



Structural Investigation of Photosynthetic Membrane Using Small-Angle Scattering

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Publication date:
2018

Document version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Jakubauskas, D. (2018). *Structural Investigation of Photosynthetic Membrane Using Small-Angle Scattering*. The Niels Bohr Institute, Faculty of Science, University of Copenhagen.
https://soeg.kb.dk/permalink/45KBDK_KGL/fbp0ps/alma99122110603605763

storage (what has also been confirmed by TEM of these samples).

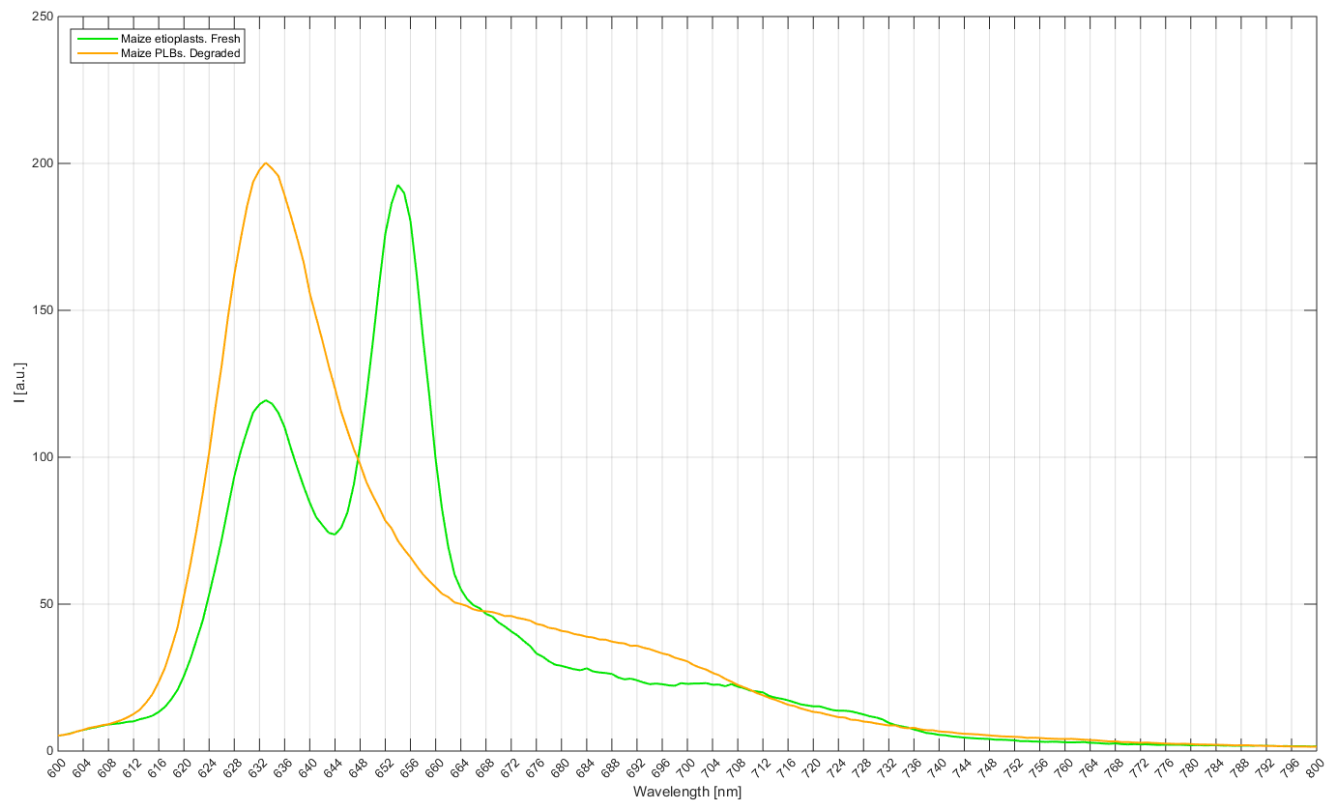


Figure 4.2: a) 77K spectra of purified *Zea mays* etioplasts - non-illuminated etioplasts (green), degraded etioplasts (orange) are visualized. Excitation wavelength - 440 nm. Performed together with Radoslaw Mazur in Faculty of Biology, University of Warsaw.

4.1.4 TEM and tomography studies of isolated etioplasts (prolamellar bodies) and etiolated leaves

The etioplast pellet was fixed in 50 % sucrose (w/v) PLB storage buffer (pH 7.6) with 2.5 % (w/v) glutaraldehyde and 50 mM sodium cacodylate in the darkness for 2 hours. Etiolated leaves were fixed in 2.5 % glutaraldehyde and 50 mM sodium cacodylate (pH 7.4) in the darkness for 2 hours. Etioplast pellet/etiolated leaves were subsequently washed with 50 mM sodium cacodylate (pH 7.4) and stained with 2 % OsO_4 in 50 mM sodium cacodylate (pH 7.4) buffer overnight at 4 ° C. The specimens were dehydrated in a graded acetone series and embedded in a low viscosity epoxy embedding medium (Sigma-Aldrich) and cut with a Leica UCT ultramicrotome. Thin sections of 250 nm were cut, put on 100 mesh formvar grids and stained with 1% aqueous uranyl acetate UranylLess (EMS, USA) for contrast-enhancement.

Tomography series and image alignment was performed in Department of Histology and Embryology, Medical University of Silesia, Zabrze, with the TECNAI G2 12 Spirit BioTWIN transmission electron microscope (FEI, Eindhoven, Netherlands) with the tomography supply, at the voltage of 120 kV. TEM images from a number of randomly selected regions per sample were captured with a Morada CCD camera (Olympus Soft Imaging System Solutions GMBH, Münster, Germany). Images for tomography were taken at a magnification of 60,000x from $+58^\circ$ to -58° at 1° intervals around one axis. Two tomograms from two PLB developmental stages were collected. Each set of aligned single-axis tomogram tilts was reconstructed using the 'TomoJ' software (the component of ImageJ) [174]. For the reconstruction, 100 iterations of the SIRT algorithm were applied [174]. Tomograms were displayed as isosurfaces of the 3D reconstructed volume and analyzed with the Imaris software (Bitplane) to create the isosurface of the external PLB membrane (Fig. 4.3 e, f). In other tomography experiments, the outlines of tomogram regions of interest were shaped with the 3DMOD software (IMOD) [139] in order to reconstruct and visualize individual thylakoids in chloroplast grana stacks.

From TEM image analysis we demonstrate that cubic (paracrystalline) PLB structure is preserved in maize leaves, grown both in Copenhagen (Fig. 4.3 a) and in Australia (Fig. 4.3 b), and in the isolated etioplast samples (Fig. 4.3 c). Although the cubic structure was preserved in the majority of isolated maize etioplasts, a small number of PLBs with tubular structures were observed in etioplast isolates (Fig. 4.3 d), showing that some PLBs were either degraded due to isolation or partially developed into etio-chloroplasts. TEM of leaves of barley seedlings grown in Australia also showed the co-existence of etioplast and etio-chloroplast stages in the same leaf; etioplast stage was predominant in leaves of maize seedlings.

By reconstructing a tomographic volume of a single [111] plane of PLB (Fig. 4.3 e), we observe a regular hexagonal arrangement with protrusions up and down, showing that maize PLB is of a closed type, having a hexagonal symmetry of the tubule arrangement. Two reconstructed PLB isosurface elements are visualized as the inset of Fig. 4.3 e). The tetrahedrally branched tubular asymmetric units form a typical, potentially diamond cubic (Fd3m), structure, similar as in pea [136, 254], although the unit cell lengths of maize PLBs are much smaller. In etio-chloroplast stage PLB, compact paracrystalline structure is absent - tubules are much less compact and longer (Fig. 4.3 f).

Overall, from 77K fluorescence emission spectras and TEM, I confirm that 1. paracrystalline prolamellar body structure of etioplasts is retained after isolation and 2. PLBs are present in etiolated leaves of maize and barley seedlings, grown in Australia. Therefore both isolated and *in vivo* samples are suitable for neutron scattering measurements.

4.1.5 Scattering studies of isolated etioplasts (prolamellar bodies) and etiolated leaves

Small angle neutron scattering measurements on isolated etioplasts and etiolated leaves were performed with 'BILBY' instrument in a ToF mode (ANSTO, Sydney, Australia)

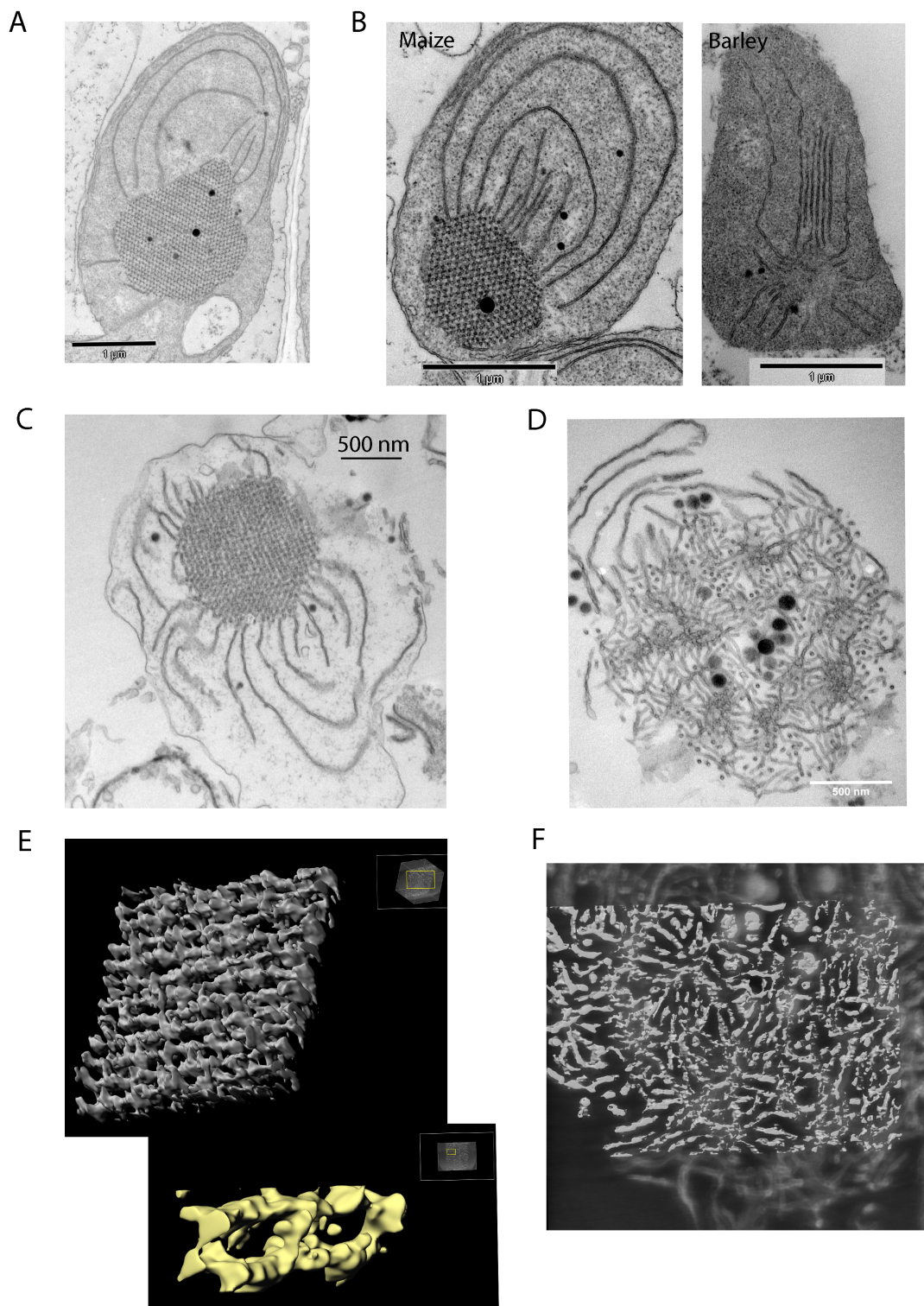


Figure 4.3: a) *Zea mays* var. 'Ambition' leaves b) *Hordeum vulgare* and *Zea mays* 'Reid's yellow dent' leaves. c) Isolated *Zea mays* var. 'Ambition' etioplasts, PLB cubic structure is preserved; d) Isolated *Zea mays* var. 'Ambition' etioplasts, PLB with degraded cubic structure; e) Tomogram surface and two hexagonal PLB units of *Zea mays* var. 'Ambition' PLBs; f) Tomogram surface of a degraded PLB of *Zea mays* var. 'Ambition'. TEM images of a,c,d) taken by Łucja Kowalewska, University of Warsaw, Dept. of Plant Anatomy and Cytology. TEM image b) taken in Center of Advanced Bioimaging, University of Copenhagen. Tomogram reconstruction is described in the text.

[269]. Experimental parameters: measuring temperature 10 °C, measuring time 60 min, q range 0.0035-0.26 Å⁻¹ (wavelength 2-20 Å), resolutions of 7% (back detector) and 9 % (front detector), transmission measurement time - 20 min. The samples were measured in demountable cells equipped with Al-sputtered quartz windows to avoid sample exposure to light (Dark measurements). Later, samples were taken out, transferred into a new demountable cell with ordinary quartz windows, illuminated for 2+ min with 50-70 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ white halogen light (KL-2500, Schott) and measured again (Light measurements, Fig. 4.5). 1 min exposure with 70 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ white light was shown to cause complete PChlide to Chlide conversion [253], but I have illuminated etiolast pellets longer, to make sure that their ultrastructure degrades as well.

Small angle neutron scattering on three biological replicates of isolated maize etioplasts (2 technical replicates for first and second isolation) and D₂O-infiltrated maize and barley leaves have been measured. The observed scattering features were distinct, although of low intensity. The peak of $q = 0.014 \text{ Å}^{-1}$ was observed in all three biological replicates, a broad peak of $q = 0.037 \text{ Å}^{-1}$ was also prevalent in the measurements of first and second isolations. Notably, the first peak has a very similar q value measured in the SAXS experiments of Eva Selstam *et al.* [254] and Williams *et al.* [304]. Due to a significant peak broadening, no further distinct peaks can be ascribed in our SANS measurement, burdening to determine the exact space group and lattice parameters. I overlay the theoretical Bragg peak q positions from different lattices having $q_1=0.0014 \text{ Å}^{-1}$ in Fig. 4.6: Cubic FCC (black), Cubic BCC (violet) and Cubic Double Diamond (dark green). No characteristic peak pattern, from which the crystal structure (space group) can be unequivocally determined, is observed in scattering curves.

For small angle neutron scattering on leaves *in vivo* (Fig. 4.7) the middle third of the leave was excised, several leaves were stacked and enclosed in demountable sample cell with Al-sputtered quartz windows. The presence of paracrystalline PLBs in the leaves of etiolated maize and barley seedlings was also confirmed by TEM (Fig. 4.3 b). Although some scattering features are visible, the leaves did not yield a sufficient ordered scattering. In principle, a baseline subtraction for individual peaks, as suggested in Ben-Nun *et al.* [29] is possible to obtain increased scattering features. However, a further optimisation of the isolation method is also necessary to obtain a more pronounced scattering signal from PLBs.

The disappearance of $q=0.0014 \text{ Å}^{-1}$ peak was observed after light exposure (Dark measurements - coloured curves, Light measurements - black curves in Fig. 4.4 a,b). Since the paracrystalline PLB disperses in light, scattering from ordered PLB structure is expected to diminish. Therefore I attribute this peak to the ordered PLB structure.

To increase the instrumental resolution, complementary SAXS experiments were carried out in the Dept. of X-ray and neutron science, Niels Bohr Institute, University of Copenhagen on isolated and concentrated etioplasts resuspended in a H₂O-based buffer. However, samples again did not yield sufficient scattering from ordered PLBs, probably due to an insufficient lipid-protein concentration - despite a prior etioplast centrifugation and SAXS measurements on a clearly visible pellet. Therefore, double-

diamond paracrystalline PLB structure cannot be determined by scattering methods yet.

To conclude, a more intensive signal is needed to investigate prolamellar bodies by means of scattering. This could be achieved by improving the isolation protocol or fine-tuning the instrumental resolution. This would eventually allow unequivocally determining the space group and the unit cell size of the PLB membranes. The hexagonal nature of maize PLBs has been confirmed by the electron tomography, these experiments could be extended by FFT studies of the obtained micrographs with the aim to determine the PLB unit cell size. The establishment of correlative FFT and scattering methods would allow the ultrastructural investigation of different PLB types from various plants, visualized in the near-native conditions.

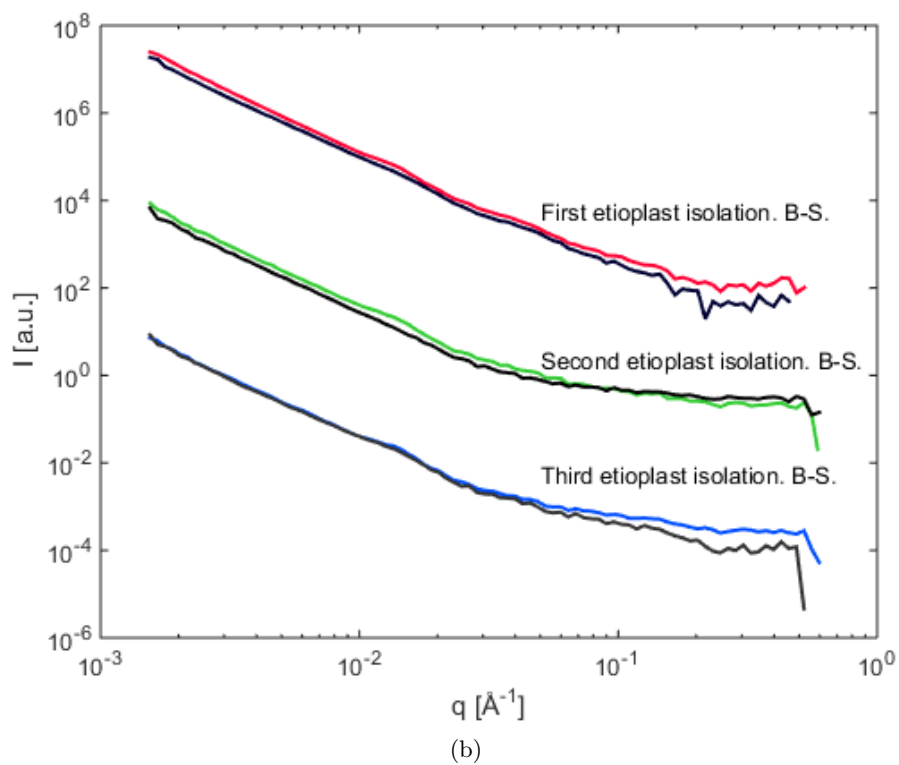
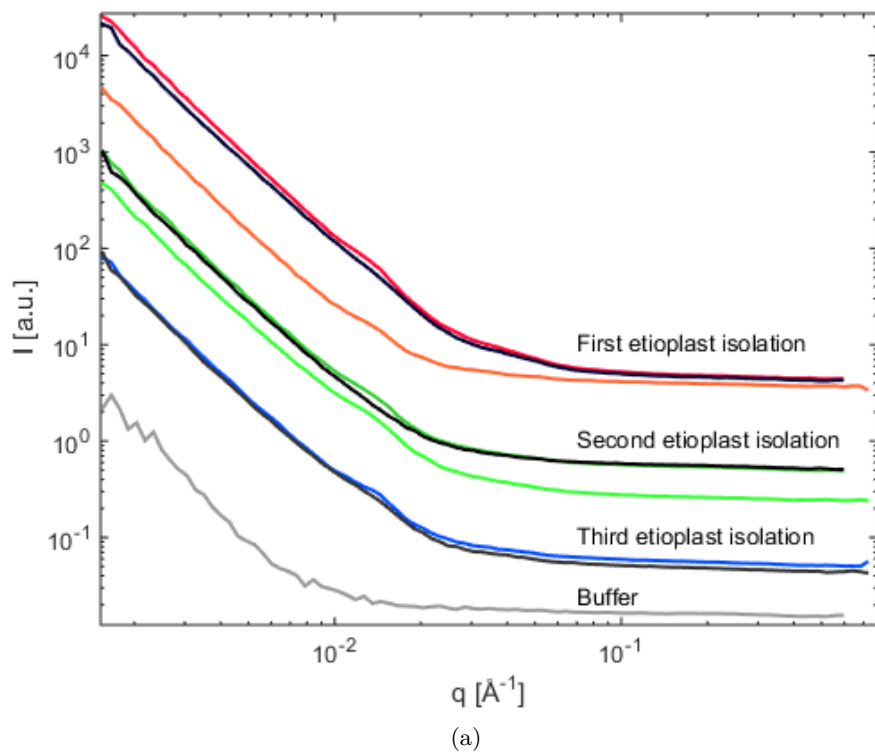


Figure 4.4: a) Three biological replicates of SANS measurements of isolated etioplasts. Coloured curves - dark-measured samples, grey-black curves - illuminated etioplasts. Arbitrary scalling; b) Background-subtracted SANS measurements of isolated etioplasts from a).

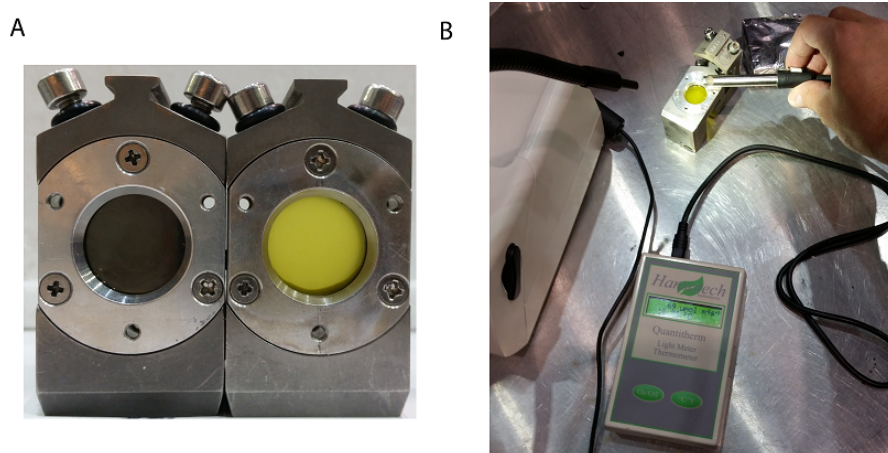


Figure 4.5: a) Dark and light measurements of the isolated etioplast sample. Left: demountable cell with Al-sputtered quartz windows. Right: ordinary quartz windows; b) The setup for illumination procedure.

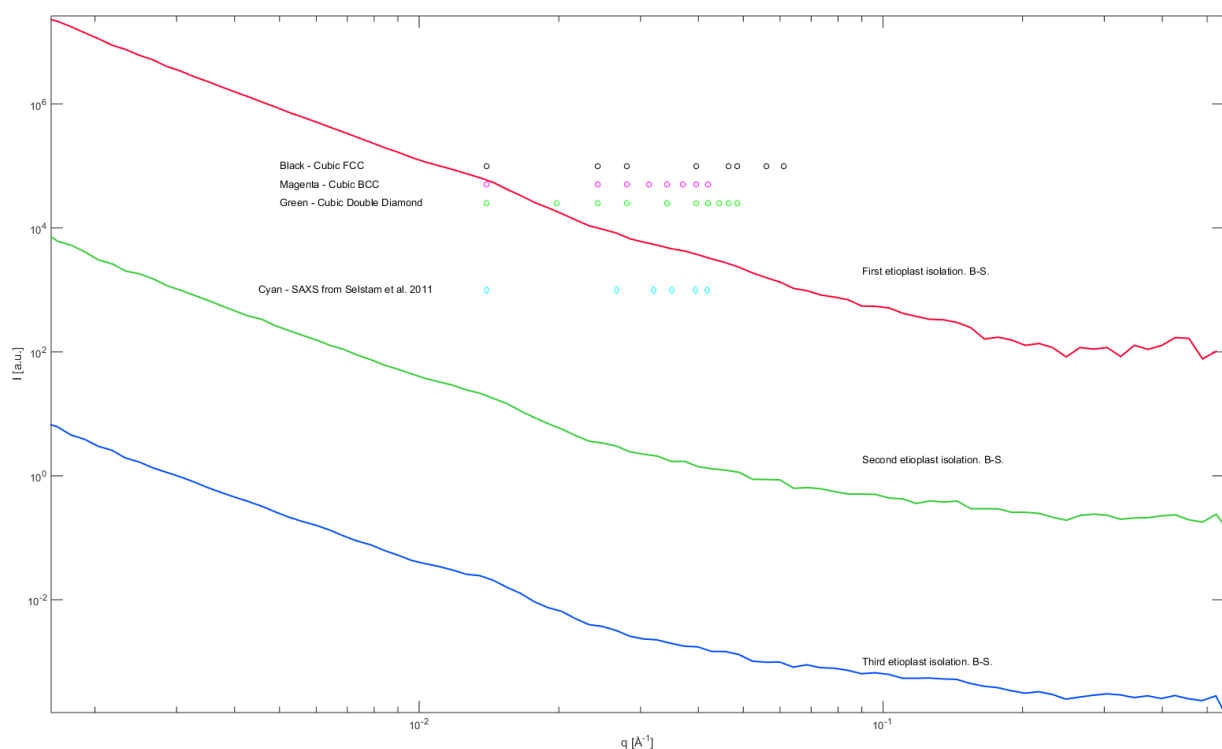


Figure 4.6: Overlay of theoretical Bragg peak q positions from different lattices with $q_1=0.0014 \text{ \AA}^{-1}$: Cubic FCC (black circles), Cubic BCC (violet circles) and Cubic Double Diamond (green circles); B-S - background-subtracted measurements. Positions of experimentally observed peaks from SAXS experiment of E. Selstam on isolated maize PLBs from [254] are depicted as diamonds in cyan.

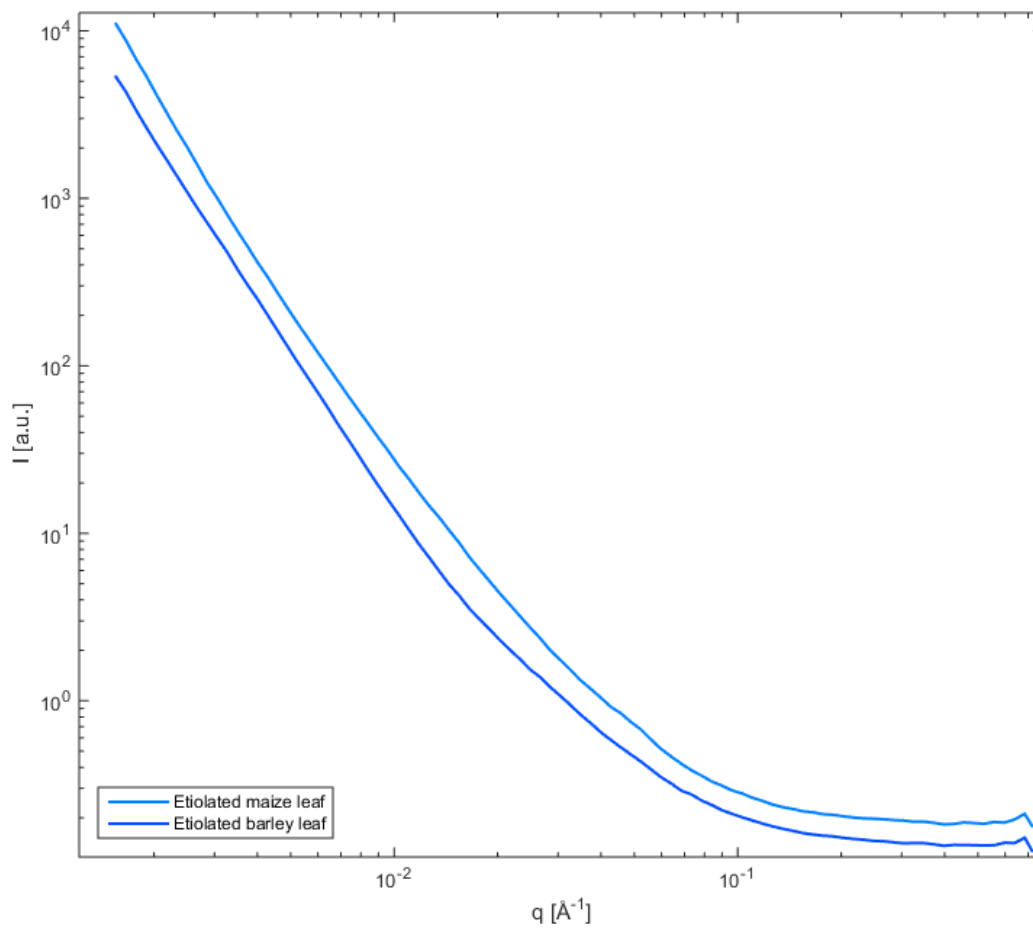


Figure 4.7: SANS of intact, D_2O -infiltrated etiolated leaves of 730 maize and barley seedlings.

4.2 Investigation of cyanobacteria

A number of previous scattering work has been performed on isolated higher plant thylakoids, but the ultrastructure of isolated thylakoids was shown to differ from their state *in vivo* [295]. As the scattering measurements of higher plants *in vivo* were considered complex as a starting point (now elaborated as Manuscript 2), the cyanobacterial work was started as an *in vivo* complement for the isolated-thylakoid measurements. Firstly, cyanobacteria have a simpler thylakoid arrangement than higher plants - only 3-6 thylakoids are stacked in a 'pseudogranum'. Secondly, the content of cyanobacterial membranes is 90 % thylakoids - therefore a high scattering signal from thylakoids is expected, even if cyanobacterial cells are measured *in vivo*. Thirdly, thylakoid arrangement in the cyanobacterial cell is uniform - in the sense that there is no different grana and stroma thylakoids regions. This solves the problem of the simultaneous need of 'stromal' and 'granal' unit cells, as presented in the PhD thesis of J. K. Holm [103]. Furthermore, cyanobacteria can also be concentrated by centrifugation, therefore one would expect an increased scattering signal, compared to higher plant leaves, where the cell is predominantly occupied by a vacuole and not chloroplasts. Lastly, cyanobacterial water content can also be easily exchanged to D₂O by resuspension the cells in a different medium, therefore no infiltration is required - this makes contrast-variation experiments feasible.

I have therefore considered cyanobacterium as a suitable organism, the scattering studies of which would well resemble an *in vivo* situation of higher plant thylakoids and would complement the existing thylakoid measurements *in vitro*. Furthermore, I consider cyanobacteria as suitable organisms to investigate thylakoid ultrastructure changes *in vivo* in relation to external stimuli - illumination intensity and temperature - modifications. These experiments could act as a 'baseline', which is suitable to compare thylakoid *in vivo* behaviour in higher plants. I have performed the complementary SAXS, SANS (including contrast variation study) and TEM investigations of three wild type (6803, 7942 and 7002) cyanobacterial strains. The explanation of cyanobacterial thylakoid ultrastructural studies by means of a scattering model and its correlation to TEM studies is given as Manuscript 1.

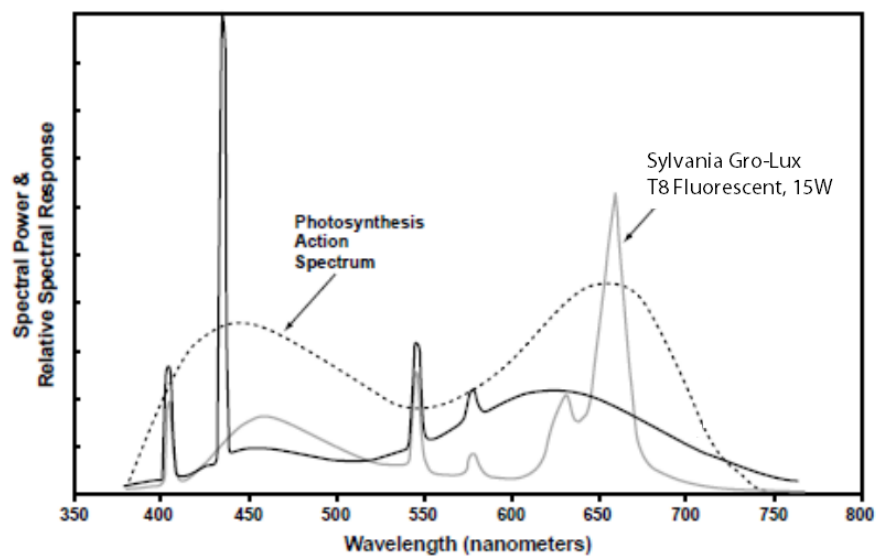
To investigate the overall variation of a scattering signal in a cyanobacterial system, I have chosen cyanobacterial strains with different cellular thylakoid arrangements - three wild types and four CURT1 protein expression mutants of *Synechocystis* sp. PCC 6803. I have performed SANS studies of wild-type cyanobacterial dark-light adaptation, *in vivo* SAXS measurements of wild-type cyanobacterial cells in different temperatures and SAXS measurements of CURT1 protein expression mutants of *Synechocystis* sp. PCC 6803. These experiments are described below. The detailed investigation and modelling of these scattering curves would be a logical continuation of this PhD project, the primary focus of which was to create a suitable scattering model to investigate such systems.

4.2.1 Cyanobacterial growth conditions

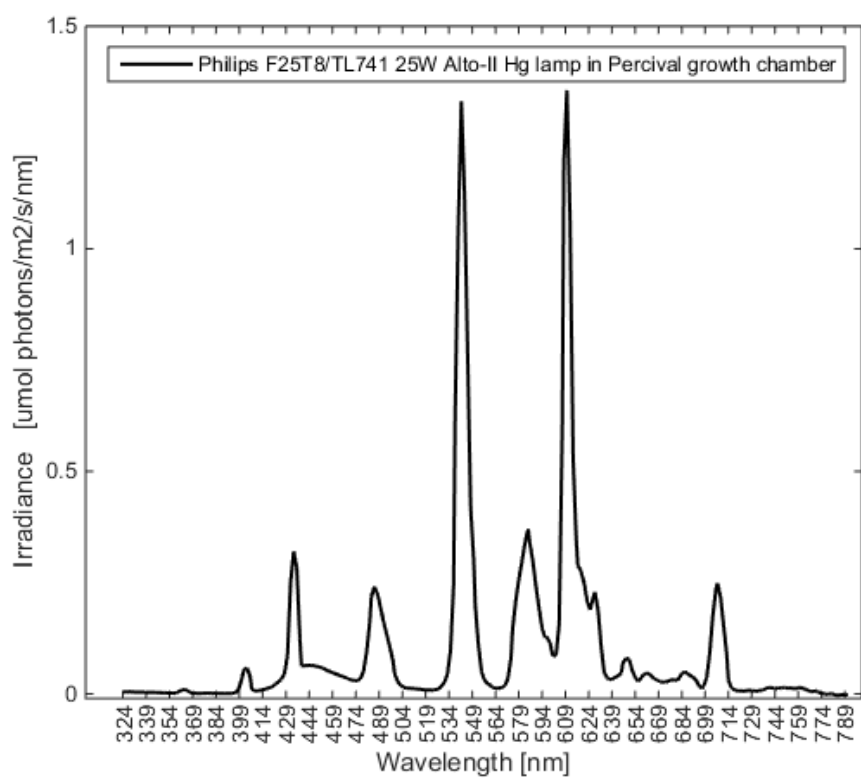
The liquid cultures of cyanobacteria *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* sp. PCC 7942 and *Synechococcus* sp. PCC 7002 were grown at 30 °C, shaking 150 rpm, continuous white light illumination of 50 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (equivalent to 10.4 W/m² or 3765 lux), humidity of 60 % (University of Copenhagen) or 30 °C, shaking 200 rpm, continuous white light illumination of 5-6 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (equivalent to 500 lux), humidity not controlled (ANSTO) in BG-11 (6803, 7942) and A+ (7002) medium. Illumination spectras are given in Fig. 4.8. Growth medium of *Synechocystis* sp. PCC 6803 strains expressing CURT1 protein variants was supplemented with 100 or 200 $\mu\text{g/mL}$ kanamycin. When a cell culture reached an exponential phase $\text{OD}_{730 \text{ nm}} = 0.5\text{-}1.2$, cells were centrifuged (5000 g, 5 min), $\text{OD}_{730 \text{ nm}}$ adjusted to 2 with BG-11 medium. OD-adjusted culture was pelleted (5000g, 5 min), cells were resuspended in small amount (300 μL) of fresh medium and subjected for small-angle scattering experiments. I have not observed that the q position of scattering peak is different in different OD (OD is correlated to cell concentration). Therefore I consider the exact OD value unimportant for scattering measurements - as long as the cells are in exponential/stationary phase and are fit (i.e., cultures look 'normal green' and are not bleached, see *e.g.* Figs. 4.9 and 4.14). For X-ray scattering measurements, the final sample volume in H₂O-based medium was 300 μL . For neutron scattering measurements, cyanobacterial pellets were resuspended in D₂O-based medium and centrifuged to exchange H₂O with D₂O. The procedure was repeated two times (medium discarded), the final sample volume in D₂O-based medium was 500 μL , final sample optical densities were $\text{OD}_{730 \text{ nm}} = 2.1\text{-}2.2$. Seven day cultivation of 6803, 7942 and 7002 cyanobacterial cells in 90% D₂O-based media showed a reduced growth (as evaluated by $\text{OD}_{730 \text{ nm}} = 0.3\text{-}0.4$), whereas growth in 75 % D₂O-based media ($\text{OD}_{730 \text{ nm}} = 1.0\text{-}1.3$) was generally comparable to cyanobacterial cell growth in 100 % H₂O-based media (having $\text{OD}_{730 \text{ nm}} = 0.85\text{-}1.3$, the OD value of cells in 100 % medium was measured one day after culture dilution with a fresh medium) (Fig. 4.9).

4.2.2 Cyanobacterial TEM investigations

The samples for TEM were prepared by pelleting 20 mL of a cyanobacterial culture (5000g, 5 min). Cyanobacteria were fixed in 2.5 % (w/v) glutaraldehyde in 5 mM cacodylate buffer (pH 7.4) in BG-11 or A+ medium for 2 h (shaking 180 rpm), washed in the cacodylate buffer without glutaraldehyde and placed in a 2 % (w/v) OsO₄ in 50 mM cacodylate buffer (pH 7.4) for about 12 h at 4 °C. The specimens were dehydrated in a graded acetone series, embedded in a low viscosity epoxy embedding medium (Sigma-Aldrich) and cut with the Leica UCT or Leica EM UC7 ultramicrotome. 300 mesh copper (Copenhagen) or 100 mesh formvar grids (Warsaw) were used. Ultrathin sections of 60-70 nm (Copenhagen) or 200 nm (Warsaw) were stained with 1% aqueous uranyl acetate UranylLess (EMS, USA); in Copenhagen grids were additionally stained with 3% aqueous Reynold's lead citrate (EMS, USA); and examined with a JEM 1400 electron microscope (Jeol, Japan) in the Nencki Institute of Experimental Biology of the Polish



(a)



(b)

Figure 4.8: a) Spectrum of photosynthetic light of Innova 43R shaker [8] (ANSTO); b) Spectrum of photosynthetic light of Percival growth chamber (University of Copenhagen).

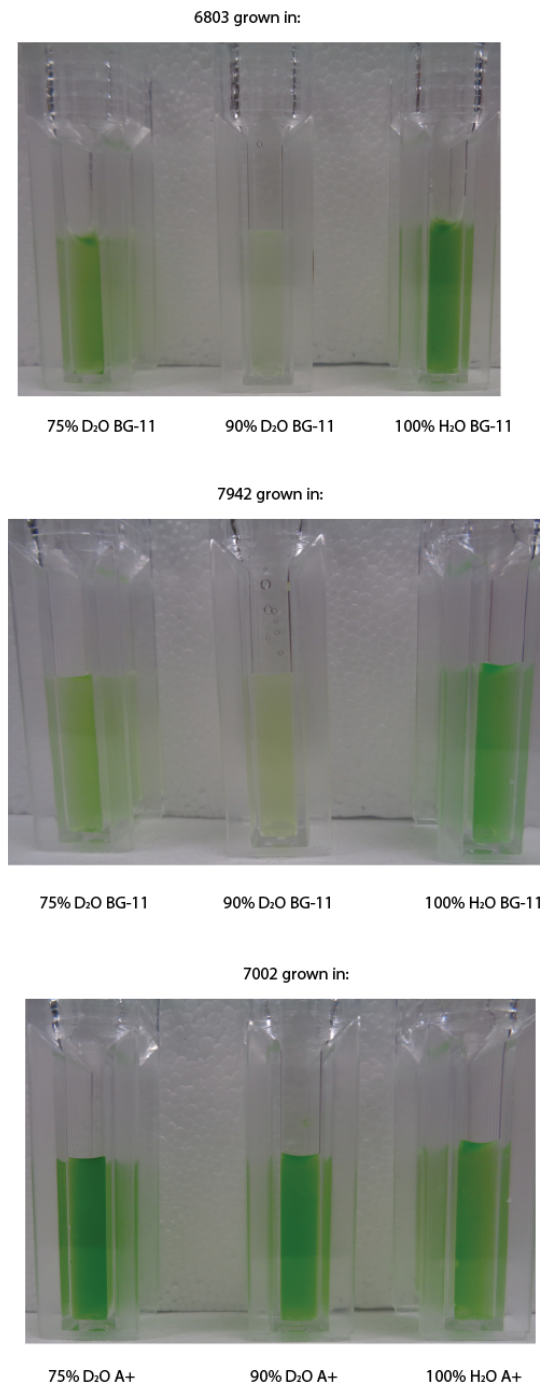


Figure 4.9: Cyanobacterial growth in 75% and 90 % D₂O-based and 100 % H₂O-based medias.

Academy of Sciences, Warsaw or Philips CM-20 electron microscope, Niels Bohr Institute, University of Copenhagen. The voltage of 120-160 kV was used. TEM images of a number of randomly selected bacteria were captured at a magnification of 48,000-78,000x (Copenhagen) or 30,000-100,000x (Warsaw).

For TEM analysis, cyanobacteria were grown in University of Copenhagen in the conditions specified above. The repeat distances of N 'pseudograna' of cyanobacterial thylakoids from three biological replicates of *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* sp. PCC 7942 and *Synechococcus* sp. PCC 7002 have been manually measured in ImageJ software from TEM images of cyanobacterial cells.

A boxplot and histogram (a binwidth of 20 Å) of RD distribution in replicates is given in Fig. 4.10. The average, median RD values, RD standard deviation and RD mean confidence limits (Table 4.1) were calculated in *R* on non-transformed data by lsmeans package. 0.95 confidence level was used, σ_{RD} is 116.5 Å (df = 1668).

The statistical comparisons of RD from different cyanobacterial species were performed in *R*: normality was checked by Shapiro-Wilk test and by evaluating residual quartile-quartile (Q-Q) plots (given in Appendix 3). As these tests indicated residual deviations from normality for some replicas, Box-Cox transformation of data ($\lambda=-0.667$) had been performed to normalize data for statistical analysis. RD outliers (extremes higher than 1.5·IQR, indicated as open circles in Fig. 4.10 a) were not omitted from data analysis. A linear mixed model on normalized data with repeat distance (RD) as a quantitative continuous variable, Species as qualitative (nominal) variable and Replicas (nominal variable) as a random effect was applied.

From the statistical analysis, average RD between three cyanobacterial species was statistically significantly different ($p<0.001$, df = 1677). However, applying *post hoc* Tukey contrast test showed that this RD difference between different species is of the order of $3 \cdot 10^{-4}$ Å ($-3.92 \cdot 10^{-4}$ Å for 7942-6803, $-7.01 \cdot 10^{-5}$ Å for 7002-6803, $3.22 \cdot 10^{-4}$ Å for 7002-7942, detailed in Appendix 3) and is therefore biologically insignificant. To conclude, statistical analysis indicates that although H_0 is disproved, the average thylakoid repeat distance is practically the same between the three investigated cyanobacterial species.

Table 4.1: Cyanobacterial repeat distances, measured from TEM images.

Bacterial strain	Replica	N pseudograna	Average RD, Å	Median RD, Å	SE, Å	Lower CL, Å	Upper CL, Å
6803	1	114	574.1	568.1	10.91	552.7	595.5
	2	198	632.5	615.0	8.28	616.3	648.8
	3	189	556.9	554.2	8.48	540.3	573.6
7942	1	114	612.8	605.5	10.91	591.4	634.2
	2	207	565.7	532.6	8.10	549.8	581.6
	3	204	572.7	582.1	8.16	556.6	588.7
7002	1	242	615.8	572.6	7.49	601.1	630.5
	2	205	622	601.1	8.14	606.0	638.0
	3	204	556.7	534.6	8.16	540.7	572.7

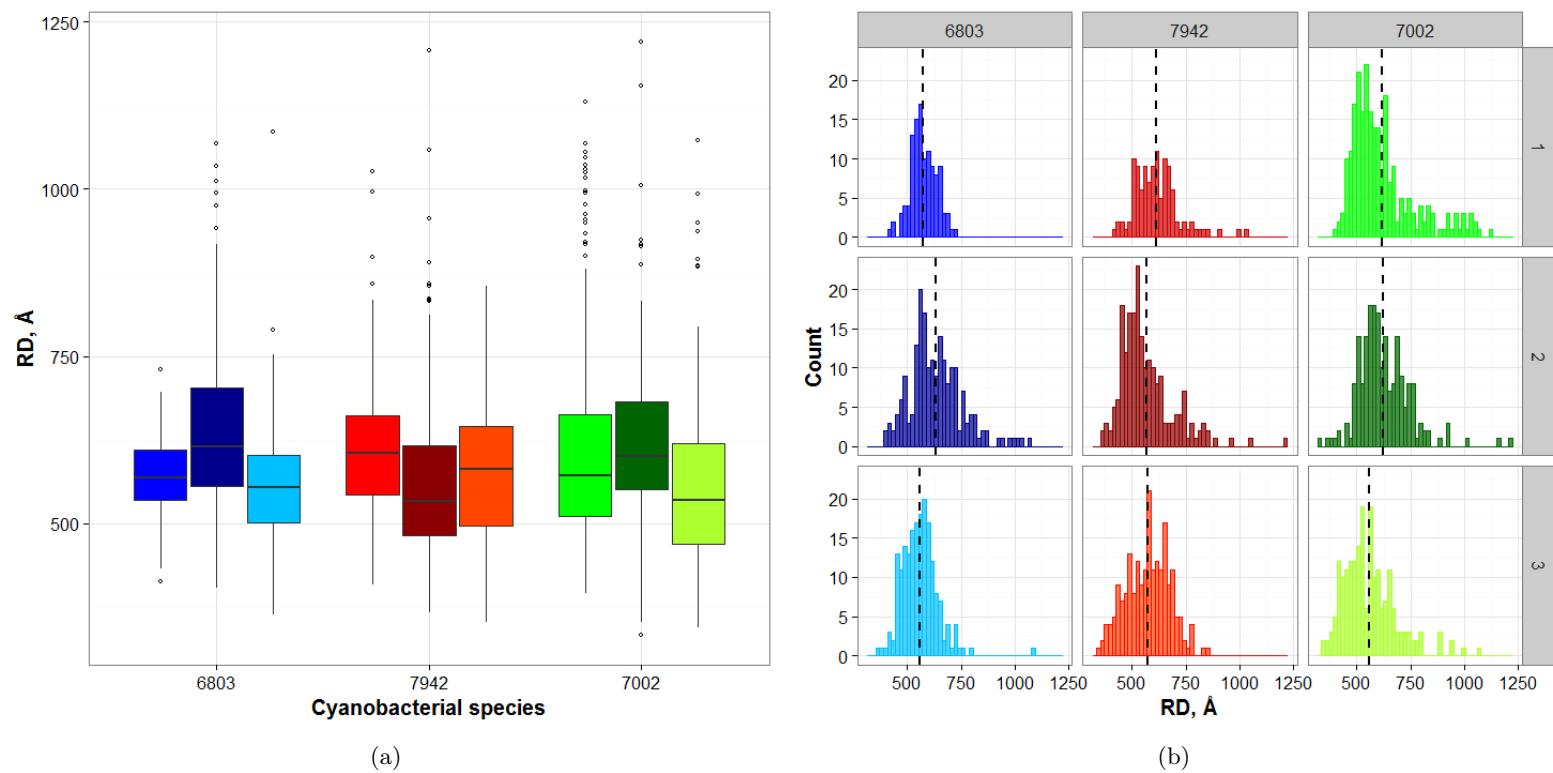


Figure 4.10: a) Boxplots of three biological cyanobacterial replicates. Outliers are depicted as black circles. The first quartile, median (vertical bar) and third quartile depicted on the boxplots; b) The repeat distance distribution of three biological cyanobacterial replicates, median RD is indicated as dashed line.

4.2.3 Cyanobacterial contrast variation

Small angle neutron scattering measurements on cyanobacteria were performed with ‘BILBY’ instrument in a ToF mode (ANSTO, Sydney, Australia) [269]. Experimental parameters: measuring temperature 20 °C, measuring times 60, 90 and 120 min, q range 0.0035-0.26 Å⁻¹ (wavelength 2-20 Å), resolutions of 7 % (back detector) and 9 % (front detector), transmission measurement times - 20 min. 24h dark-adapted cyanobacterial pellets were measured in 1 mm path length demountable cells with Al-sputtered quartz windows. Data reduction and transmission correction was performed in ANSTO with Mantid software [223], a preliminary data analysis was performed with Matlab.

Cyanobacteria grown in 100 % H₂O medium were exchanged with 100, 85, 42 and 21 % D₂O-based BG-11 and A+ medias for contrast variation measurements, cyanobacterial OD_{730 nm} was adjusted to 2.2. The contrast of cyanobacterial cells in 21 % D₂O-based medium were measured 2 weeks later due to instrumental issues (cyanobacteria were grown in the same temperature and illumination conditions), their OD was not adjusted to 2.2 before the experiment. Their OD_{730 nm} (measured on 10x diluted samples) were 1.849 (6803), 1.785 (7942) and 1.446 (7002). For data analysis, transmission-corrected buffer measurements were scaled by a factor of 0.95-1.33 and subsequently subtracted from transmission-corrected sample measurements. Buffer-subtracted cyanobacterial scattering curves are shown in Fig. 4.11.

The approximate peak position $q_1=0.0096$ Å⁻¹ (654 Å) for *Synechocystis* sp. PCC 6803, 0.0085 Å⁻¹ (738.8 Å) for *Synechococcus elongatus* sp. PCC 7942 and 0.0103 Å⁻¹ (609.7 Å) for *Synechococcus* sp. PCC 7002. q_1 and subsequently $2q_1$, $3q_1$ and $4q_1$ positions, indicating lamellar order structure factor peak positions, are plotted as vertical red dotted lines. No peak is contrasted in 85 % (90 and 75 % - not shown) media, suggesting that the lamellar structure does not arise from deuterated polysaccharides or DNA. Inside the cell, H-D exchange of these moieties is possible after cell infiltration with D₂O. The intensity of a scattering is decreased in 42 % D₂O media, where hydrogen-rich proteins are masked out. Only the first peak is distinguished in these curves, whereas the intensities of $2q_1$, $3q_1$ and $4q_1$ peaks are reduced. In 21 % D₂O medium, where lipids are masked out, no defined peaks are visible in 6803 curve, similarly as in Liberton *et al.* [154]. For 7942 and 7002, the q_1 peak was still observed after cell resuspension in 21 % D₂O-media.

As the scattering signal of q_1 peak is distinguished both when hydrogenated proteins and hydrogenated lipids are masked out, this confirms that the observed membrane has both protein and lipid components. In conclusion, contrast variation experiments confirm, that the scattering peaks originate from thylakoid membrane lamellae, which are composed of both lipid and protein components.

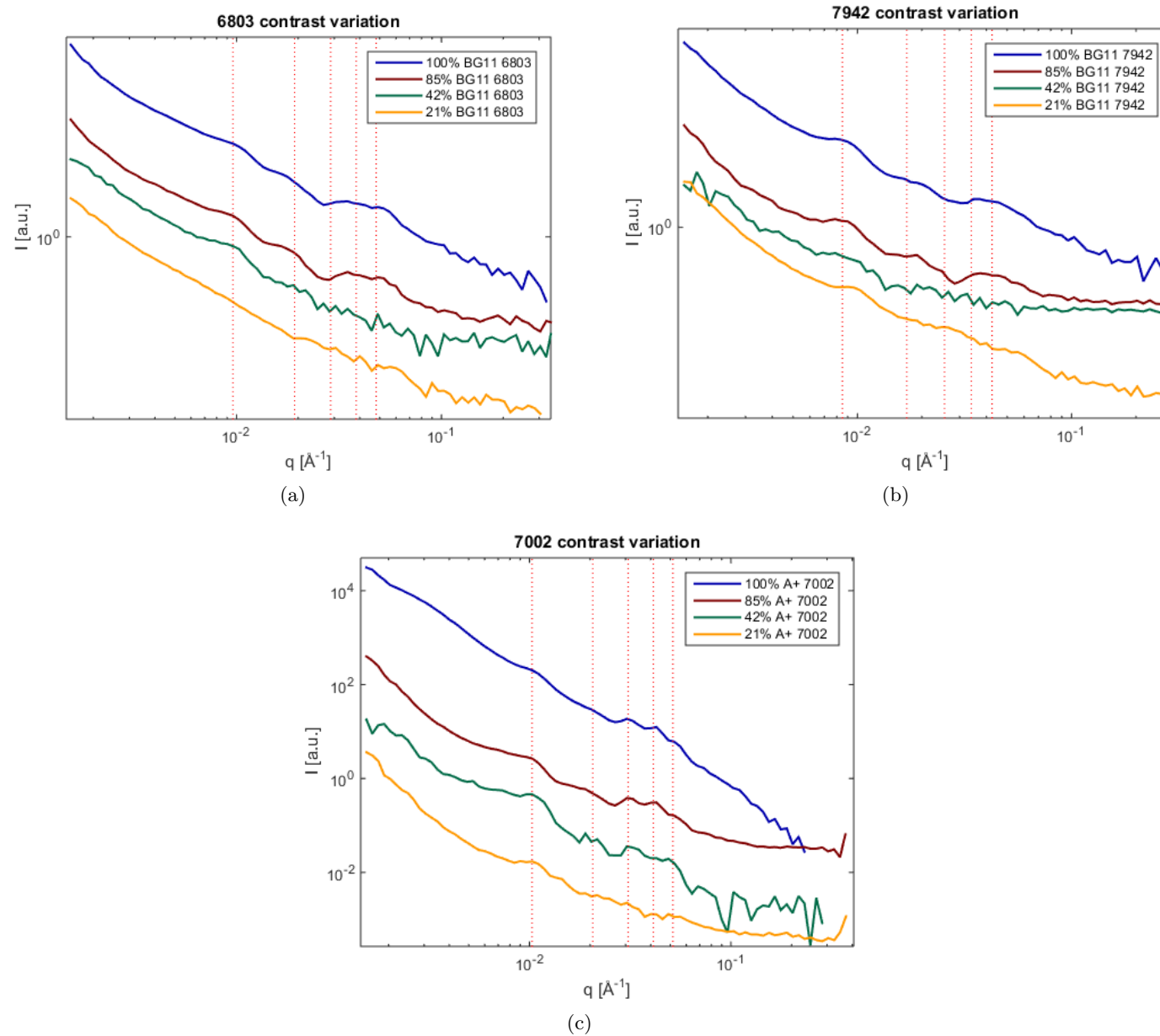


Figure 4.11: a) *Synechocystis* sp. PCC 6803 ; b) *Synechococcus elongatus* sp. PCC 7942; c) *Synechococcus* sp. PCC 7002 contrast variation series.

4.2.4 Cyanobacterial illumination studies using small angle neutron scattering

For illumination studies, the scattering of cyanobacterial cells resuspended in 100 % D₂O-based media was measured. In the first experiment, cyanobacterial cells were measured in the 1 mm pathlength Banjo cuvettes, cell culture OD_{730 nm} was adjusted to 0.65 in order for the light to fully penetrate the sample. Scattering measurements were performed under 3 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Dark measurement 1) and 50 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ intensity white halogen (KL-2500, Schott) light (Light measurement 1). Samples were pre-illuminated for 20 min and then continuously illuminated for additional 60 min during the entire data acquisition. After illumination, cyanobacterial cell aggregates were observed in Banjo cuvettes (Experiment 1, Fig. 4.13 a). Therefore, different cuvettes of dark-adapted 6803 and 7942 cyanobacterial cells with OD_{730 nm}, also adjusted to 0.65, were measured as Dark measurement 1 - in the ambient illumination in 'BILBY' instrument enclosure of 3 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ intensity. Since the 7002 cell sample did not show any aggregation, the same sample was measured again after dark-adaptation. Scattering peak positions were identical in dark and light measurements.

As 50 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ did not cause any change, a higher light intensity was used during Experiment 2. Cyanobacterial OD_{730 nm} was adjusted to 2.21-2.24, the samples did not penetrate light. In Experiment 2, 1 mm pathlength demountable cells with Al-sputtered quartz windows containing 24 h dark-adapted cyanobacterial cell cultures were measured (Dark experiment 2). The same samples were put into demountable cells with normal quartz windows, samples were pre-illuminated with 20 min (during transmission measurement) and then continuously illuminated for additional 60 min during the entire data acquisition (Light measurement 2) with 200 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ white halogen light (KL-2500, Schott). Both Experiment 1 and Experiment 2 were performed at room temperature of 21-23 °C.

In Experiment 2, illuminated cells sedimented and bubbles were observed in the outer edges of demountable cells (Fig. 4.13 b). The origin of bubbles was not investigated, although my hypothesis is that they contain oxygen (what indicates cyanobacterial photosynthetic activity) and air from gas vacuoles. It has been shown, that gas vacuoles occupy around 0.8 % cellular volume and are important for gas storage, cyanobacterial cell buoyancy and cell light shielding, as they strongly scatter light. Gas vacuoles increase back-scattering and therefore reduce the amount of light, penetrating cyanobacterial suspension and absorbed by the thylakoid membranes. Photosynthetic activity due to gas vacuole scattering remains unchanged [68, 159].

Gas vacuoles indirectly sense light intensity: when the cyanobacterial cells are transferred to high irradiance (above 35 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), gas vacuoles are destructed and cell buoyancy is lost. Therefore cyanobacterial cells sink down and sediment on the bottom [159], exactly as is observed in Experiment 2 (Fig. 4.13 b). Gas vacuoles are normally restored after cyanobacterial cell incubation in low illumination and cyanobacteria float again, although this was not observed after Experiment 2, keeping the

cells in ambient illumination for 24 h. Importantly, the overall neutron scattering signal quality of illuminated cyanobacteria was not worsened by the presence of gas bubbles and parasitic air scattering.

In conclusion, scattering peak positions from dark-adapted cyanobacterial cells *in vivo* do not change upon their subsequent illumination, indicating no RD changes upon illumination (Fig. 4.12). M. Liberton *et al.* and G. Nagy have also not observed q_1 position changes of 6803 wild type cells upon illumination [154, 192].

4.2.5 Cyanobacterial temperature studies by molecular biology methods and TEM

Cyanobacterial cultures were grown as described in the subsection 'Cyanobacterial growth conditions'. 20 mL culture was split in two equal volumes, $OD_{730\text{ nm}}$ was measured. 10 mL of culture was subjected to 60 °C treatment in a water bath for 60 min. The temperature-treated cells have bleached to some extent (Fig. 4.14 a). For comparison, cyanobacterial cell cultures, which were subjected to $2000\text{ }\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in room temperature for 8 hours bleached even more (Fig. 4.14 b). TEM samples were prepared from non-treated and treated cells as described in the subsection 'Cyanobacterial TEM investigations'.

For the immunoblot analysis, cyanobacterial pellets were collected from 20 mL of cyanobacterial cell culture by centrifugation (5000 g, 5 min), resuspended in 1 mL of 50 mM Tris-HCl (pH = 8.0) with the protease inhibitor. 500 μL of solution was lysed by 5 min sonication (power: 10), with 0.15 mm diameter zirconium oxide beads (Bullet Blender Storm 24, Next Advance, US). Broken cell lysates were centrifuged for 5 min at 3000 g in 4 °C in order to remove the cell debris, supernatant was subjected to immunoblot analysis. To determine chlorophyll *a* concentration, 100 μL of cyanobacterial culture were spun down (15 000 g, 5 min) and supernatant was removed. 1 mL 100 % DMSO was added, everything was subjected to 70 °C for 5 min, shaking 1700 rpm. The solution was spun down (15 000 g, 1 min), Chl *a* concentration [$\mu\text{L Chl } a/\text{mL}$] = 12.9447 ($A_{665\text{ nm}} - A_{720\text{ nm}}$) was determined as in Ritchie *et al.* [231].

Samples (equivalent to 0.3-1 μg of Chl *a*) with SDS were heated to 90 °C for 10 min, spun down 10 000 g for 3 min and subjected to SDS-PAGE using 12 % Bis-Tris Criterion XT precast gels (Bio-Rad, US) in MES buffer at 150 V for 35 min. Proteins were transferred to 0.2 μm PVDF membrane using Trans-Blot Turbo transfer system (Bio-Rad, US) according to the manufacturers protocol. The membrane was blocked for 1 h at RT with 5 % (w/v) skimmed milk in TGS-T buffer and incubated overnight with primary antibodies in 5 % (w/v) skimmed milk in TGS-T. The blot was washed with TGS-T buffer and incubated with a secondary swine anti-rabbit horseradish HRP-conjugated antibody (Dako, Denmark) for 1 h at RT. The membrane was washed again with TGS-T buffer and secondary antibody detected using SuperSignal West Dura Chemiluminescent Substrate (Pierce, US) and developed with a ChemiDoc MP imaging system (Bio-Rad, US).

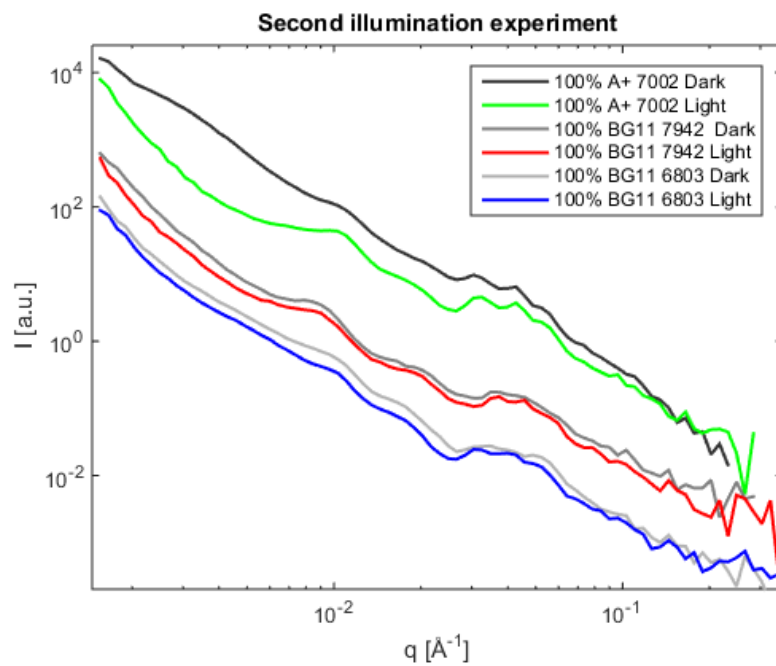
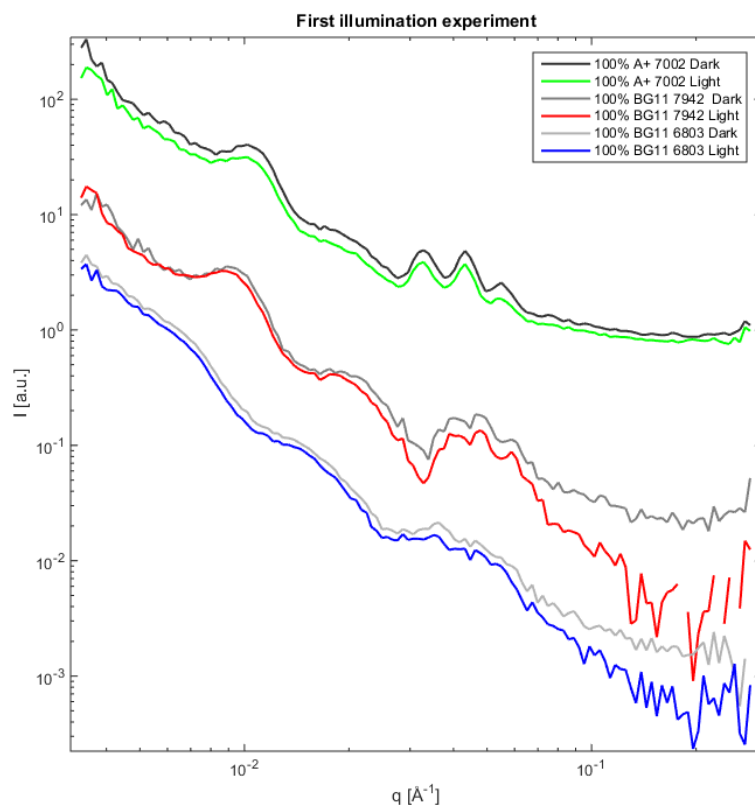
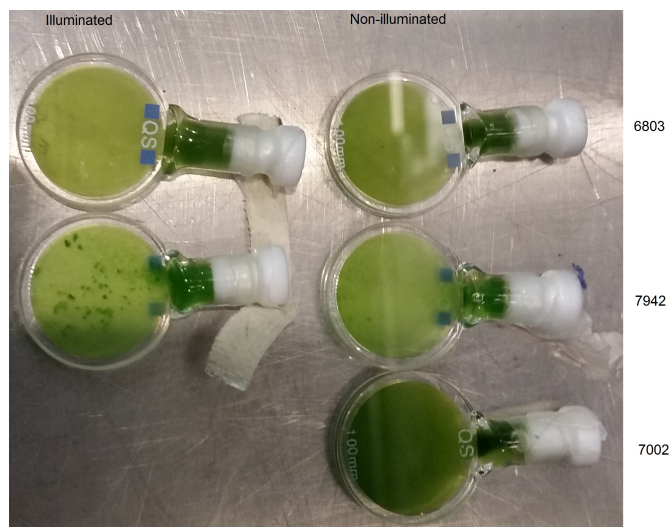


Figure 4.12: Illumination experiments. a) Experiment 1; b) Experiment 2. Dark measurements (black, grey) and subsequent light measurements (coloured). Colour code is the same as in Manuscript 1. *Synechocystis* sp. PCC 6803 - blue *Synechococcus elongatus* sp. PCC 7942 - red, *Synechococcus* sp. PCC 7002 - green.



(a)



(b)



(c)

Figure 4.13: Cyanobacterial cells after white halogen light illumination for 20 min + 60 min. a) First experiment. $50 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ illuminated cells; b) Second experiment. $200 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ illuminated cells. Cell sedimentation and gas bubbles are evident after illumination; c) General illumination setup.

The SDS-PAGE gels of photosynthetic organisms are normally loaded according to chlorophyll content. Since chlorophyll is partially degraded after heat treatment, either the same volume of heat-treated cells, which corresponds to 1 μg Chl in non-treated cyanobacterial cell lysate (lanes 1-6), or 30 μL of heat-treated cyanobacterial cell lysate (lanes 7-12) has been loaded (Fig. 4.16). Although protein amounts are lower in heat-treated cyanobacteria, bands corresponding to non-degraded photosystem subunits are still observed. This is confirmed by Western blot with primary antibodies against PSI core and PsbA (D1 N-term, 28-30 kDa). Chemiluminescent bands, corresponding to full-length proteins are observed both in non-treated and heat-treated cell lysates (Fig. 4.16 b). For PsbA, bands of 24 kDa (and possibly 10 kDa), corresponding to C-terminal fragments are observed as well. Although Western blots need to be properly repeated, the overall band pattern between non-treated and heat-treated cell lysates looks similar - what indicates that thylakoid proteins are not fully degraded after the heat treatment.

The complementary TEM images of the cyanobacterial cells (Fig. 4.14) show that cells remain intact after heat treatment and their thylakoids are much less organized, although the lamellar pattern is still preserved. Compared to non-treated cells, two-three adjacent thylakoid membranes are located very close to each other in heat-treated cells, most probably due to an increased thylakoid membrane adhesion *via* hydrophobic interactions. It is also observed that individual thylakoids of heat-treated cells are more undulated and have a variable lumen thickness. However, a continuous thylakoid membrane bilayer and a confined lumenal space are observed, indicating that individual thylakoids are intact.

4.2.6 Cyanobacterial temperature studies using small angle X-ray scattering

Small angle X-ray measurements of the cyanobacterial cells *in vivo* in different temperatures were performed in X-ray and neutron science group, Niels Bohr Institute, University of Copenhagen. Cyanobacterial cells in exponential phase were pelleted (5000 g, 5 min). By adding medium to the cyanobacterial pellet, $\text{OD}_{730\text{ nm}}$ of the cyanobacterial cells was adjusted to 2 in order to equalize the OD between different samples, pelleted again and then resuspended in a small (around 100 μL) volume of BG-11 or A+ medias. Cyanobacterial cell samples were injected into a quartz capillary of 0.15 mm diameter and measured with the GANESHA instrument (SAXSLAB, Denmark), equipped with a 40 W micro-focus Cu source Micromax 002+ of $\lambda = 1.54\text{ \AA}$ (Rigaku) and 300k Pilatus pixel detector. Sample-detector distances: 690 and 1490 mm, fluxes: 62 or $17 \cdot 10^6$ photons/s, covered q range: $0.007 - 0.4\text{ \AA}^{-1}$. Silver behenate standard was used for q calibration, scattering data reduction was carried out using SAXSgui platform, preliminary data analysis was performed in Matlab.

The second SAXS experiment was carried at P12 beamline, PETRA III synchrotron (DESY), Hamburg, Germany. Cyanobacterial pellet in BG11 or A+ media was injected into a quartz capillary of 1.8 mm diameter for measurement. Sample-detector distance: 3 m, $\lambda = 1.24\text{ \AA}$, flux 10^{13} photons/s and 2D photon counting Pilatus 2M detector

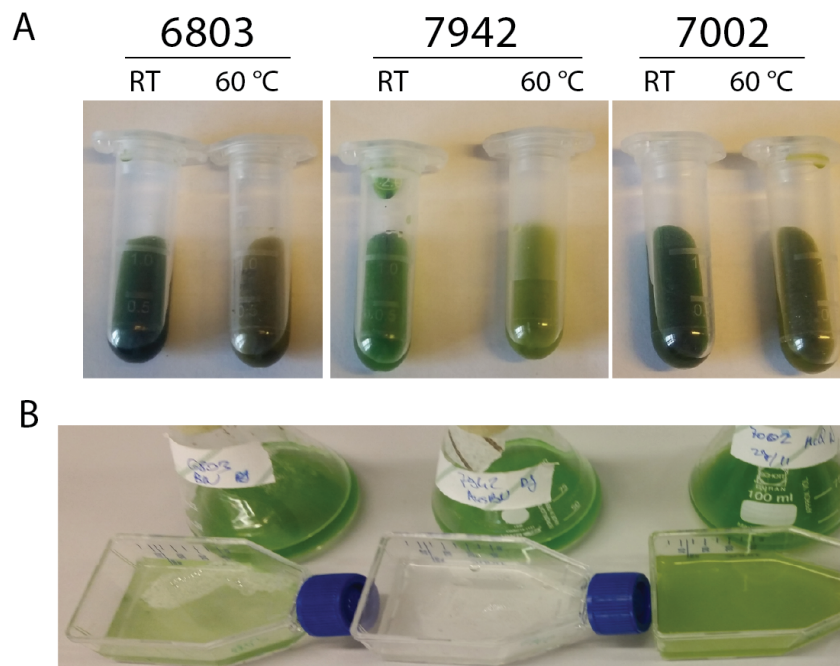


Figure 4.14: a) 6803, 7942 and 7002 cyanobacterial cultures before (left) and after (right) 1 h 60 °C temperature treatment; b) 6803, 7942 and 7002 cyanobacterial cultures before (above) and after (below) 2000 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ white light treatment.

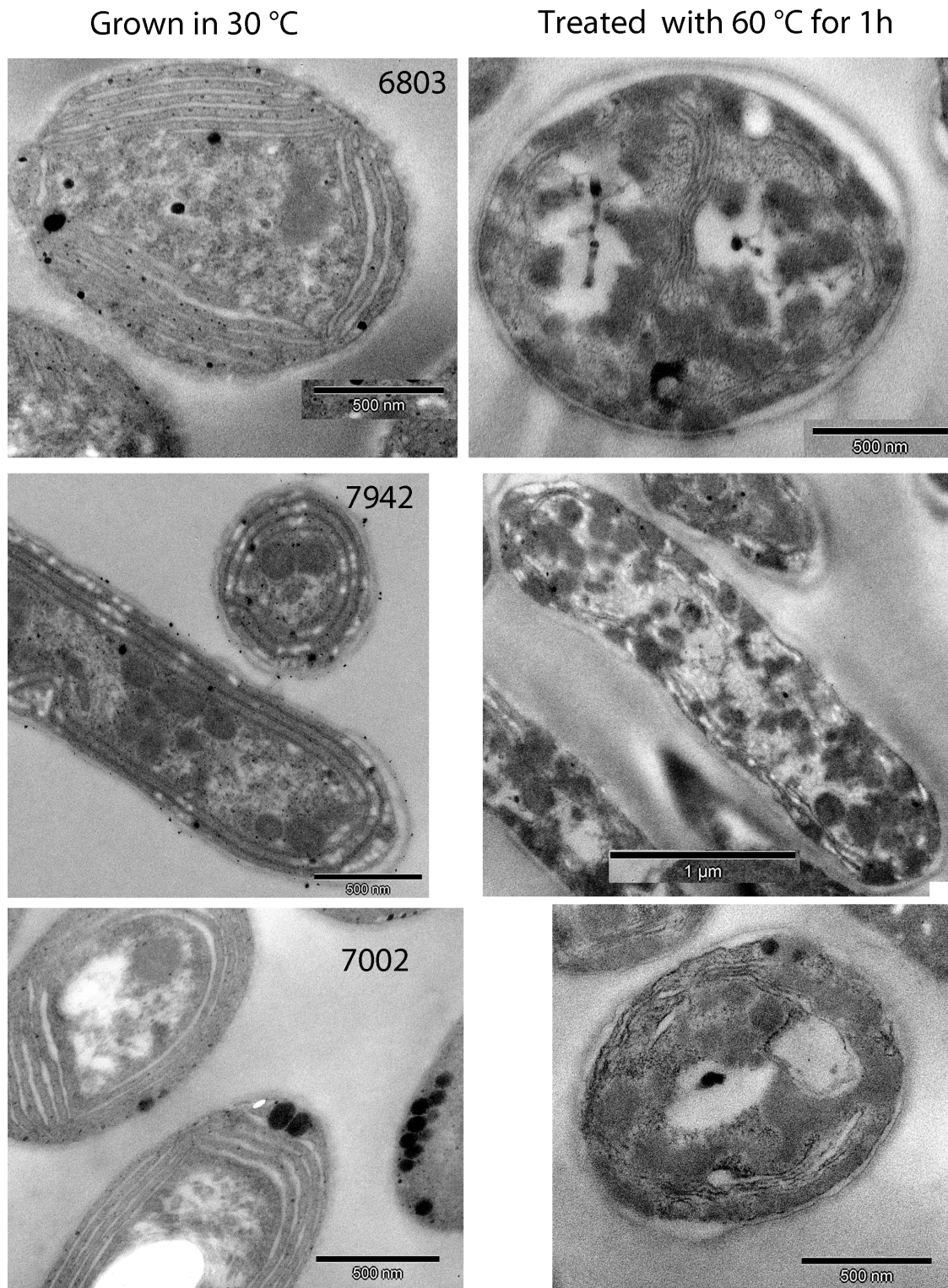


Figure 4.15: Left column: Cyanobacterial cells grown in 30 °C; Right column: Cyanobacterial cells grown in 30 °C and placed into a 60 °C water tank for 1 h. Increased disorder in thylakoid ultrastructure is observed.

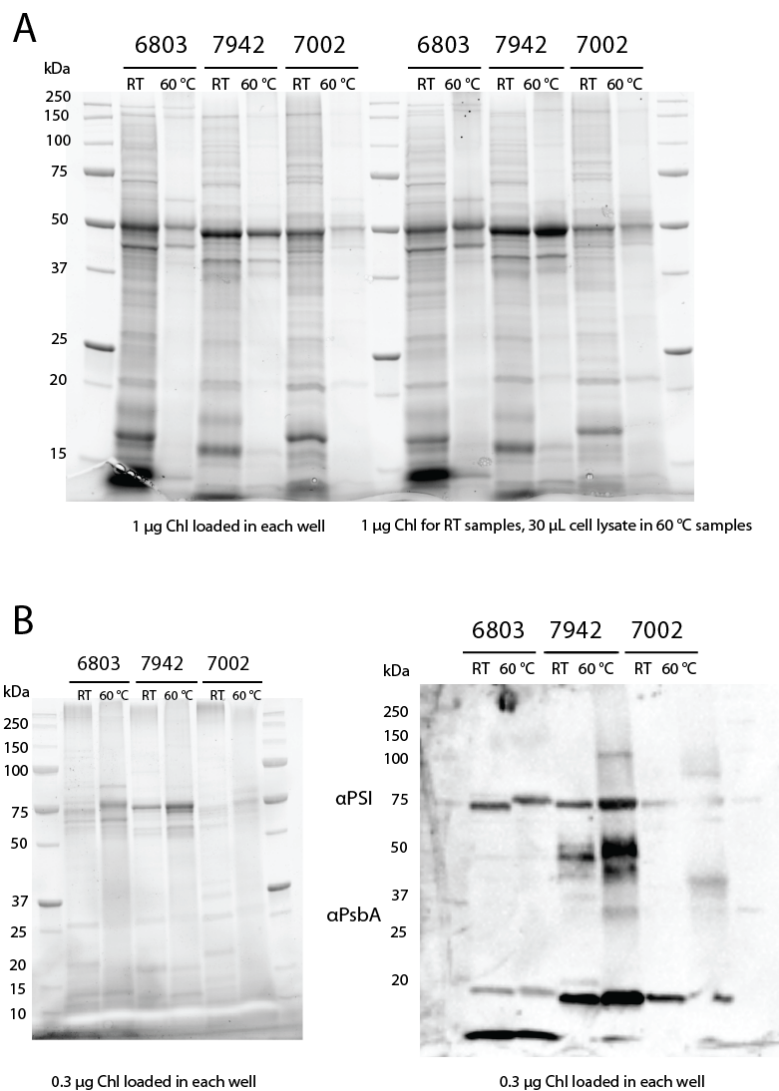


Figure 4.16: a) SDS-PAGE of cyanobacterial cell lysates. For RT samples, Chl *a* concentration was measured and 1 μ g Chl was loaded. In temperature-treated cyanobacteria chlorophyll was degraded. Therefore, either the same volume, corresponding to 1 μ g Chl (left) or 30 μ L (right) of a lysate was loaded; b) Loading control (0.3 μ g Chl) and Western Blot with PSI core (since *PsaA* was not available) and *PsbA* primary antibodies.

(Dectris), covered q range: $0.0026 - 0.5 \text{ \AA}^{-1}$. $\text{OD}_{730 \text{ nm}}$ of cyanobacteria was adjusted to 2.3. Measuring temperature in the sample holder and capillary was $10 \text{ }^\circ\text{C}$. Overall, the SAXS curves obtained from PETRA III measurements and 'Ganesha' instrument are identical, therefore synchrotron measurements are not discussed separately.

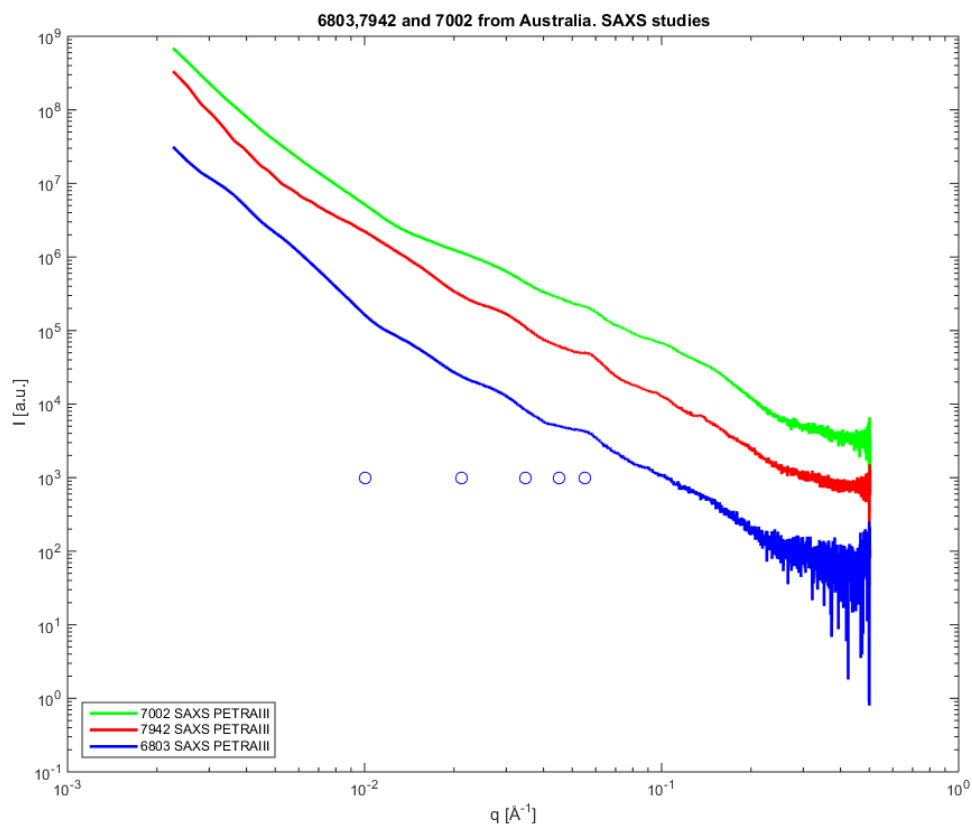
The temperature series of small angle neutron scattering measurements on cyanobacterial cells, although planned, was not measured, because the temperature-control block (Julabo) was out of service. The only performed temperature SANS measurement was on the same 6803 cyanobacterial cell sample, equilibrated to $10 \text{ }^\circ\text{C}$ and $20 \text{ }^\circ\text{C}$. No difference was observed (Fig. 4.17 b).

No difference was observed between SAXS curves of $30 \text{ }^\circ\text{C}$ and $4 \text{ }^\circ\text{C}$ -incubated cyanobacterial cells (Fig. 4.19). As no changes have been observed, the hypothesis that thylakoid membrane undergoes phase separation (liquid crystalline to gel phase) in temperatures below $15 \text{ }^\circ\text{C}$ has not been confirmed.

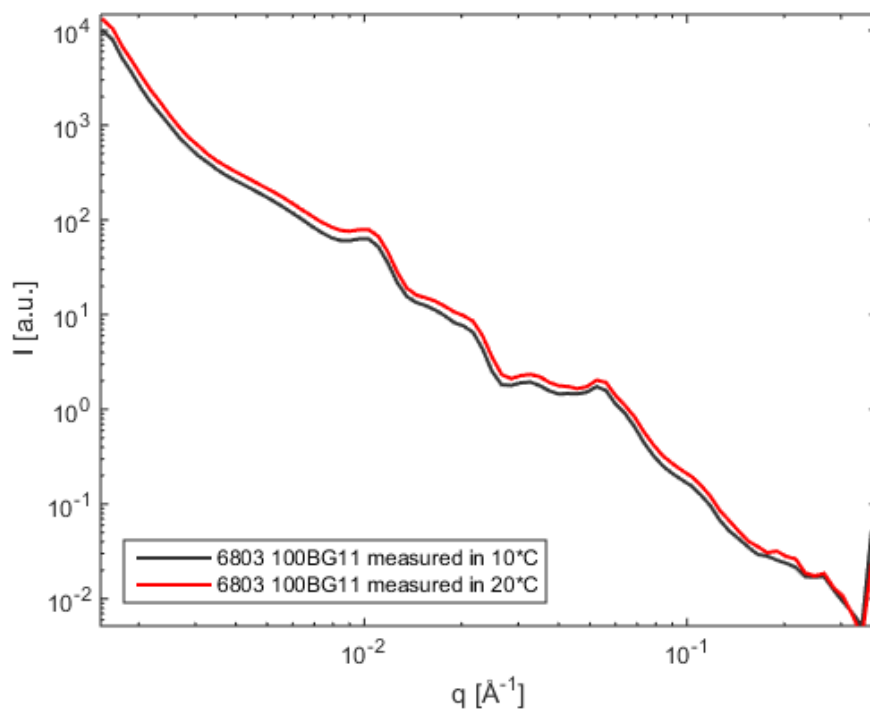
On the other hand, in SAXS experiments where cyanobacterial cells have been heated, exhibit a consistent significant peak change to higher q values in temperature interval $30\text{-}60 \text{ }^\circ\text{C}$. This indicates a decrease of the thylakoid repeat distance in all investigated cyanobacterial strains. A general flattening of the scattering curve also suggests an increase in thylakoid lamellar system polydispersity. Furthermore, a hypothesis of thylakoid membrane thickness decrease in higher temperatures is proposed (Fig. 4.18). This hypothesis is based on the SAXS study on temperature effect on charged synthetic membrane structure, where similar changes are observed in rigid lipid-only membrane systems [282].

The general lamellar scattering profile from cyanobacterial cells remains largely unchanged in $60 \text{ }^\circ\text{C}$ and even in $70 \text{ }^\circ\text{C}$ measurements of *Synechococcus elongatus* UTEX2973 (not shown; this strain genomically and ultrastructurally almost identical to *Synechococcus elongatus* sp. PCC7942). This indicates, that the lamellar scattering profile of thylakoid membranes is preserved upon heating (as is also inferred from complementary TEM pictures and blots) even in physiologically-limiting temperatures. A peak around $q=0.09 \text{ \AA}^{-1}$ ($70 \text{ \AA}$), apparent after 6 h treatment with $60 \text{ }^\circ\text{C}$ in one experiment with 6803 cells (Fig. 4.19) could be attributed to the appearance of non-bilayer phases in membrane lipids, as has been previously hypothesized by G. Garab [76], although this needs to be unequivocally confirmed.

The main questions remaining to be answered in order to experimentally confirm the hypothesis of a temperature-dependent thylakoid membrane behaviour suggested above is to investigate the causes of a repeat distance change, including the effect of transmembrane proteins, and to investigate whether the RD decrease in the temperatures above $30 \text{ }^\circ\text{C}$ is correlated to the change of lipid phases.



(a)



(b)

Figure 4.17: a) SAXS measurements of cyanobacteria (6803 - blue, 7942 - red, 7002 - green) in PETRAIII. Blue circles - peak q positions observed by M. Liberton et al. [154]. SANS measurement of *Synechocystis* sp. PCC 6803 in 20 °C (red) and 10 °C (black). No difference observed.

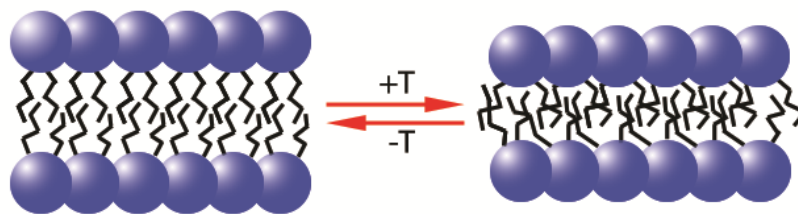


Figure 4.18: The hypothesis of the thylakoid membrane thickness changes upon temperature treatment.

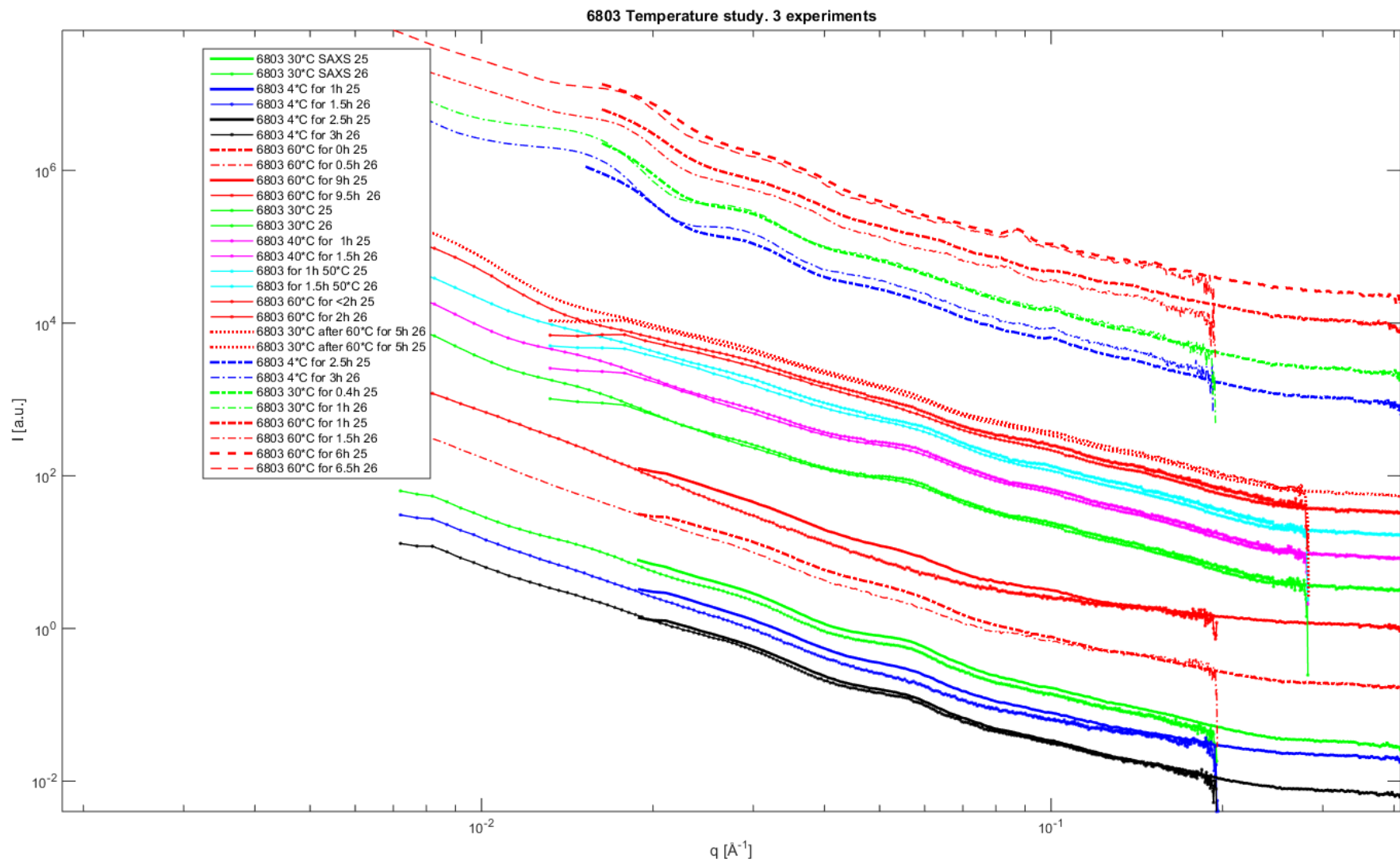


Figure 4.19: 6803 temperature study.

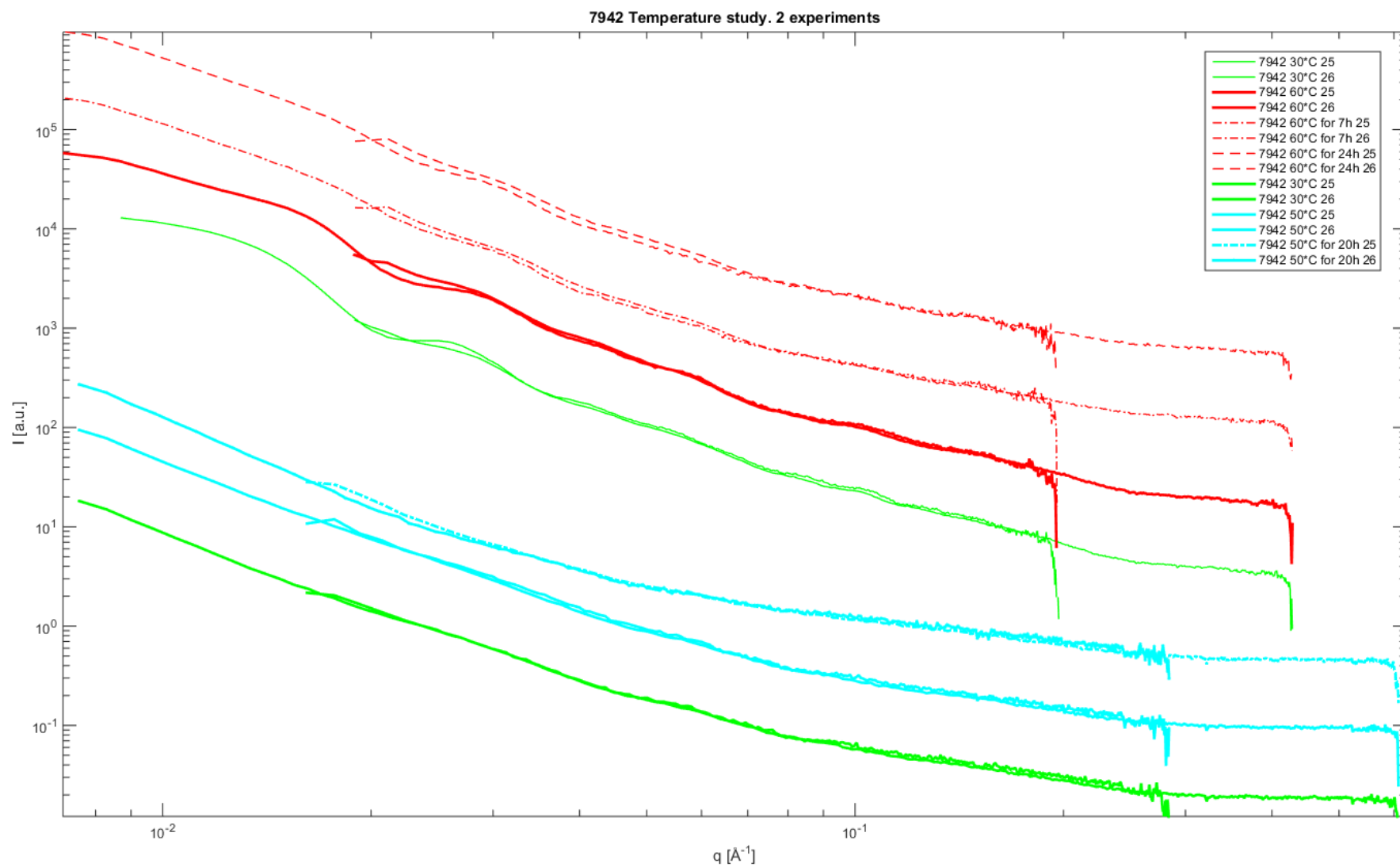


Figure 4.20: 7942 temperature study.

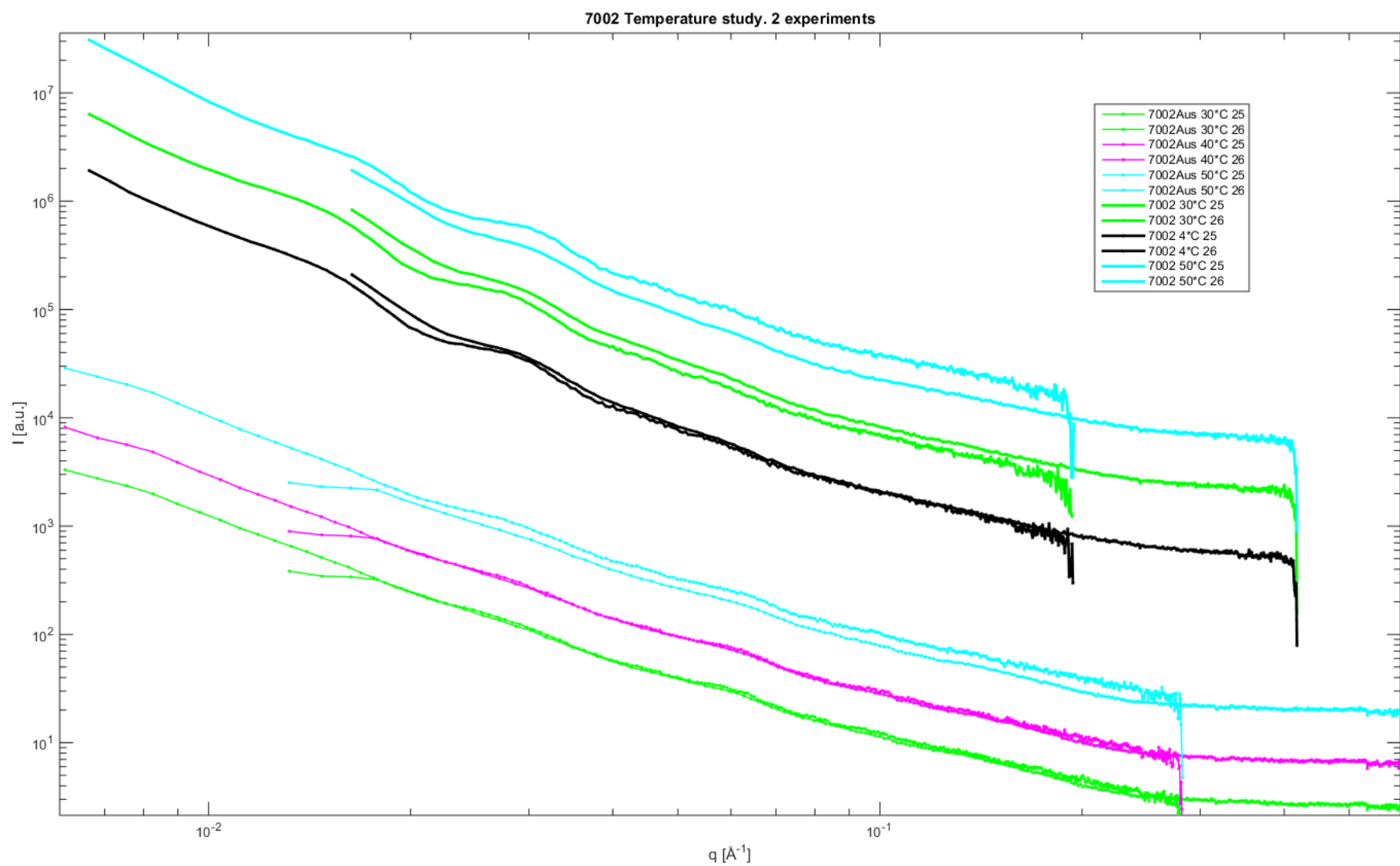


Figure 4.21: 7002 temperature study.

Temperature dependence - discussion

Cyanobacteria can serve as model systems to study low- and high- temperature stresses *in vivo*. Small angle scattering technique is routinely used to investigate lipid phase transitions of synthetic lipid mixtures, isolated cyanobacterial thylakoids [300, 293] or *E. coli* membranes [199]. Lipid phase transition temperature of isolated thylakoid membranes was determined by following a disappearance of a sharp $q = 1.5 \text{ \AA}^{-1}$ ($4.2 \text{ \AA}$) reflection and its change to broad $4.5 \text{ \AA}$ peak [232]. In the case of cyanobacterial samples in a water-based medium, it is impossible to investigate the phase changes *in vivo* by directly following $q = 1.5 \text{ \AA}^{-1}$ reflection behaviour, as it is completely overshadowed by a scattering peak of water background at $q = 2.0 \text{ \AA}^{-1}$.

The phase transition temperature of cyanobacterial membranes varies from 26 to 16 °C (14-23 °C [300]) and depends on cyanobacterial growth conditions, but is generally comparable to phase transition temperature of cyanobacterial MGDG, which is around 30 °C [294]. From a FTIR experiment, a lamellar gel-to-liquid crystalline L_β - L_α phase transition of isolated thylakoid membranes from *Synechocystis* 6803 is in the range of 14.8-19.6 °C, for cyanobacterial cell membranes it is 14.2-22.4 °C [147].

Such biophysical studies, although performed in a broad temperature range, used isolated thylakoid membranes [281, 147] and do not necessarily reflect *in vivo* situation, since thylakoid membranes are more susceptible to heat if they are isolated from the cellular environment; furthermore, heat treatment was usually short [206]. Indeed, determining a temperature of a membrane phase separation state for 28 °C grown *Synechococcus elongatus* sp. PCC 7942 intact cells yields equivocal results: microscopy experiments yield 5 °C for cell membranes, freeze-fracture experiments yield 0 °C (this method can be unreliable on thylakoid membranes and disproved in [186, 187]), chlorophyll fluorescence yields or 13-16 °C for thylakoid membranes. To compare, later articles give a thylakoid membrane phase separation state temperature interval between -5 and 16 °C, it increases during senescence [186, 187]. Some references claim, that it is unlikely that true gel-phase lipids exist in thylakoid membranes at temperatures above 0 °C [228].

In comparison, isolated spinach chloroplast membrane lipids DGDG undergoes phase transition at -50 °C and MGDG at -30 °C [264]. Interestingly, no phase change in bulk pea thylakoid lipids was observed by DHP technique in the temperature range of -20 °C to 55 °C [170, 25]. To conclude, the exact membrane phase separation and phase transition temperature *in vivo* is unknown, although it was shown to be well below 30 °C [293, 300]; literature investigations indicate a very broad interval.

In order to investigate thylakoid membrane behaviour at different temperatures and maybe to observe ultrastructural thylakoid membrane changes *in vivo*, which are related to phase transitions, I performed small-angle scattering on intact cyanobacterial cells in 4-60 °C range. I did not observe differences in SAXS curves between 4 °C and 30 °C measurements after the temperature equilibration for all three cyanobacterial species, although a slight peak intensity decrease was observed for peaks around $q = 0.1 \text{ \AA}^{-1}$ at 4 °C. Since the scattering at low q exhibits lamellar features and does not show any

change in q values, I conclude that cyanobacteria do not change average thylakoid repeat distance in low temperatures. The complementary SANS measurement was performed in 10 °C, but no difference was observed either (Fig. 4.19 b).

I was unable to actually observe the phase transition in low temperatures due to the sample and method limitations. Although it seems that if the phase transition does occur, it does not impact overall layered thylakoid ultrastructure. It shall be said, that our scattering experiments involving 4 °C incubation did not last more than 4 hours, therefore an increased membrane fluidity due to the action of acyl-lipid desaturase enzyme, is highly unlikely [162, 242, 186, 248, 247]).

The situation is different upon prolonged heating of cyanobacterial cells - a clear increase of q values is observed in cyanobacteria *in vivo*. Overall, I observe a clear correlation between SAXS and TEM experiments in higher temperatures. To my knowledge, only a single heat-stress experiment, where *Synechocystis* cells were visualized by TEM has been performed, showing only minor thylakoid ultrastructure changes due to a putative PBS redistribution [298]. Here, cells were visualized using TEM after 2-24 h incubation in 44 °C.

Several works following membrane ultrastructure changes were performed with isolated plant chloroplasts - in 38-46 °C grana reduced in size, at a temperatures of ≥ 50 °C adjacent thylakoids fused to form pseudograna - local fusions of several individual thylakoids [256]. This correlates well with my TEM observations. The formation of inverted micelles and non-bilayer structures in chloroplasts was also observed above 45-55 °C, although some thylakoids remained intact and well preserved even after 60 °C treatment. In 70 °C, thylakoids already formed bubbles [86, 87, 256]. I cannot confirm the presence of non-lamellar structures from TEM due to insufficient resolution, but their putative presence can be questioned by the appearance of a new scattering peak.

Cyanobacterial short-term heat stress investigations in a molecular level [107, 14, 31] indicate that cyanobacterial cell membranes become leaky in 48-50 °C and 72 °C [107, 106, 198]. In comparison, *Synechococcus vulcanus*, thylakoids denature at 75 °C, phycobillisomes (PBS) detach from PSII reaction centers at 70 °C, whereas phycobiliproteins denature at 76-80 °C and PBS disassemble at 80 °C [106]. In plant leaves, a complete loss of PSII photochemical activity occurs at 43-45 °C (PSI loses activity in 43-53 °C) [165, 291, 228]. In mesophilic cyanobacteria *Synechocystis* sp. PCC 6803, this temperature is even higher - 52-54/57 °C [106, 107, 14] for thermophilic *Synechococcus vulcanus* it is 75 °C [106]. Isolated-thylakoid-membrane proteins denature in two steps in high temperatures: at 65 °C (protein structural changes) and 87-90 °C (protein aggregation and dissolution of membrane structure) [147]. These experiments nicely complement our TEM investigations, showing that thylakoid ultrastructure remains intact and is preserved in the temperatures up to 70 °C, despite that photosynthetic activity is lost.

To conclude, cyanobacterial strains of *Synechococcus* sp. can survive and adapt extreme environments and temperatures -60 to 74 °C [229]. In physiological sense, it

is advantageous that thylakoid ultrastructure and its membrane integrity is preserved even for mesophilic cyanobacteria [94]. This means that thylakoid can act as a functional scaffold of photosynthetic proteins even in extreme conditions, despite that photosynthetic activity of proteins is severely reduced due to denaturation. From SAXS and TEM experiments I conclude that thylakoid membranes are stable in a wide temperature range and they preserve ordered lamellar structure inside a cyanobacterial cell.

4.2.7 Cyanobacterial CURT1 mutant' studies using small angle X-ray scattering

Thylakoid curvature protein family is responsible for thylakoid bending and stacking [19]. To investigate the thylakoid ultrastructure variations in cyanobacteria, CURT1 protein cyanobacterial mutants were used. The following CURT1 protein expressing strains have been obtained from Steffen Heinz, Mathias Labs and Dario Leister: AtCURT1A (also called CURT1A OI), heterologously expressing *Arabidopsis thaliana* CURT1A protein in *Synechocystis* sp. PCC 6803 wild type genetic background, Δ synCURT1, a knockout of endogenous *Synechocystis* sp. PCC 6803 CURT1 variant (also called curT^- [95]), AtCURT1A+ Δ synCURT1, *Arabidopsis thaliana* CURT1A protein in endogenous CURT1 knockout of *Synechocystis* sp. PCC 6803 and oe synCURT1 - an overexpressor of endogenous *Synechocystis* sp. PCC 6803 CURT1 variant [19, 95]. Cyanobacterial cultures have been grown under antibiotic selection of 100 or 200 $\mu\text{g/mL}$ in 30 °C as already described.

From Western blots, I confirm that endogenous CURT1 is absent from *Synechocystis* sp. PCC 6803 in knockout strains (Fig. 4.24). Evaluating AtCURT1A expression from Western Blots is slightly ambiguous and shall be repeated. TEM analysis of the same cyanobacterial mutants, performed by the group in Munchen (Fig. 4.23), shows that thylakoids have a crumpled appearance, a decreased lumen width, phycobilisomes are detached from thylakoid membranes [19]. In Δ synCURT1 (curT^-) mutant thylakoids appear as disordered continuous rings, which lack thylakoid biogenesis centers [95]. It has been shown, that AtCURT1 can partially replace endogenous CURT1, but a disordered thylakoid phenotype is still observed [19].

Small angle X-ray scattering of CURT1 mutants confirms a reduced cellular thylakoid order. *Synechocystis* sp. PCC 6803 WT is depicted in Fig. 4.22 in blue, $1-4 \cdot q$ positions of lamellar structure factor ($q_1=8 \cdot 10^{-3} \text{ \AA}^{-1}$ are drawn as vertical dashed blue lines). Compared to WT, the Δ synCURT1 curve is flatter (Fig. 4.22, brown), indicating a high polydispersity and suggesting that thylakoids in the cell are not ordered; though thylakoid RD, similar to 6803 WT, is observed (elucidated from q_1 peak position). Although thylakoids seem stacked in TEM images, the AtCURT1A curve (Fig. 4.22, orange), is flatter - probably because of the variation in thylakoid thickness; thylakoid RD is also similar to WT. oe synCURT1 (Fig. 4.22, green) curve is the most similar to 6803 WT - it contains more defined scattering features, although q of the peaks is lower (indicating smaller average thylakoid RD) and the peaks are slightly broader. The

scattering curve of AtCURT1A protein introduced in $\Delta\text{synCURT1}$ genetic background (Fig. 4.22, red) also exhibits defined scattering peaks, what supports a partial rescue of $\Delta\text{synCURT1}$ phenotype by the expression of AtCURT1A protein in the cyanobacterial cell (compared to $\Delta\text{synCURT1}$ curve).

For more detailed structural analysis, SAXS dataset of these CURT1 cyanobacterial mutants still needs to be fitted with the scattering model, described in Chapter 3. For contrast variation and complementary SANS measurements, D33 beamline in ILL (Grenoble, France) can be considered, as the import of these cell strains for SANS measurements with 'BILBY' beamline in ANSTO is burdensome due to Australian GMO regulations.

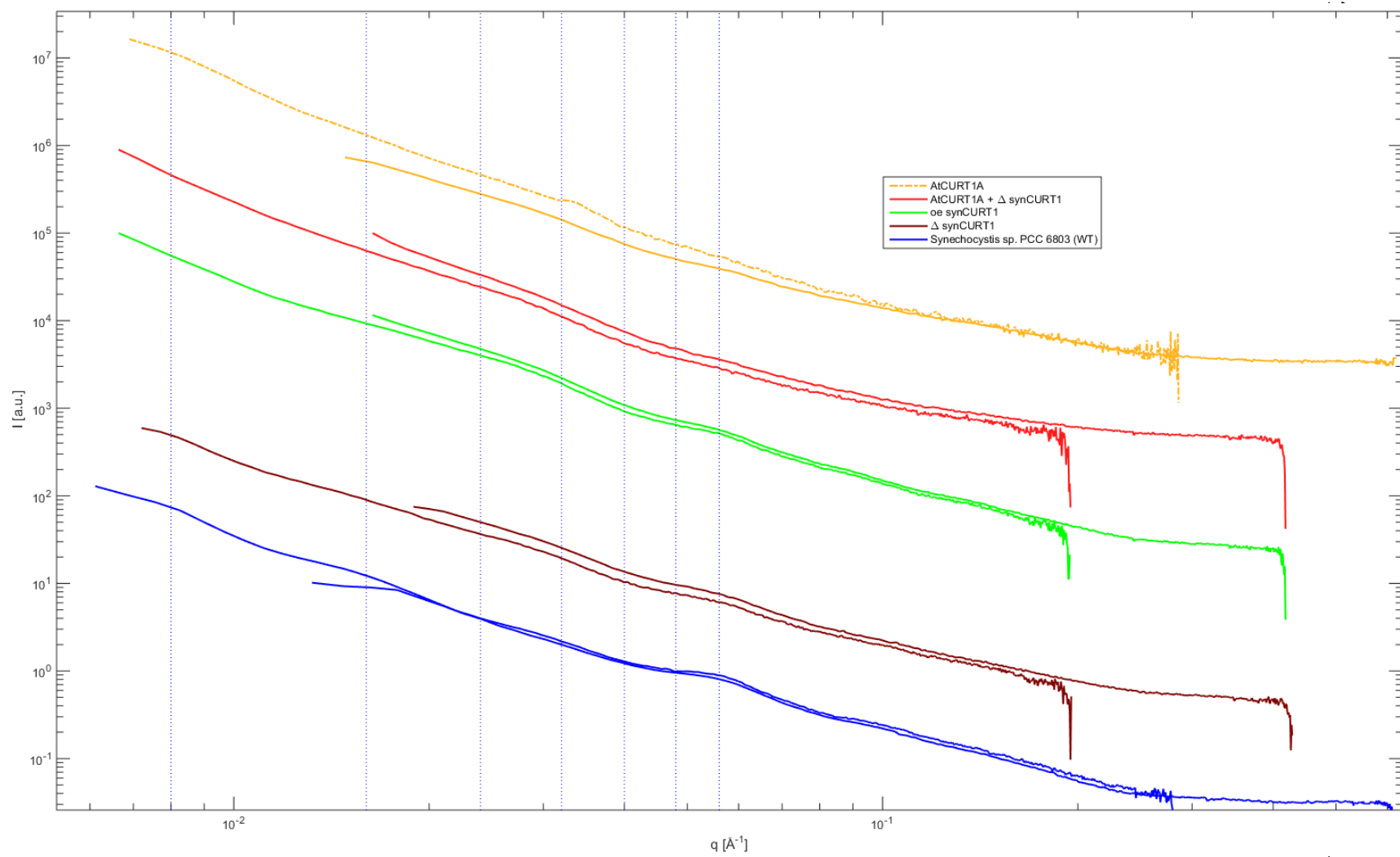


Figure 4.22: SAXS curves of cyanobacterial cells expressing different CURT1 variants.

To conclude, cyanobacteria do not exhibit light-induced ultrastructural changes under the investigated conditions. The temperature-induced thylakoid repeat distance shrinkage in cyanobacterial cells is pronounced in higher temperatures, the shrinkage is confirmed both by small angle scattering and electron microscopy methods. The shrinkage is hypothesized to be caused by the increase of hydrophobic interactions between adjacent thylakoid membranes, what remains to be confirmed experimentally. Lastly, CURT1 protein expression mutants of 6803 with different thylakoid ultrastructures exhibit a decreased thylakoid order, compared to wild-type cyanobacterial species, based on broader scattering peaks. SANS studies of these mutants as well as the investigation of the obtained SAXS curves would be a logical continuation of this PhD project.

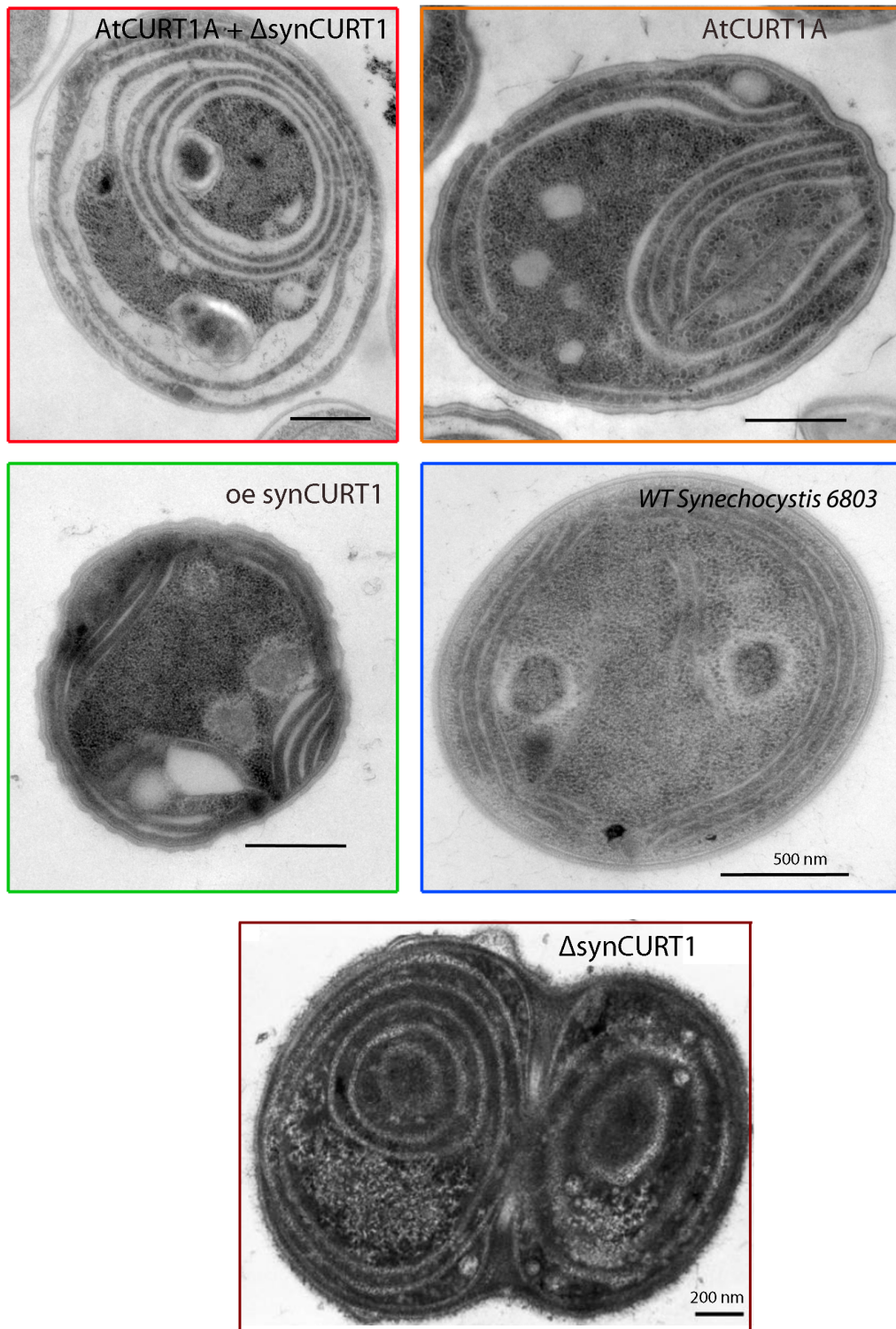


Figure 4.23: TEM of cyanobacterial cells expressing different *CURT1* variants. Scale bars of 500 nm. With the courtesy of Mathias Pribil, Department of Plant and Environmental Sciences, University of Copenhagen. [19, 95].

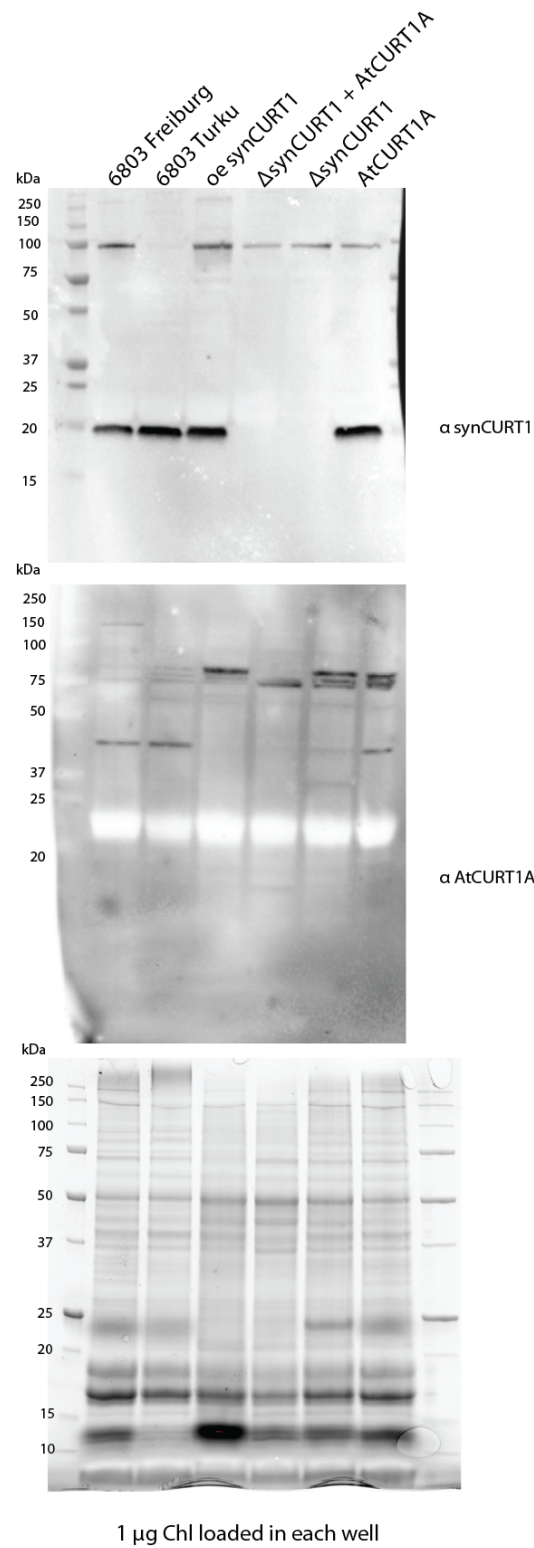


Figure 4.24: Western blots with α synCURT1, α AtCURT1A and loading control. 1 μ g Chl loaded in each lane.

Objectives

This PhD project has focused on investigating thylakoid dynamics by means of scattering and modelling scattering curves.

The objectives of the primary project (Manuscript 1) were:

- To measure scattering curves on cyanobacteria in biologically relevant conditions.
- To derive the mathematical apparatus of the thylakoid unit cell.
- To model the entire scattering curve and extract thylakoid physical parameters, to show that all scattering features can arise from a single thylakoid repeat distance.

An extension of the primary project (Manuscript 2), objectives to eukaryotic organisms were:

- To obtain higher-plant thylakoid scattering *in vivo* and to adapt the cyanobacterial scattering model to higher plants.
- To explain and compare plant leaf scattering results to the results obtained by transmission electron microscopy and confocal laser scanning microscopy methods.
- To compare illumination-induced thylakoid dynamics in plants with a conventional grana-stroma thylakoid arrangement (*Arabidopsis* Col0 and *Chlorophytum comosum*) and shade tolerant (high-grana) plants.

Side project objectives (Additional experiments) were:

- To obtain scattering signal from isolated etioplasts and etiolated leaves in order to confirm a (double-)diamond cubic structure of prolamellar bodies and to follow a light-induced decay of paracrystalline structure.
- To investigate cyanobacterial thylakoid ultrastructure changes upon illumination and high/low temperature treatments.

As the use of scattering methods in biological structure studies is expected to increase, a thorough compilation of scattering works performed on photosynthetic organisms (Manuscript 3) and the evaluation of their ultrastructure changes in different conditions has been a logical side project. This review explains physical scattering concepts in a biologist-accessible way, evaluates the current status of scattering experiments and explains recurrent issues of scattering data interpretation in a biological context. In my opinion, the critical value of this review is that scattering results are discussed in the context of open biochemical questions on thylakoid ultrastructure changes. Lastly, a

personal perspective on experimental preparation and technicalities, based on expertise obtained during this PhD project is provided.

Publication list and author contributions

Publications

Manuscript 1. D. Jakubauskas, Ł. Kowalewska, A. V. Sokolova, C. J. Garvey, K. Mortensen, P. E. Jensen and J. J K. Kirkensgaard. "Ultrastructural modelling of small angle scattering from cyanobacterial thylakoid membranes". *In preparation*.

Author contribution: In this publication I have designed the biological part of experiment, I have performed SANS and SAXS measurements on cyanobacteria *in vivo* with 'BILBY' SANS-TOF instrument in the Australian Nuclear Science and Technology Organization (ANSTO), bioSAXS beamline P12 in PETRAIII (DESY) and 'Ganesha' instrument in University of Copenhagen. I have calculated theoretical scattering length densities, have been involved in writing a C++ code of cyanobacterial thylakoid unit cell for 'Will It Fit?', data reduction and the final data fitting. I have prepared TEM samples and measured cyanobacterial thylakoid RD from images. I have taken part in drafting and writing the manuscript and designing figures.

Manuscript 2. D. Jakubauskas, Ł. Kowalewska, R. Mazur, A. V. Sokolova, C. J. Garvey, K. Mortensen, P. E. Jensen and J. J K. Kirkensgaard. "Light-induced thylakoid dynamics of higher plants". *In preparation*.

Author contribution: In this publication I have designed the biological part of experiment, I have performed TOF-SANS measurements on plant leaves *in vivo* using 'BILBY' instrument (ANSTO). I have been involved in adjusting the C++ code for plant thylakoid unit cell for 'Will It Fit?', data reduction and the final data fitting. I am responsible for the critical content of the publication, have selected and compiled literature and drafted the manuscript.

Manuscript 3. D. Jakubauskas, K. Mortensen, P. E. Jensen and J. J K. Kirkensgaard. "Small angle scattering on photosynthetic membranes - a critical review". *In preparation*.

Author contribution: In this publication I have compiled and reviewed the relevant literature, drafted the manuscript and designed figures.

The results of this publication are not discussed in this PhD thesis:

Paper 4. S. B. Mellor, A. J. Z. Nielsen, M. Burow, M. S. Motawie, D. Jakubauskas, B. L. Møller, P. E. Jensen. "Fusion of ferredoxin and cytochrome P450 enables direct light-driven biosynthesis". ACS chemical biology, 2016, Vol. 11, Issue 7, Pages 1862-1869, DOI: 10.1021/acscchembio.6b00190

Conference contributions

"Cyanobacteria: scattering studies". D. Jakubauskas, K. Mortensen, P. E. Jensen, M. Pribil and J. J K. Kirkensgaard, in "Photosynthesis research on sustainability". Puschino, Russia, 19-26 June 2016. [Conference talk and Poster]

"Small angle scattering study of cyanobacterial thylakoid membranes". D. Jakubauskas, K. Mortensen, P. E. Jensen, M. Pribil and J. J K. Kirkensgaard, in "Animal, vegetal, mineral". Yallingup, Australia, 19-23 September 2016. [Poster]

"Applying small angle scattering to investigate thylakoid membranes". D. Jakubauskas, Ł. Kowalewska, K. Mortensen, P. E. Jensen and J. J K. Kirkensgaard, in "SEB 2017". Gothenburg, Sweden, 3-6 July 2017. [Poster and Poster presentation]

Conclusions

- 1. Small angle scattering from cyanobacteria and plant leaves *in vivo* can be modelled by the thylakoid form factor and the lamellar structure factor.
- 2. Cyanobacterial thylakoid parameters, extracted from a small angle neutron scattering curve by means of fitting, are: thylakoid repeat distance (597-693 Å), number of layers in pseudogranum (3.4-4.3), lumen height (62-84 Å), thylakoid membrane thickness (34-42 Å), inter-thylakoid space height (429-555). Repeat distance obtained from SANS and TEM measurements are comparable. Calculated protein content of cyanobacterial lumen is 82 %.
- 3. Complementary SANS, CLSM and TEM experiments show that thylakoid dynamics of dark-adapted shade-tolerant plants with extensive grana stacking is significantly suppressed compared to sun species. The stacking repeat distance remains unchanged upon dark-to-light transition of shade-tolerant plant *Ficus elastica*, but is reduced in sun plant *Arabidopsis thaliana* Col0.
- 4. Cyanobacterial thylakoid repeat distance decreases upon heating to 40-60 °C. The lamellar nature of cyanobacterial thylakoids is preserved upon heating.
- 5. Electron tomography of isolated maize etioplasts confirms the cubic geometry of PLB membranes. A reproducible small angle neutron scattering signal from isolated maize etioplast samples in D₂O-based buffer has been obtained, but the exact space group of PLB membranes could not be ascribed.

Perspectives

In my opinion, the most accurate way to look into the perspective is a retrospective -

During the poster discussion in the conference 'Animal, vegetal, mineral' in Yallingup (September 2016), Robert Corkery expressed his thought 'I have tried to fit a lamellar structure factor for cyanobacterial scattering curves, but it did not work'. After some time and additional work we can say that we have constructed a model, which explains scattering patterns *in vivo* without undue parametrization and we can elucidate dimensions of the entire thylakoid system solely from a scattering pattern. This model is implemented in a user-friendly 'WillItFit?' platform. Therefore, the biggest personal perspective is that the model will be routinely employed to explain scattering patterns from other photosynthetic organisms in relation to thylakoid dynamics, as there are several groups working on these matters and the model will be freely available. This would include investigating diatoms with more diverse thylakoid ultrastructures, structural and functional photosynthetic cyanobacterial and plant mutants, halophilic, extremophilic and shade tolerant photosynthetic organisms. Scattering experiments shall be carried out in different illumination intensities, fluctuating light intensities, different light qualities - causing different photosynthetic states and following the change from State 1 to State 2 or in the NPQ conditions. In this way thylakoid dynamical changes can be measured in more 'biochemically precise' conditions and therefore be related to underlying biochemical processes more exactly.

Also, although scattering curves of wild-type cyanobacteria are somewhat similar to each other, SAXS studies of cyanobacterial CURT1 protein-mutant strains show a large variation of thylakoid polydispersity. The investigation of CURT1 thylakoid ultrastructure mutants with neutron scattering and subsequent modelling would greatly complement the understanding of the overall role of thylakoid curvature-inducing proteins, also in the light of grana stacking in higher plants. That said, we continue to develop our scattering model for plants having high grana. Ultimately, this model will be a big achievement in the current era of elucidating putative thylakoid system dynamic changes based solely on the change of the q value of the peak.

If this scattering model is accepted by the physics and biology scientific communities, the second perspective is that small angle scattering method will become a reference technique in plant ultrastructural biology, as it virtually requires no sample preparation.

Scattering can be therefore considered a technique, where samples are measured in near-*in vivo* conditions. Having said that, large scale facilities shall be proactive in establishing a suitable working environment for biologists - to employ biologically-trained personnel, to have biological laboratories with the necessary GMO certification, growth chambers suitable for plant adaptation and the access to dark rooms. Hopefully, these requirements are accounted for during the construction of the new large-scale experimental facilities.

Manuscript 1

Ultrastructural modelling of small angle scattering from cyanobacterial thylakoid membranes

Ultrastructural modelling of small angle scattering from cyanobacterial thylakoid membranes

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Abstract

The last decade has seen a range of studies using non-invasive neutron and x-ray scattering techniques to probe the ultrastructure of photosynthetic membrane systems from both plants, cyanobacteria and other organisms. The common denominator in this work is an unpretentious approach to analysis based on simple peak position readings with no underlying structural model, ultimately leading to questionable peak interpretations. Here we formulate and implement a full mathematical model of the scattering from a stacked double bilayer thylakoid membrane system taking instrumental resolution and polydispersity into account. We validate our model by direct simulation of scattering patterns from 3D structural models. Most importantly, we demonstrate that the full scattering curves from three different, but structurally typical, cyanobacterial thylakoid membrane systems can all be described within this framework. The model provides realistic estimates of key structural parameters in the thylakoid membrane, in particular the overall repeat distance but also how this distance is divided between membranes, lumen and cytoplasmic liquid. Finally, on the basis of scattering length density fitting parameters, we propose an estimate of the lumenal protein concentration. SLD calculations derived from combining SANS and SAXS model fits results in the conclusion that the protein content in the lumen as measured in dark adapted samples is lower than in the cytoplasmic liquid and constitutes about 82% of the available lumen volume. We anticipate that the model will be applied to analyse other stacked photosynthetic membrane systems.

Keywords

Cyanobacteria — Small angle scattering — Photosynthesis

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1. Introduction

Oxygenic photosynthesis in cyanobacterial ancestors started 3.4-2.3 billion years ago [1, 2, 3]. Photosynthetic electron transfer takes place in the thylakoids - a highly specialised membrane system which accommodates photosynthetic and cellular respiration protein machinery. Cyanobacterial thylakoids are composed of ca. 70 % proteins [4] - photosystems (PSI, PSII), antennae (phycobilisomes), cytochrome *b₆f* (Cyt *b₆f*), ATP synthase (ATPase), cellular respiration enzymes and of 10-28 % lipids (MGDG, DGDG, SQDG and PG) [5, 6]. In cyanobacteria, several stacked sheet-like thylakoid membrane (TM) layers are arranged as 'pseudograna' in cell periphery and are confined by the surrounding plasma membrane. Cyanobacterial thylakoid arrangements are strain-dependent [7, 8]. In Figure 1 a) we present thin-section electron micrographs of three cyanobacterial wild-types: these are typical variations in cyanobacterial thylakoid ultrastructure arrangement. In the round *Synechocystis* sp. PCC 6803 cell, 3-6 layers of parallel flattened thylakoids localise in close proximity of the cell membrane and occasionally converge to the thylakoid centers [9, 10].

Elongated fresh-water *Synechococcus elongatus* sp. PCC 7942 cell contains 3-6 layer concentrically arranged thylakoids, which run through the entire cell length without convergence sites [11, 12]. Halotolerant *Synechococcus* sp. PCC 7002 cell has three to four thylakoid stacks, each composed of 3-6 flattened thylakoid sheets, which converge at the edges [13].

Cyanobacterial antennae - phycobilisomes (PBS) - are hemispherical phycobiliprotein-pigment structures. Phycobilisomes are densely packed between two thylakoid membranes in a zig-zag pattern [14, 7, 15, 16]; phycobiliproteins account for 24 % of the cell dry weight and around 50 % of the total cell protein content [17]. Phycobilisomes are composed of 3-4 core cylinders and 6-10 peripherally radiating rods and have overall dimensions (*w* · *l* · *h*) of 550-680 Å · 180-450 Å · 250-390 Å [18, 14, 19].

Whereas the cyanobacterial thylakoid thickness is similar between species (2·TM thickness of 30-40 Å + lumen thickness of 50-100 Å), thylakoid center-to-center repeat distance (RD) differs for species or mutants [9, 20, 21, 22, 16, 23, 24] and also depend on illumination conditions and

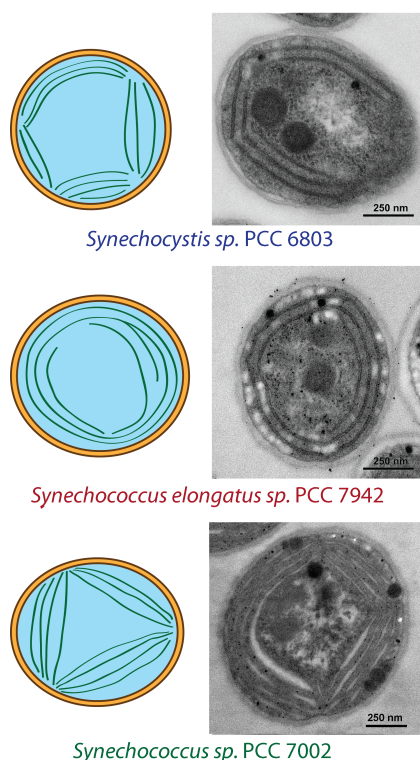


Figure 1. Transmission electron micrographs with transverse views of the cyanobacterial species investigated, showing schematics and microscopy images representative of the typical thylakoid arrangements in the cells. The color code of the three species is maintained throughout the paper.

hydration [20, 25, 21, 24]. *Synechocystis* sp. PCC 6803 PAL mutant without phycobilisomes has a repeat distance of 340 Å [26], whereas RD of *Synechocystis* sp. PCC 6803 WT and *Leptolyngbya ohadii* ranges from 600-800 Å [26, 27, 24].

While light microscopy is of insufficient resolution to visualize thylakoids in micrometer resolution [28, 29, 22], transmission electron micrographs can reveal many details on the three-dimensional organisation of thylakoid membranes and their repeat distances (Fig. 1 b). However, thylakoid membrane shrinkage occurring from sample fixation [30] and inherently static images (also after cryo-freezing) limits the applicability of microscopic methods in dynamic studies. On the contrary, X-ray and neutron scattering methods are devoid of sample fixation and gives structural information which is a statistical and spatial average of the total irradiated volume of the sample. Thus, scattering and microscopy are complementary methods and can, if properly utilised and combined, provide strong and reliable structural information. For this reason there has been several studies using scattering techniques to gain information on photosynthetic membranes, including cyanobacterial thylakoid membranes.

Focusing on scattering studies on cyanobacteria, the wild type strain *Synechocystis* sp. PCC 6803 is by far the most studied cyanobacteria [27, 26, 31, 30, 32]. Liberton *et al.* recently described small-angle neutron scattering (SANS) on *Synechocystis* sp. PCC 6803 and three mutant strains with various degrees of phycobilisome deficiency [31]. Stingaciu *et al.* [32], has shown that thylakoid membranes are less flexible in the light, correlating the membrane mobility with photosynthetic activity, supporting results for *Chlamydomonas* [33]. In Li *et al.* a SANS study on *Halomicronema hongdechloris* on intact cells complements microscopy work and indicates a far-red light induced decrease of the thylakoid repeat distance [21]. In Eyal *et al.* scattering work on intact cells from *Leptolyngbya ohadii*, also complements microscopy work, showing dehydration induced decrease of the thylakoid repeat distance [24].

Common to all the SANS studies done over the last decade on both plants and cyanobacteria is that the data interpretation and analysis is either based on simple peak position readings or on using some *ad hoc* model expression to extract these peak positions [34, 27, 35, 36, 37, 26, 31, 38, 39, 30, 21, 24]. None of the studies makes use of an underlying structural model, based on the clear fact that the scattering in all cases originates from a stacked membrane system. Instead, the interpretation of the scattering curves has leaned on more or less educated guesses regarding peak origin or has treated all peaks as 1st order Bragg peaks, which we will argue below to be simply erroneous.

In this work we formulate a full structural model, explaining cyanobacterial scattering. We account for the double bilayer nature of the photosynthetic membrane stacks, instrumental resolution effects and inherent polydispersity of the system. We validate the structural model by direct scattering pattern simulations using full 3D models and explore model predictions to aid the scattering pattern interpretation. Further, we employ detailed calculations of scattering length

densities from existing knowledge of cyanobacterial cell composition to help define relevant molecular constraints. We demonstrate that the entire neutron scattering curve from three different, but structurally typical cyanobacterial thylakoid membrane systems, are fully described within our model framework. Finally, we estimate cyanobacterial luminal protein content solely from the scattering model. We further argue that this framework can be applied in the analysis of other stacked photosynthetic membrane system, such as grana stacks in higher plants.

2. Methods

2.1 Strains and growth conditions

Strains used in this experiment: *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* sp. PCC 7942, PCC 7002. For growth of cyanobacteria on plates, liquid BG-11 or A+ (supplemented with 4 mg/L vitamin B₁₂) growth medium was supplemented with 15 g/L bactoagar and autoclaved; plates were kept at 30 °C at 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Liquid cultures of cyanobacteria (20 mL flasks) for TEM experiment were grown at 30 °C at 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ white light, flasks were shaken 150 rpm. Liquid cultures of cyanobacteria (20 mL flasks) for TEM experiment were grown at 30 °C at 5–6 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, flasks were shaken 150 rpm. When a cell culture reached a logarithmic phase $\text{OD}_{730 \text{ nm}} = 0.8\text{--}2$, cells were centrifuged (5000 g, 5 min), resuspended in a small amount of fresh 100 % D₂O BG-11 or A+ medium and subjected for small-angle neutron scattering experiments.

2.2 Small angle neutron scattering

For SANS measurements and contrast variation experiments, cyanobacterial cells were centrifuged and pellet was resuspended in 21, 42, 85 or 100 % (v/v) D₂O-based media, final $\text{OD}_{730 \text{ nm}}$ was adjusted to 2.2. Cells were measured in 1 mm pathlength demountable cells with quartz windows, ambient illumination was 2–3 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ white light.

SANS measurements were performed at the "Bilby" beamline in ANSTO, Sydney, Australia, which is operated in time-of-flight data collection mode [40]. Neutron beam was defined by 15 mm radius sample aperture, source-sample distance 10.77 m, sample-detector distance 10 m, beamstop radius 40 mm. Transmission measurement - 20 min, data integration time - 1.5 to 3 hours. Neutrons with a range of wavelengths from 2 to 20 Å ($\Delta\lambda/\lambda = 7.3\%$) were used to cover a q -range from 0.0035 to 0.26 Å⁻¹. Data reduction of two-dimensional time-of-flight SANS was performed using Mantid software [41]. Correction for detector efficiency and absolute calibration was performed using transmission (3rd order polynomial fit), empty beam, blocked beam, empty cell and buffer measurements.

2.3 Small angle X-ray scattering

$\text{OD}_{730 \text{ nm}}$ of exponential cyanobacterial liquid cultures was routinely adjusted to 2, cells were pelleted (5 min, 5000 g) and resuspended in 150 μL fresh growth medium. Small

angle X-ray scattering of cyanobacterial cell suspension was measured using 0.15 mm diameter quartz capillaries with GANESHA instrument (SAXSLAB, Denmark) in University of Copenhagen. GANESHA instrument was equipped with 40 W micro-focus Cu source Micromax 002+ of $\lambda = 1.54$ Å (Rigaku) and 300k Pilatus pixel detector. Sample-detector distances measured in: 690 and 1490 mm, photon fluxes: 62 or $17 \cdot 10^6$ photons/s, covered q range: 0.007 – 0.4 Å⁻¹. Silver behenate standard was used for q calibration, scattering data reduction was carried out using SAXSgui platform.

2.4 Transmission electron microscopy

Cyanobacterial cells were centrifuged and the pellet was fixed in 2.5 % (w/v) glutaraldehyde and 50 mM cacodylate in either BG-11 or A+ media for 4 h (room temperature), washed with 50 mM cacodylate buffer (pH 7.4) and placed in a 2 % (w/v) OsO₄ at 4 °C in 50 mM cacodylate buffer (pH 7.4) for about 12 h. The specimens were dehydrated in graded acetone series, embedded in a low viscosity epoxy resin and cut on a Leica EM-UC7 ultramicrotome. Thin sections stained with uranyl acetate were examined with a JEM 1400 electron microscope (Jeol, Japan) in the Laboratory of Electron Microscopy, Nencki Institute of Experimental Biology of PAS, Warsaw, Poland. Repeat distances of cyanobacterial thylakoids have been manually measured in ImageJ software from TEM images of cyanobacterial cells.

2.5 Statistical analysis

Statistical comparisons of RD from different cyanobacterial species were performed in R software. A number (N) of 'pseudograna' was measured for each replicate, outliers were not omitted from data analysis. Normality was checked by Shapiro-Wilk test and by evaluating residual Q-Q plots. As tests indicated residual deviations from normality for some replicas, Box-Cox transformation of data ($\lambda = -0.667$) had been performed to normalize data for statistical analysis. Linear mixed model on transformed data with repeat distance (RD) as a quantitative variable, Species as qualitative variable and Replicas as a random effect was derived, *post hoc* Tukey contrast test was applied, 0.95 confidence level was used. Average, median RD values, RD standard deviation and RD mean confidence limits (Table 2) were calculated in R on non-transformed data using lsmeans package.

3. Theory and mathematical modeling

3.1 Model details

We follow the classic work of Nallet *et al.* [42] on lyotropic liquid crystalline lamellar phases to derive a model expression for the stack. In Figure 2 we illustrate the basic scattering density profile model of the cyanobacterial thylakoid membrane stack. The difference between our approach here and the classical lamellar stack is that for the thylakoids, the basic building block of the stack is not a single lamellar membrane sheet, but the double bilayer thylakoids. Denoting the lamellar repeat distance D , the overall model expression for the measured powder averaged intensity takes the form:

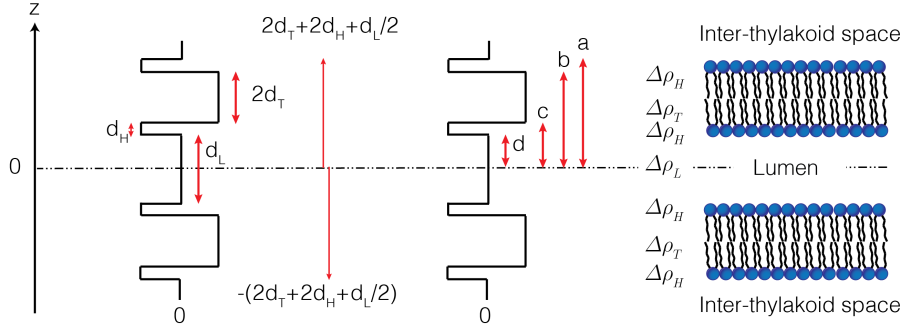


Figure 3. Details of the structural parameters of the double bilayer unit cell used in the expression for the form factor, Equation 10.

$$I(q) = 2\pi \frac{P(q) \cdot S(q)}{D \cdot q^2} \quad (1)$$

where $P(q)$ is the unit cell form factor of the thylakoids, i.e. the double bilayer, and $S(q)$ is the structure factor describing the stacking of these unit cells. From [42] $S(q)$ is given by

$$S(q) = 1 + 2 \sum_{n=1}^{N-1} \left(1 - \frac{n}{N}\right) \cos(nq \cdot D) \exp(-q^2 D^2 \alpha(n)) \quad (2)$$

with

$$\alpha(n) = \frac{\eta_{cp}}{4\pi^2} (\ln(\pi n) + \gamma_E) \quad (3)$$

where γ_E is Euler's constant. The Caillé parameter η_{cp} describes the bending and compression energetics of the sheets and is given by

$$\eta_{cp} = \frac{q_0^2 k_B T}{8\pi \sqrt{KB}} \quad (4)$$

Here q_0 is the primary 1st order Bragg peak value, k_B the Boltzmann constant, T the temperature and K and B the membrane bending elasticity and membrane compression modulus respectively. Thus, the Caillé parameter measures the rigidity of the lamellar membranes, with a low value indicating a stiff membrane with a high bending modulus, and high values indicating more flexible bilayers. Setting $\eta_{cp} = 0$ equals perfectly ordered flat sheets which we will use below for model validation purposes. The form factor of the double bilayer is built from a series of square box functions as illustrated in Figure 2. Denoting the direction normal to the membrane planes z , the scattering length density of a single box of thickness 2δ is

$$\rho_0(z) = \Delta\rho \quad \text{for} \quad -\delta \leq z \leq \delta \quad (5)$$

and zero elsewhere where $\Delta\rho$ is the contrast of the material represented by the box, i.e. $\Delta\rho$ is the scattering length density difference relative to some chosen reference, in this case the surrounding cytoplasm (inter-thylakoid space) liquid (see below). The scattering amplitude is the Fourier transform of the scattering length density function

$$A(q) = \int \rho_0(z) \exp(iqx) dx \quad (6)$$

and the form factor is the absolute square of the amplitude

$$P(q) = |A(q)|^2. \quad (7)$$

To construct the full unit cell we exploit that we can add and subtract boxes on the amplitude level to build up a double bilayer profile. The Fourier transform of a single box of width 2δ is

$$A_{box}(q) = \int_{-\delta}^{\delta} \Delta\rho \exp(iqx) dx = \frac{\Delta\rho}{q} 2 \sin(q\delta) \quad (8)$$

giving the box form factor

$$P_{box}(q) = \frac{\Delta\rho^2}{q^2} 4 \sin^2(q\delta) \quad (9)$$

With reference to Figure 3 we then define the distances a, b, c, d to arrive at the total double bilayer form factor

$$P(q) = \frac{4}{q^2} [\Delta\rho_H (\sin(qa) - \sin(qb) + \sin(qc) - \sin(qd)) + \Delta\rho_T (\sin(qb) - \sin(qc)) + \Delta\rho_L \sin(qd)]^2 \quad (10)$$

with $\Delta\rho_H$, $\Delta\rho_T$ and $\Delta\rho_L$ being the contrasts of the head group regions, tail regions and lumen region respectively. Thus, we allow for the lumen to have a scattering length