protecting the protein from unfolding during the drying process by substituting for the hydrogen bonding of water. Strong interaction with the protein should mean there is little chance of the protein and sugar separating into different phases.

A bulking agent is often added to a freeze-dried formulation for mechanical strength of the cake and for aesthetic reasons to produce an elegant cake (2). Mannitol is the most common bulking agent, largely because it crystallizes easily to form a rigid and "elegant" cake, and the high eutectic temperature means primary drying is simple and fast. The addition of a bulking agent to a phase-separating hemoglobin/PEG/dextran model system prevented the structural damage to hemoglobin (12). That is, a system with mannitol remaining amorphous [2% (w/w)] had a greater loss in secondary structure than a system with crystalline mannitol [5% (w/w)]. Both formulations were annealed at −7°C, allowing for crystallization of the mannitol in the 5% (w/w) sample. The authors speculated that crystallization of mannitol inhibited the formation of multiple amorphous phases, assumed to be damaging to protein structure, by dividing the amorphous matrix into smaller volumes, thereby kinetically preventing nucleation and growth of two amorphous phases. The remaining amorphous phase, once mannitol crystallizes out, consists of PEG and dextran, a well-studied phase-separating system. Previous studies reported on the phase separation of protein out of the stabilizer phase (PEG) and into the dextran phase resulted in loss in protein secondary structure (36). Thus, the formation of small volumes prevented the separation of protein into a non-PEG phase.

Nonionic surfactants are an important excipient class in freeze-dried formulations and are typically added to protect the protein from denaturation at interfaces, such as the ice-water interface. At least one study has demonstrated the potential for phase separation to occur between surfactants and proteins (50). The authors demonstrate the propensity for bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) to phase separate under specific conditions of high ionic strength and low pH. Although this system is not a typical freeze-dried formulation, since SDS is not an acceptable surfactant for parenteral use, the observation does suggest that under certain conditions phase separation may occur in protein-surfactant systems. It should be noted that since high surfactant concentrations can have a negative effect on protein stability (59), a phase-separated protein-surfactant system may be destabilizing. That is, if the protein were to separate into a phase rich in surfactant that protein would likely be destabilized.

The active molecule and its properties may play a role in phase separation. For example, the isoelectric point of the protein is important when choosing the formulation pH and when choosing excipients. Phase separation has been shown to occur in protein-polysaccharide mixtures at pHs above the protein's isoelectric point because of repulsive electrostatic interactions and altered affinities to water (21). Additionally, the molecular weight or size may be a significant factor in formulation compatibility. As previously stated, molecular weight is a critical parameter in the phase separation of polymer systems (11) and could also be a significant variable in formulating proteins, particularly very high–molecular weight proteins such as antibodies. Again, although structurally different than polymers, proteins can phase separate at low temperatures and high concentrations, which is a desirable attribute when attempting to crystallize proteins (56,57).