Experimental report

15/02/2021

Proposal: 9-13-941 **Council:** 4/2020

Title: Dehydration-induced structural transitions of proteins in solid stateformulations

Research area: Chemistry

This proposal is a new proposal

Main proposer: Tuan PHAN

Experimental team:

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Samples: Deuterated sucrose
lysozyme powder

Instrument	Requested days	Allocated days	From	To
D16	5	5	02/09/2020	07/09/2020

Abstract:

We propose to study the structural transition of proteins at low water contents and the effect of carbohydrates that are used as pharmaceutical excipients. Our previous Small and Wide angle X-ray scattering experiments on solid state protein samples showed a range of structural information which appears in a form of broad peaks in the q-range 1 - 20 nm-1. The peak at q = 2.5 nm-1 arises from the protein - protein correlations in the crowded amorphous state. The other peak at q = 4.5 nm-1 is however unclear: either it is related to the local packing arrangement, or to a conformational change outlining a domain-domain correlation. In this SANS proposal, using contrast-variation, we aim at disentangle conformational signatures from the packing structure and the localisation of (deuterated) sucrose in solid formulations as well as possible preferential absorption of water around sugar or proteins. This knowledge is crucial to develop understanding the mechanisms of destabilisation of proteins in solid-state formulations.

Abstract:

The aim of the experiment was to study the effect of the hydration level on the structural transitions of a model protein, lysozyme, in the solid amorphous state and its effect on the destabilisation of the protein molecules in solid/semi solid formulation for therapeutic applications. The presence of water in the solid formulation has been identified as a critical factor for long term stability. In this experiment, we attempted to study the structural change of lysozyme at different hydration levels using SANS. Doing this together with our previous SAXS/WAXS and thermodynamic studies on the same system, we aimed to explore the relationship between protein structure, excipients, and moisture in relation to the protein's stability.

Goals:

We would like to reveal the structural transitions of protein at low water contents together with the distribution of proteins and carbohydrate in the formulation and try to learn more about the interaction between water and these components. This knowledge is crucial to develop understanding the mechanisms of destabilisation of proteins in solid-state formulations.

Experimental results:

1. **Sample preparation for neutron experiment:**

Purification: Lysozyme powder from chicken egg white was dissolved in mili-Q water. The solution was then dialysed and lyophilised to remove the impurities as described in (1).

Preparation of rehydrated lysozyme: The rehydration of lysozyme was carried out as follows: dried lysozyme powders, with and without sucrose, were placed in a desiccator with a vapor atmosphere from various saturated salt solutions in 3 different solvent of 100% H2O, 100% D2O and H2O/D2O 40/60 (protein contrast matching condition) at ambient temperature for a period of 10 days until the sample's mass reached stable values. The amount of water uptake was determined by measuring the total mass of the powder and the slide before and at the end of the incubation. The powder was then transferred to glass capillaries and sealed before shipping to ILL. A list of samples that were sent to ILL is shown in the table 1. The amount of water uptake for different desiccator environments are shown in figure 1.

Table 1: List of samples that were prepared for SANS experiment.

	desiccator	H2O	D2O	40/60 vol % H2O/D2O
Dialysed Lysozyme	Freeze dry powder	LH0	LD0	--
	MgCl2	--	--	LM1
	NaCl	LH2	LD2	LM2
	KCl	LH3	LD3	LM3
	K2SO4	LH4	LD4	LM4

		H2O	D2O	40/60 vol % H2O/D2O
Lys/suc 40/60	Freeze dry powder	MD0	MD0	--
	MgCl2	MH1	MD1	MM1
	NaCl	MH2	MD2	MM2
	KCl	MH3	MD3	MM3
	K2SO4	MH4	MD4	MM4

LH, LD,LM: lysozyme in H2O,D2O and matching point D2O/H2O desiccators respectively.

MH, MD, MM: lysozyme/sucrose mixture in H2O,D2O and matching point D2O/H2O desiccators respectively.

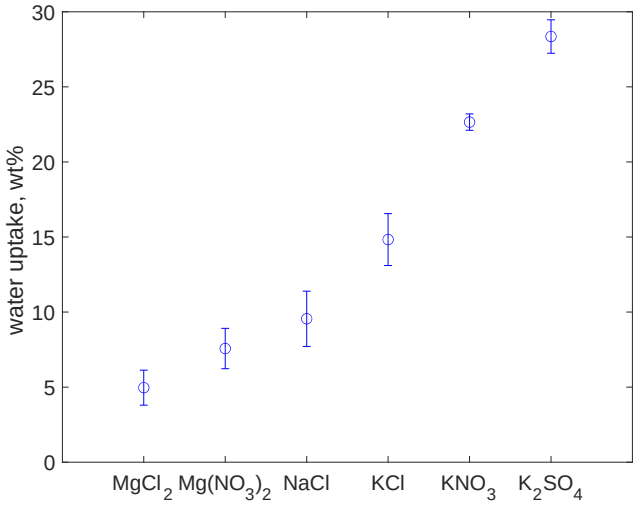

Figure 1: Evolution of the amount of water uptake after incubating dried powder of lysozyme in different saturated salt solutions

2. SAXS/WAXS precharacterisation:

Recently we have systematically investigated structural changes of a therapeutic protein (palifermin) as well as of a model protein (lysozyme) at different hydration levels using SAXS/WAXS. In dried state, the scattering patterns showed a clear distinct feature of structural information which appears in a form of single broad peak at q = 2.5 nm^{-1} (or d_{dried} = 2.5 nm in term of distance) (figure 2). This peak is attributed to a packing arrangement, characterizing the averaged center-to-center distance or a lattice plane of a local arrangement.

In addition to the pronounced peak at 2.5 nm^{-1}, another peak at a higher q-value (q = 4.5 nm^{-1}) is seen in many samples with water contents below 35 wt% which has never been reported in literature (figure 2b). The origin of this peak is unclear: either it is related to the local packing arrangement, or to a conformational change outlining a domain-domain correlation.

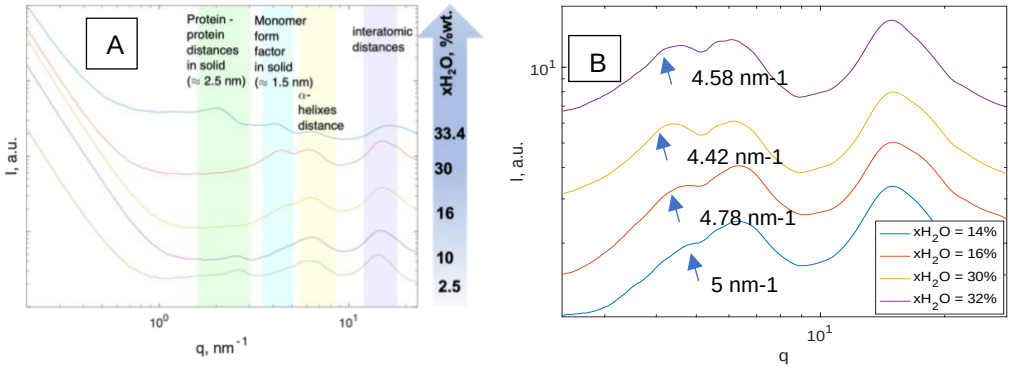

Figure 2: (A) Hydration level dependence of scattered intensity I(q) of lysozyme at 25oC in the solid state and (B) Evolution of the peak position at q range 4 – 5 nm^{-1} . Data collected at a synchrotron source (ALBA, Spain).

We also performed a SAXS pre-characterisation study on the same samples using our inhouse X-ray lab-source instrument (figure 3). The data showed very similar features of samples hydrated by H_2O or D_2O which are also in good agreement with our previous synchrotron data.

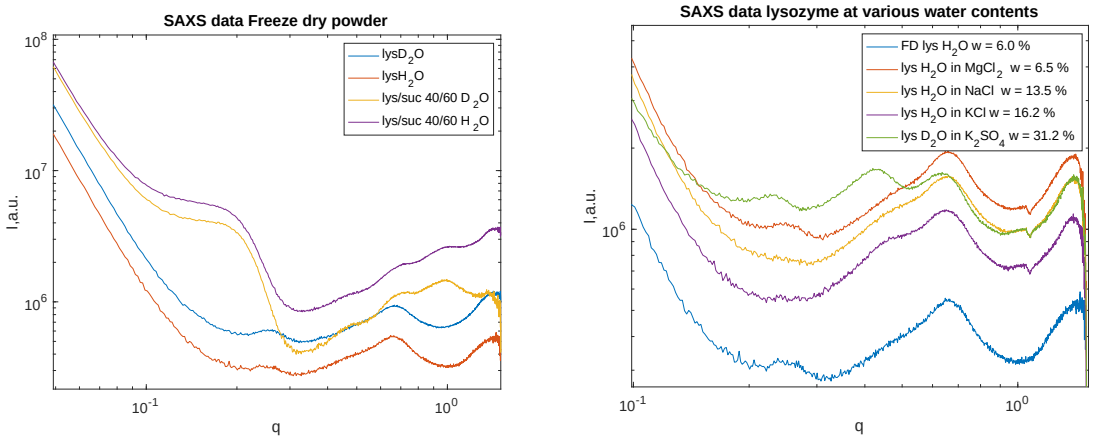

Figure 3: (A) SAXS patterns of lysozyme samples prepared for neutron scattering experiment. Data collected using our inhouse X-ray lab-source instrument.

3. SANS investigation:

The experiment was performed at D16 with a diffraction geometry set-up with 3 detector positions in order to cover the q range of 0.1 – 10 nm^{-1}. Furthermore, the low $\Delta\lambda/\lambda = 0.01$ is important to resolve the peak structure to compare to results obtained from SAXS.
Due to the CoViD-19 pandemic, the experiment was done via mail-in operation with the help of the beamline scientist. A diffraction geometry set up at D16 with 3 detector positions in order to cover a q-range of 0.1 – 10 nm^{-1} was used for the experiment.
Figure 4 is an example of the SANS patterns for dialyzed lysozyme samples at different contrast variations indicated in the figure. We have observed a pronounced correlation peak that appeared in the similar q-range compared to our SAXS data for sample of lysozyme in at D_2O = 32 %wt. A less pronounced peak in the same q-range was also observed for lysozyme in H_2O = 15%.
We will continue to work on the analysis of the SANS data with the help of the beamline scientist to for more extended q-range to understand more about the contribution from the packing arrangement of the protein in the amorphous solid state.

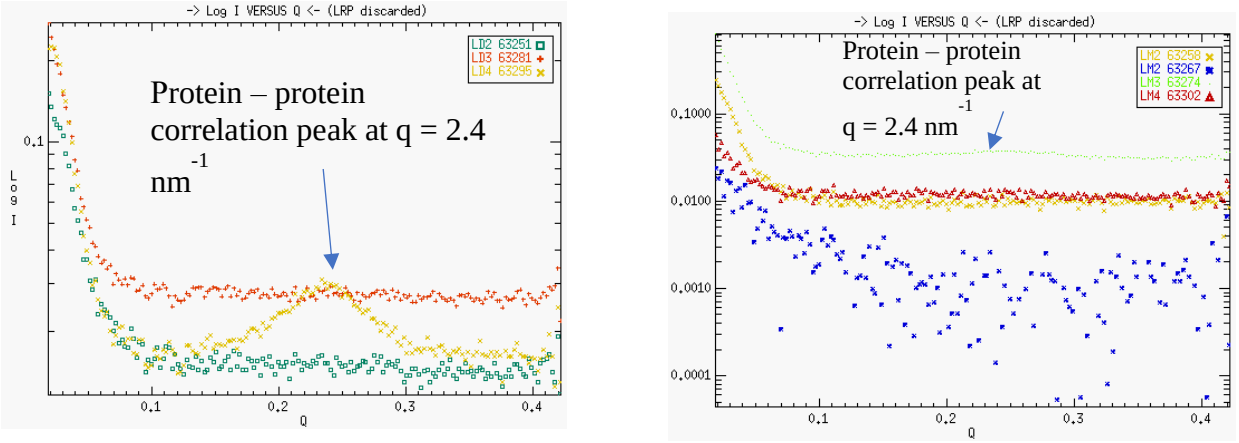

Figure 4: An example of the SANS patterns at a detector position that cover the q-range up to 4 nm^{-1}