Supplementary Information.

Two populations of protein molecules detected by small-angle neutron and X-ray scattering (SANS and SAXS) in lyophilized protein : lyoprotector (disaccharide) systems.

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Materials and Methods.

Preparation of freeze-dried mixtures.

D-(+)-Trehalose dihydrate, sucrose, BSA (lyophilized powder, >96% agarose gel electrophoresis), and D_2O are sourced from Sigma-Aldrich. BSA:sucrose and BSA:trehalose mixtures are lyophilized from D2O solutions at protein:sugar weight/weight (w/w) ratios of 1:1 and 1:3 containing 3 % (w/w) BSA using Alpha 1–2 LD plus freeze-dryer from Christ. As a control, BSA sample without sugar is also prepared by lyophilization from D_2O solution at BSA concentration 40 mg/ml. In this process, EAppendorf tubes are filled with 20 ml of solutions, frozen in liquid nitrogen, and connected to the vacuum system of the lyophilizer to set up the sublimation process. Once the drying process is finished, the tubes are sealed with parafilm film. Neutron and X-ray scattering measurements are carried out shortly after the sample preparation.

SANS and SAXS/WAXS measurements

SAXS/WAXS experiments are carried out at the ID02 beamline of the European Synchrotron Radiation Facility, Grenoble, France. The powders are loaded in quartz capillaries and sealed with wax; SAXS/WAXS patterns are collected at a sample-to-detector distance of 1 m using an X-ray wavelength of 1 Å 912.46 keV). Each sample is measured at 10 positions along the capillary, and the measurements are averaged and background is subtracted.

The small-angle neutron scattering experiments are carried out on the D16 cold neutron diffractometer at the Institut Laue-Langevin (ILL) in Grenoble (France). The instrument is configured to operate at a wavelength of 4.5 Å. The diffraction pattern is measured on a q range of 0.02–0.5 Å. The powder samples were inserted in Hellma cell of 1 mm. The scattering intensities are normalized with a standard calibration of water run to correct by the non-uniform detector response and to normalize in the intensity at cm^{-1} unit. Further corrections take into account the empty cell, ambient background, sample transmission and the thickness of the sample. The data are radially averaged to obtain the scattered intensity I(q) versus q curves, where $q = 4\pi\sin(\theta)/\lambda$ and 2θ is the scattering angle.

Analysis of the SAXS & SANS curves.

SAXS and SANS patterns are fitted to Eq 1 using Prism software

$I(q) = A*q^{-p} + B$ Eq S1

where A is proportional to surface area, number density and contrast of the scatterers, B is baseline and p is Porod invariant. 95% confidence intervals from the fitting procedure are estimated to be 0.02 nm (d1), 0.06 nm (d2) and 0.05 (I1/I2) for SAXS and 0.05 nm (d1),0.09 nm (d2), and 0.07 (I1/I2) for SANS.

The p value are between 3.8 and 4.1 for the curves having enough data points for fitting (all 4 curves in SAXS and 3 SANS curves). Such p values correspond to sharp interfaces [S1], probably solid/air interfaces resulted from sublimed ice crystals, and are typically observed in freeze-dried materials [S2, S3].

The baseline is subtracted from SAXS and SANS curves. After baseline subtraction, the SANS/SAXS patterns are fitted using two Gaussian curves

$$I(q) = I_1 e^{-\left(\frac{q-q_1}{\sigma_1}\right)^2} + I_2 e^{-\left(\frac{q-q_2}{\sigma_2}\right)^2} \quad \text{Eq S2}$$

In all cases, 2-peak fitting procedure gives better description of the data than a single-peak curve. Fitted curves using 2-peaks fitting are shown in Figures 1a,b and Figure S2. There are large variations on the curve fitting if the peak width ratio (σ_2/σ_1) is used as adjustable parameter, due to the overlaps of the two peaks. To reduce the variations, the σ_2/σ_1 is kept constant: 2.5 for SANS and 2 for SAXS. These fixed peak ratio values were selected empirically based on fit quality for SANS and SAXS patterns, with the fit quality checked at different ratios.

The d-space is calculated using q_1 and q_2

$$d_1 = \frac{2\pi}{q_1}; \quad d_2 = \frac{2\pi}{q_2}; \quad \text{Eq S3}$$

Size exclusion chromatography (SEC).

SEC for the BSA solution iin 10 mM phosphate buffer pH 6.8 was conducted using a Tosoh Bioscience TSKgel G2000SWxl column (7.8mmX30cm, 5u) at column temperature of 30°C. The mobile phase was 20mM phosphate in 200mM NaCl. The UV detection wavelength was 280nm. Injection volume was 20 uL and concentrations repeated with 10 and 1mg/mL. Representative SEC curve for the starting BSA material is provided in Figure S5. The monomer content is approx. 90% and dimer approx. 10%.

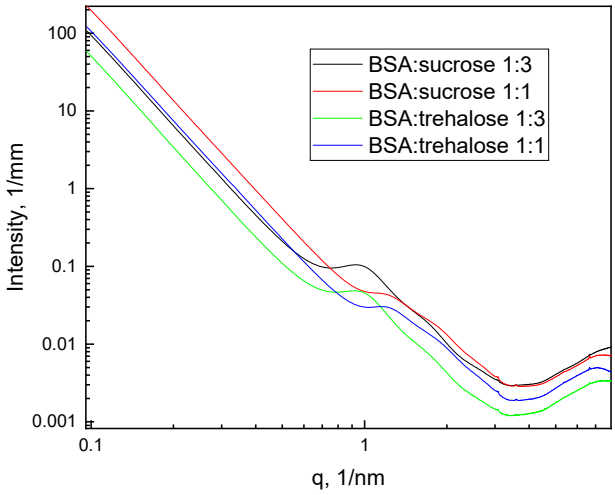

Figure S1. SAXS/WAXS patterns of lyophilized BSA:sucrose and BSA:trehalose mixtures.

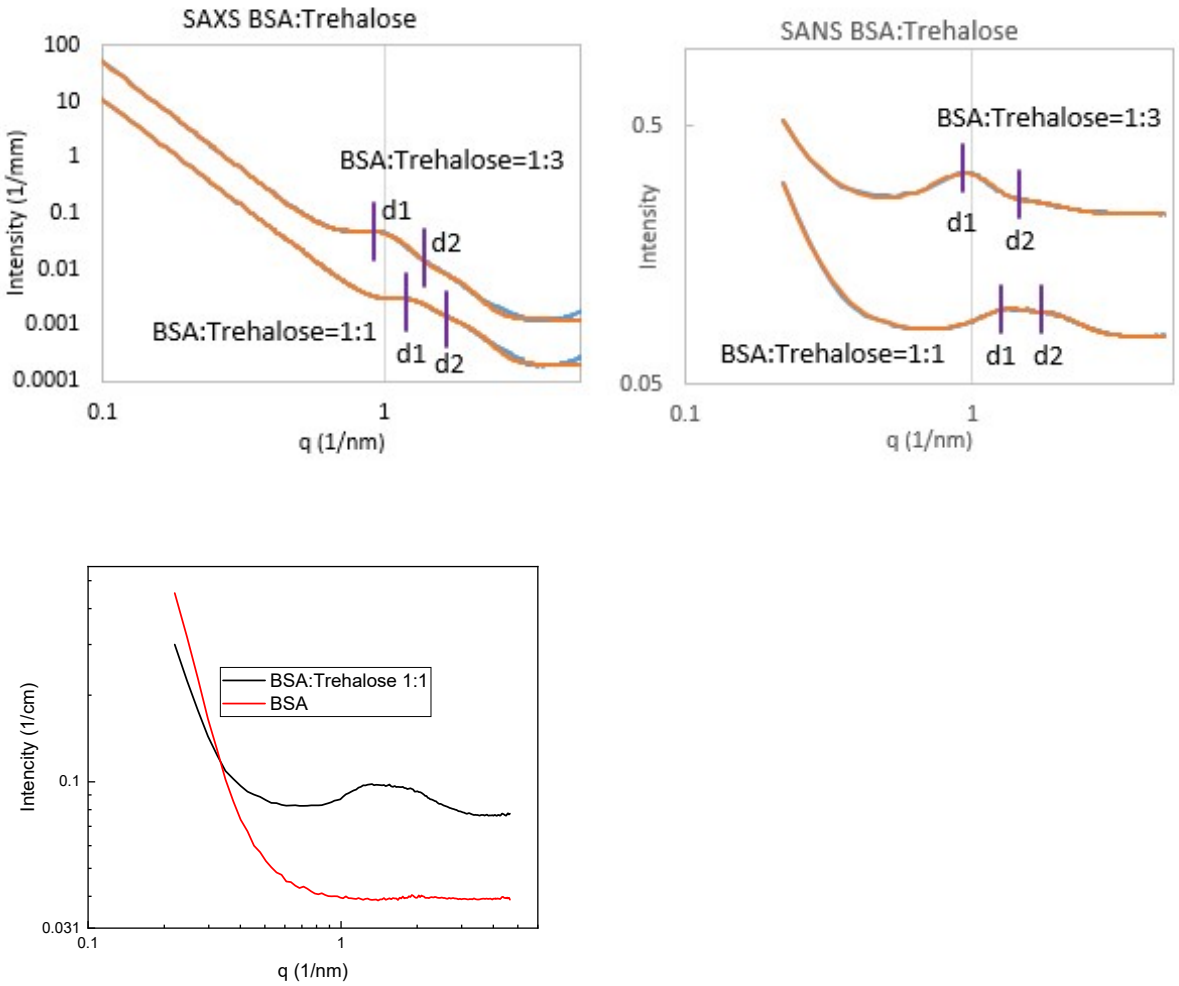

Figure S2. SAXS (upper left) and SANS (upper right) curves for freeze-dried BSA:trehalose mixtures. The blue and the orange curves show experimental and fitted curves, respectively Overlay of SANS curves for BSA and BSA:trehalose = 1:1 is provided in the bottom graph. As expected, SANS protein interaction peaks in pure freeze-dried BSA sample is not observed, because there is no contrast with the D2O (D2O was removed during freeze-drying) and the incoherent part of the signal is very high. Similarly, the "disappearance" of the interaction peak was reported in SAXS study of drying of pure BSA, when water content was reduced below 40 wt % [N Begam, S Da Vela, O Matsarskaia, M K Braun, A, F Zhang, F Schreiber. Packing and dynamics of a protein solution approaching the jammed state. Soft Matter, 2020,16, 7751].

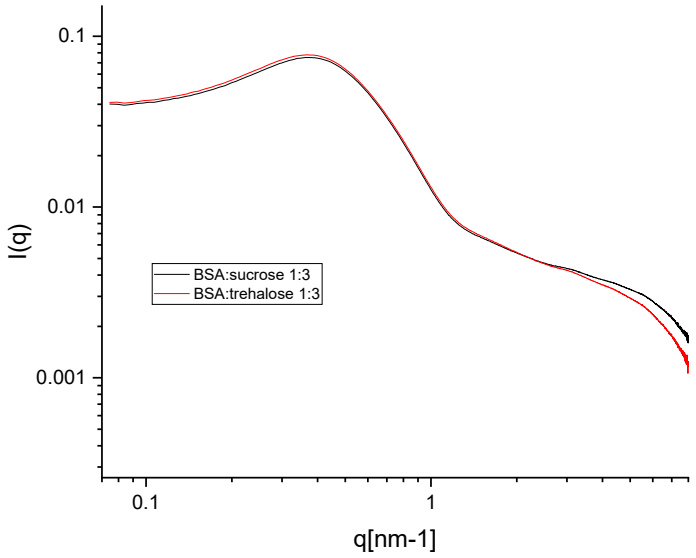

Figure S3. SAXS/WAXS for BSA:sucrose 1:3 and BSA/trehalose 1:3 solutions in D2O. The peak maxima d-spacing is 16.7 and 16.8 nm for BSA:sucrose 1:3 and BSA/trehalose 1:3 solutions, respectively, which is consistent with a literature SAXS study of BSA in water, with the protein-protein positional correlation peak observed at approx. 0.4 1/nm (d=16 nm) at similar protein concentration [S4].

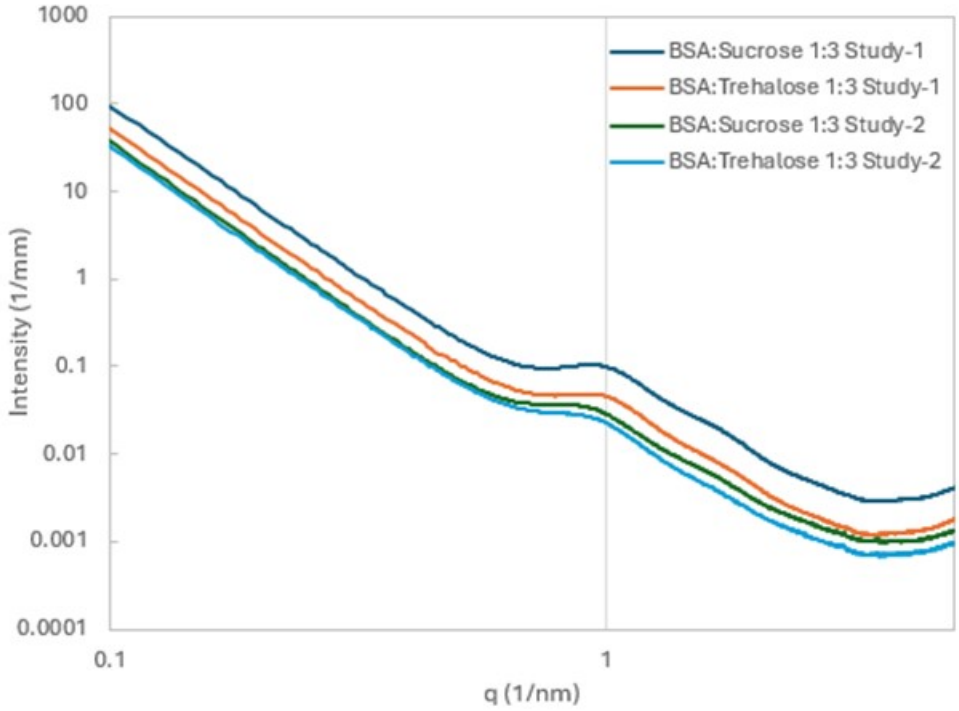

Figure S4. Comparison of SAXS/WAXS patterns for independently prepared lyophilized BSA:disaccharide samples.

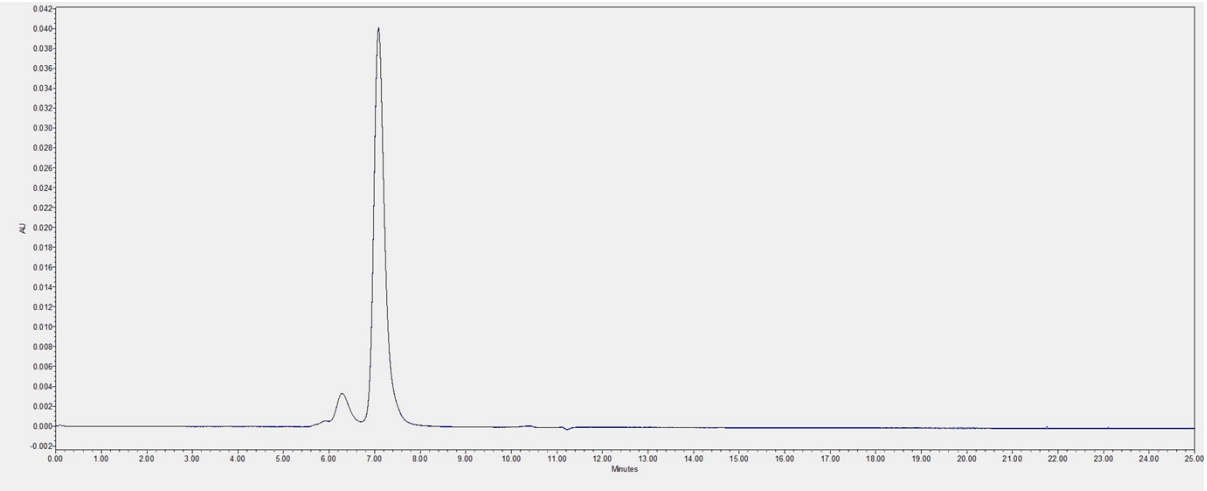

Figure S5. Representative size exclusion chromatogram of starting BSA material.

Table S1. d-spacing of protein interaction peaks from SAXS curves for freeze-dried BSA:sugar 1:3 mixtures and ratios of the peaks intensity, I, from two independent sample preparations and SAXS tests.

Sample	Study-1			Study-2		
	d1, nm	d2, nm	I1/I2	d1, nm	d2, nm	I1/I2
BSA:trehalose 1:3	6.85	4.54*	4.7	7.32	4.72*	5.2
BSA:sucrose 1:3	6.77	4.55*	3.8	7.35	4.72*	4.2

*weak peak and therefore larger uncertainty

Table S2. d-spacing of protein interaction peaks from SAXS and SANS curves for freeze-dried BSA:sugar mixtures and ratios of the peaks intensity, I. Note that, while both SAXS and SANS data show higher fraction of the d1 population at the higher sugar content (1:3 mixtures), the I1/I2 values are different between SAXS and SANS. The difference in the peak ratios between SAXS and SANS may be related to the strong q^{-4} slope closely below the peaks in the SAXS patterns, while the background for the SANS peaks is flat in the q-range of interest.

We also estimated the protein center-of-mass distances in the d1 population using a simple cubic lattice packing model of BSA molecules homogeneously distributed in sugar materials, with the densities of BSA and sugar are 1.36 and 1.5 g/cm^3, respectively. The estimated d values are 5.37 nm and 6.71 nm for the weight ratios of BSA:sugar are 1:1 and 1:3, which are consistent with measured d1 values.

Sample	SAXS			SANS		
	d1, nm	d2, nm	I1/I2	d1, nm	d2, nm	I1/I2
BSA:trehalose 1:1	5.24	3.80	1.8	4.99	3.62	0.5
BSA:sucrose 1:1	5.25	3.86	1.5	4.94	3.62	0.3
BSA:trehalose 1:3	7.09±0.33**	4.63±0.13**	5.0±0.4**	6.80	4.36*	3.0
BSA:sucrose 1:3	7.06±0.41**	4.64±0.12**	4.0±0.3**	7.00	4.42*	2.8

*weak peak and therefore larger uncertainty

**average between two independently prepared samples in two SAXS experiments

References.

S1. T. Narayanan. In: *Soft Matter Characterization*; Borsali, R., Pecora, R., Eds.; Springer-Verlag: Berlin, Heidelberg, Germany, 2008; pp 899−952.

S2. T. Phan-Xuan, E. Bogdanova, A. Millqvist Fureby, J. Fransson, A.E. Terry, V. Kocherbitov, *Mol. Pharm.* 2020, https://doi.org/10.1021/acs.molpharmaceut.0c00351

S3. V. Cristiglio, M. Sztucki, C. Wu, E Shalaev, *Biochim. Biophys. Acta (BBA) - General Subjects,* 2022, **1866**, 130101.

S4. K. Yanase, R. Araib, T. Sato. J. Mol. Liquids, 2014, **200**, 59–66.