Heterogeneity of Protein Environments in Frozen Solutions and in the Dried State

Maya Salnikova, Dushyant Varshney and Evgenyi Shalaev

Introduction

Typical solutions for freeze-drying contain 80–95 wt.% of water and several solutes, including an active ingredient and excipients, such as buffer components, lyoprotector or/and bulking agent, and a stabilizer such as surfactant. Behavior of such systems during freezing and freeze-drying is commonly described with the aid of supplemented phase diagrams, also known as solid–liquid state diagrams and extended phase diagrams. Use of the state diagrams for cryobiology and freeze-drying was pioneered by Luyet, Rasmussen, MacKenzie, and Franks, based on the evaluation of binary water–sucrose system and similar systems in which solutes do not crystallize [1–3]. Solid–liquid state diagrams of aqueous systems containing both crystalline and amorphous solutes were introduced for cryobiology [4] and freeze-drying applications [5]. In particular, the state diagrams allowed a generalized description of the phase behavior of typical aqueous solutions used in freeze-drying [6, 7], as follows. When an aqueous solution is cooled below its equilibrium melting point, a fraction of water molecules is isolated in a separate phase as hexagonal ice, leaving behind amorphous freeze-concentrated solution

E. Shalaev (✉)
Allergan, Irvine, CA 92612, USA
e-mail: Shalaev_Evgenyi@Allergan.com

M. Salnikova
Novartis Vaccines and Diagnostics, 475 Green Oaks Parkway,
Holly Springs, NC 27540, USA
e-mail: mayadusha@gmail.com

D. Varshney
Novartis Vaccines and Diagnostics, 475 Green Oaks Parkway, Holly Springs, NC 27540, USA
e-mail: dushamaya@gmail.com

Hospira, Inc. 275 N. Field Drive Lake Forest,
Lake Forest, IL 60045, USA

D. Varshney, M. Singh (eds.), *Lyophilized Biologics and Vaccines,*
DOI 10.1007/978-1-4939-2383-0_2

consisting of all the solute molecules and residual water. At this point, a two-phase system is formed consisting of hexagonal ice and freeze-concentrated solution. Upon further cooling, a behavior of such a two-phase system follows one of three scenarios below, depending on the solutes, cooling rate, and other variables such as the presence and properties of interfaces (e.g., particles) which can serve as nucleation centers: (i) the freeze-concentrated solution forms a kinetically stable amorphous phase, the so-called maximally freeze-concentrated solution; (ii) the freeze-concentrated solution forms a "doubly unstable" glass (i.e., unstable in both kinetics and thermodynamics sense), in which solute + water crystallization may occur later in the process, during annealing or drying; (iii) a secondary solute + water crystallization may occur during further cooling, resulting in a three-phase system of hexagonal ice, crystalline excipient, and the remaining freeze-concentrated solution.

Overall, the solid–liquid state diagrams have been extensively and successfully used to represent fundamentals of the freeze-drying processes [1, 2, 5–7]. It should be recognized, however, that the solid–liquid state diagrams reflect phase behavior under either equilibrium or metastable conditions. In particular, the assumptions of thermal equilibration across the sample and a sufficiently fast mass transfer between phases as related to the rate of temperature changes apply. In many real systems, however, these conditions are not satisfied and specific details of the freezing process need to be taken into consideration. For example, it was demonstrated using a carefully designed cryo-microscope and a model system (aqueous solution of $NaMnO_4$) that equilibrium conditions as assumed in phase diagrams do not always represent a good approximation. Deviations from equilibrium was more prominent at higher cooling rates where the redistribution of solute in front of the advancing ice–liquid interface was observed [8, 9]. The nonequilibrium features of the freezing process, including events on the ice/solution interface have been reviewed extensively [10–12]. In particular, an existence of the concentration gradients (for both neutral molecules and ions) on the ice/solution interfaces is commonly acknowledged [13–15]. Such concentration gradients could lead to significant inhomogeneity in the environment of an active pharmaceutical ingredient, including variations in the environment of protein molecules. Furthermore, as the protein stability depends on the composition and properties of their immediate environment, the heterogeneity would result in different populations of protein molecules, all having different stability characteristics, leading to a distribution of the degradation rates. As a result of the heterogeneity, shelf life of a pharmaceutical protein formulation would be limited by the most unstable population of protein molecules, which may represent a relatively minor fraction. Identifying this least stable portion of protein molecules and targeting formulation development efforts on this fraction, rather than going after the main (and potentially the most stable) part would allow a formulator to optimize stabilization and formulation development efforts.

An obvious practical challenge in studying heterogeneity in protein systems is that the majority of experimental tools provides an average measure of a property (e.g., structure), and may not have sufficient sensitivity or resolution to detect the

presence of protein molecules in different environments or conformational states. For example, Fourier transform infrared spectroscopy (FTIR), the now-standard formulation tool, would reflect destabilization of secondary structure [16], but only if the majority of molecules are affected. Also, measurements of storage stability include reconstitution of the freeze-dried cake and analysis of the resulting solution using stability-indicating methods (e.g., size-exclusion chromatography). Such tests provide sample-averaged degradation extent but would not allow extracting information about potential heterogeneity. Such challenge in the detection of heterogeneity in protein formulations represents probably the main reason why the subject of heterogeneity has not attracted much of attention in the biotech community until recently, although a few exceptional studies should be noted [17, 18]

In this chapter, we first discuss examples of experimentally determined heterogeneity of protein environment in frozen solutions and freeze-dried preparations, following by a discussion of several mechanisms leading to such heterogeneity. These mechanisms are predominantly related to events during freezing, and include concentration gradients created due to difference in the diffusion coefficients of proteins and other solutes, redistribution of the charged species and electric potential on the ice/solution interface [19, 20], and solution inclusions by ice crystals [21]. It should also be added that heterogeneity is a fundamental property of amorphous systems including both solutions and glasses, as was previously discussed in some details [22]. This chapter is focused on heterogeneity which can be expected within a single container (e.g., vial). Discussion of vial-to-vial variability is outside of the scope of the present study.

Experimental Evidences of Heterogeneity of Protein Environment in Frozen Solutions and Dried Solids

An extreme case of heterogeneity would be a phase separation between a protein and excipients, resulting in two amorphous phases, protein-rich and excipient-rich [23–25]. A potential protein/polymer phase separation in human brain-derived neurotropic factor (BDNF) and BDNF-polyethylene glycol (PEG) co-lyophilized with dextran was suggested based on scanning electron microscopy [26]. Additional evidences of phase separation between proteins and polymers were obtained using Raman mapping, which detected amorphous/amorphous phase separation between a protein and a lyoprotector, e.g., in lactoglobulin–dextran system [27–29]. It was also shown that trehalose had a greater propensity for phase separation from protein than sucrose, with phase separation detected for lysozyme–trehalose and lactoglobulin–trehalose (but not for protein–sucrose) systems. The occurrence of phase separation was correlated to higher instability of proteins.

Raman and FTIR spectroscopy were used to detect the heterogeneity and adsorption of proteins to ice surfaces [30]. It was shown that concentration of albumin in dimethyl sulfoxide (DMSO)/water solutions was high at the ice interface at low temperatures and as much as 20 % of the albumin (for 32–53 mg/mL solutions) can

be adsorbed on the ice or entrapped in the ice phase. In a recent study of the freeze-dried recombinant human growth hormone (rhGH) [31], the amount of protein on the surface of the freeze-dried cake was determined using electron spectroscopy for chemical analysis in formulations with sucrose, trehalose and hydroxyethyl starch (HES). The freeze-dried formulations were prepared at five different freezing conditions that include standard lyophilization cycle with slow freezing, pre-annealing before primary drying, post-annealing after secondary drying, fast freezing by immersion of vials into liquid nitrogen, and fast freezing of droplets by pipetting solution into immersed in liquid nitrogen vial. The surface concentration of rhGH was higher than in the bulk and was related with the rate of freezing and the use of annealing in frozen solids prior to drying, or annealing in glassy solids after secondary drying. Lower fraction of the protein was observed on the surface after slow freezing and annealing. In the same study, the average degradation rate was separated into two contributions, from bulk and surface degradation. It was shown that the degradation of protein molecules on the interface was approximately two orders of magnitude faster than the bulk degradation for chemical processes (deamidation and oxidation), whereas bulk versus surface difference for the aggregation rate was even more pronounced. Similar impact of the heterogeneity on stability was observed in the earlier studies for methionyl human growth hormone formulations prepared by freeze-drying, spray-drying, and film-drying [17].

In another important study, it was shown that protein concentration on the air/solid interface was higher than in the bulk for both spray-drying and lyophilization processes in trehalose/potassium phosphate formulations. [18]. The addition of polysorbate 20 reduced protein surface adsorption and decreased (but did not completely prevent) aggregation.

Appearance of two populations of protein molecules in the frozen state was detected in lysozyme/sorbitol/water system by small-angle neutron scattering (SANS). In that study, two populations of the protein were observed in frozen samples whereas the initial solution consisted of a single population of protein molecules [32], as illustrated in Fig. 1. In one of the populations (with intermolecular center-to-center distance of approximately 3 nm), protein molecules were in close

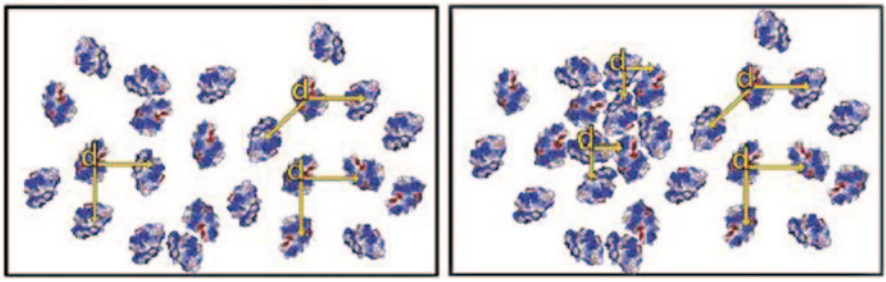

Fig. 1 Schematic drawing of increase in lysozyme crowding from solution (*left*) to freeze-concentrated solution (*right*), showing reduced protein–protein distance (*marked as d*) in one of the two populations of protein molecules, as a precursor for aggregation. The figure is reproduced from [32]

contact and interaction with each other, thus creating favorable spatial conditions for agglomeration and aggregation.

It was noted also that many chemical and physical processes in freeze-died formulations, both proteins and small molecules, do not follow simple kinetics law, and suggested that such observations can serve as an indirect evidence for heterogeneity of the local environments [22]. Indeed, heterogeneity would result in different populations of molecules of the active ingredient with different individual rate constants. As the common experimental methods (e.g., high-performance liquid chromatography) would measure bulk-averaged concentration of the reaction products or the extent of the conversion of a reactant, the apparent rate constant would represent a weight-averaged sum of the individual rate constants. In this case, even if the kinetics of each individual reaction corresponds to a simple reaction order, e.g., first-order, the average kinetic curve would reflect distribution of the individual rate constants, resulting in a more complex kinetic curve.

Mechanisms for the Inhomogeneity

Common mechanisms for the inhomogeneity (heterogeneity) are related to freezing (ice formation), resulting in redistribution of solutes via, e.g., inclusion inside ice crystals. In addition, we note that heterogeneity is a general property of solutions in both liquid and solid state (glasses). One specific case of heterogeneity was reviewed [33], where water clustering in solutions and amorphous solids was discussed as a probable case of heterogeneity on the sub-nanometer to nanometer-length scale. In the following two sections, we consider large-scale heterogeneities which are directly related to ice/solution interfaces.

Protein Sorption on Interfaces

Protein partitioning between bulk solution and interfaces would be an obvious case of heterogeneity, with properties of protein molecules on the interface be different from the bulk phase. Interaction of proteins with ice surface was studied for antifreeze proteins in some details. The propensity of some proteins to interact with ice surfaces is one of the defense mechanisms in nature that prevents ice growth due to increase in the curvature of the ice–water interface and thus resulting in non-colligative local freezing point depression [34–36]. The interaction of the antifreeze proteins with ice is mainly based on hydrogen-bonding mechanisms. Propensity of antifreeze proteins to ice/solution interface was used to purify antifreeze proteins, to separate them from other proteins present in *Escherichia coli* lysate [37]. In this case, non-antifreeze proteins were actually excluded from ice interface. Such exclusion of “common” proteins from ice interface is an important observation, considering that pharmaceutically relevant proteins are obviously not antifreeze proteins and

that the exclusion would not be consistent with a hypothesis of "pharmaceutical" proteins interacting with ice surface.

As evidence to the contrary, i.e., of interaction of non-antifreeze-proteins with ice interface, studies reported in [38, 39] are commonly invoked, and discussed below in some detail. Conformational changes of globular proteins were studied by employing the phosphorescence emission of tryptophan (Trp) residues as a monitor of the conformational changes of six proteins in response to variations in conditions of the medium [38]. Changes in well-structured compact cores of the macromolecules were monitored by the direct correlation between the phosphorescence lifetime τ and the rigidity of the protein matrix surrounding the chromophoric probe. The solidification of water markedly decreased τ and indicated unfolding related changes in conformation of the proteins, which was related primarily to the protein–ice interaction. Additionally, tryptophan florescence was employed to monitor unfolding of azurin mutant C112S from *Pseudomonas aeruginosa*. The thermodynamic stability (ΔG^0) of the macromolecule in frozen aqueous solutions was studied by introducing guanidinium chloride and monitoring tryptophan fluorescence for native and denatured states. The evaluation of the guanidinium chloride-induced unfolding in the frozen state allowed assessment of the thermodynamic stability of proteins in frozen solutions. The results obtained with C112S azurin demonstrated that the stability of the native fold may be significantly perturbed in the frozen solutions depending mainly on the size of the liquid solution pool in equilibrium with the solid phase. It was proposed that the effectiveness of stabilizing additives in preventing protein unfolding in the frozen state will be a combination of two influences: the ability to stabilize the N-state at low temperature and high-solute concentration (a preferential hydration mechanism), plus a specific action of the additive to contrast perturbations deriving from protein–ice interactions. We should note that, while these studies provided convincing evidence that destabilization of protein can be induced by formation of ice, it is not obvious if such destabilization is the results of the direct protein/ice interaction, as other mechanisms can be invoked.

In another study, concentration of the freeze-dried human interferon-γ on the surface of freeze-dried and spray-lyophilized materials was measured by electron spectroscopy for chemical analysis [18]. A higher concentration of the protein was observed on the surface of the dried materials, whereas the use of a surfactant (polysorbate 20) minimized the surface excess. The observations of the high surface concentration of a protein on the surface of freeze-dried cakes were taken as an evidence of protein sorption on solution/ice interface during the freezing step. This would certainly be a logical conclusion, but these studies did not provide a direct proof of protein sorption on the surface of ice crystals.

To reconcile the studies which showed rejection of non-antifreeze proteins from ice [37] with reports of both protein destabilization due to ice formation and the higher concentration of proteins on the surface of dried materials, we note that the formation of ice crystals during freezing usually results in formation of air bubbles [40], thus greatly increasing solution/air interface and potential for protein destabilization. Protein sorption on solution/air interface is well-documented, e.g., [41], and will not be discussed here. Therefore, protein sorption on the air bubbles created as

a result of freezing can be expected, with a corresponding increase in the surface concentration of protein in dried material and protein destabilization. Another possible explanation for the two apparently conflicting observation, that is, exclusion of non-antifreeze proteins from ice interface in the presence of antifreeze proteins versus destabilization of proteins by ice, is that the antifreeze proteins compete with other proteins for the interface, whereas in the absence of antifreeze proteins, other proteins might indeed be sorbed by ice surface. In either case, the use of nonionic surfactants to stabilize proteins, presumably by preventing the binding of the proteins to air/liquid surfaces, and also possibly ice/liquid interfaces, was demonstrated in many systems, e.g., in the example of recombinant human factor XIII [42].

Solute Inclusion Inside Ice Crystals

Solubility of essentially all common freeze-drying solutes in hexagonal ice is negligible, in other words, one can expect that the ice phase consists of 100% water. However, on a macroscopic scale, solution can be entrapped by growing ice crystals under certain conditions. In this section, several examples of such entrapment are discussed.

Entrapment of a solution phase by growing ice crystals depends on the freezing conditions, i.e., geometry of the crystallization front, rate of progression of the ice/solution interface, and macroscopic viscosity of the solution phase. In one study, freezing of small droplets of solutions containing sucrose, pullulan, bovine serum albumin (BSA), antifreeze glycoprotein, polyvinyl alcohol (PVA), and PEG was studied using optical refractometry [43]. Relatively diluted solutions, with the solute concentration of <5 wt.% (for sucrose) and <1 wt.% (for other solutes), were used. A concentration gradient of solute was observed at the ice/solution interface (length scale up to 200 μm) for all solutes but antifreeze glycoprotein, for which the concentration measured at the ice/solution interface, was the same as in the bulk. No incorporation of the solution phase into ice crystals was observed at the growing speed of 2 μm/s, when the ice/solution interface remained approximately planar, whereas at the higher growth speeds dendritic ice morphology was observed with a significant amount of solution trapped between the dendrites as liquid inclusions.

In a recent report, freezing behavior of ternary system water–DMSO–albumin was studied using FTIR and confocal Raman microscopy [30]. Solutions with different albumin/DMSO ratios were equilibrated at various subzero temperatures to create a two-phase (ice + freeze-concentrated solution) system. The albumin/DMSO ratios in the freeze-concentrated solution (FCS) were measured using FTIR and confocal Raman microscopy. In such a two-phase system, one would expect that the ratio would not change from the original single-phase solution, as can be shown using the temperature–composition phase diagram of a ternary system [7]. It was observed, however, that the ratio changed in a complicated manner. In particular, the albumin/DMSO ratio increased at relatively higher temperatures of -4 and -6 °C, which was interpreted as due to trapping DMSO inside ice crystals, whereas the

trend switched at lower temperatures, with FCS containing lower fraction of albumin as related to the initial solution.

An indirect evidence of trapping of protein molecules by ice crystals, and the heterogeneity associated with this was obtained [44] where the bulk concentration of *lactate dehydrogenase* (LDH) in the frozen sample was measured as a function of the distance from the container wall, with resolution of several mm. The concentration of LDH was the same across the sample, whereas there was a noticeable concentration gradient for small molecular weight solutes ($NaMnO_4$ and NaCl). A natural interpretation of these results is that, while small solutes are expelled from the ice crystallization front, larger protein molecules are trapped because of their slower diffusion rate. Therefore, local concentration of salts around protein molecules is likely varies with the position across a frozen sample.

Furthermore, solute inclusion was also invoked to explain the "double T_g" events, which are commonly observed in differential scanning calorimetry (DSC) studies of aqueous solutions. It should be stressed that the physical nature of these two events is still controversial and a subject of a number of publications [1, 21, 45–51]. The lower-temperature event (so-called T_g') has been attributed to a glass transition of the freeze-concentrated solution, whereas the second event (T_g') is proposed to be due to either the onset of ice melting/dissolution in the freeze-concentrated solution, or a glass transition of the freeze-concentrate. In the case of the latter interpretation, i.e., under the assumption that both events are the glass transitions, it was proposed that the two T_gs are due to the existence of two freeze-concentrated solutions with different concentrations of a solute (e.g., sucrose) in the same sample [45].

In order to consider this hypothesis, one would need to answer a question-why would there be two freeze-concentrated solutions with different sucrose concentration in the same sample? For a multicomponent system, heterogeneity in the composition of the freeze-concentrated solution is indeed possible, due to differentiation of the solutes by growing ice crystal because of differences in the diffusion coefficients or/and interaction with the ice surface. However, binary sucrose–water system has only one solute, and a sucrose solution trapped inside an ice crystal would achieve the same sucrose concentration as the solution outside of ice crystal which is also in direct contact (and local equilibrium) with ice, under a reasonable assumption that both temperature and pressure are the same in the trapped versus expelled parts of the solution. As a potential resolution of this problem, we hypothesize that the volume expansion due to water-to-ice transformation may result in differences in local pressures in different parts of the sample. A higher pressure would change the T_g of the freeze-concentrated solution either due to lower solute concentration as a result of the pressure-depression of the ice melting temperature (shift in the water liquidus), or increase in the T_g due to higher pressure. An indirect evidence of elevated local pressures was obtained in a study in which simple aqueous solutions were studied by synchrotron X-ray diffraction [52]. In that study, complex X-ray diffraction (XRD) patterns, with two or more poorly resolved peaks in place of each of the four diagnostic peaks of hexagonal ice, referred to as "splitting," were observed in the majority of cases. Deformation of the lattice of hexagonal ice, probably due to local stress created on the ice/ice or ice/container interface during water-to-ice transformation, was proposed as a possible mechanism for the

observed splitting of XRD peaks. It was also estimated using molecular modeling that the observed shifts in the peak positions are equivalent to applying a hydrostatic pressure of 2–3 kbar.

Furthermore, in a separate and a carefully designed study of water–sucrose system it was shown that, when the trapping (and therefore a probability of creating regions with elevated pressure) was minimized, a single-glass transition event was observed, followed by the onset of ice melting [21]. In that investigation, solution enclosure by ice crystals was prevented by using either scraped-surface freezing process or slow growing of ice crystals from solution containing ice nucleus.

To summarize the discussion on heterogeneity of protein environments due to solution inclusion by ice crystals, we note that the absolute majority of pharmaceutical formulations contain more than one solute. The solutes have different diffusion coefficients and/or interaction with ice surface, and therefore can be expected to develop variable extent of spatial heterogeneity under nonequilibrium freezing conditions. Protein environment in such materials will obviously be different in different parts of the sample, which could lead to distribution of degradation rates. An additional mechanism for the heterogeneity, i.e., due to local pressures as a result of volume expansion during water-to-ice transformation, should also be taken into consideration.

Inhomogeneity as a General Property of Solutions and Glasses

Inhomogeneity on the length scale of sub-nanometer to nanometer is a fundamental property of amorphous liquids (solutions), as summarized earlier [33]. Moreover, even a single-component system such as liquid water is nonhomogeneous on the molecular level, as illustrated by the Frank and Wen's model [53] (Fig. 2).

Many important events in solutions, such as crystal nucleation are expected to be dependent on local heterogeneity, with small clusters of molecules serving as nucleation centers. In addition, larger-scale heterogeneities (on the scale of hundreds of nanometer) were observed in solutions under certain conditions, [55] although the origin of the driving force for such large-scale heterogeneity is obscure.

For amorphous solids (glasses), their intrinsic heterogeneity and its pharmaceutical significance were discussed in [22], and briefly outlined below. The heterogeneous nature of glasses is reflected in non-exponential behavior of the structural relaxation, as expressed in the well-known Kohlrausch–Williams–Watts (KWW) equation:

$$X(t) = \exp\left(-\frac{t}{\tau_{KWW}}\right)^{\beta_{KWW}}$$

where X(t) is a property of the material, τ_{kww} and β_{kww} measure the average relaxation rate and the extent of non-exponentiality and the distribution of relaxation times, respectively. Values of β_{kww} vary from 0 to 1, with 1 representing a single-exponential relaxation process. Typically, many organic amorphous materials have

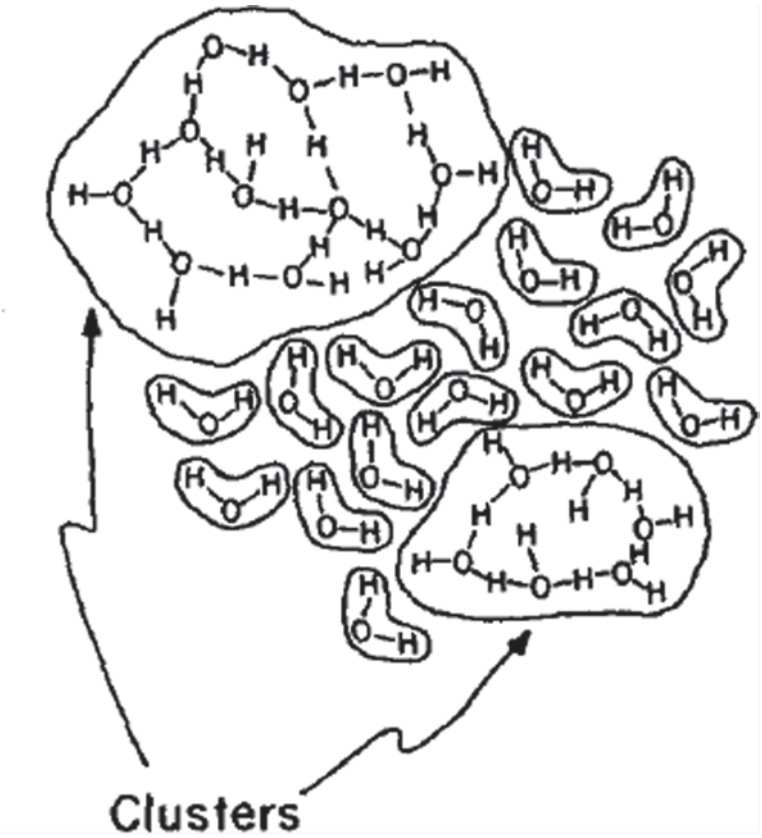

Fig. 2 Schematic representation of the model of liquid water, showing hydrogen-bonded clusters and unclustered water molecules. The molecules in the interior of the clusters are tetracoordinated, but not drawn as such in this two-dimensional diagram. The representation is based on the Frank–Wen model of liquid water. (Reproduced with permission from [54]. Copyright 2014, AIP Publishing LLC)

values of β_{kww} from 0.3 to 0.8, indicating a broad distribution of relaxation times and significant inhomogeneity.

Domains with different relaxation times may also have different degradation rates. Note that heterogeneity in freeze-dried amorphous materials can be easily observed by DSC. Indeed, DSC traces of such samples are commonly reveal sub-T_g transition, which can be eliminated by thermal cycling (i.e., heating above the T_g followed by quenching) or annealing below the T_g [56]. In this respect, it is possible that the high-temperature annealing, which was shown to improve stability of both proteins and small molecules, [57–62] reduces population of the least stable molecules thus resulting in the decrease of an average degradation rate.

An experimental observation of large-scale heterogeneities developed after cooling a model system (concentrated sorbitol/water solution) below its T_g was reported

http://www.springer.com/978-1-4939-2382-3

Lyophilized Biologics and Vaccines
Modality-Based Approaches
Varshney, D.; Singh, M. (Eds.)
2015, XI, 401 p. 99 illus., 68 illus. in color., Hardcover
ISBN: 978-1-4939-2382-3