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