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

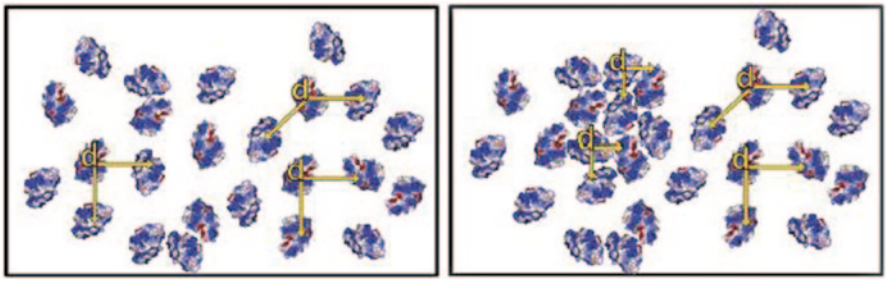

Fig. 1 Schematic drawing of increase in lysozyme crowding from solution (*left*) to freeze-concentrated solution (*right*), showing reduced protein–protein distance (*marked as d*) in one of the two populations of protein molecules, as a precursor for aggregation. The figure is reproduced from [32]