trend switched at lower temperatures, with FCS containing lower fraction of albumin as related to the initial solution.

An indirect evidence of trapping of protein molecules by ice crystals, and the heterogeneity associated with this was obtained [44] where the bulk concentration of *lactate dehydrogenase* (LDH) in the frozen sample was measured as a function of the distance from the container wall, with resolution of several mm. The concentration of LDH was the same across the sample, whereas there was a noticeable concentration gradient for small molecular weight solutes ($NaMnO_4$ and NaCl). A natural interpretation of these results is that, while small solutes are expelled from the ice crystallization front, larger protein molecules are trapped because of their slower diffusion rate. Therefore, local concentration of salts around protein molecules is likely varies with the position across a frozen sample.

Furthermore, solute inclusion was also invoked to explain the "double T_g" events, which are commonly observed in differential scanning calorimetry (DSC) studies of aqueous solutions. It should be stressed that the physical nature of these two events is still controversial and a subject of a number of publications [1, 21, 45–51]. The lower-temperature event (so-called T_g') has been attributed to a glass transition of the freeze-concentrated solution, whereas the second event (T_g') is proposed to be due to either the onset of ice melting/dissolution in the freeze-concentrated solution, or a glass transition of the freeze-concentrate. In the case of the latter interpretation, i.e., under the assumption that both events are the glass transitions, it was proposed that the two T_gs are due to the existence of two freeze-concentrated solutions with different concentrations of a solute (e.g., sucrose) in the same sample [45].

In order to consider this hypothesis, one would need to answer a question-why would there be two freeze-concentrated solutions with different sucrose concentration in the same sample? For a multicomponent system, heterogeneity in the composition of the freeze-concentrated solution is indeed possible, due to differentiation of the solutes by growing ice crystal because of differences in the diffusion coefficients or/and interaction with the ice surface. However, binary sucrose–water system has only one solute, and a sucrose solution trapped inside an ice crystal would achieve the same sucrose concentration as the solution outside of ice crystal which is also in direct contact (and local equilibrium) with ice, under a reasonable assumption that both temperature and pressure are the same in the trapped versus expelled parts of the solution. As a potential resolution of this problem, we hypothesize that the volume expansion due to water-to-ice transformation may result in differences in local pressures in different parts of the sample. A higher pressure would change the T_g of the freeze-concentrated solution either due to lower solute concentration as a result of the pressure-depression of the ice melting temperature (shift in the water liquidus), or increase in the T_g due to higher pressure. An indirect evidence of elevated local pressures was obtained in a study in which simple aqueous solutions were studied by synchrotron X-ray diffraction [52]. In that study, complex X-ray diffraction (XRD) patterns, with two or more poorly resolved peaks in place of each of the four diagnostic peaks of hexagonal ice, referred to as "splitting," were observed in the majority of cases. Deformation of the lattice of hexagonal ice, probably due to local stress created on the ice/ice or ice/container interface during water-to-ice transformation, was proposed as a possible mechanism for the