

# Benefits of combined SAXS and UV/Vis measurements for the analysis of biological samples

Relevant for: BioSAXS, Proteins, Polymers

Small-angle X-ray scattering (SAXS) and UV/Vis spectroscopy are highly complementary methods for the characterization of e. g., biological molecules in solution. While SAXS probes structural features of the molecules such as size, shape, and folding state, UV/Vis can be used to measure agglomeration and concentration. This report shows the advantages of doing an *in-situ* combination of both methods probing the exactly same sample volume.



## 1 Introduction

Small angle X-ray scattering (SAXS) is a powerful method in structural biology enabling characterization of molecular structure in solution, i.e. in native state. SAXS often fully utilizes its potential in combination with other methods that help interpreting the data or assessing the requirement of particle monodispersity.

Absorption spectroscopy of UV and visible light is such a complementary technique. Typically, it is used for measurements of molecule concentration or monitoring sample changes (e. g., aggregation) under various conditions. UV/Vis spectroscopy can be performed with very low sample volumes and often in confined space, which makes it ideal for *in-situ* applications in combination with other methods.

Combining SAXS with *in-situ* UV/Vis absorption spectroscopy enables the characterization of samples using both methods on the same sample volume at the same time. As an example, this report highlights concentration measurement and temperature induced aggregation monitoring of a protein sample.

### 1.1 Concentration series

Protein molecules are charged particles that tend to self-organize in solution. Due to the charge this results in a repulsive inter-particle interaction. In order to reduce this effect on the SAXS data, a series of different concentrations are often measured. The measured data is then extrapolated to zero concentration. Thus, the precise concentration of the particular samples has to be known.

The concentration of a protein in solution can be measured using the absorption of UV light at a wavelength of 280 nm. The concentration can be directly connected to the absorption by using Lambert-Beer's law:

$$A = \epsilon l c$$

where  $A$  is the absorption,  $\epsilon$  is the molar attenuation coefficient,  $l$  is the optical path length and  $c$  is the concentration. Therefore, measuring UV/Vis absorbance *in-situ* also translates to the concentration of the actual sample subjected to a SAXS measurement.

### 1.2 Temperature stability

Protein molecules can also undergo attractive inter-particle interaction, e. g. aggregation. Aggregation often results in precipitation of the molecules from the solution, which can be observed optically. In absorption spectroscopy, it translates to an increase of the absorption in the visible range as a result of light scattering on the particles. For SAXS, the aggregation is most often the effect hampering the requirement of monodispersity; however, from SAXS data only, it is sometimes difficult to distinguish aggregation from other effects.

In this work, effect of thermally induced aggregation on UV-Vis absorption and SAXS is shown.

## 2 Experimental details

### 2.1 Experimental setup

The measurements were performed using an Anton Paar SAXSpoint instrument equipped with an Excillum MetalJet X-ray source (Figure 1), a Dectris EIGER R 1M detector and a customized UV/Vis sample stage. The sample stage is a modified flow-through capillary, which enables usage of an autosampler. The temperature of the sample can be controlled using a Peltier element integrated into the sample stage. UV/Vis absorption is measured orthogonally to the X-ray beam at the same position at sample. An Agilent CaryUV 60 spectrometer is used to generate monochromatic light (190 – 1100 nm) which is brought to the sample with fiber optics. Light transmitted through the sample is recorded using a dedicated detector that is mounted to register the amount of absorbed light directly at the beam position, making sure to probe the same sample volume by both SAXS and UV/Vis measurements.

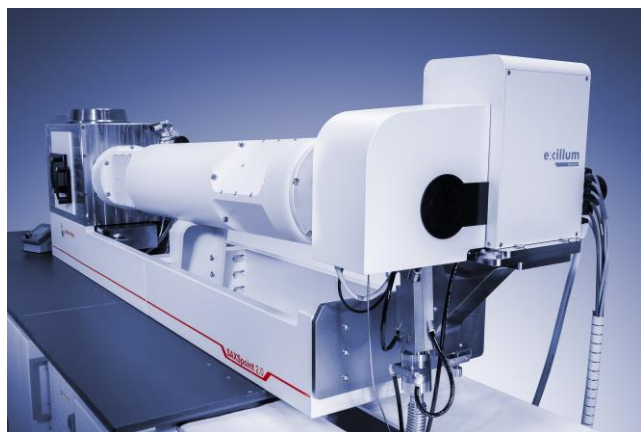


Figure 1: SAXSpoint with MetalJet source.

### 2.2 Measurement

Thyroglobulin (TG) from bovine thyroid (Sigma-Aldrich) was dissolved in 20 mM Tris, 100 mM NaCl, pH 7.4 to a final concentration of 20 mg/ml. Further decreased concentrations were produced by adding corresponding amounts of buffer. The samples were measured in a 1 mm quartz capillary, in which 25  $\mu$ l of sample was loaded automatically using the ASX(-c) autosampler. SAXS data and UV/Vis data were then measured simultaneously.

### 2.3 Concentration measurement

The stock solution was dissolved to 2.5 mg/ml, 5 mg/ml, 10 mg/ml and 20 mg/ml. For SAXS, each sample was for exposed for 6 x 30 s and data was recorded in the  $q$ -range from 0.08  $\text{nm}^{-1}$  to 4  $\text{nm}^{-1}$ . UV/Vis spectra were collected each 60 s for wavelengths between 200 nm and 500 nm with an averaging time of 0.2 s per point. The equivalent buffer was measured under the same conditions.

### 2.4 Temperature stability

Thyroglobulin at 10 mg/ml was measured. The sample was heated at rate of 5 K/min with 5 min of waiting time before the each SAXS measurement. For each temperature point (between 293 K and 343 K), the sample was exposed for 12 x 30 s. After the heating period, the sample was also cooled down in few steps. The UV-Vis spectra were collected each 30 s for wavelengths between 200 nm and 500 nm with averaging time 0.2 s per point. The equivalent buffer was measured for 30 x 30 s at 293 K.

## 3 Results

### 3.1 Concentration measurement

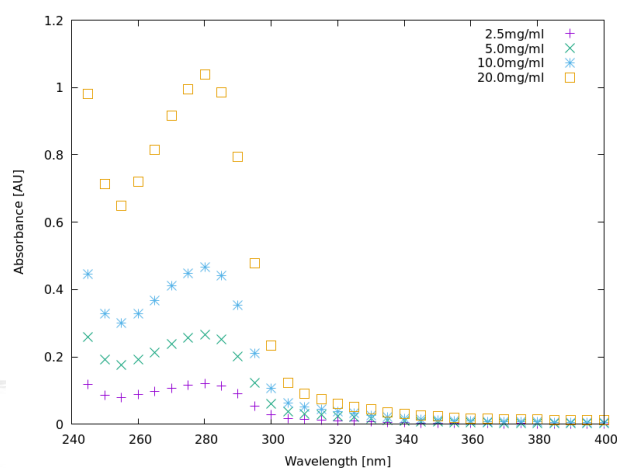


Figure 2: UV/Vis spectra of the differently concentrated TG solutions.

The measurements of the individual samples were averaged and corrected for buffer, both UV-Vis spectra (Figure 2) and SAXS (Figure 3A). Both methods show clear effect of the concentration on the measured data. The measurement capillary has cylindrical shape, and the actual optical path is difficult to estimate due to varying thickness of the sample and re-

flection effects of the capillary walls. Therefore, SAXS data were scaled only using the measured absorbance values at 280 nm. The scaled curves show good agreement and could be used for further analysis (Figure 3B).

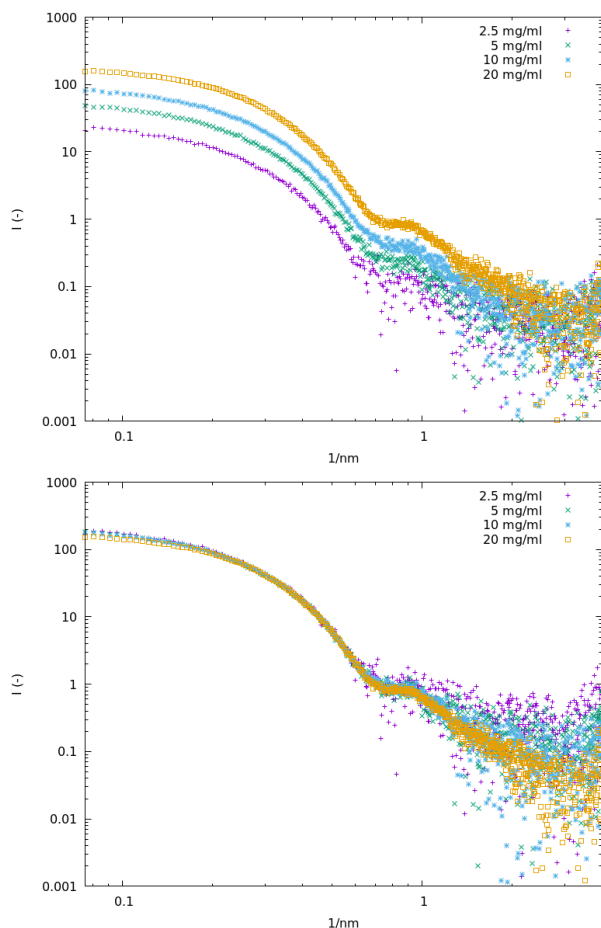


Figure 3: Scattering curves before (A) and after (B) scaling using the UV/Vis intensity at 280 nm.

### 3.2 Temperature stability

The aggregation in the absorption spectroscopy is typically shown as non-zero absorbance at 350 nm. For SAXS, bigger aggregated particles causes an increase of the scattered intensity in the low angle region (Figure 5A). Therefore, for each measured UV/Vis spectrum and SAXS curve, absorbance at 350 nm and average scattering intensity in  $q$ -range (0.1; 0.15) were extracted (Figure 5B). Both curves show typical behavior of melting proteins however, the SAXS plot is slightly offset to the plot obtained from the UV/Vis experiment. This indicates that melting may start already at lower temperatures and suggests that SAXS is more sensitive towards early signs of protein denaturation as it detects structural changes at an early stage.

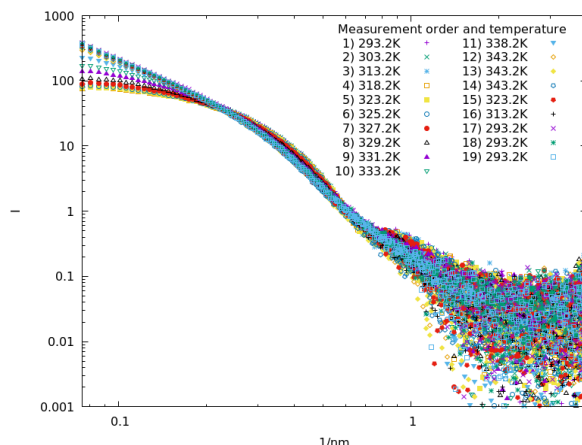


Figure 4 (A): SAXS scattering curves of a heating series of the protein solution showing aggregation of the protein molecules.

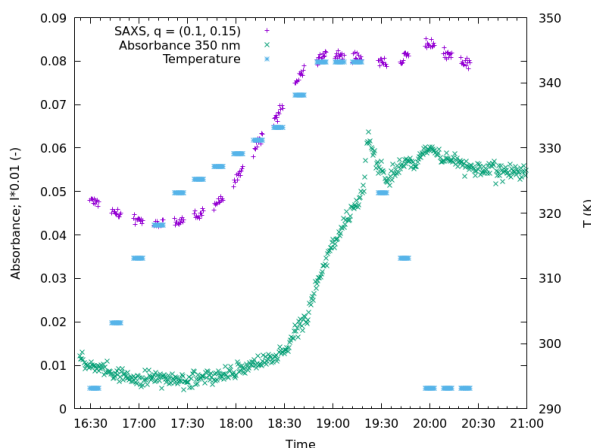


Figure 5 (B): Plot of the scattered SAXS intensity in the range from  $q = 0.1 \text{ nm}^{-1}$  to  $0.15 \text{ nm}^{-1}$  of a heating series of the protein solution showing aggregation; absorbance at 350 nm vs. experiment time.

## 4 Conclusion

Coupling SAXS with *in-situ* UV/Vis absorption spectroscopy allows monitoring the sample's state by complementary means. This gives information of the precise concentration at the beam position during a SAXS measurement, which is helpful for e.g., size-exclusion chromatography (SEC) experiments coupled with SAXS.

The two methods also complement each other in studying protein aggregation, as they interact with different types of particles. Apart of the direct scientific significance, the UV/Vis absorption can help the operator of SAXS with diagnosing day-to-day issues, such as cleanliness of the capillary, positional stability of the sample in the beam, and bubble formation.

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