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