

Accepted Manuscript

Combination of acoustic levitation with small angle scattering techniques and synchrotron radiation circular dichroism. Application to the study of protein solutions

Viviana Cristiglio, Isabelle Grillo, Margarita Fomina, Frank Wien, Evgenyi Shalaev, Alexey Novikov, Séverine Brassamin, Matthieu Réfrégiers, Javier Perez, Louis Hennet

PII: S0304-4165(16)30138-6
DOI: doi: [10.1016/j.bbagen.2016.04.026](https://doi.org/10.1016/j.bbagen.2016.04.026)
Reference: BBAGEN 28474

To appear in: *BBA - General Subjects*

Received date: 22 January 2016
Revised date: 25 April 2016
Accepted date: 27 April 2016



Please cite this article as: Viviana Cristiglio, Isabelle Grillo, Margarita Fomina, Frank Wien, Evgenyi Shalaev, Alexey Novikov, Séverine Brassamin, Matthieu Réfrégiers, Javier Perez, Louis Hennet, Combination of acoustic levitation with small angle scattering techniques and synchrotron radiation circular dichroism. Application to the study of protein solutions, *BBA - General Subjects* (2016), doi: [10.1016/j.bbagen.2016.04.026](https://doi.org/10.1016/j.bbagen.2016.04.026)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Combination of acoustic levitation with small angle scattering techniques and synchrotron radiation circular dichroism. Application to the study of protein solutions

Viviana Cristiglio^a, Isabelle Grillo^a, Margarita Fomina^b, Frank Wien^d, Evgenyi Shalae^c, Alexey Novikov^e, Séverine Brassamin^e, Matthieu Réfrégiers^d, Javier Perez^d, Louis Hennet^{e,*}

^a Institut Laue Langevin, 71 avenue des Martyrs, 38000 Grenoble cedex 2, France

^b Department of Physics and Chemistry, University of Palermo, via Archirafi 36, 90123 Palermo, Italy

^c Allergan Inc., 2525 Dupont Drive, Irvine, CA 92612, USA

^d Synchrotron Soleil, L'Orme des Merisiers, BP 48 91192 Gif sur Yvette Cedex, France

^e CNRS-CEMHTI, 1D, avenue de la Recherche Scientifique, 45071, Orléans cedex 2

* Corresponding author: louis.hennet@cnrs-orleans.fr

Abstract

Background: The acoustic levitation technique is a useful sample handling method for small solid and liquids samples, suspended in air by means of an ultrasonic field. This method was previously used at synchrotron sources for studying pharmaceutical liquids and protein solutions using x-ray diffraction and small angle x-ray scattering (SAXS).

Methods: In this work we combined for the first time this containerless method with small angle neutron scattering (SANS) and synchrotron radiation circular dichroism (SRCD) to study the structural behavior of proteins in solutions during the water evaporation. SANS results are also compared with SAXS experiments.

Results: The aggregation behavior of 45 µl droplets of lysozyme protein diluted in water was followed during the continuous increase of the sample concentration by evaporating the solvent. The evaporation kinetics was followed at different drying stage by SANS and SAXS with a good data quality. In a prospective work using SRCD, we also studied the evolution of the secondary structure of the myoglobin protein in water solution in the same evaporation conditions.

Conclusions: Acoustic levitation was applied for the first time with SANS and the high performances of the used neutron instruments made it possible to monitor fast container-less reactions in situ. A preliminary work using SRCD shows the potentiality of its combination with acoustic levitation for studying the evolution of the protein structure with time.

General Significance: This multi-techniques approach could give novel insights into crystallization and self-assembly phenomena of biological compound with promising potential applications in pharmaceutical, food and cosmetics industry.

Highlights

- First measurements made by small angle neutron scattering of protein solutions using acoustic levitation
- In-situ fast kinetics of drying protein solutions
- First results from circular dichroism spectroscopy of myoglobin protein using acoustic levitation.

Keywords: Acoustic levitation, Small angle scattering, Circular dichroism spectroscopy, Lysozyme, Myoglobin, Aggregation

Introduction

The drying process is a critical final stage in various manufacturing processes. Due to its influence in specifying the quality of a product, drying is one of the most important operations and has probably the widest range of industrial application, in particular in the food and pharmaceutical fields. Freeze drying (lyophilization) is one of the drying methods where the solvent is frozen prior to drying and is then sublimed. This method is commonly used to preserve the integrity and the bioactivity of protein drugs minimizing chemical and physical degradation during their shelf life. In addition, the spray-drying process is attracting an increasing attention in biopharmaceutical industry, as an alternative for freeze-drying process.

Moreover, various pharmaceutical formulations are developed to avoid protein degradation and irreversible aggregation during freeze-drying and storage, as the use of cryo and lyo-protectors polyhydroxy compounds [1].

It has been observed that the storage in freeze-dried states does not guarantee long-term stability, and aggregation effects are often observed after freeze-thaw or reconstitution of freeze-dried powder. In particular, formation of ice per se was shown to have destabilizing effect on protein molecules during freezing [2]. An essential step is to understand the biophysical mechanisms involved in the conservation and stabilization processes during the freeze-drying and the crystallization dynamics is not completely elucidated.

In this work, we focused our interest on the study of the structural evolution of protein solutions up to supersaturation conditions. These are created by partial evaporation of the solvent to mimic the drying process conditions. Our objective is to develop an appropriate experimental set-up to make time-resolved structural analysis during this drying process. Future advances of this set-up will permit to be able to introduce a new procedure to work at low temperature and to reproduce the freeze-drying process.

The sample environment used to reproduce the evaporation conditions on the sample is a critical point. Heterogeneous nucleation or chemical contamination could be induced by the sample containers avoiding the follow up of the crystallization in supersaturated solutions.

In the past, various methods based on contactless techniques have been developed to overcome these problems [3,4]. Among them, acoustic levitation is an interesting tool for container-free sample handling. Using this contactless method, solid and liquid samples can be positioned in the surrounding medium (ambient air or defined gas) by means of a stationary ultrasonic field creating a pressure gradient in the medium. A single droplet is held in a node of a standing acoustic wave [5], avoiding hereby any contact with a thermally-conducting holding-device. The droplet size can be controlled in a wide range from about 1 to 3 mm. The surrounding drying-gas can be conditioned such that its temperature, relative humidity, and flow rate past the droplet are accurately controlled. This technique was already employed to study the evaporation rate of pure solvent droplets suspended in a dry gas, but was later extended to solution droplets.

Evaporation of the solvent during levitation gradually decreases the volume of the droplet and therefore increases the corresponding concentration of the solute. Thus, formation of aggregates and processes of crystallization could be followed in situ and on live finding suitable crystallizing conditions.

A large number of studies using acoustic levitation has been published by Lee and coworkers targeted to understand drying processes in carbohydrates, proteins and enzymes solutions looking the formation of structure during the different steps of evaporation [6-10]. In particular, they made an extensive research on the parameters involved in the evaporation

process during the levitation, measuring the drying rates using several external conditions [11].

In this work small angle neutron (SANS) and x-ray (SAXS) scattering was used to probe structural larger-scale fluctuations and interactions of a model protein, lysozyme, in aqueous solution as a function of the concentration in drying conditions using acoustic levitation. Small-angle scattering provides information on the concentration, sizes and shapes of nanosize objects and thus, is a very useful technique for studying proteins in a variety of phases. Neutrons are particularly sensitive to hydrogen atoms and by a substitution of hydrogen by deuterium in the aqueous solutions, we can reduce the incoherent scattering effect and highlight features in the neutron scattering curves.

A considerable number of studies have been done on lysozyme solutions [12-15]. The existence of equilibrium clusters depending on the concentration and the temperature was a controversial question [12,13]. In the paper of Porcar et al. [14], the authors have shown that at relatively low concentrations (approximately 5 to 10 mg/ml) there are no clusters in lysozyme solutions and at higher concentrations (> 15 mg/ml) dynamic clusters can form. This study will offers complementarities to the previous results obtained by other experimental techniques and gives us the possibility to validate this methodology to investigate bio-based materials structures during the drying process.

There are very few works using acoustic levitation on biological compound devoted to lipids properties, protein crystals grown and small molecular weight pharmaceutical drugs crystallization [16-18]. Recently, the acoustic levitation technique was used to produce new solution-derived amorphous forms of drugs and glassy forms of organic materials [19].

Up to now, at large scale facilities, it has been only used at synchrotron sources to study soft matter systems, including protein aggregation in solution and other materials science applications [18,20].

With this article, we propose to show the capabilities of this novel instrument set-up based on acoustic levitation using in situ time-dependent neutron scattering measurements to monitor structural changes of the lysozyme protein solutions in drying conditions. To our knowledge this is the first time that acoustic containerless processing has been used in a neutron source. Our results are also compared to SAXS measurements.

Moreover, first results of this levitation technique using synchrotron radiation circular dichroism (SRCD) spectroscopy is reported as new perspectives for the investigation of protein solutions which would be complementary to small angle scattering (SAS). With this technique it is possible to follow up the secondary structure of proteins during drying in levitation mode. In this part we will take in consideration a solution of myoglobin in water, as model protein solution. Myoglobin is present in almost all mammals and responsible for the red color of raw meat. The solubility of this protein in water brought the interest of researchers to understand the mechanism of protein dynamics in water and his biological activity in different solvents. [21]

Experimental

Reagents

Lysozyme from chicken egg white ($> 90\%$) was purchased in powder form from Sigma–Aldrich and used without further purification. Lysozyme protein, well known as globular, enveloped inside an ellipsoid of size $45 \times 30 \times 30 \text{ \AA}^3$, can be approximated by a sphere model with a radius of $15.5 \text{ \AA}$.

Lysozyme solutions were prepared directly dissolving lysozyme powder into pure 99.9% D₂O (Cambridge Isotope Labs, Inc.) at protein concentrations of 58 mg/ml and 100 mg/ml for SANS measurements, 10 mg/ml, 60 mg/ml and 90 mg/ml for SAXS.

Myoglobin from horse skeletal muscle (salt-free, lyophilized powder; BioUltra ≥98%) was purchased from Sigma–Aldrich. The solution was prepared by mixing the powder in Milli-Q water (Merck) at a concentration of 1.5mg/ml.

SANS and SAXS

The small-angle neutron scattering experiments were carried out on the D33 and D16 instruments at the Institut Laue-Langevin (ILL) in Grenoble (France). Measurements on D33 were performed at room temperature, using 2 and 1.2 m sample-to-detector distances for the rear and front panels respectively. We used a 6 Å wavelength with a collimation setup of 2.8 m enabling to cover the q range of 0.01 - 0.3 Å⁻¹. A 1 mm diameter diaphragm was placed in front of the levitating droplet. The data were placed on an absolute scale by normalizing the scattered intensity to the incident beam flux.

On D16 we used a pyrolytic graphite monochromator giving a working wavelength of 4.5 Å. The diffracted beam was measured over an angular range of 2-10°, corresponding to a q range 0.02–0.3 Å⁻¹. A 4 mm diameter diaphragm was placed before the levitating droplet position.

All SANS data were recorded in both instruments for one minute and the acquisition was repeated during half an hour. The scattering intensities were normalized with a standard calibration of water run to correct by the non uniform detector response and to normalize in absolute unit. Further corrections take into account the ambient room background (levitator without the sample), the buffer contributions (measurement of a droplet of D₂O in the same sample condition), the transmission and the thickness of the sample (monitored by the video images) measurements acquired each minute on the levitated sample.

SAXS experiments were carried out at the Small and Wide angle x-ray scatterING (SWING) beamline at the SOLEIL synchrotron (Saint-Aubin, France). The incident beam energy was 12 keV ($\lambda = 1.033$ Å) and the sample-to-detector (Aviex 17x17 cm² CCD) distance was set to 1835 mm. The corresponding usable scattering-vector range was $0.007 < q < 0.60$ Å⁻¹. During the experiment the beam size was 400 x 100 μm². In order to avoid any damages of the protein from the intense beam, each measurement corresponds to an average of 10 acquisitions of 100 ms separated by a dead time of 2.4 s which gives a total counting time of 25 s / scan. For each composition, the total duration was 40 minutes including a delay of 90 s between 2 records. All measurements are normalized by the transmission and background subtracted. For each sample, the curves are further normalized to the intensity of the form factor peak of the first measurement found at about 0.35 Å⁻¹. This corresponds to a normalization to the concentration and the sample size and the obtained scaling factor enables to get an estimation of the concentration according to:

$$\frac{I_i(q = 0.35\text{Å}^{-1})}{I_1(q = 0.35\text{Å}^{-1})} = \frac{C_i}{C_1} \frac{r_i}{r_1} = \left(\frac{C_i}{C_1} \right)^{2/3} \quad (1)$$

where i is the scan number, C the concentration and r the optical path in the sample. This assumes that the sample is always centered in the beam.

For all small angle scattering measurements, the integrated 2D data produce the scattered intensity versus q , $I(q)$, where $q = 4\pi\sin\theta/\lambda$ where 2θ is the scattering angle. For a monodisperse particle solution the $I(q)$ can be defined by the product of the form factor $P(q)$ and the structure factor $S(q)$:

$$I(q) = P(q).S(q) \quad (2)$$

For a diluted system (concentrations up to approximately 3 mg/ml) the structure factor is a constant set to 1 and the $I(q)$ represents the factor form and depends on the shape of the particles.

At higher concentration the structure factor become more determinant with the appearance in the $I(q)$ of a peak in q region between $0.01 < q < 0.6 \text{ \AA}^{-1}$. The position of the peak is linked to the distance between particles by the equation $d = 2\pi/q_p$, where q_p is the peak position.

This peak becomes more intense and narrow with the increase of the particle concentration in the solution.

As the data analysis is still under processing, due to the several effects presents in the levitation process, we don't treat this part in this article. A qualitative discussion on the experimental results will be made in the following sections to show the potentiality of the levitation environment applied to technique for large-scale structures investigation.

SRCD spectroscopy

As a prospective work, far ultra violet SRCD spectroscopy with a spectral domain ranging from 170 to 320 nm, was used for secondary structure determination of proteins (α -helices, β sheets and turns). The principle is to measure the differential absorption of left- and right-circularly polarized light passing through the protein solution. Compared to standard circular dichroism, SRCD improves the information content obtainable from the extended spectral range, with a better signal to noise ratio and faster acquisition times. The circular dichroism experiments were performed at the Dicroism, Imaging, mass Spectrometry for Chemistry and biOlogy (DISCO) beamline at the SOLEIL synchrotron (Gif sur Yvette, France) [22,23].

Acoustic levitator

A Single-axis Acoustic Levitator device is available at the ILL and Soleil facilities for the user community. This device is designed and manufactured by MDI (Materials Development, Inc) in Illinois (US) and a full description can be found elsewhere [5]. Two opposite transducers operated at a frequency of 22 kHz generate plane acoustic waves and a single droplet is held in one of the nodes of the resulting standing wave.

The vertical sample position can be monitored by electronically adjusting the relative acoustic phases of the two transducers, enabling the levitated object to be moved along a vertical axis. This is useful for readjusting the sample position in the beam. Liquids samples with sizes ranging between 1 and 3 mm are introduced in the acoustic field using a micropipette injection. A video camera was used to record images and video and to help the sample positioning. The device is relatively compact and can be easily combined with various analysis techniques at large scale facilities.

Due to the increase of the incoherent background during long neutrons scattering acquisition times, for the experiment on D16 SANS instrument, we use an argon gas flow directed onto the levitated sample to create a neutral gaseous atmosphere around it in order to minimize the exchange from deuterium to hydrogen of the sample solutions.

Pictures of the levitation set-up installed at the different neutron and x-ray facilities are shown in figure 1a, 1b and 1c respectively.

Despite the small quantities of product that is possible to suspend (just few microliters in volume), the high flux and performance of neutron instruments make it possible to perform in-situ monitoring of fast container-less reactions and follow drying processes at the nanoscale changes.

Results and discussion

To perform the measurements, a droplet of 45 μl was injected in the sound field of the acoustic levitator and left to evaporate in air at room temperature for 20 minutes.

A characteristic increase of the protein concentration with time is observed. To have an estimation of the evolution of the concentration during the drying process we extracted the images of the droplet from the video camera after each minute (a snapshot of the droplet is shown in figure 1d) and we made an analysis on the volume changes as described in ref [18]). We also estimated the concentration from the SAXS measurements using equation (1). As shown in the inset of figure 5, which present the evolution of the concentration with time, we obtained a reasonable agreement between the two methods. Some differences can be attributed to a bad estimation of the optical path (see equation 1) but we can consider that our concentration values are reasonable. Finally, to confirm the analysis on the sample volume, we also measured the weight of the sample before and after the evaporation process to estimate the loss of solvent. An additional coupling with optical density measurements is under development and will help to have a better control of the concentration in the future.

From our estimation of the concentration during the drying process of the lysozyme solutions at room temperature, the sample reduces by a bit less of half of its volume in 20 minutes of evaporation. A starting concentration of 60 mg/ml increases to approximately 111 mg/ml.

SANS scattering profiles obtained starting with the lysozyme concentration at 58 mg/ml in D_2O solution are plotted on the $\log(I)$ vs $\log(q)$ scale in figure 2a.

From these scattering curves we were able to monitor structural changes looking the evolution of the main characteristic interaction peak of the lysozyme particles with the solvent evaporation. We can observe that, in the first stage of the levitation, the peak position is centered approximately at $0.085 \text{ \AA}^{-1}$ and then shifts to higher q values (slightly above $0.09 \text{ \AA}^{-1}$) after 20 minutes of evaporation. The intensity of the peak decreases constantly and it becomes broader after 15 minutes.

In particular for SANS, with the decrease of the D_2O content, the signal is mainly dominated by the incoherent contribution coming from the hydrogen present in the sample. Hence, the interaction peak of the lysozyme disappears faster compared to the SAXS measurements, which are not affected by the incoherent contribution.

A more detailed analysis of the SANS signal will permit us to better understand the intensity changes behavior during the evaporation.

In the SAXS measurements on lysozyme solutions at 30 and 60 mg/ml in D_2O (figure 2b and 2c), we observe the same trend as with SANS. For the sample with a concentration of 30 mg/ml the peak shift is more evident starting from $0.07 \text{ \AA}^{-1}$ up to $0.09 \text{ \AA}^{-1}$ (decrease of the distance d between proteins from 90 down to 70 $\text{\AA}$) and the peak appears well defined even after 20 minutes. It is possible to see a less intense peak at $q \sim 0.35 \text{ \AA}^{-1}$, corresponding to the characteristic oscillation of the form factor. The sample at 60 mg/ml shows a similar evolution during the drying as the one at lower concentration, i.e, an intensity decrease and broadening of the interaction peak.

As mentioned previously, in the SANS data there is an evident increase in the background signal with the sample evaporation. This raise is not only given by the change of the sample conditions, but since the sample is not in a confined environment, this is also due to the incoherent contribution coming from the exchange of deuterium of the solution with the hydrogen from the ambient air, To validate our assumption we have studied the evolution of

the SANS signal, from a D₂O droplet levitated in air. This measurement will be further used to correct the data from the buffer scattering during evaporation.

In figure 3a, we reported the evolution of the SANS signal as a function of time. We can see that the constant incoherent signal is increased by a factor 5 after 20 minutes, which is a clear evidence of the H/D exchange. To reduce this effect we decided to make an inert environment around the sample using an argon gas flow. As seen in figure 3b, this procedure leads to slow down the H/D exchange limiting the increase of the background contribution to a factor of about 2 after 20 minutes. For future experiments, a specific chamber will be used to confine the sample from external conditions and create an appropriate inert atmosphere around the sample.

In figures 4a and 4b we reported the SANS and SAXS data of the concentrated lysozyme solution at 100 mg/ml. During the neutrons experiment, we used the Ar gas flow to reduce the incoherent background. At this concentration the peak do not shift as observed for the low concentrated samples but remains in the same position at $q_p \sim 0.1 \text{ \AA}^{-1}$. However the peak intensity decreases faster with the sample evaporation.

For the lysozyme solution at 100 mg/ml, we highly concentrate the sample up to 220 mg/ml after 20 minutes. In these drying conditions we could also have a partitioning of lysozyme molecules into larger clusters, not visible in this SANS/SAXS q -range.

Comparing our SANS results with those presented in previous works, for low lysozyme concentrations (figure 2), we observe a behavior of the interaction peak position as a function of the concentration similar to ref [13]. In particular, we observe a clear sign of interparticle interferences with a systematic shift toward higher q values with increasing concentration. The center-to-center distances between molecules become smaller due to the evaporation inducing a concentration increase. At high lysozyme concentrations we found that the peak remains at the same position exhibiting only a decrease of its intensity. Thus, the distance between molecules seems unchanged and no clustering effect is observed.

We could speculate that in such high concentration regime, we have a coexistence of two phases and during the evaporation process we move along a tie-line, changing the relative proportion of the phases but not their composition. Such liquid-liquid or liquid-solid coexistences have been reported for lysozyme solution in presence of salt during the crystallization process [24].

For an ideal solution the distance d ($2\pi/q_p$) between molecules is proportional to $C^{-1/3}$. In the figure 5, we have then represented the evolution of the peak position q_p derived from the SAXS measurements as a function of $C^{1/3}$ for the four studied lysozyme solutions (10, 30, 60 and 90 mg/ml). For the lowest concentration the q_p position is at $0.053 \text{ \AA}^{-1}$ corresponding to a distance d between proteins of about $119 \text{ \AA}$. By increasing the concentration, the evolution is quasi linear up to a concentration of approximately 150 mg/ml where q_p the position reaches a maximum at $q_p \sim 0.093 \text{ \AA}^{-1}$ corresponding to $d \sim 68 \text{ \AA}$. At concentrations above 230 mg/ml, a continuous decrease of the peak position is observed giving a position $q_p \sim 0.090 \text{ \AA}^{-1}$ at the highest concentration (454 mg/ml). The origin of this effect is not clear and a precise data analysis is current underway to model the SANS/SAXS data to have a clear picture of the behavior of the system at the different evaporation states.

We have to emphasize that the acoustic field induces an internal mixing of the liquid droplet by the air convection and therefore the sample is continuously homogenized preventing large aggregates. Nevertheless, the acoustic field could cause effects of unexpected denaturation of the protein or phase transition, as already observed in anionic surfactant solution [25].

New perspectives using SRCD spectroscopy

In addition to SAS techniques, it can be interesting to use other techniques enabling to study the protein structure. As a prospective work, we combined the acoustic levitation with SRCD in order to evaluate the potentiality of this association. Unfortunately measurements were not performed on Lysozyme but on myoglobin. In this section we present the first results obtained using this combination.

Figure 6 presents five consecutive SRCD spectra measured on bovine myoglobin suspended in water with an initial concentration of 1.5 mg/ml. Each curve is the average of 2 scans and the total duration of the measurement was 40 minutes. The curve 1 shows a denaturation of the protein probably due to a sonication effect by the 22 kHz frequency generated by the levitator, as explained before. Upon consecutive scans the protein concentration increases and restructures itself in the drying droplet. Evidence for this is the increasing amplitude and change of the frequency of the spectral minimum.

A preliminary analysis of the spectral evolution has been performed using the BeStSel algorithm [26], which allows an optimization of the spectral scaling factor providing the lowest fitting NRMSD. Results are summarized in the table I. We observe an increase of α -helices content from 4.7 to 12.6% with time and the full loss of parallel β -sheets (initially 5.7%). The antiparallel β -sheets are almost stable. The number of turns decreases with time, indicating longer lengths of the α -helix chains and β sheets. These changes are significant and thus are the signature of structural rearrangements occurring during the drying of the droplet and the protein agglomeration.

We showed here that the combination of SRCD with acoustic levitation is an interesting method to follow up the evolution of the secondary structure of a protein as a function of the concentration. However, sonication effects on the studied protein have to be evaluated before any measurements. From the SAXS measurements, it appears that the Lysozyme is not denatured during the acoustic levitation (no modification of the form factor). It will be then interesting to complete the SAS study with SRCD measurements.

Conclusions

We present for the first time, to our knowledge, a small angle neutron study on proteins solution using an acoustic levitator. Despite the small quantities of product that it is possible to suspend (just few tens of microliters in volume), the high performances of neutron instruments make possible in-situ monitoring of fast containerless reactions, providing detailed molecular and structural information.

A new system integrating a cryostream device to be able to work at lower temperature and an adapted chamber to preserve the sample from external conditions are under implementation.

For a better determination of the concentration at the different drying phase of the sample, we are working in coupling acoustic levitation with UV measurements of the sample during the evaporation.

The combination of neutron and x-ray scattering techniques using this contactless technique will give access to novel insights into aggregation and crystallization phenomena and open the way to promising potential applications in industrial processes such as spray-drying and spray-freeze-drying in a wide range of chemical structures with varying pharmaceutical uses.

The results discussed here prove that the acoustic levitator can be used as a tool for structure analysis and that it easily permits the contactless study of many other kinds of samples. Detailed SANS and SAXS data analysis regarding proteins structure changes and interaction

will be the main topic of our future work. When possible, a comparison with Dynamic Light Scattering measurements could be interesting to get more information about the size of the aggregates during the evaporation.

This work shows also that SRCD is an interesting technique for studying the protein behavior during acoustic levitation. However further analysis are necessary to well understand the effect of the ultrasonic excitation on the protein. The combined use of SRCD with SAS techniques will provide important tools for studying the structural evolution of the protein during the drying process.

Acknowledgements

We thank the technical staff of SWING and DISCO beamlines at the SOLEIL Synchrotron and of D16 and D33 instruments at the Institut Laue Langevin for assistance with the experiments. We gratefully acknowledge financial support from the Regional council of the Region Centre (grant no. 201100070584).

References

- [1] J. C. Lee, S. N. Timasheff, *J. Biol. Chem.* 256 (1981) 7193
- [2] G. B. Strambini, M. Gonnelli, *Biophys. J.* 92 (2007) 2131
- [3] S. Krishnan, J. J. Felten, J. E. Rix, J. K. R. Weber, P. C. Nordine, M. A. Beno, S. Ansell, and D. L. Price, *Rev. Sci. Instrum.* 68 (1997) 3512
- [4] L. Hennet, V. Cristiglio, J. Kozaily, I. Pozdnyakova, H. E. Fischer, A. Bytchkov, J. W. E. Drewitt, M. Leydier, D. Thiaudière, S. Gruner, S. Brassamin, D. Zanghi, G. J. Cuello, M. Koza, S. Magazu, G. N. Greaves, D. L. Price, *Eur. Phys. J. Special Topics*, 196 (2011) 151
- [5] R. J. K. Weber, C. A. Rey, J. Neuefeind, C. J. Benmore, *Rev. Sci. Instrum.* 80 (2009) 083904
- [6] E. Lorenzen, G. W. J. L. Lee, *J. Pharm. Sci.*, 102 (2013) 4268-4273
- [7] E. Lorenzen, G. W. J. L. Lee, *J. Pharm. Sci.*, 101(2012) 2239-2249
- [8] G.W.J.Lee* & H. Schiffter, 'Acoustic Levitation' in: *Modern drying Technology. Volume 2: Experimental techniques'* (2008) Eds. Tsotsas, Mujamdar, Wiley VCH, Weinheim, Germany.
- [9] H. Schiffter, G.W.J. Lee, *J.Pharm. Sci.*, 96 (2007) 2284-2295
- [10] H. Schiffter, G.W.J. Lee, *J. Pharm. Sci.*, 96 (2007) 2274-2283
- [11] J. Sloth, S. Kiil, A. Jensen, S. Andersen, K. Jörgensen, H. Schiffter, G.W.J. Lee, *Chem Eng Sci*, 61 (2006) 2701-2709.
- [12] A. Stradner, H. Sedgwick, F. Cardinaux, W. C. K. Poon, S. U. Egelhaaf and P. Schurtenberger, *Nature* 432 (2004) 492
- [13] A. Shukla, E. Mylonas, E. Di Cola, S. Finet, P. Timmins, T. Narayanan and D. I. Svergun, *Proc. Natl Acad. Sci. USA* 105 (2008) 5075
- [14] L. Porcar, P. Falus, W. R. Chen, A. Faraone, E. Fratini, K. Hong, P. Baglioni and Y. Liu, *J. Phys. Chem. Lett.* 1 (2010) 126–129
- [15] J. E. Curtis, H. Nanda, S. Khodadadi, M Cicerone, H. J. Lee, A. McAuley, S. Krueger, *J. Phys. Chem. B*, 116 (2012) 9653
- [16] U. Varanasi, R. E. Apfel, and D. C. Malins, A Novel Microtechnique for the Measurement of Acoustic Properties of Lipids, *Chem. Phys. Lipids* 19 (1977) 179
- [17] S. K. Chung and E. H. Trinh, Containerless Protein Crystal Growth in Rotating Levitated Drops, *J. Cryst. Growth* 194 (1998) 384
- [18] J. Leiterer, F. Delißen, F. Emmerling, A. F. Thunemann, and U. Panne, *Structure Analysis Using Acoustically Levitated Droplets*, *Anal. Bioanal. Chem.* 391 (2008) 1221

- [19] C. J. Benmore and J. K. R. Weber, Phys rev X, 1 (2011) 11004
- [20] F. Delißen, J. Leiterer, R Bienert, F. Emmerling, A. F. Thünemann, Anal. Bioanal. Chem. 392 (2008) 161
- [21] F.-X. Gallat, A. P. S. Brogan, Y. Fichou, N. McGrath, M. Moulin, M. Härtlein, J. Combet, J. Wuttke, S. Mann, G. Zaccai, C. J. Jackson, A. W. Perriman, M. Weik, J. Am. Chem. Soc., 134, 32, (2012) 13168-13171
- [22] A. Giuliani, F. Jamme, V. Rouam, F. Wien, J.-L. Giorgetta, B. Lagarde, O. Chubar, S. Bac, I. Yao, S. Rey, C. Herbeaux, J.-L. Marlats, D. Zerbib, F. Polack, M. Réfrégiers, J Synchrotron Rad. 16 (2009) 835–841
- [23] M. Réfrégiers, F. Wien, H.-P. Ta, L. Premvardhan, S. Bac, F. Jamme, V. Rouam, B. Lagarde, F. Polack, J.-L. Giorgetta, J.-P. Ricaud, M. Bordessoule, A. Giuliani, J Synchrotron Rad. 19 (2012) 831–835
- [24] Yingxin Liu, Xiujuan Wang,* and Chi Bun Ching, Crystal Growth & Design, Vol. 10, No. 2, 2010
- [25] V. Cristiglio, I. Grillo, A. Novikov, L. Hennet, ILL Annual Report 2014
- [26] A. Micsonai, F. Wien, L. Kernya, Y.-H Lee, Y. Goto, M. Réfrégiers, J. Kardos, Proc. Natl. Acad. Sci. USA. 112, (2015) E3095-E3103

Table

curve	α -helices	β sheets		Turns	Others
		Parallel	Anti parallel		
1	4.7%	5.7%	21.3%	15.9%	52.3%
2	7.8	6.3%	17.8%	15.4%	52.8%
3	6.5%	5.7%	17.3%	19.0%	51.4%
4	10.3%	0.0%	20.2%	16.9%	52.7%
5	12.6%	0.0%	23.7%	7.9%	55.8%

Table I. Structural information of the 1.5 mg/ml myoglobin in water solution obtained from the analysis of the SRCD spectra using the BeStSel algorithm.

Figures

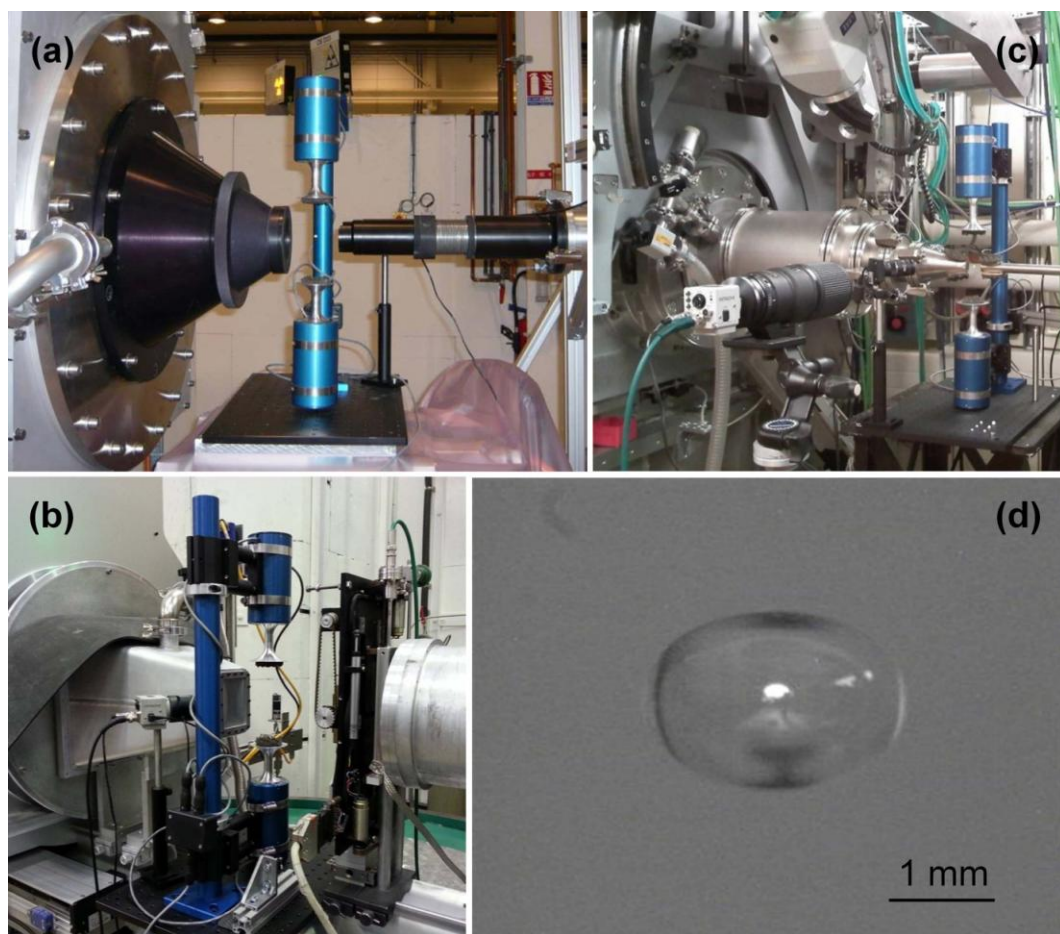


Figure 1. Acoustic levitation apparatus installed on the different X-rays and neutrons installations: D33 (a) and D16 (b) instruments at ILL; SWING beamline at Soleil (c). Snapshot of 45 μ l water droplet suspended by the acoustic field at room temperature (d).

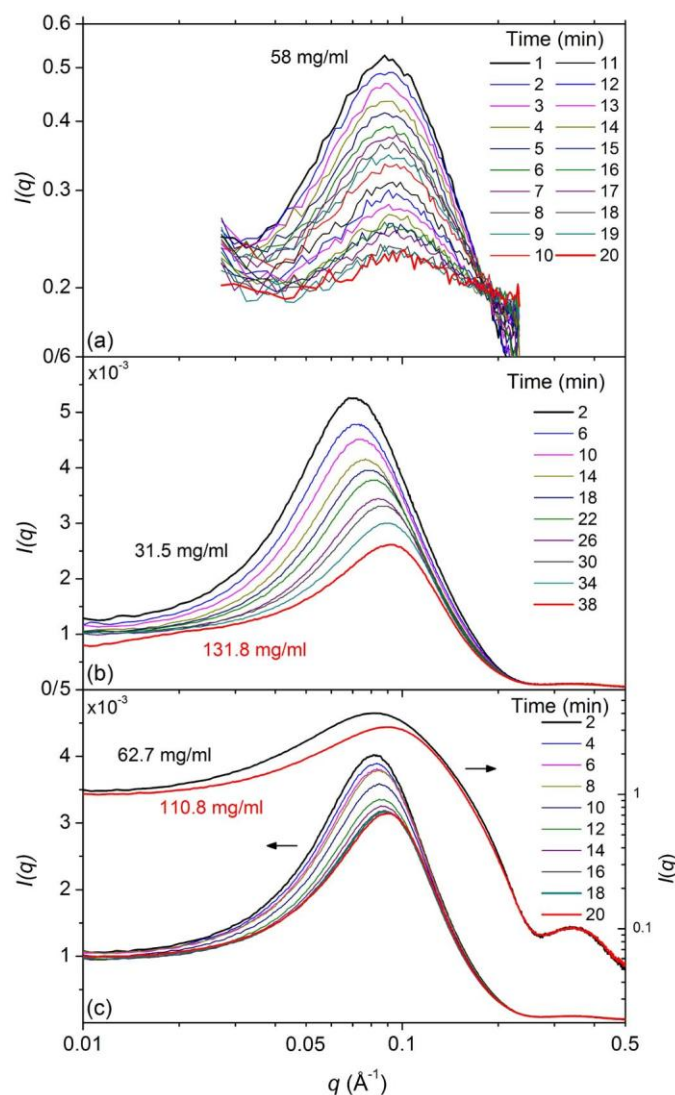


Figure 2. Neutrons and X-rays small angle scattering profile in $\log(I(q))$ vs. $\log(q)$ scale during the evaporation of a $45 \text{ }\mu\text{l}$ droplet. (a) SANS data (D33, ILL) from 58 mg/ml lysozyme in D_2O solution. SANS patterns were recorded during 1 minute for a total time of 20 minutes of droplet evaporation. SAXS patterns in $I(q)$ vs. $\log(q)$ scale of droplets of lysozyme solutions with initial concentrations of 30 mg/ml (b) and 60 mg/ml (c). For each SAXS curve, 10 acquisitions of 100 ms separated by a dead time of 2.4 s were recorded with a total counting time of 25 s per scan. For the 30 mg/ml solution, the total duration of the acquisition was 40 minutes; for the 60 mg/ml only the first 20 minutes of measurements have been reported in the plot in order to compare with the SANS data.

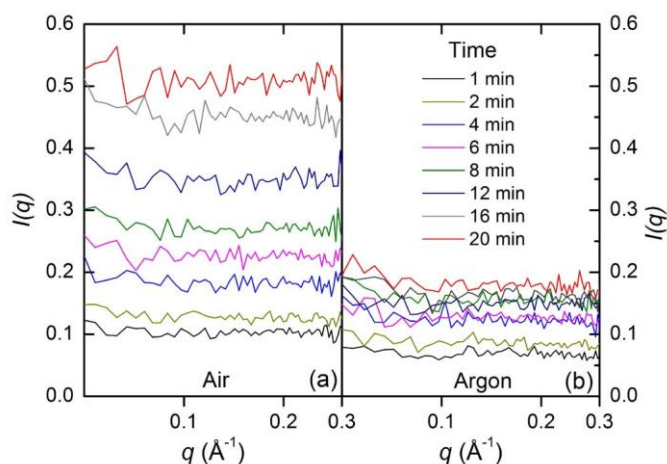


Figure 3. SANS data recorded during the evaporation of a 45 μl droplet of D_2O at room temperature in air (a) and surrounded by an Ar gas flow (b). The evolution of the scattering profile of the D_2O droplet (a) is attributed to the exchange from deuterium to hydrogen of the sample in air which is considerably reduced using an argon atmosphere (b).

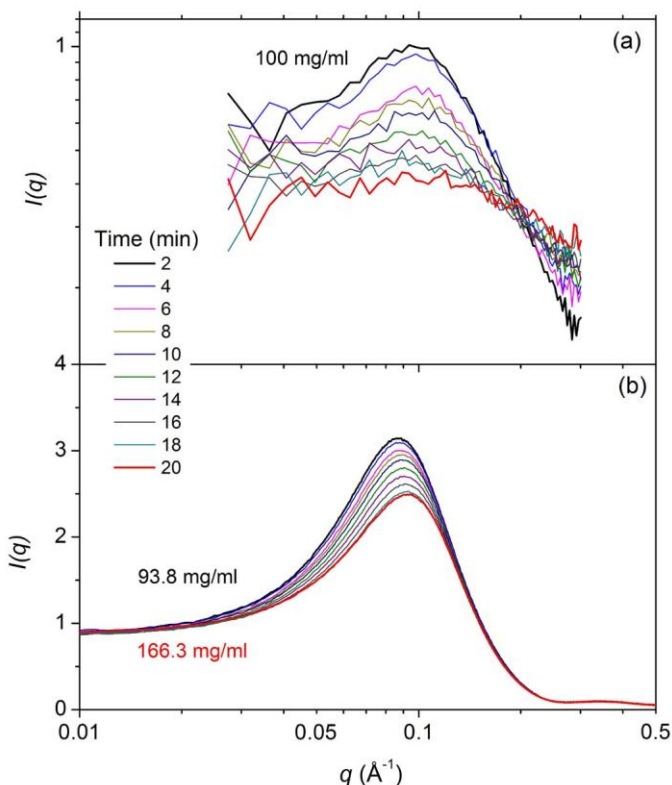


Figure 4. Scattering profiles obtained during the evaporation of 45 μl droplets of lysozyme solutions: (a) SANS Data recorded at D16 (ILL) in $\log(I(q))$ vs. $\log(q)$ with an initial concentration of 100 mg/ml with the sample exposed to an argon atmosphere (1 min/run for a total acquisition time of 20 minutes). (b) SAXS patterns in $I(q)$ vs. $\log(q)$ scale measured with an initial concentration of 90 mg/ml (25 sec/run for a total acquisition time of 20 minutes).

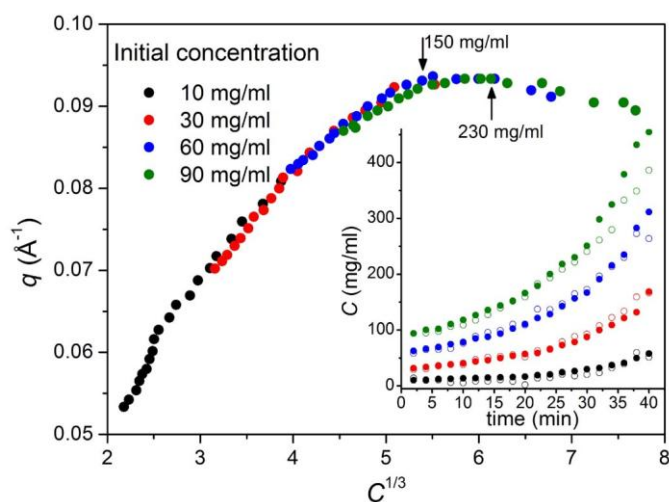


Figure 5. Plot versus $C^{1/3}$ of the peak position q derived from SAXS measurements from samples at different initial protein solutions concentration. The curves of the 4 studied samples (10, 30, 60 and 90mg/ml) follow the same trend whatever the initial concentration in the beginning of the levitation, with the proteins distances varying between 119 Å and 68 Å. Inset: Comparison between the concentration determined from the video analysis (plain circles) and from SAXS measurements (open circles).

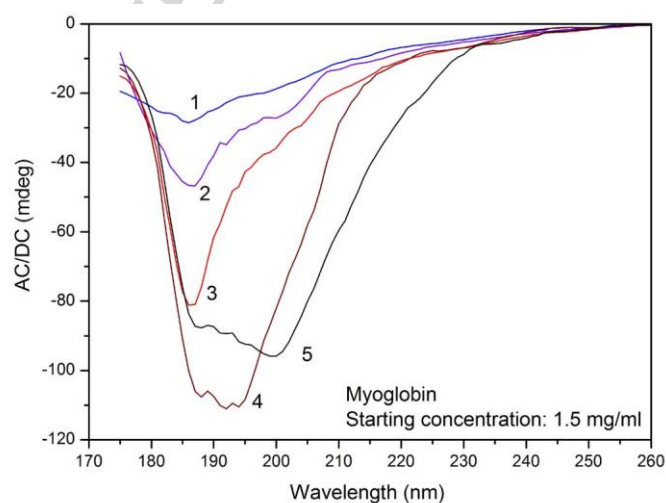


Figure 6. SCRD spectra measured during the levitation of a myoglobin drop on the DISCO beamline at the Soleil synchrotron facility. The initial concentration was 1.5 mg/ml dispersed in water solution. 5 consecutive scans are presented and tagged on the corresponding curve.

Highlights

- First measurements made by small angle neutron scattering of protein solutions using acoustic levitation
- In-situ fast kinetics of drying protein solutions
- First results from circular dichroism spectroscopy of myoglobin protein using acoustic levitation.