using SANS [63]. In that study, significant increase in the scattering (which is an indication of increased inhomogeneity) was observed during cooling 70% sorbitol solution below its glass transition temperature. Note that the sample retained its amorphous structure (i.e., no sorbitol or water crystallization) during the experiment. It was also observed also that this increase in the scattering was partly reversed during heating, although some hysteresis persisted. A power-law analysis of the SANS data indicated the existence of domains with well-defined interfaces on the submicrometer length scale, probably as a result of the appearance and growth of microscopic voids in the glassy matrix. The SANS results provided an example of long-range inhomogeneity in aqueous glasses, and also suggested an intriguing possibility of a thermal memory retained by the glasses even after heating above the glass transition temperature. The large-scale heterogeneity in glasses was also reported for another system, lysozyme/sorbitol/water mixtures, in which heterogeneity was detected in freeze-concentrated solutions [32]. It was suggested that the interfaces, which were detected by SANS below the T_g, can impose additional stresses on proteins, resulting in destabilization and degradation.

Conclusions

Heterogeneity in protein environment can be created by several mechanisms including sorption of proteins on ice crystals, inclusion of freeze-concentrated solution by ice crystallization front, and amorphous/amorphous phase separation, as well as due to creation of voids and corresponding interfaces in freeze-concentrated solutions. Also, note that crystallization of a solute (such as buffer or lyoprotector) would be an additional source of a heterogeneity, solute crystallization and its relevance to stability of frozen and freeze-dried formulations was discussed elsewhere [5, 10, 11, 64] and not considered here. Difference in the stability of different populations of proteins was convincingly demonstrated for the case of proteins on air–solid interface in freeze-dried cakes, [31] with the degradation rates on the interface exceeding the rate of bulk molecules by at least two orders of magnitude. As a result of such heterogeneity, shelf life of a pharmaceutical protein formulation would be limited by the most unstable population of protein molecules. Identifying the least stable portion of protein molecules and targeting formulation development efforts on elimination or stabilization of this fraction would allow a formulator to optimize formulation development efforts. Use of surfactants is an example of such strategy, allowing minimizing protein sorption on the interface and therefore reducing concentration of the least stable fraction of protein molecules. Phase separation of lyoprotector from protein, either due to crystallization or amorphous/amorphous phase separation represents another commonly accepted mechanism of heterogeneity and creation of a less-stable fraction of protein molecules. Overall, the identification of heterogeneity of protein molecules should be an essential part of a scientific design of freeze-dried and frozen protein formulations.