass transition temperature (T_g) on the amount of activity loss of lyophilized invertase at near-zero residual water content following 20 h incubation at 90 °C. The polymers examined in the study and their respective T_g values are indicated in the figure.
Data adapted from Schebor et al. [215].

5.3. Mechanism

Extensive analysis of the effects of surfactants will be described in Section 8 and it may suffice to mention here that the fundamental mechanism of aggregation suppression by surfactants is the prevention of surface denaturation [222,227,228]. Protein–surfactant interactions have been studied by various indirect methods including surface tension [229], viscosity [230], and dye solubilization [231], and by direct measurements such as dialysis [232–236] and ion-selective electrodes [237–240]. Surfactants compete with protein for container surface, air–water interface, ice–water interface, or any other solid surfaces, and prevent the protein from non-specific adsorption [241–243]. Although it is possible that surfactants bind to the protein at the hydrophobic sites and reduce their tendency to aggregate [244–246], it is difficult to distinguish such direct binding from inhibition of surface denaturation. Various surfactants have been reported to be effective in preventing freeze-thaw-induced damage to proteins [223,242,247]. Examples include, Tween 80, Brij 35, Brig 30, Triton X-10, Pluronic F127, and SDS. Surfactants can also prevent aggregation by serving as chaperones and foster protein refolding [223,224,247–250].

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There are various mechanisms by which surfactants exert their protective effects on proteins during lyophilization. The mechanisms include: 1) prevention of surface-induced unfolding and aggregation during mixing, filtration, and filling operations prior to lyophilization [241,245,251]; 2) prevention of structural damage and aggregation at the ice–water interface during the freezing step [222]; 3) protection against aggregation during the drying step (although they are not as effective as disaccharides) [223]; and 4) prevention of aggregation during the rehydration step [31,223,224,247]. The fact that surfactants are effective in preventing aggregation during reconstitution of lyophilized formulations [224,225,247] suggests the reversible nature of the surfactant–protein interaction. It is also possible that the presence of a surfactant at the solid–air interface of the lyophilized sample retards the dissolution rate during reconstitution, thus allowing for sufficient time for protein refolding [247].

Although the widespread use of non-ionic surfactants reflects their effectiveness in preventing surface- and stress-induced aggregation of proteins, they must be used with caution. Polysorbate can undergo auto-oxidation [178,252,253], hydrolysis of the fatty acid ester bond [178], or in some cases increase thermally- [254] and denaturant-induced [247] aggregation. In addition, surfactants alone may be insufficient to confer stability during long term storage [255].

6. Arginine

6.1. Solution

The effects of amino acids in general were described earlier in Section 2. Here, the focus is placed on one specific amino acid, arginine. Arginine is not a protein-stabilizing excipient, but is highly effective in suppressing protein aggregation. Due to this effect and its safety in humans, arginine is frequently used for enhancing the shelf life of proteins. The aggregation-suppressing effect of arginine was accidentally observed by Rudolph and Fischer [256] during their attempt to prevent the auto-catalytic digestion of refolded tissue-type plasminogen activator. Inclusion of arginine during refolding led to increased recovery of the protein by suppressing the aggregation of folding intermediates, without imparting any stabilizing effect on the native structure itself. Arginine does not enhance protein stability [35] and is also not utilized by osmo-tolerant organisms [42], and thus does not belong to the class of osmolytes. However, it does increase the solubility of proteins and suppresses aggregation [34–37,257–262]. Here, the term “stability” is used with more specificity: under conditions in which the co-solvent increases the stability of the proteins by increasing the equilibrium concentration of the native state (see Fig. 3). Certain co-solvents, e.g., arginine, suppressed protein aggregation, while not increasing such equilibrium. For example, ciliary neurotrophic factor (CNTF) readily aggregates when subjected to heat stress, however aggregation is completely suppressed in the presence of arginine [263]. Arginine has been shown to inhibit the aggregation of lysozyme during refolding following heat denaturation, although it did not enhance its thermal stability [264]. Arginine has been reported to reduce the aggregation of heat- or urea-denatured lysozyme [36,264], interleukin-6, and antibodies [265]. An interesting application of arginine is its ability to synergistically inhibit aggregation of insulin in the presence of α-crystallin, which functions as a chaperone [266]. Application of arginine in the suppression of protein aggregation is rapidly growing.

FGF20 is an investigational therapeutic protein for oral mucositis [267,268] and also a candidate for Parkinson's disease [269–271]. Similarly to other FGF family members, the handling of FGF20 is problematic in that its solubility is very low [272]. Fig. 15A shows the solubility of recombinant *E. coli*-derived FGF20 in 50 mM phosphate as a function of pH. The solubility data exhibits a typical bell-shaped curve with minimal solubility of ~0.02 mg/ml observed at pH close to the pI of FGF20 (~pH 7.0). Even at pH values far removed from the pI, the solubility was increased to only ~0.25 mg/ml, which is still too low for processing. Fig. 15B shows the effects of arginine (arginine sulfate, to be exact) on its solubility, expressed as the ratio of the protein solubility in the presence of arginine to the solubility in its absence at each pH value. Arginine concentration monotonically increased the solubility of FGF20. The effects were insignificant at pH 8.0 and the effects were maximum at pH 6.0, leading to ~1000-fold increase in solubility. As the charged state of both arginine and FGF20 changes with pH, it is evident that the charges on arginine, protein, or both play a key role in increasing the solubility of FGF20.

FGF family members are characterized by their ability to bind to heparin and poly-anions, as described for KGF [26,71,168]. As the arginine salt (i.e., arginine sulfate) was used above to test for the effects of arginine on FGF20 solubility, the effects of sulfate anion cannot be ruled out. Consistent with this notion, sodium sulfate, typically classified as a salting out salt, significantly increased the solubility by approximately 20-fold, as shown in Fig. 16. However, the effects are much weaker than that of arginine sulfate, clearly indicating the contribution from arginine. However, arginine alone is less effective, as observed for arginine chloride and phosphate. Thus, it appears that arginine and sulfate synergistically affect the solubility of FGF20.

Similar effect of arginine on protein solubility has been observed for recombinant plasminogen activator (rPA), when the equilibrium solubility was measured by two different methods, as described in Fig. 17. This protein has an extremely low aqueous solubility, less than 1 mg/ml. The solubility of rPA increased upon the addition of arginine in a concentration-dependent manner, leading to >50 mg/ml at 1 M concentration, as shown in Fig. 17. In contrast, NaCl at 1 M concentration and a combination of 0.5 M NaCl and 0.5 M glycine demonstrated marginal improvement (Fig. 17). These results clearly demonstrate the unique nature of arginine in that neither changes in concentration nor ionic strength using other salts and amino acids is sufficient to increase the protein solubility.

High concentration antibody formulations often become too viscous to inject. It takes several minutes to inject a small volume of the formulation, as shown in Fig. 18. Addition of arginine at ≥0.15 M reduced the viscosity, and its inclusion may allow for shorter administration time. Such effect of arginine on high protein concentration formulation may be a viable approach for reducing the formulation viscosity.

6.2. Dry state

Mattern et al. [90] examined the physical state of several amino acids following lyophilization, and reported that only a handful of amino acids, including arginine, formed an amorphous solid. The rest was crystalline solids. For L-arginine, 1.2 wt.% residual water content and T_g of 42 °C were noted (although wide-angle X-ray diffraction revealed the partially crystalline nature of arginine), while the vast majority of other amino acids examined exhibited no detectable T_g and were apparently fully crystalline following lyophilization. The crystallization propensity of amino acids justifies the frequently cited use of amino acids as crystalline bulking agents [87]. The addition of HCl, H_3PO_4, or H_2SO_4 to arginine, sufficient to form the respective salt, produced amorphous solids following vacuum drying, however, all salts retained high residual water contents and consequently exhibited relatively low T_g. For example, an equimolar solution of L-arginine and HCl resulted in a greater than 2-fold increase in the residual water content, and T_g was reduced from 42 to 18 °C. In contrast, a solution of H_3PO_4 and L-arginine at 1:2 molar ratio resulted in increased T_g from 42 to 93 °C, despite the increase in residual water content. Furthermore, the dried product of arginine-PO_4 was fully amorphous. Similar behavior of T_g-enhancing capability of PO_4 following its addition to sugars has been reported by Ohtake et al. [273].

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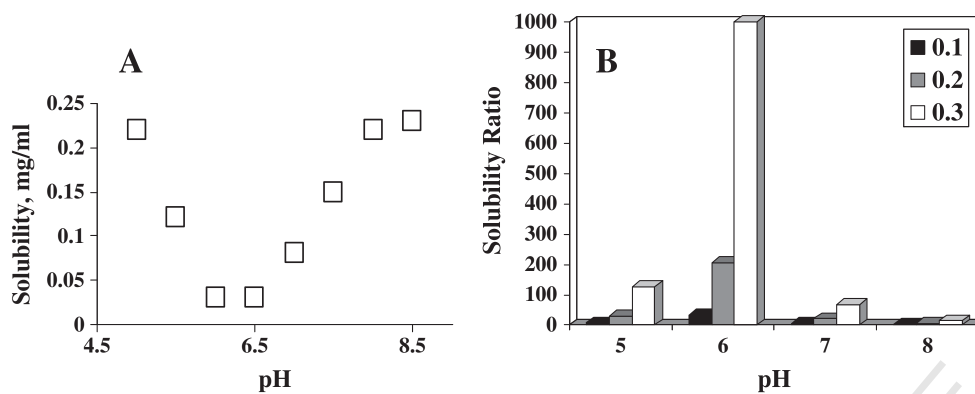

Fig. 15. Solubility of FGF-20 in 50 mM sodium phosphate buffer in the (A) absence and (B) presence of arginine sulfate at various solution pH values. Solubility ratio in (B) represents the ratio of FGF-20 solubility in the presence and absence of arginine sulfate at each pH.
Data adapted from Maity et al. [272].

The rate of water removal has an impact on the physical structure of dried arginine salts. L-arginine on its own was crystalline following vacuum drying (i.e., without freezing), although it was found to be amorphous following lyophilization (Table 2). The residual water content of the crystalline arginine was higher than that of the amorphous form (1.3 and 0.5%, respectively). The addition of HCl or H_3PO_4 suppressed arginine crystallization during vacuum drying, which can be attributed to the reduced tendency for nucleation (due to salt formation) during desiccation. Interestingly, the T_g of freeze dried arginine salts was higher than those prepared by vacuum drying. Suppression of arginine crystallization during vacuum drying was also shown to be partially suppressed upon the addition of phenylalanine (Phe). Furthermore, arginine in combination with Phe and mineral acid inhibited the aggregation of vacuum dried recombinant human granulocyte colony-stimulating factor (rhG-CSF) and lactate dehydrogenase (LDH) during storage at 40 °C [90].

β-galactosidase freeze dried with L-arginine HCl demonstrated no loss in activity following storage at 70 °C for 7 days (Fig. 19) [80]. In contrast, the enzyme lyophilized without additives only retained approximately 20% of its initial activity following storage under similar conditions. Interestingly, β-galactosidase lost its activity completely if freeze dried with arginine, and this may be attributed to the increased pH of the solution during desiccation. X-ray diffraction determined the samples lyophilized with arginine and arginine-HCl to be both amorphous (though it is not clear to the authors how the free arginine base was incorporated into the formulation prior to lyophilization) [80].

The crystallization propensity of arginine is partly dictated by the amount of interactions present between the amino acid and the protein. In one example, the freeze dried mixture containing arginine with anti-CD11a antibody demonstrated melting endotherms at 12% antibody concentration using DSC [274]. Upon increasing the antibody concentration beyond 20%, the melting peak was no longer detected, which suggests the presence of direct interactions between the antibody and L-arginine upon lyophilization.

6.3. Mechanism

How does arginine suppress aggregation of proteins? Similar to the protein stabilizing co-solvents, arginine is more effective at higher concentrations, e.g., above 0.1 M, although its effectiveness at lower concentrations, e.g., ~15–50 mM, has recently been reported. Although arginine is a natural amino acid, it is not an osmolyte, unlike many other amino acids. Yancey et al. [42] examined the effects of osmolytes, arginine, and salts on the enzymatic function of LDH for nicotinamide adenine dinucleotide and observed no adverse effects upon osmolyte addition. However, the authors reported a significant inhibition of substrate binding for arginine and salts, as plotted in Fig. 20. It has been shown that arginine does not bind strongly to proteins, nor is it strongly

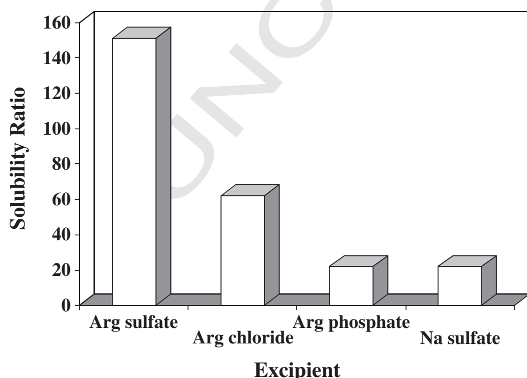

Fig. 16. Solubility of FGF-20 in 50 mM sodium phosphate buffer in the presence of various arginine salts, including 0.4 M arginine sulfate, 0.4 M arginine chloride, and 0.4 M arginine phosphate. 0.185 M sodium sulfate was included as control. Solubility ratio represents the ratio of FGF-20 solubility in the presence of excipients to that in their absence at pH 7.0.
Data adapted from Maity et al. [272].

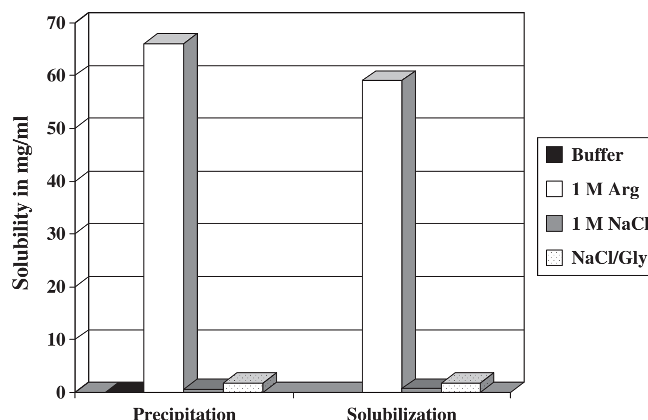

Fig. 17. Solubility of recombinant plasminogen activator in the absence and presence of various co-solvents, including 1 M arginine, 1 M NaCl, and NaCl/glycine (0.5 M each). Equilibrium solubility was measured by precipitation of protein from a concentrated solution or solubilization of the protein precipitate.
Data adapted from Tischer et al. [262].

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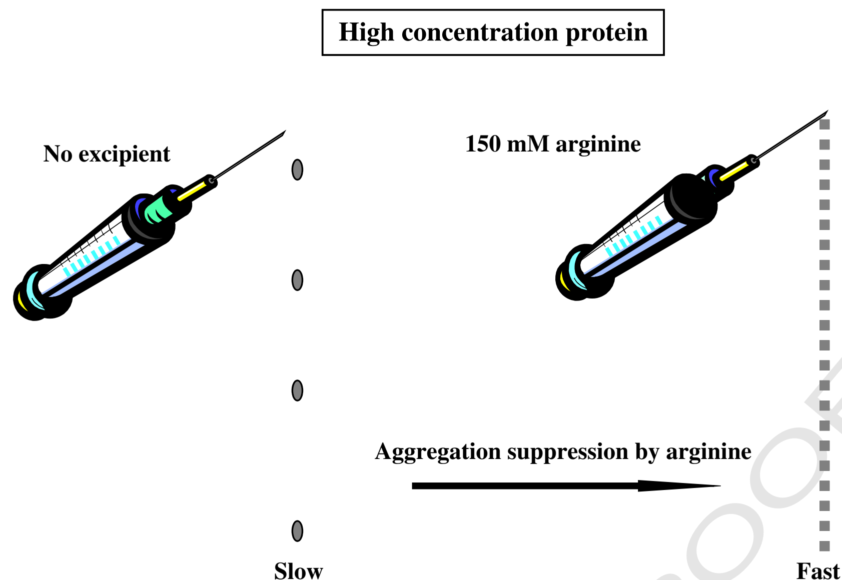

Fig. 18. Illustration of the effect of arginine on the reduction of viscosity of a high concentration protein formulation.

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excluded from the protein surface [174,275]. Its interaction with protein is dependent on the co-solvent concentration, buffer concentration, the pH, and the protein itself [174,276]. Nevertheless, arginine does demonstrate weak affinity for proteins [174,277]. Amino acid solubility measurements revealed that arginine and GdnHCl interact in a similar manner with the amino acid side chains and peptide backbones, suggesting that arginine has affinity for side chain groups, most significantly for aromatic side chains [277]. In fact, binding of arginine to small aromatic compounds has been suggested in several reports [246,252,278]. The interaction is schematically illustrated in Fig. 21, which compares the mode of protein–solvent interactions for various co-solvents, including arginine and a specific ligand. Specific ligands in general bind to the functional, and hence, native structure, thereby stabilizing the protein. Case A depicts the interaction of structure-stabilizing osmolytes with a protein. The interaction with the native protein is unfavorable, although to a lesser extent than with the unfolded state. Thus, unfolding free energy becomes greater in the presence of stabilizing co-solvents (see Fig. 9), relative to that in their absence. Unlike protein stabilizers, protein denaturants bind to the native protein [279,280], and even more so with the unfolded state (case C). The interaction of arginine with the native protein varies depending on the solution condition, and thus their interaction with the unfolded state cannot be inferred readily (case B). However, the fact that arginine has little effect on the stability of protein suggests neither strong binding nor strong exclusion from the unfolded state. With regard to protein aggregation, arginine may play a role in influencing the kinetics of aggregation reaction by its enhanced binding to the dissociated state, which contains more arginine binding sites. Consistent with its binding affinity for proteins, arginine has also been shown to decrease their surface adsorption [281]. Arginine may also affect the kinetics of protein–protein association by destabilizing the intermediate structures or oligomers [282].

Table 2
Influence of counterions on the freeze dried and vacuum dried behavior of L-arginine.
Adapted from Mattern et al. 1999 [90].

Arginine salt	Vacuum dried		Freeze dried	
	% H_2O	T_g (°C)	% H_2O	T_g (°C)
L-arginine	0.5 ± 0.1	NA	1.3 ± 0.2	42 ± 2.0
L-arginine-HCl	6.5 ± 0.1	3.5 ± 0.3	3.5 ± 0.2	18 ± 0.2
L-arginine-H_3PO_4	3.3 ± 0.2	5.2 ± 0.6	2.2 ± 0.1	93 ± 1.0

L-arginine present at 0.24 M, HCl at 0.24 M and H_3PO_4 at 0.12 M.

The mode of interaction between arginine and protein is still under extensive investigation. As with other protein formulation excipients, the interaction of arginine with simple model compounds provides an insight into the possible mode of interaction between arginine and protein surface. Fig. 22 demonstrates such an example on the solubility of coumarin [283]. Arginine increases its solubility in a concentration-dependent manner and is more effective than GdnHCl, urea, and the other co-solvents examined. Recently, Hirano et al. [284] examined the effects of arginine on the solubility of aromatic alkyl-gallate compounds. Arginine, but not lysine, also greatly increased the solubility of four alkyl-gallates (methyl-, ethyl-, propyl- and butyl-gallate). Molecular dynamics (MD) simulation revealed the free energy change to be negative as arginine approached the surface of ethyl-gallate, with a minimum observed at 5 Å and a shoulder between 5 and 10 Å, indicating the presence of favorable interaction between gallate and arginine [285]. As the 5 Å is most likely due to direct contact, the shoulder may reflect weak, momentary interactions. MD simulation also showed that arginine interacted with the aromatic moiety of ethyl-gallate through both π–π and cation–π interactions, consistent with previous observations [37,286,287].

Hydrophobic interaction between protein and arginine has also been proposed as a potential mechanism of aggregation suppression. According to Das et al. [288], arginine forms clusters through its methylene groups and creates a larger hydrophobic surface than does a

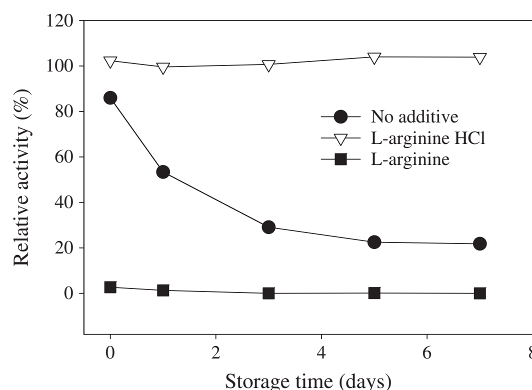

Fig. 19. Relative activity of β-galactosidase freeze dried in the absence and presence of L-arginine HCl and L-arginine, following storage at 70 °C.
Data adapted from Izutsu et al. [80].

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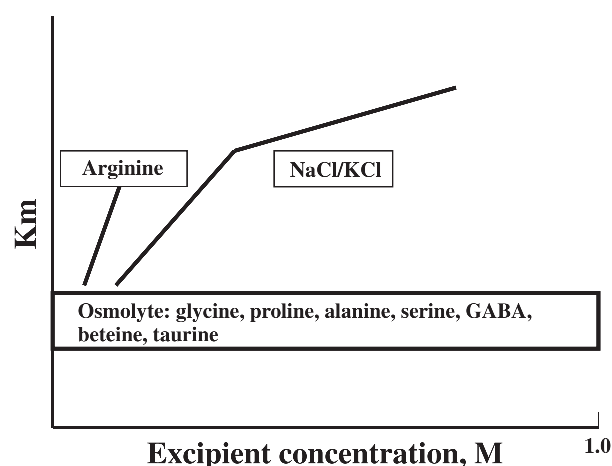

Fig. 20. The effects of osmolytes, arginine, and salts (NaCl/KCl) on the rate constant of enzymatic function.
Data adapted from Yancey et al. [42].

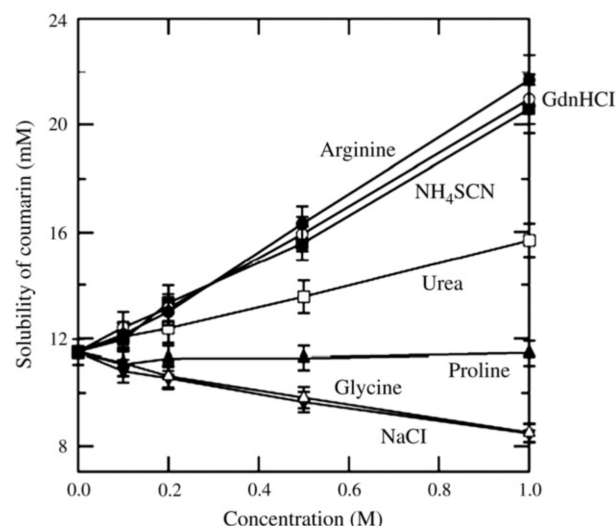

Fig. 22. Solubility of aromatic coumarin in the presence of various co-solvents.
Data adapted from Hirano et al. [283].

single arginine molecule. However, such clustering may also occur through the stacking of guanidinium groups, as demonstrated by the MD simulation of guanidinium ions [289]. Alternatively, clustering of arginine through electrostatic interactions between the carboxyl group and the guanidinium group, enhanced by double hydrogen bonds, has also been shown by MD simulation [285]. Accordingly, lysine should not form such clusters, as the electrostatic interactions are not enhanced by strong hydrogen bonds. Li et al. [285] proposed that arginine interacts with hydrophobic groups through both dispersion and hydrophobic interactions of the guanidinium group, and forms a cage-like network of arginine molecules in the vicinity of hydrophobic groups, further enhancing arginine binding and consequently solubilizing the hydrophobic compounds [290]. On the other hand, arginine solubilizes aromatic groups primarily through dispersion interactions between the aromatic and guanidinium groups. Section 6 will go into more detail on the interaction of arginine with proteins.

In the solid state, direct non-covalent interactions (i.e., hydrogen bonds and ion–dipole interactions) were shown to be present between arginine and antibodies (anti-CD11a and anti-IgE) through the use of solid-state NMR and ^{13}C and ^{15}N solid-state NMR spectroscopy [146]. In the ^{13}C NMR spectra, the chemical shift of C_ε (the carbon at the end of guanidine side chain of arginine) was shown to shift approximately 2 ppm downfield. This change is, in fact, consistent with the weak intermolecular interaction between the arginine side chain and the protein (i.e., ion–dipole and hydrogen bond). In the ^{15}N NMR spectra, the chemical shift of the N_1 on the arginine backbone remained unchanged, confirming the absence of interactions between the protein and the backbone of arginine, suggested by ^{13}C NMR. The other three nitrogen molecules on the guanidine side chain of arginine were observed to shift, and these changes have been explained by the authors to stem from the conjugated resonance of the 3 nitrogen molecules; the interaction of one of the nitrogen molecules with the antibody will perturb the other two, resulting in the observed shifts. In a separate study, the presence of increasing amounts of L-arginine was shown to inhibit the alterations in the secondary structure of the anti-CD11a antibody upon lyophilization, as determined by FTIR spectroscopy [146] (Table 3). It should be noted that in comparison to carbohydrates, higher concentrations of arginine are required to stabilize protein conformation during lyophilization.

7. Overall discussion on mechanism

The mechanism of each class of excipients for their effects on protein stability, solubility, and aggregation in both liquid and lyophilized formulations has been described above based on their interactions with proteins. In liquid formulations, there are primarily two different modes of interactions present between excipients and proteins, or container surfaces. Those that enhance protein stability demonstrate an

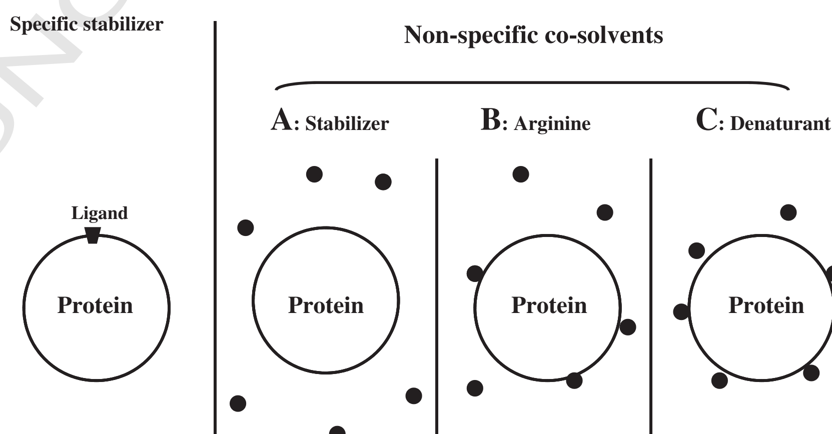

Fig. 21. Various modes of co-solvent interaction with a protein. Examples include the effects of a stabilizer through specific interaction, and those from non-specific interaction, including (A) stabilizer, (B) arginine, and (C) denaturant. Protein molecule is represented by the white circle and the additives by the filled circles.

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t3.1 **Table 3**
Secondary structure contents of anti-CD11a with various concentrations of arginine (arg) based on the Amide I region.
t3.2 Reformatted from Tian et al. [146].

	Samples	β-sheet intermolecular (%)	β-sheet intramolecular (%)	Other structures (%)
t3.4	Solution	0	67	33
t3.5	Freeze dried (FD)	28	23	49
t3.6	FD + 15% arg	24	26	50
t3.7	FD + 51% arg	11	39	50
t3.8	FD + 71% arg	7	53	40

1383 unfavorable interaction with the protein. Fig. 9 demonstrates how such
1384 an interaction leads to enhanced protein stability. Conversely, those that
1385 suppress protein aggregation or surface denaturation bind to the surface.
1386 Thus, they occupy the container surface, which can cause protein
1387 denaturation. Thus, understanding the cause of protein instability or
1388 aggregation should aid in the design of an appropriate formulation.
1389 The excipients that stabilize proteins in solution also confer
1390 stability during freezing. This should not be unexpected, however, as
1391 water is still present during the freezing process, and thus those
1392 excipients that require water for stabilization can operate under the
1393 same mechanism. However, freezing causes several stresses, includ-
1394 ing freeze concentration, ice crystal formation, salt and/or excipient
1395 crystallization, and pH shift, which are not encountered in solution
1396 and hence may alter the ability of the protein-stabilizing co-solvents.
1397 Lyophilization requires an entirely different spectrum of stabilizing
1398 mechanisms, as there is essentially no water. In addition, the effects of
1399 excipients on the physical state of the dried material become critically
1400 important for the long term storage stability of proteins.

1401 ## 8. Conclusion

1402 We have shown here the effects of four classes of co-solvents
1403 (excipients), i.e., protein-stabilizers, polymers, surfactants, and
1404 arginine on the formulation and stability of proteins in solution and
1405 dry state. The efficacy of these excipients in conferring stability to
1406 proteins has been approached from the mechanistic point of view,
1407 highlighting the various interaction forces present under different
1408 protein environmental conditions. Typical protein formulation con-
1409 tains several components, and it is through the fundamental
1410 understanding of the various interaction forces present between the
1411 formulation components and the protein that we can make further
1412 improvements in the stability of protein therapeutics.

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