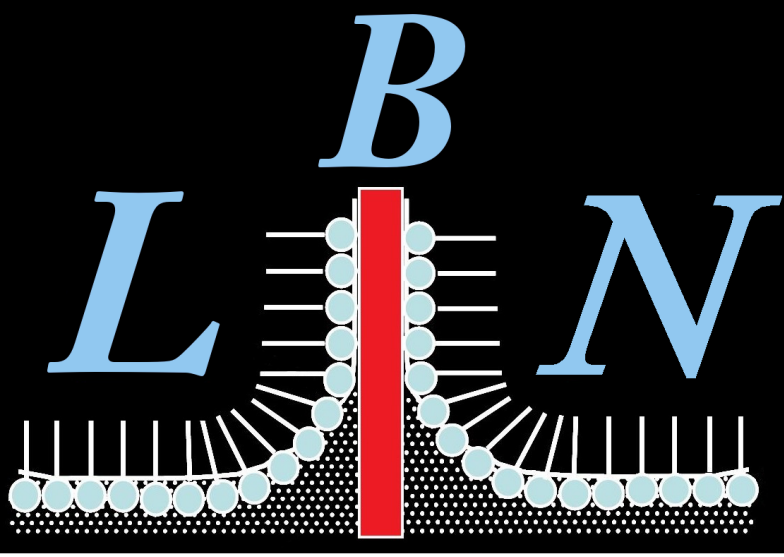


Laboratories of Biophysics and Nanotechnology

Eugenia Pechkova

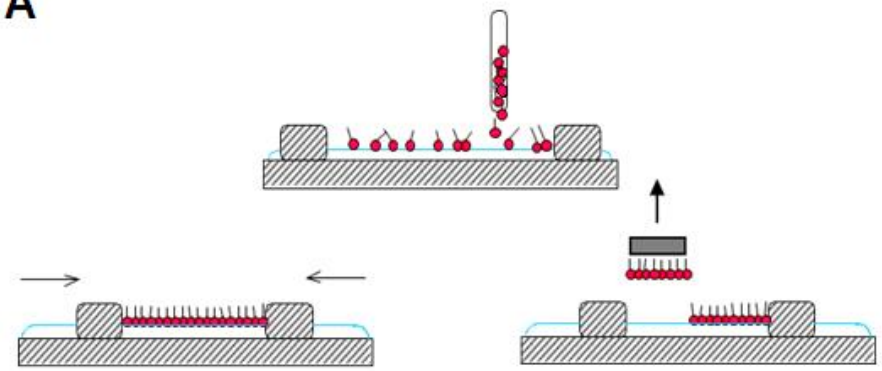
Labs of Biophysics and Nanotechnology,

Department of Experimental Medicine
University of Genova, Italy

Phycocyanin intermolecular order in nanostructured Langmuir-Blodgett (LB) films – methods and biosensor applications.

Langmuir-Blodgett thin film nanotechnology

A



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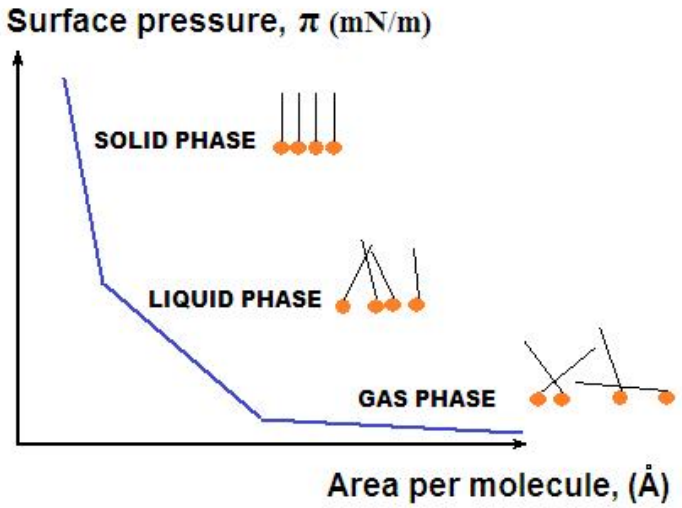

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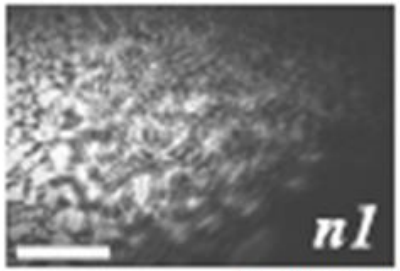
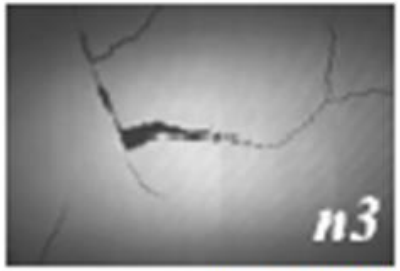


π = 0 mN/m

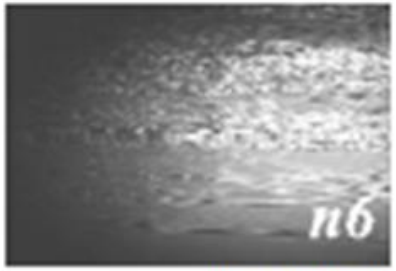
π = 25 mN/m

π = 2 mN/m

π = 42 mN/m

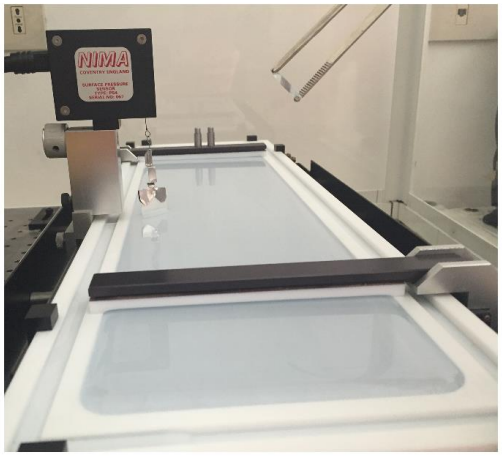


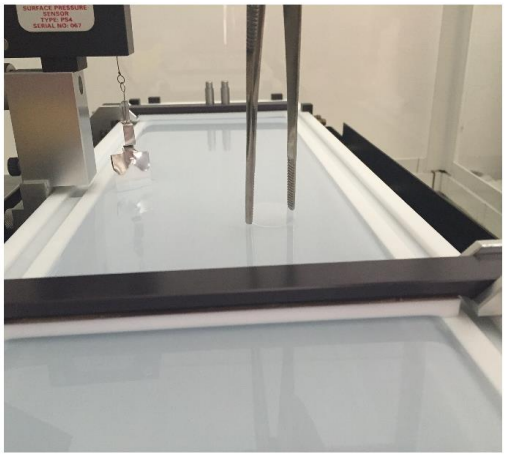
π = 15 mN/m

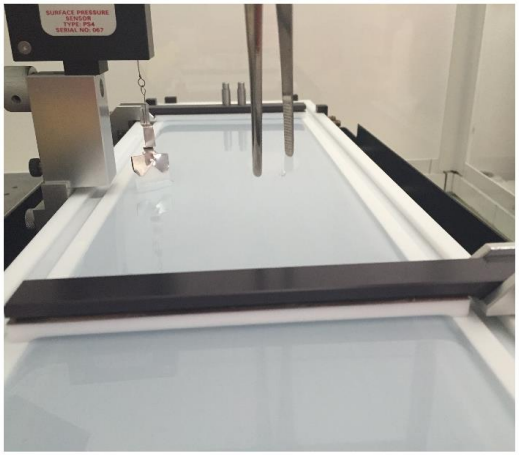


π = 50 mN/m

A


NIMA
COVENTRY ENGLAND





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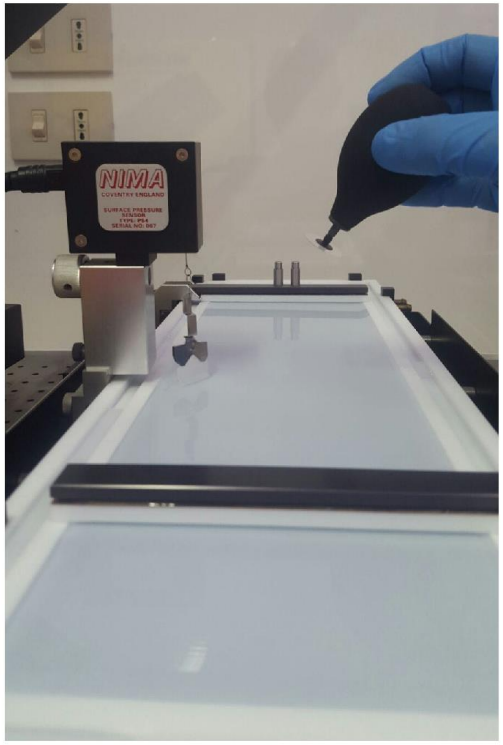
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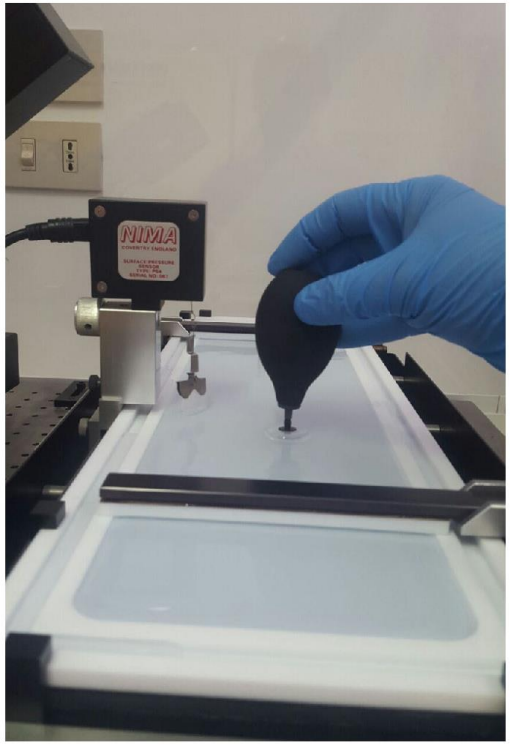
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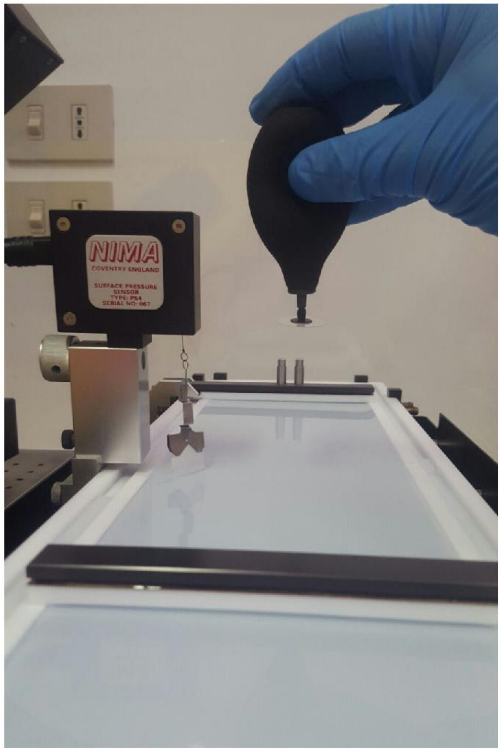
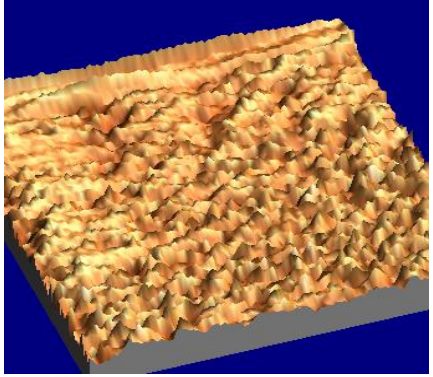
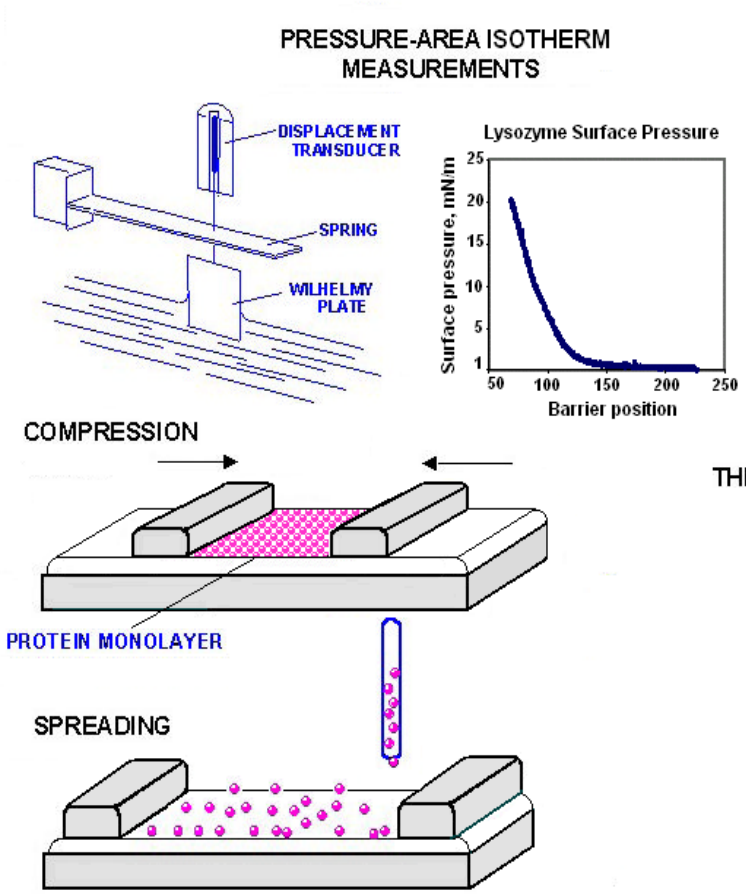
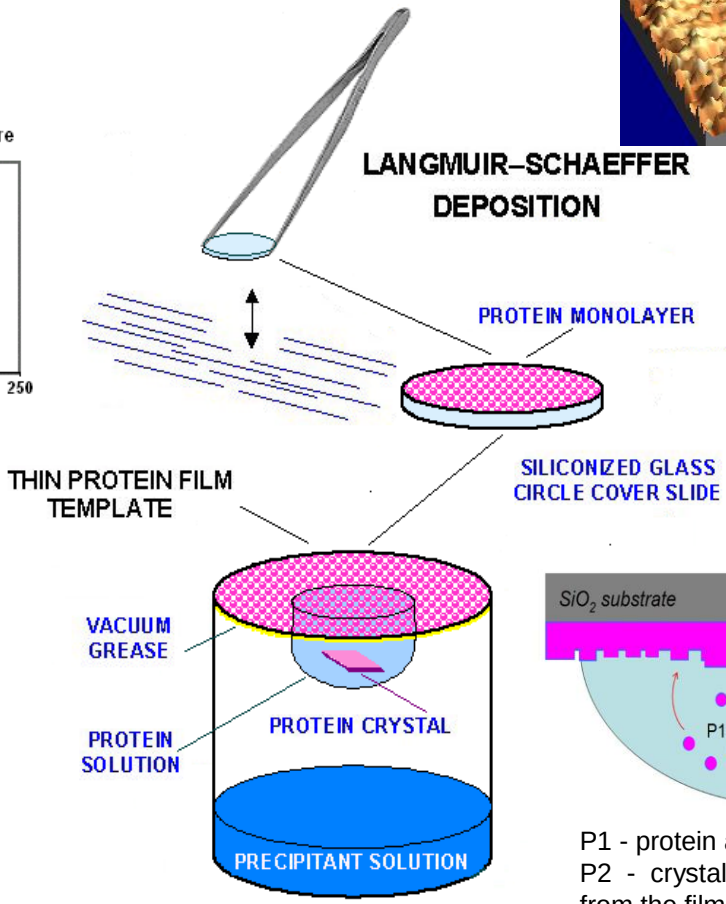
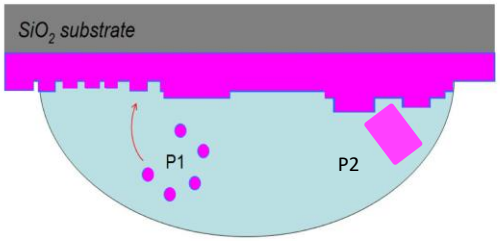

NIMA
COVENTRY ENGLAND

Langmuir-Blodgett protein nanotemplate for protein crystallography.





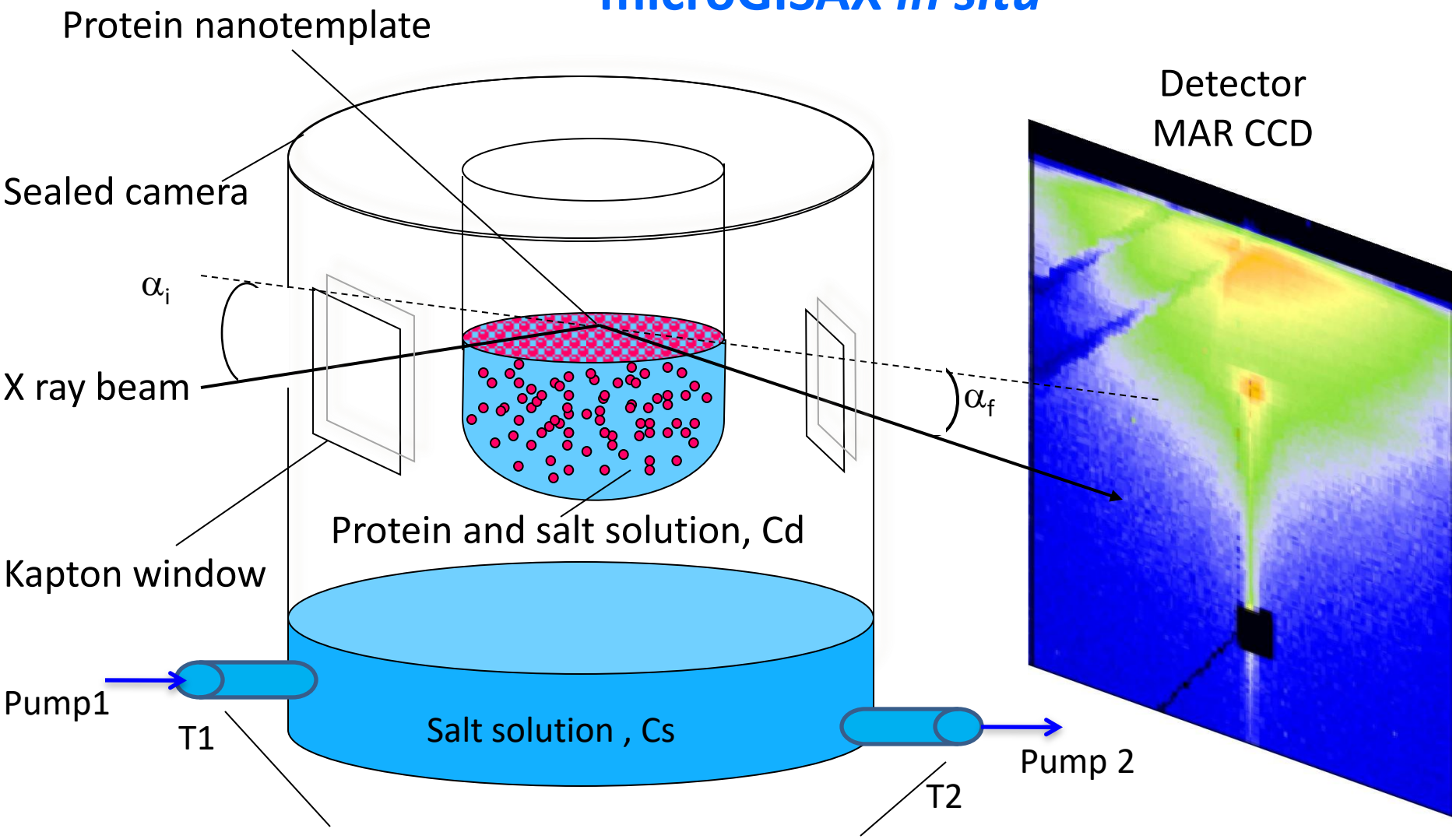




P1 - protein association to the LB film
P2 - crystal formation and detaching from the film into the drop.

Pechkova E., Nicolini C. Langmuir-Blodgett nanotemplates for protein crystallography, ***Nature Protocols*****,** 12, 2570-2589, 2017 and references therein

microGISAX *in situ*



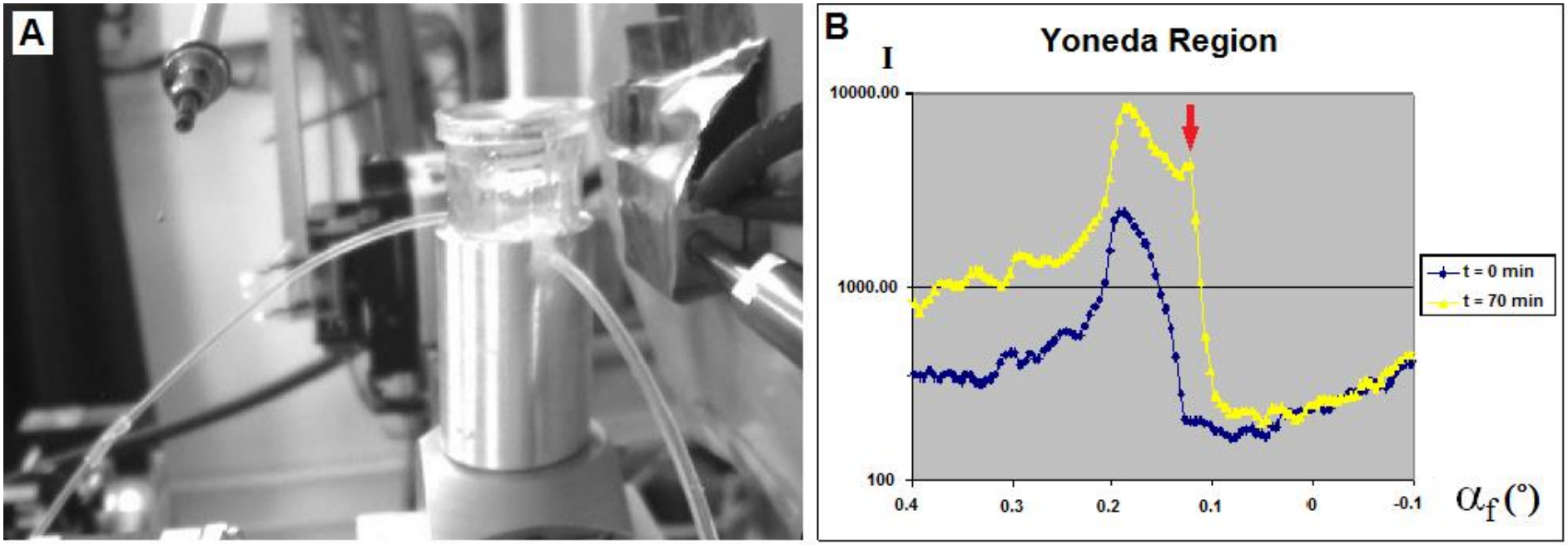
The tubes connected with pamps fo the salt solution exchange:

Cs=Cd "stop solution" for aliement procedure

Cs»Cd for accelerated nucleation

Cs=2Cd for controlled grouth

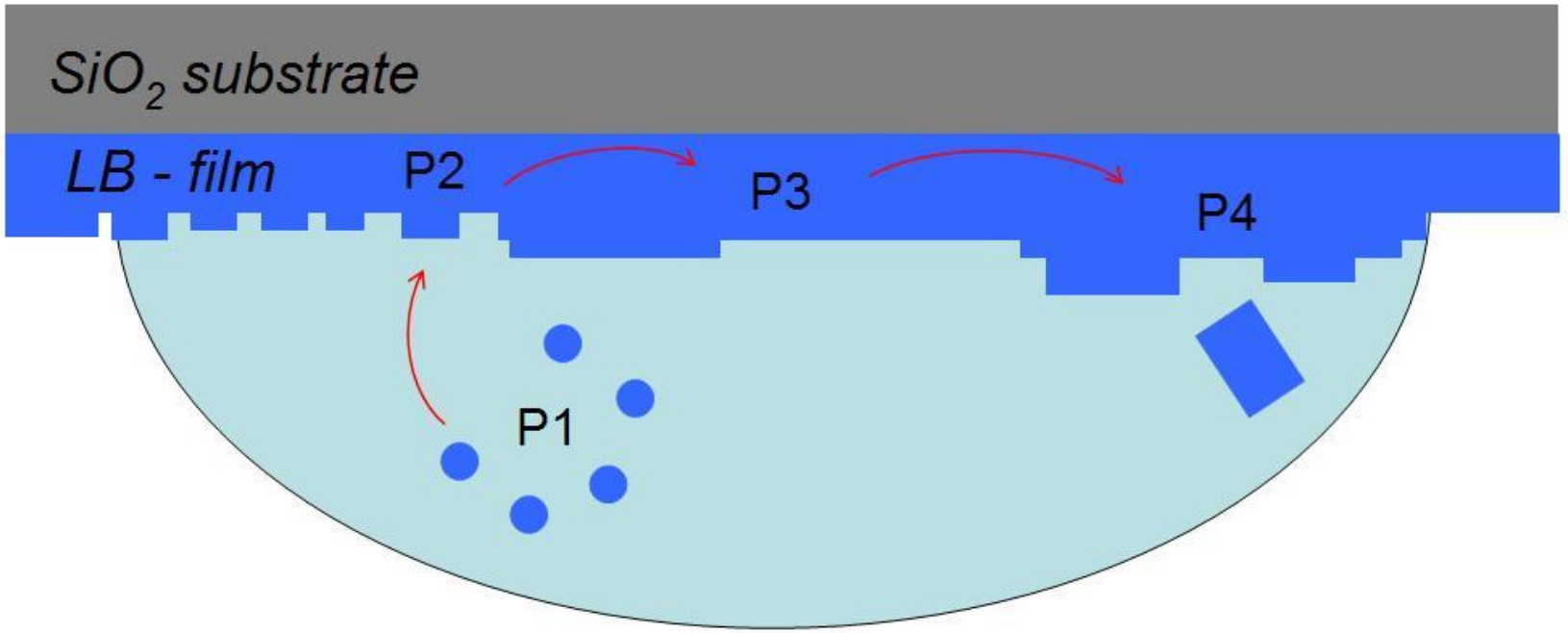
In situ submicron GISAXS of LB nanotemplate crystallization



(A) Actual experimental setup of *in situ* submicron GISAXS.

(B) The vertical cut is shown to demonstrate the time evolution of the **Yoneda-peak** formation for lysozyme crystal, with the appearance of a new peak (red vertical arrow) 70 minutes after plating, not present at 0 minutes after plating. This detector cut at fixed qy reveals information about the structures vertical to the sample surface.

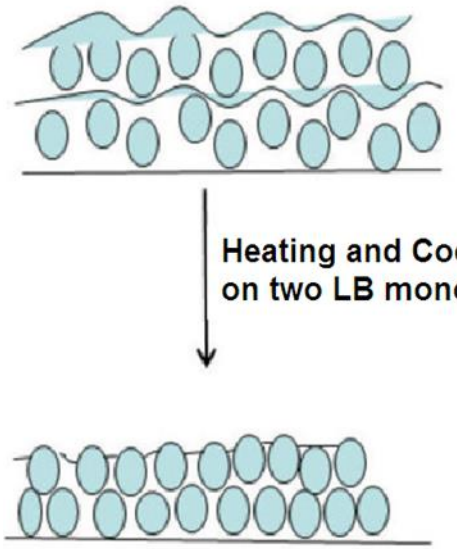
The temporal model of LB nanotemplate method for crystal formation



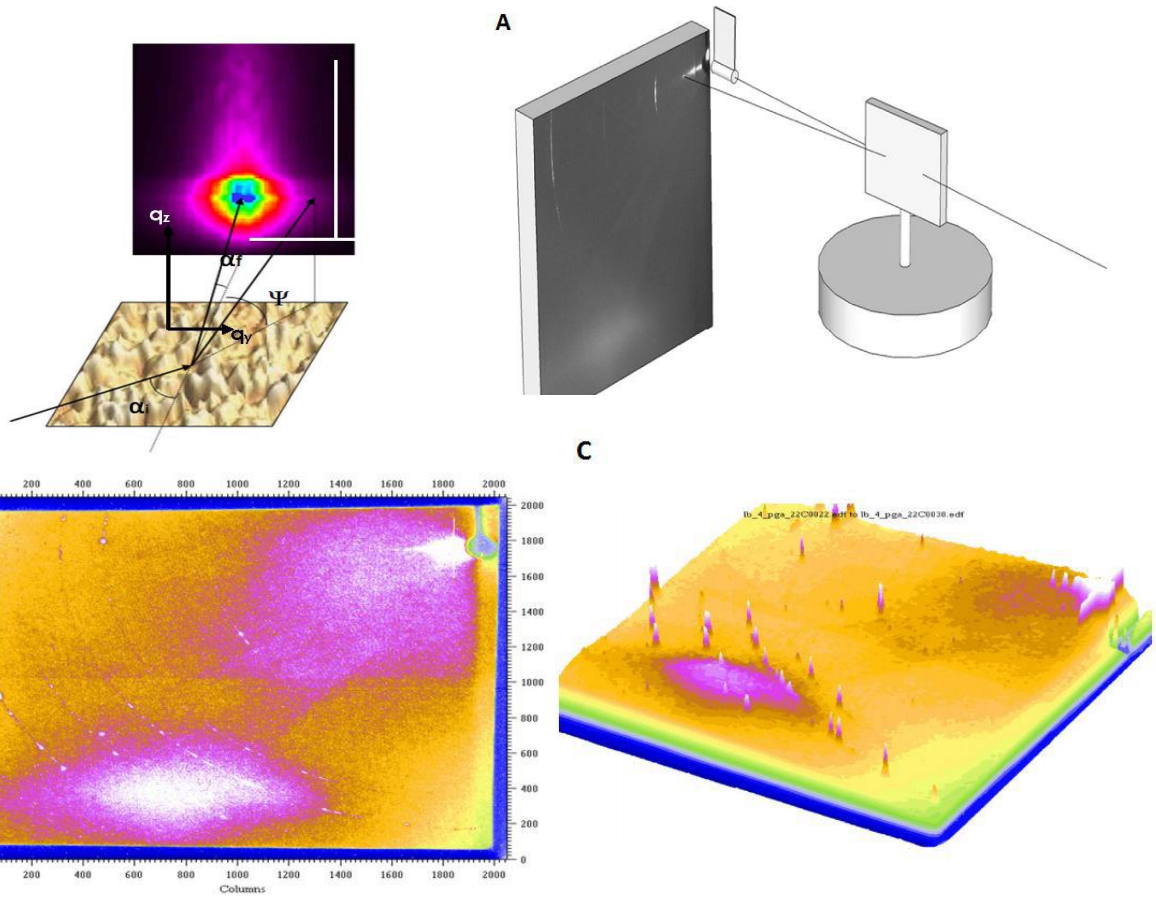
Protein solution P1 leads to protein association on the LB film states, P2 and P3, and to the crystal formation P4 detaching from the film in the drop.

Pechkova, Gebhardt, Riekel, Nicolini, Biophysical Journal, Part I, 2010
Gebhardt, Pechkova, Rielel, Nicolini, Biophysical Journal, Part II, 2010

Decreasing of film thickness and its re-ordering (increasing of structural correlation between two protein monolayers) upon heating up to 150°C and cooling to room temperature.



microGISAX (ID13) and Powder Diffraction (ID11) at ESRF

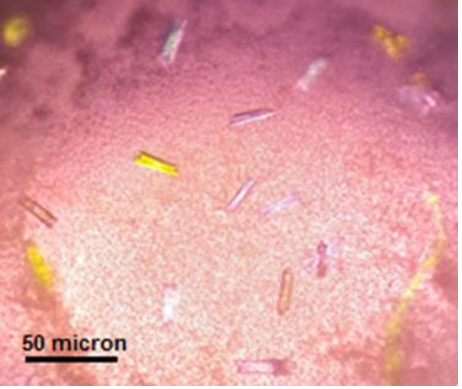


The edge of the detector is (roughly) 95 mm from the center **Θ is about 7.5° that corresponds to 2.6 Å d-spacing. The peaks are therefore between 3 Å and 10 Å in d-spacing.** (Nicolini, Whright, Pechkova, NWJ, 2015)

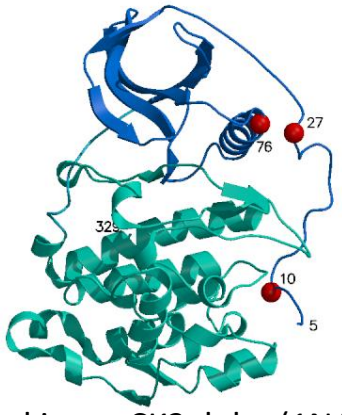
(A) 2D X-ray diffraction images were recorded at the ID11 ESRF beamline using the setup shown above and wavelength of 0.3444 Å. The sample was rotated to vary the grazing angle.

(B) The full area detected during the measurement.

(C) 3D representation of the area detector data for higly ordered protein (PGA) mulilayes at 22°C showing clear diffraction peaks. The spots visible in (B) and (C) are due to the reorganized multilayer film which shows now long range order.



Advantages of LB nanotemplate:



Human kinase CK2alpha (1NA7)

LB protein nanofilms (1-2 layers):

- Trigger protein crystallization and accelerate crystal growth
- Template for crystallization of proteins that cannot be crystallized by classical methods
- The crystals are more ordered and radiation stable in comparison with those obtained by classical methods

LB protein multilayers (20 – 100 layers):

✓ Achieve long range order after heating and cooling, including amyloidic structures

✓ Can be studied by advanced methods as X-ray nanodiffraction, Cryo-EM, XFEL

✓ Could allow to avoid the bottleneck of protein crystallization

Pechkova E., et al. **Appl. Phys. Lett.** 117, 053701, 2020.
Pechkova E., et al, **Langmuir,** 38, 86-91, 2022.

Nature Protocols, 12, 2570-2589, 2017

PROTOCOL

Langmuir–Blodgett nanotemplates for protein crystallography

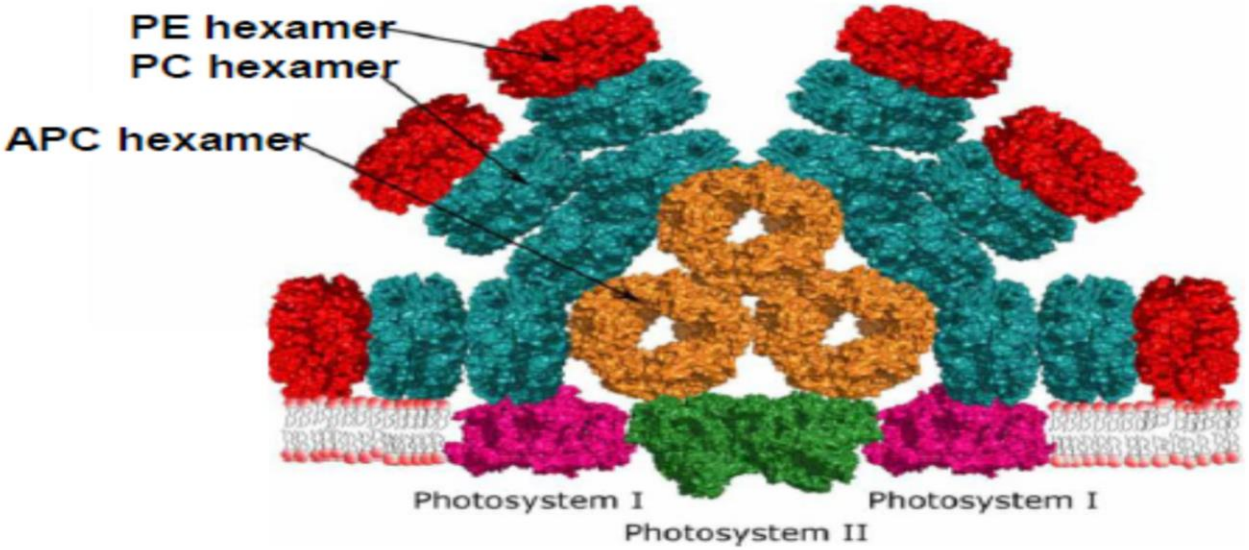
Eugenia Pechkova[1,2] & Claudio Nicolini[1,2]

[1]Laboratories of Biophysics and Nanotechnology, University of Genoa Medical School, Genoa, Italy. [2]Fondazione EL.B.A. - Nicolini, Pradalunga, Italy. Correspondence should be addressed to E.P. (eugenia.pechkova6@gmail.com).

Published online 30 November 2017; doi:10.1038/nprot.2017.108

The new generation of synchrotrons and microfocused beamlines has enabled great progress in X-ray protein crystallography, resulting in new 3D atomic structures for proteins of high interest to the pharmaceutical industry and life sciences. It is, however, often still challenging to produce protein crystals of sufficient size and quality (order, intensity of diffraction, radiation stability). In this protocol, we provide instructions for performing the Langmuir–Blodgett (LB) nanotemplate method, a crystallization approach that can be used for any protein (including membrane proteins). We describe how to produce highly ordered 2D LB protein monolayers at the air–water interface and deposit them on glass slides. LB-film formation can be observed by surface-pressure measurements and Brewster angle microscopy (BAM), although its quality can be characterized by atomic force microscopy (AFM) and nanogravimetry. Such films are then used as a 2D template for triggering 3D protein crystal formation by hanging-drop vapor diffusion. The procedure for forming the 2D template takes a few minutes. Structural information about the protein reorganization in the LB film during the crystallization process on the nano level can be obtained using an *in situ* submicron GISAXS (grazing-incidence small-angle X-ray scattering) method. MicroGISAXS spectra, measured directly at the interface of the LB films and protein solution in real time, as described in this protocol, can be interpreted in terms of the buildup of layers, islands, or holes. In our experience, the obtained LB crystals take 1–10 d to prepare and they are more ordered and radiation stable as compared with those produced using other crystallization methods.

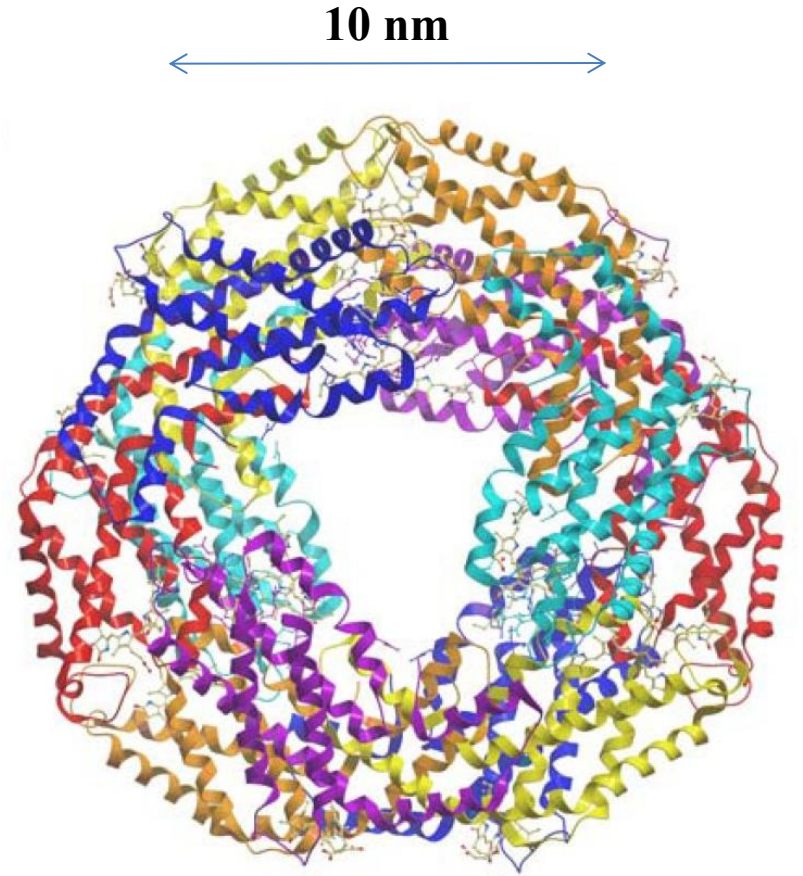
The observed natural structures of cyanobacterial light harvesting complex suggest that rather than single molecules - irrespective of their molecular complexity - hierarchical macromolecular architectures are necessary to perform photosynthetic energy conversion.



Simplified scheme of a cyanobacterial light harvesting complex (phycobilisome), PE: phycoerythrin, PC: phycocyanin, APC: allophycocyanin.

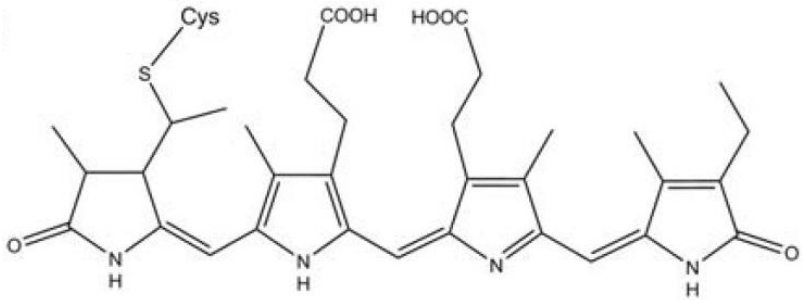
Adapted from Saer, R.G.; Blankenship, R.E. Light harvesting in phototrophic bacteria: Structure and function. Biochem. J. ***2017****, 474, 2107–2131*

Phycocyanine from cyanobacteria

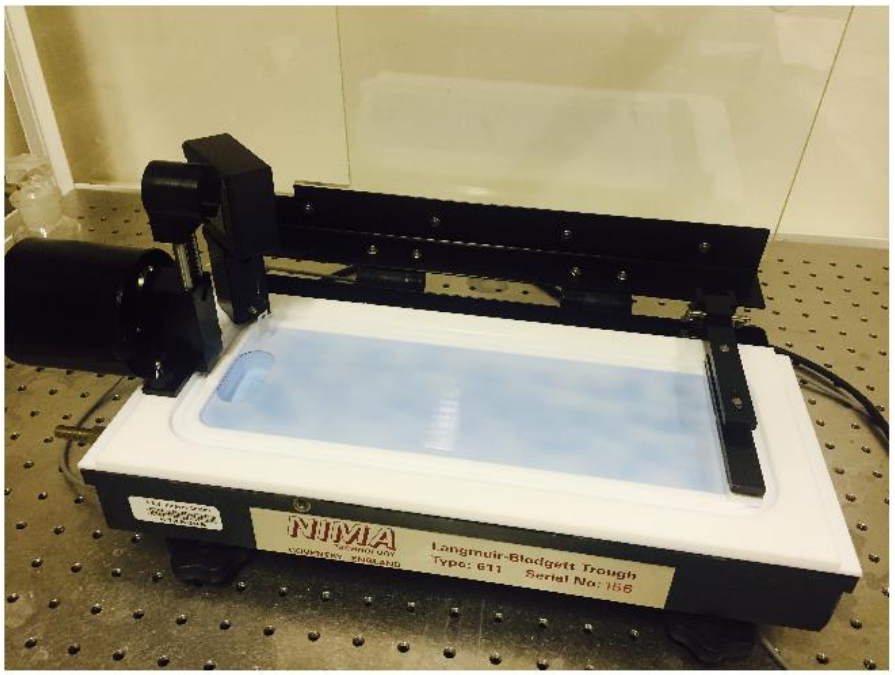


120 kDa

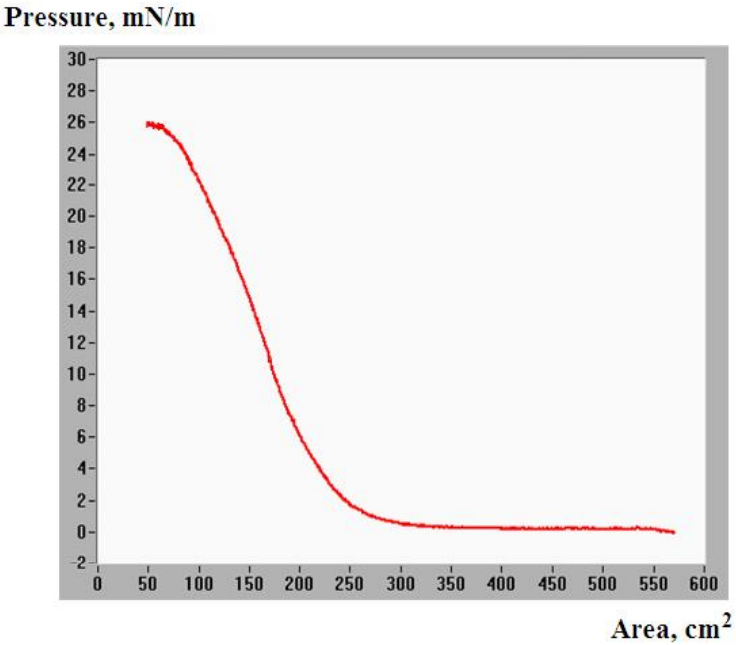
Crystal structure PDB No. 1GH0 of a c-phycocyanin hexamer of *Arthrospira platensis* α-subunits are colored red, yellow and orange, β-subunits are colored blue, cyan and violet, phycocyanobilin co-factors are depicted as atom-colored ball-stick representations.



Chemical structure of the phycocyanobilin chromophore in the open form, attached to the cysteine of the carrier protein.



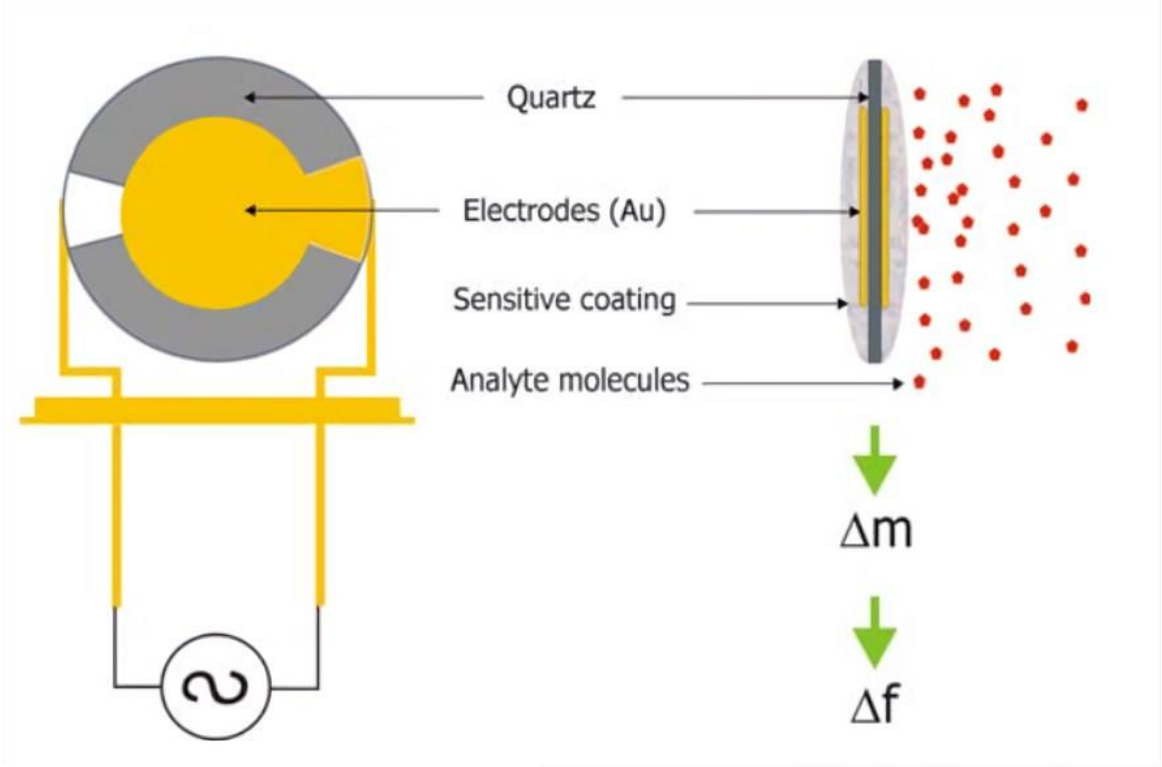
(a)



(b)

(a) LB trogh with PC on the air-water interface

(b) PC LB isotherm at r.t. The surface pressure of PC LB MLs deposition was 26 mN/m.

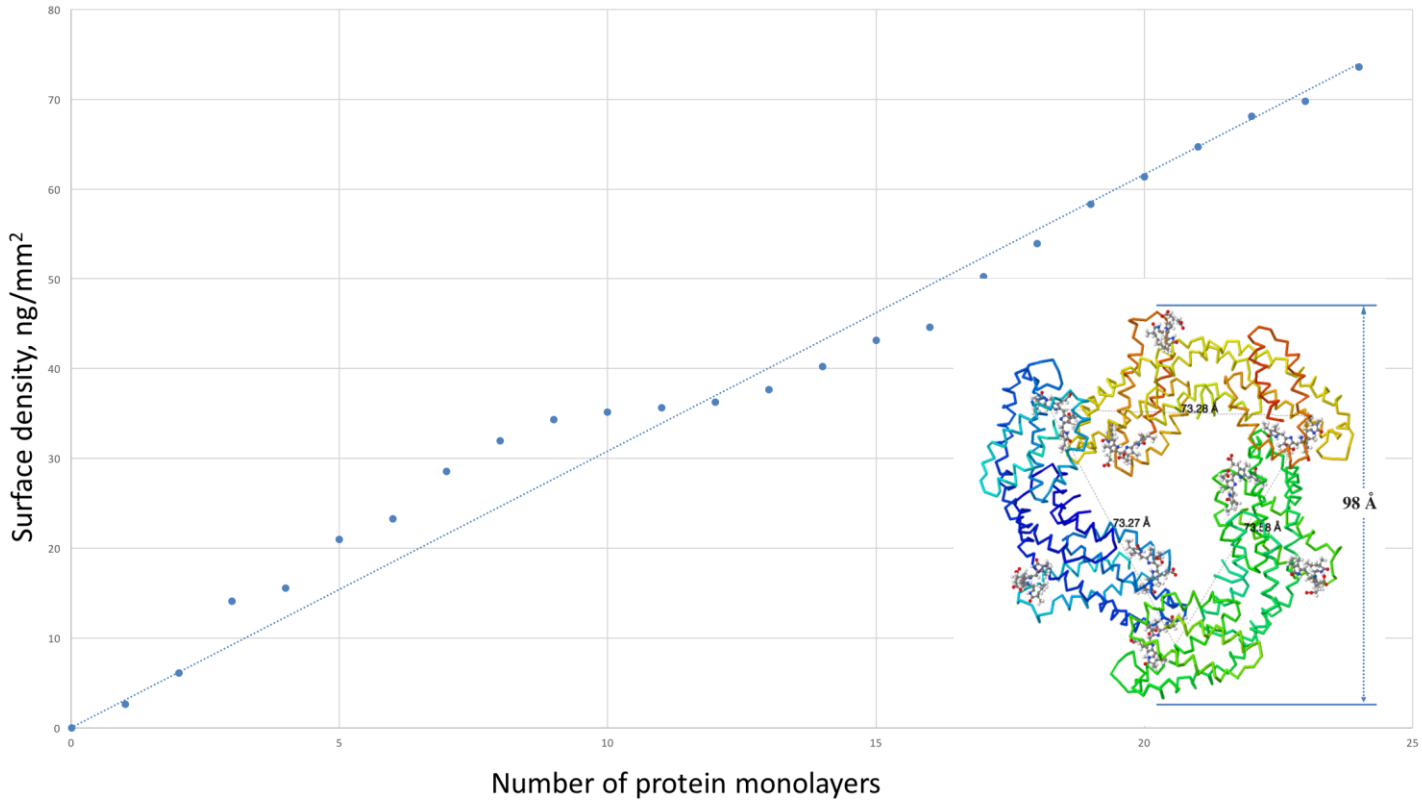


The basic principle of QCM is the piezoelectric effect of quartz crystal.

Upon an alternating voltage applied to the two poles of the quartz crystal, the quartz crystal of QCM produces mechanical vibration or oscillation.

This resonance is greatly sensitive to the thickness of the crystal/electrode system and the frequency of the acoustic frequency. Once the mass on the quartz crystal increases or decreases, the resonance will be disturbed and the resonance frequency of

QCM measurements of subsequent 24 PC LB layers deposition onto a quartz oscillator substrate resulting in a linear surface mass density increase with the number of PC monolayers deposited. Each data point corresponds to a deposited monolayer. The inset shows the PC molecule hexamer (PDB code 3L0F)



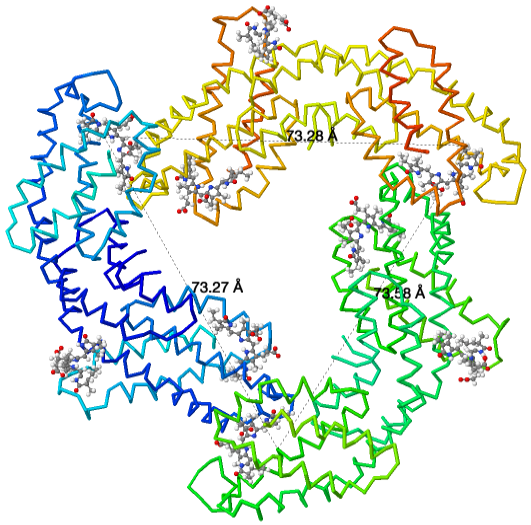
Dependence of the surface density of PC protein on the quartz oscillator upon the number of transferred protein monolayers, the surface density of one PC monolayer corresponds to 3 ng/mm^2

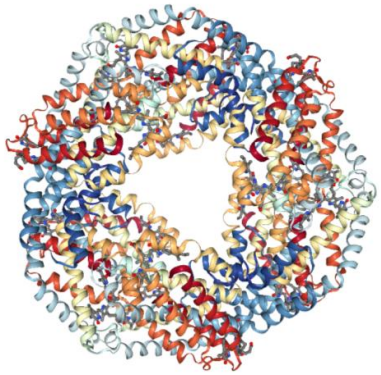
Knowing PC **Molecular Weight (120 kDa),** experimental **surface density** value can be used for estimation of the number of protein molecules for one millimeter square (N) and, consequently, the area for one molecule, A [nm^2] in the LB monolayer:

$$N = (\Delta s \times N_A)/ MW \times 10^{12}$$

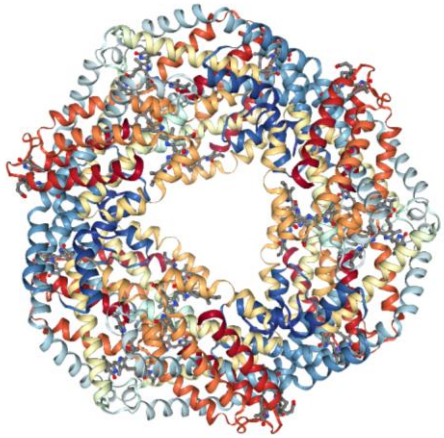
$$A = 10^{12}/ N$$

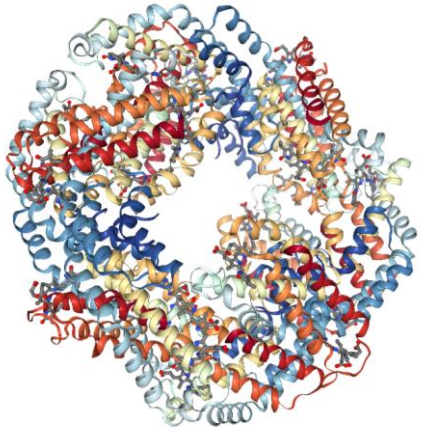
equal to **67 nm^2.** Taking into consideration the geometric features of PC protein molecule (PDB code, see Figure 4), one can conclude that deposited protein monolayers are highly packed homogeneous and deposition of each layer is reproducible. Indeed, from the PC PDB structure's geometric features the area of one PC molecule is about **70 nm^2.**

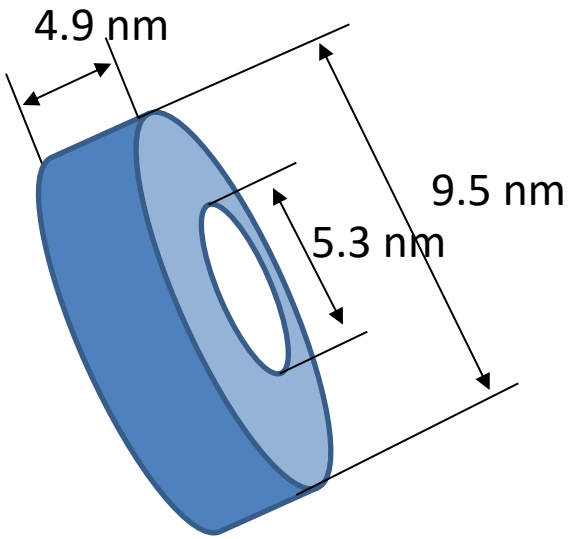




Phycocyanin 3D structure (3L0F)







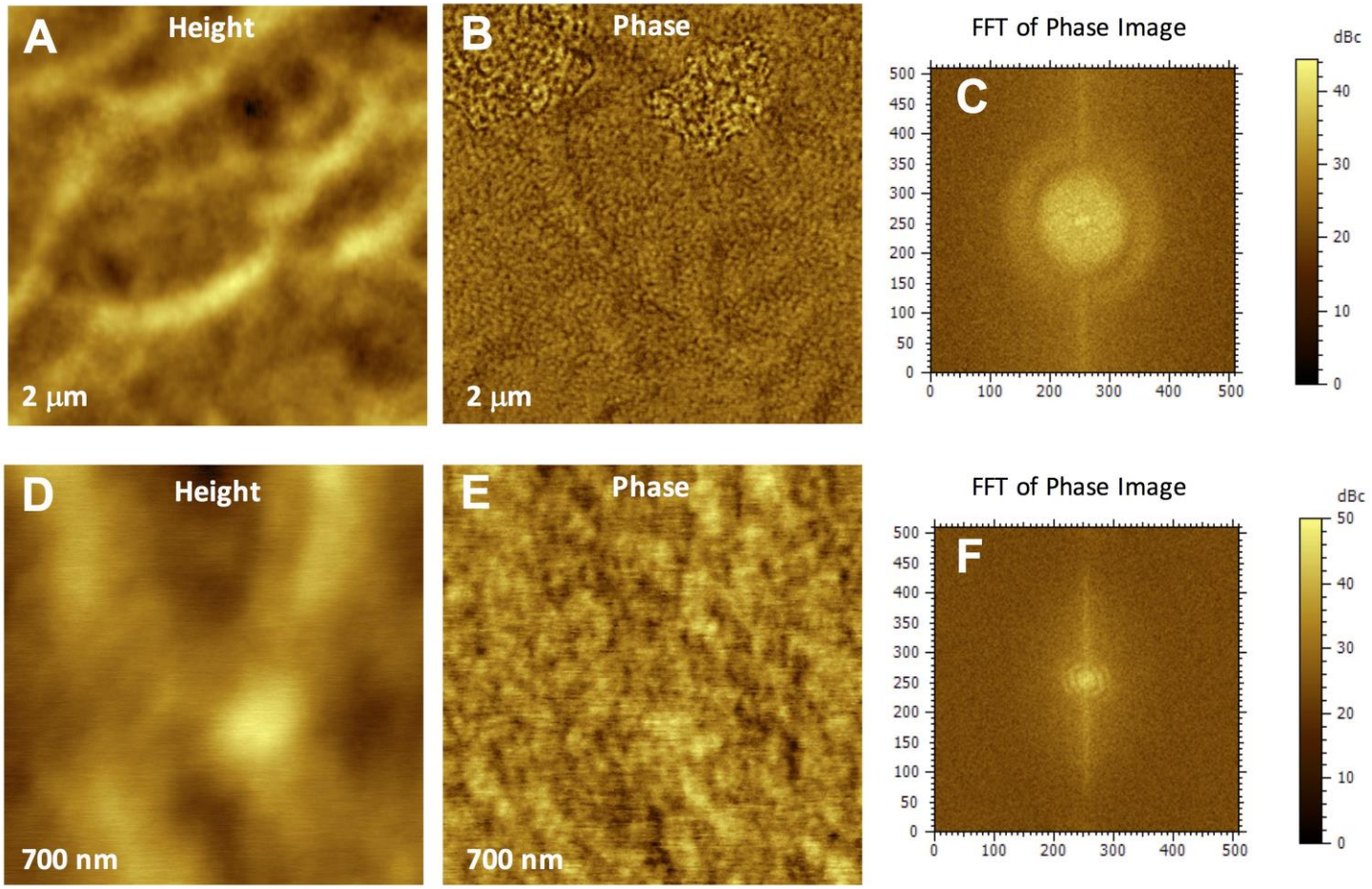
70 nm^2 (R= 4.75 nm)

LB Phycocyanin monolayer on quartz surface

The number of PC protein molecules in 1 mm ^{2} of one LB protein monolayer

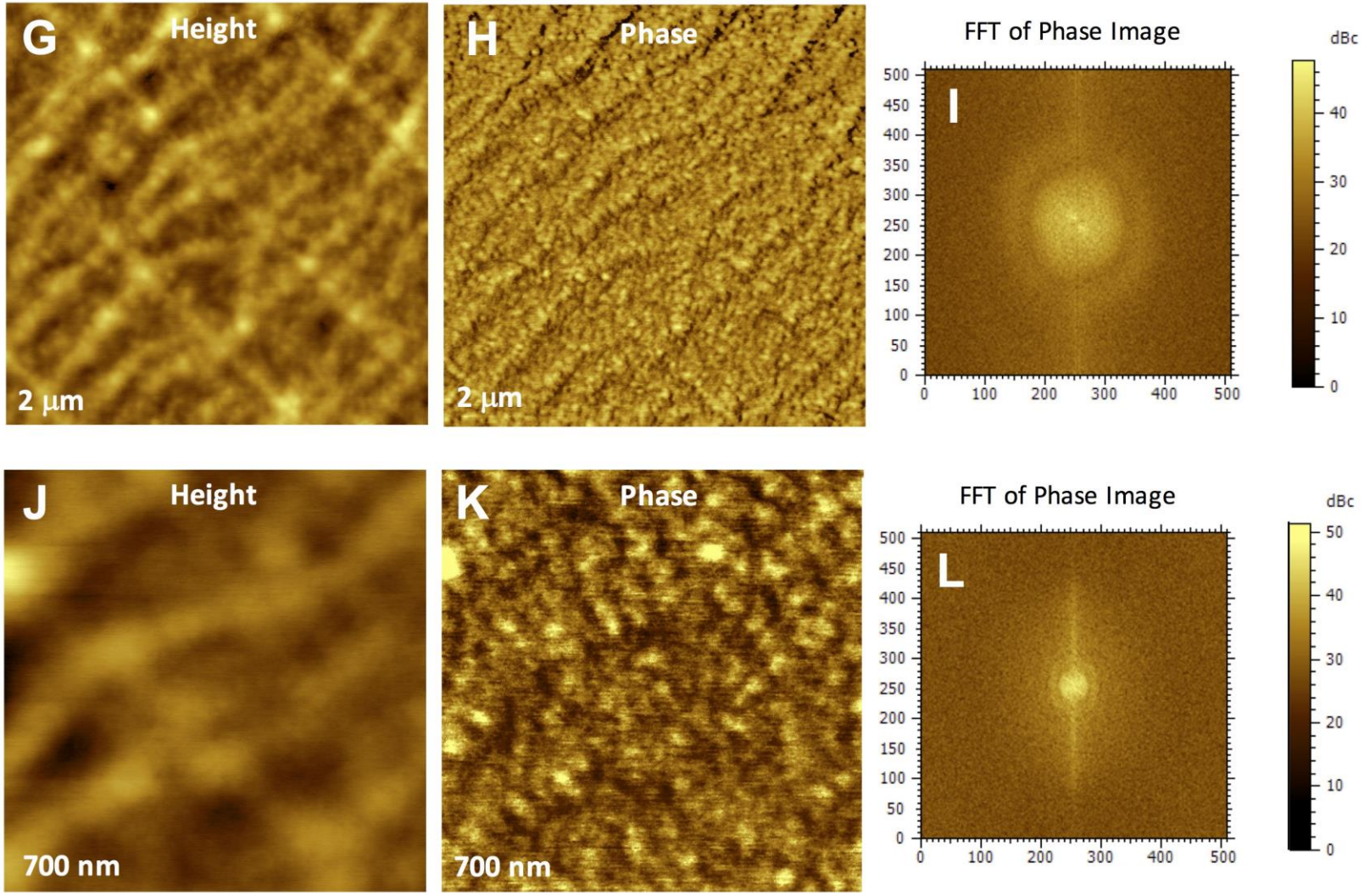
3x 6,02214076×10^{23}/120 10^{12} = 0,15 x 10^{11} = **1,5 x 10^{10} molecules**

AFM measurements



(A-F) Height and phase AFM images of PC LB-MLs (20 layers) at r.t. (before heating), the square measures 2x2 microns for upper and 700x700 nm for lower images. The corresponding Fast Fourier Transform (FFT) of phase images are shown on the right.

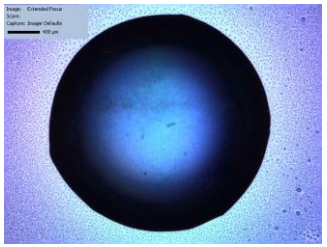
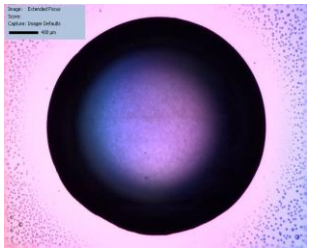
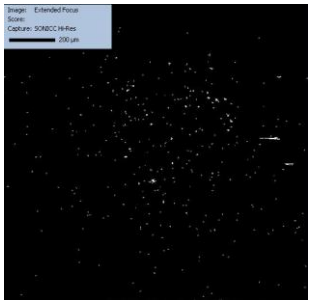
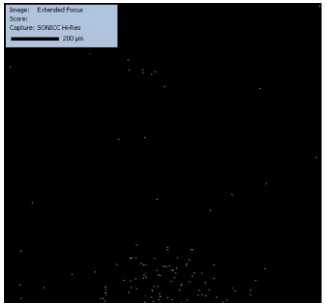
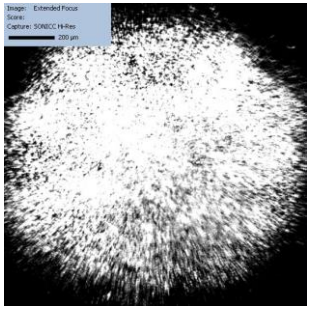
AFM measurements



(G-L) Height and phase AFM images of PC LB-MLs (20 layers) after heating to 150˚C for 10 minutes, the square measures 2x2 microns for upper and 700x700 nm for lower images. The corresponding Fast Fourier Transform (FFT) of phase images are shown on the right.

SONICC analysis: Initial step of phycocyanin crystallogenesis

Second-order harmonic generation (SHG) spectroscopy, in particular the **SONICC (second order non-linear imaging of chiral crystals)** instrument invented by G. Simpson. SONICC can identify nanocrystals of chiral molecules as small as 100 nm. When a chiral crystal is exposed to two 1024 nm photons in a strong field, frequency doubling occurs due to inherent polarization anisotropy, allowing a detector to measure the 512 nm photon output.

	Classical HD	LB nanotemplate
		
0.5 hours		
22 hours		

PC from *Thermosynechococcus elongatus*
deposied onto the Si_3N_4 membrane
(SILSON Ltd, 5x5 mm, thickness of 500nm) studied by:

	microGISAX	nanoXRD	Micro ED	XFEL
Phycocyanin ***Thermosynechococcus elongatus***	120 layers	120 layers	20 layers	20 layers

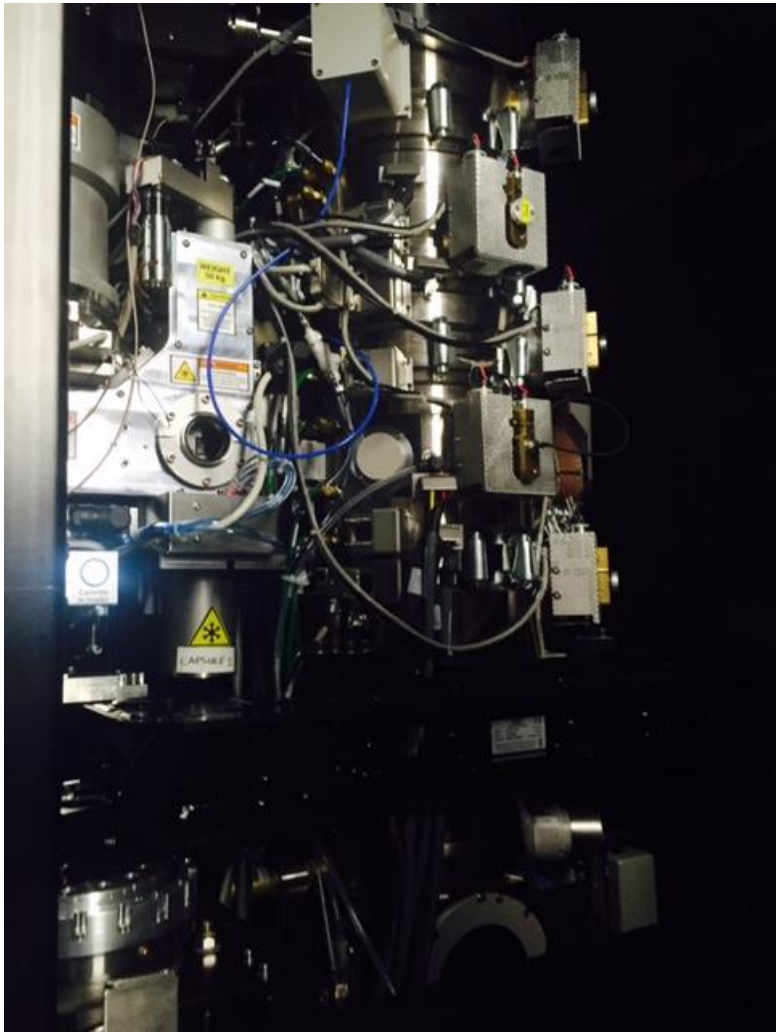
microGISAX – microfocus Grazing Incidence Small Angle scattering - ID13 ESRF

nanoXRD - X-ray nanodiffraction – ID13 microfocus beamline ESRF

MicroED - cryo-electron microscopy in microbeam electron diffraction (microED) mode, Titan Krios electron microscope (FEI) operating a 300 keV (λe = 0.0022 nm), ASU, USA

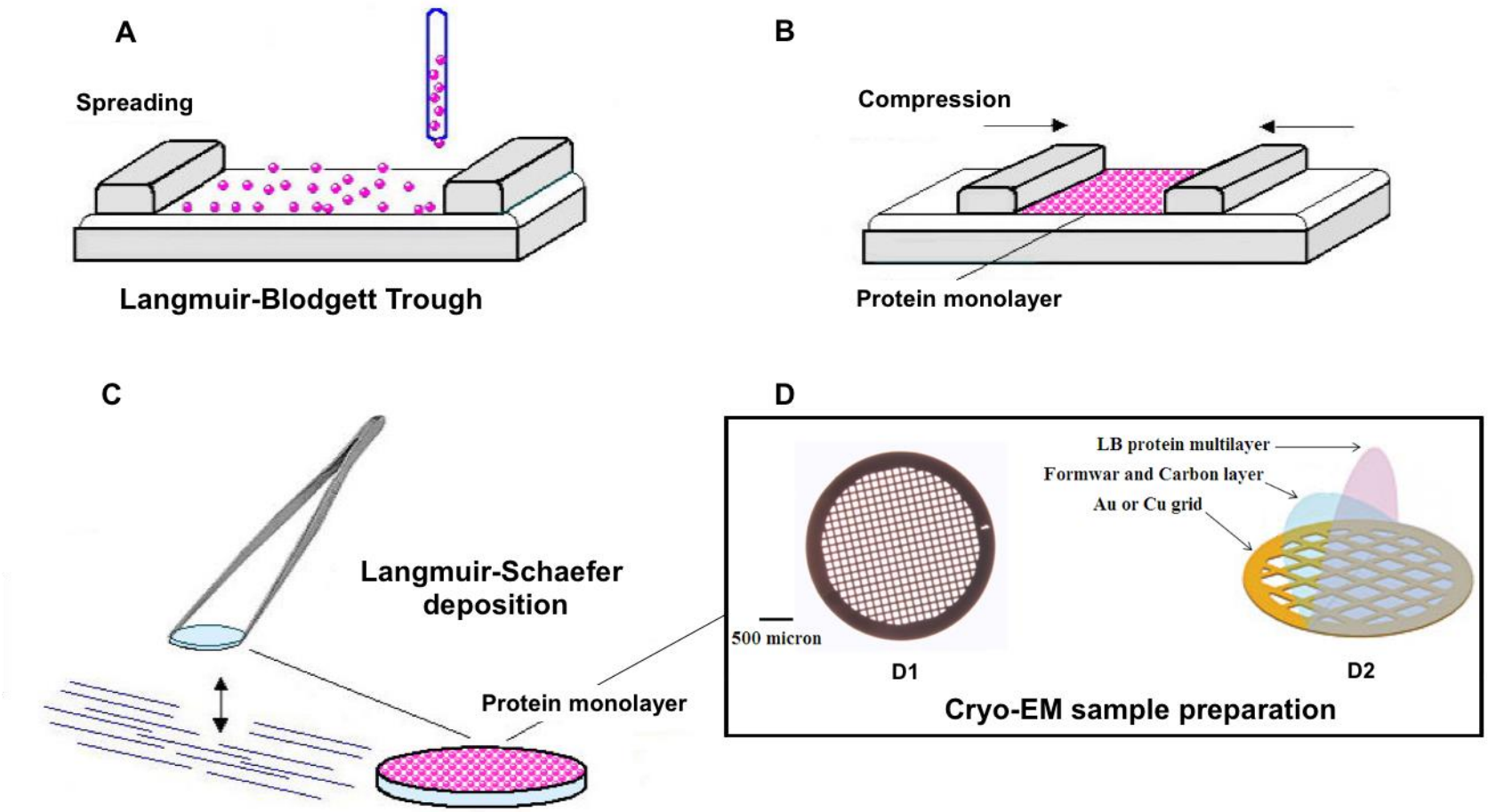
XFEL – X-ray Free Electron Laser, LCLS, SLAC, Stanford University, USA

Cryo-Electron Microscopy of LB protein multilayers



•Cryo Electron Microscopy was performed on a **Titan Krios transmission electron microscope (FEI)** equipped with Gatan K2 Summit direct electron detector and operating at 300 kV (λe = 0.0022 nm). Cs for this instrument 2.7 mm. Electron diffraction images were recorded with Ceta camera that is a hybrid CMOS 4K x 4k camera. Cryogenic sample-grids were rapidly transferred and cooled in liquid ethane (−188°C). Electron beam was estimated as 5.6 ± 0.1 µm.

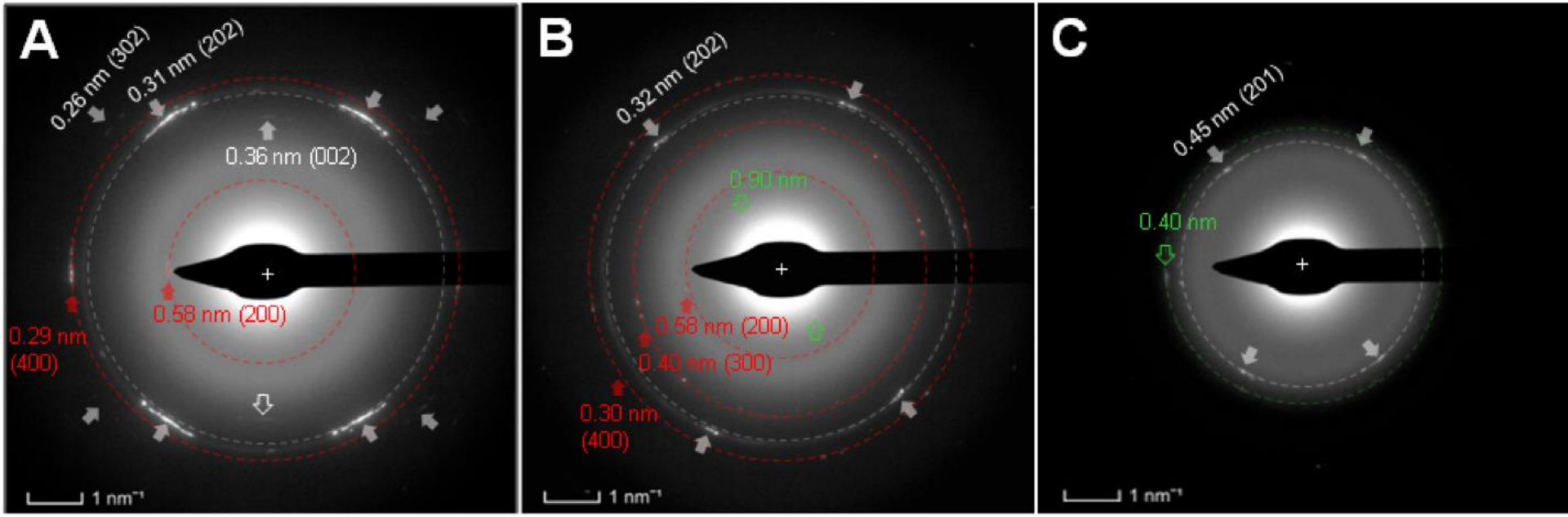
Pechkova E. Emerging advanced techniques for the protein nanofilms characterization. NanoWorld J, 7, p. 33-34, 2021.



Schematic design of Langmuir−Blodgett technique. (A) Spreading and (B) compression of the protein monolayer on the LB trough surface. (C) Solid substrates (quartz, mica slide, cryo-EM grid, Si3N4 membrane) are held with forceps for multiple manual LB deposition (LS variation). Inset D: (D1) Microscopic image of cryo-EM copper mesh grid used for the LB protein multilayer deposition. (D2) Schematic illustration of the protein multilayer sample for Micro-ED: the protein multilayers (20 layers) deposited on the copper or gold grids, previously coated with formware and carbon layer.

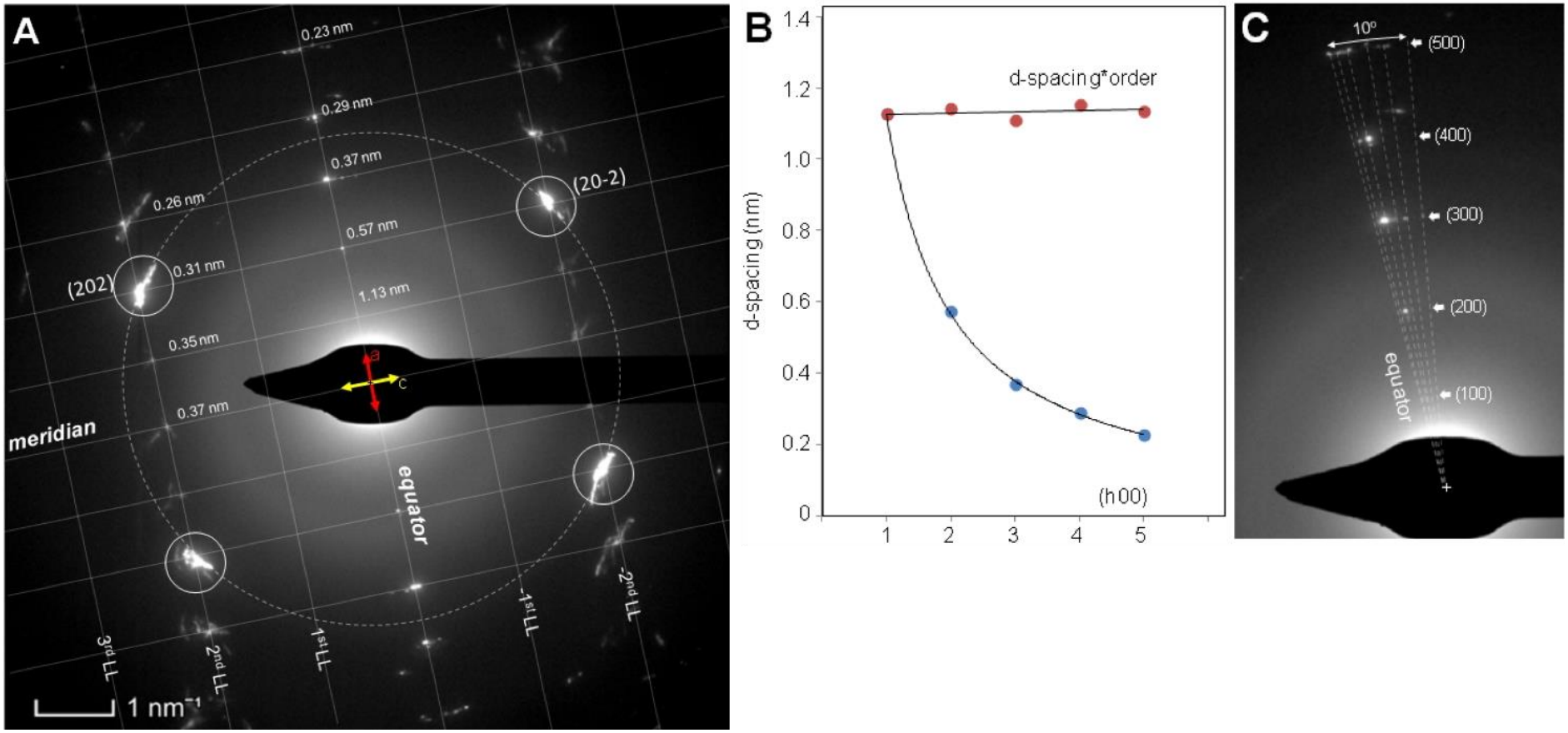
MicroED measurements

PC patterns with extensive disorder. Reflections observed also in (A) are marked by red (h00) and white (h0l) filled arrows. Textured powder rings are depicted by dashed rings. The radii of dashed rings for the (202) peaks in (A,B) have been slightly reduce to reveal the coaxial continuous powder rings. Peaks which do not match the (h0l) plane are marked by green open arrows.



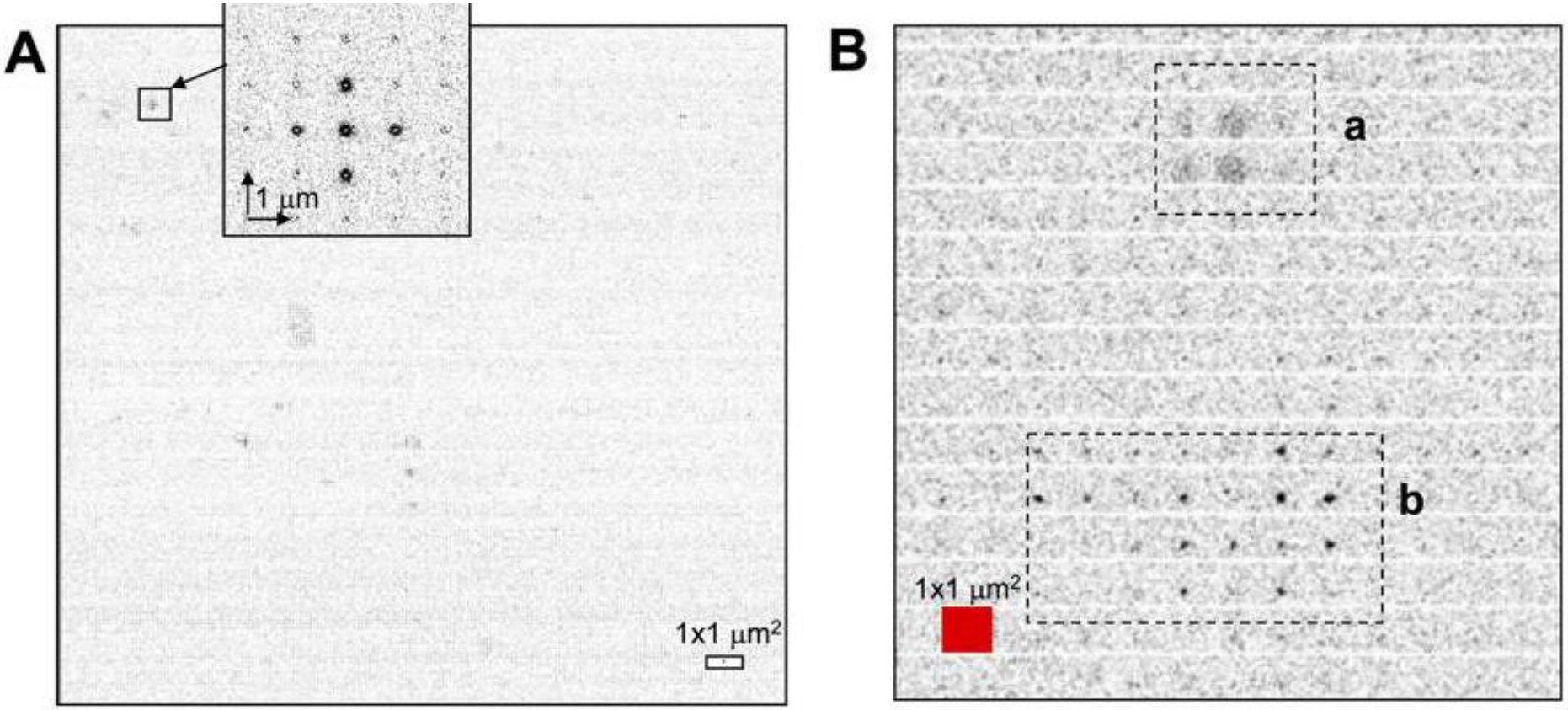
Pechkova E., Burghammer M., Nicolini C., Riekel C. New structural features appear in thermally treated Langmuir-Blodgett protein multilayers. ***NanoWorld J*** 6(3), 66-67, 2020.

MicroED measurements



(A) PC fiber texture pattern with meridional (c-axis) and equatorial (a-axis) axes. d-Spacings are indicated for selected reflections.
(B) Determination of equatorial period from five (h00) orders.
(C) Zoom into equator showing azimuthal splitting of (h00) orders into subpeaks.

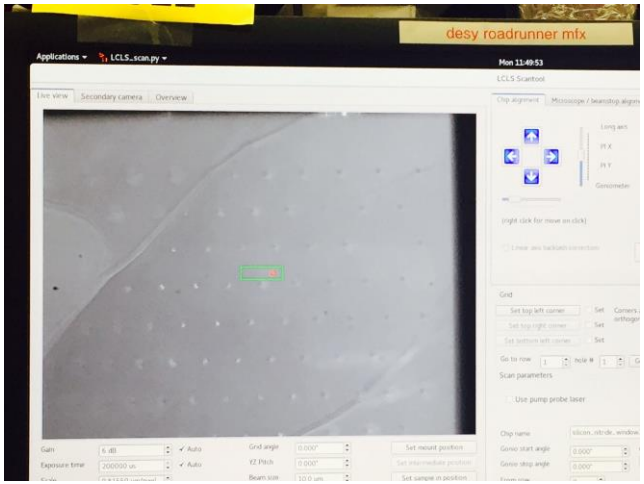
Pechkova E., Nicolini C., Fiordoro S., Riekel C. Mesoscale ordering of Phycocyanin molecules in Langmuir-Blodgett multilayers. ***Langmuir***, 38, 86-91, 2022



(A) Composite X-ray density map of PC MLs based on 1 µm step increments revealing patches with increased SAXS around the beamstop, suggesting an onset of large-scale assembly. (B) Enlarged composite density map showing patches (a) with extended angular scattering up to the d ~ 1.6 nm resolution limit and (b) with central scattering around the beamstop.

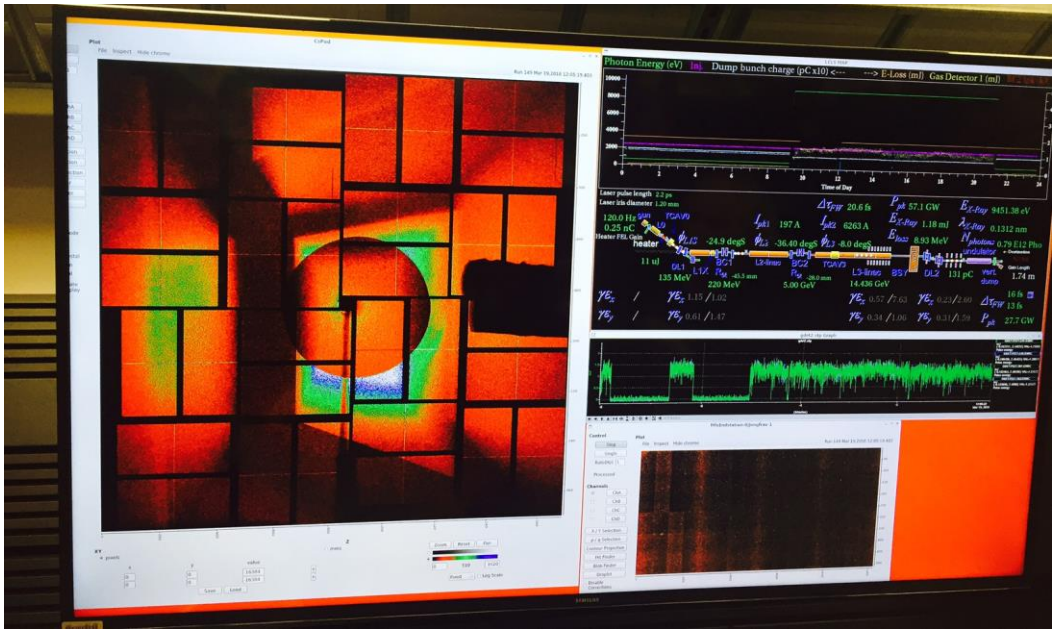
Pechkova E., Nicolini C., Burghammer M., Riekel C. Emergence of amyloidic fibrillation in 2D-ordered Langmuir-Blodgett protein multilayers upon heating. ***Appl. Phys. Lett***. 117, 053701, 2020.

LSLS XFEL experiments on protein multilayers

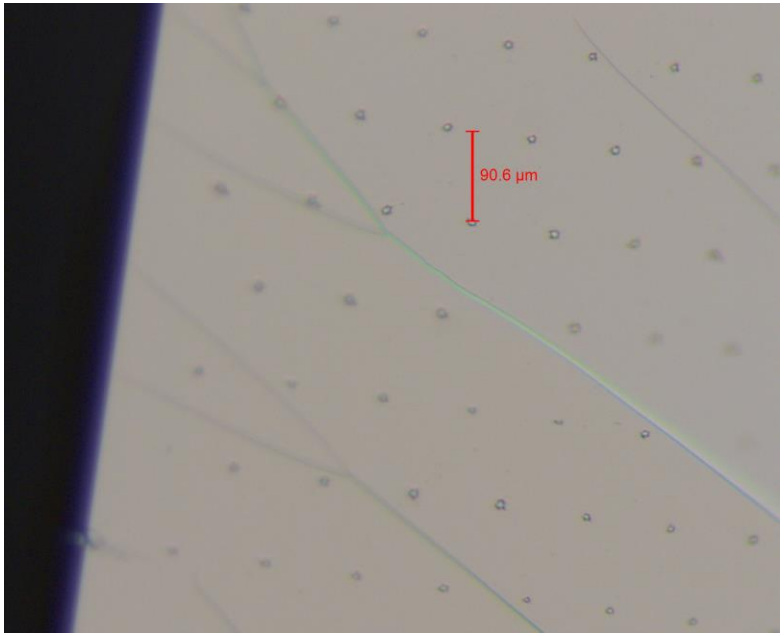


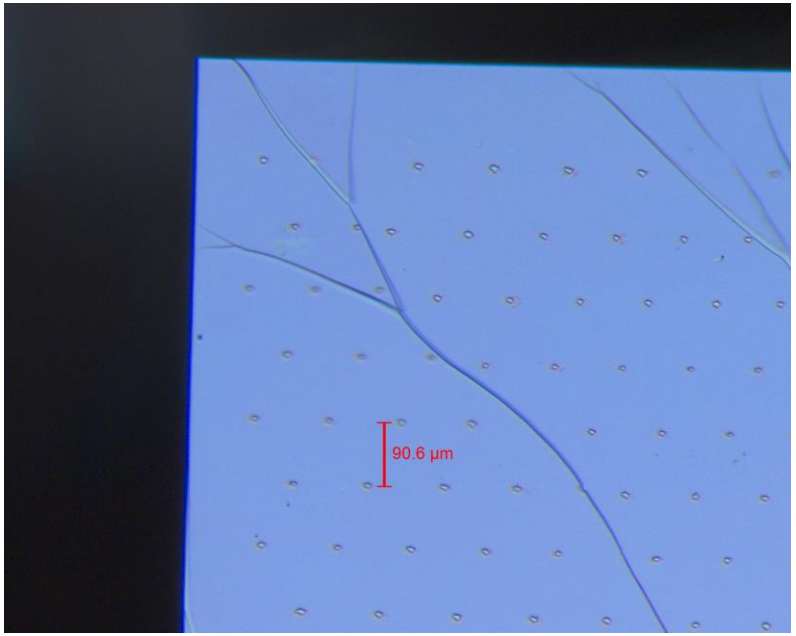
Roadrunner system

Phycocyanin and PSI LB films
5, 10, 15, 20 layers on
Si_3N_4 membranes:
1. Stored at r.t.
2. Heated to 150 ˚C and cooled to r.t.



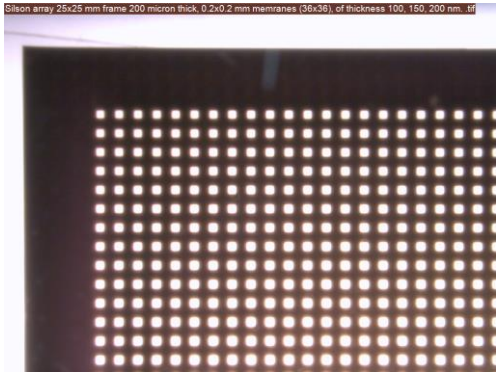
LB phycocyanine multilayers by XFEL (LCLS, SLAC, USA)





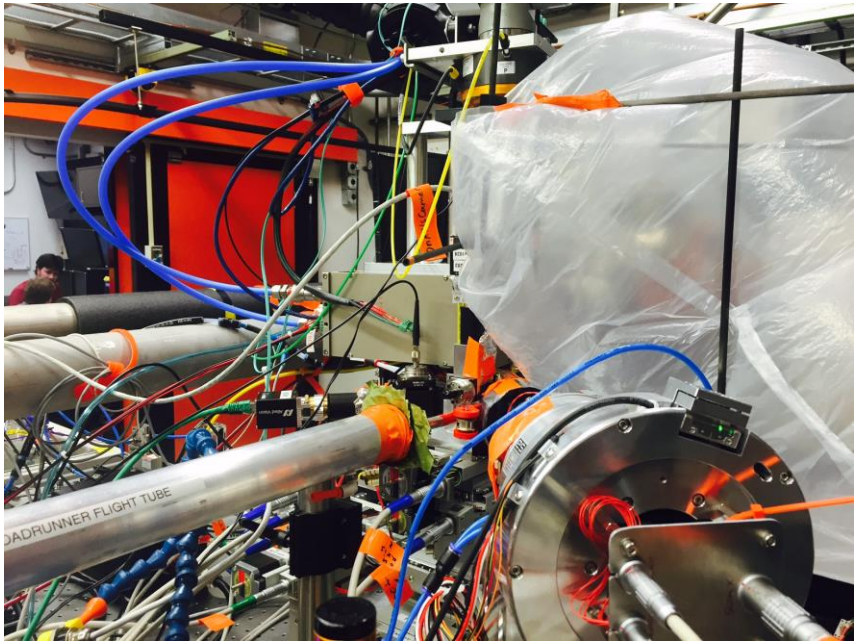
Left - XFEL scanning of the Si_3N_4 membrane of 200nm thickness.
Right - XFEL scanning of the Phycocyanin LB multilayered film (20 monolayers), heated up to 150°C and cooled down to the room temperature.

Another oprtion is to use the Si_3N_4 arrays
(e.g. 0.2x0.2 mm 36-window array of 200 nm thickness)



Pechkova E., Nicolini C. Langmuir-Blodgett protein multilayer nanofilms by XFEL. ***NanoWorld J*** 4(4): 48-53, 2018.

XFEL data collection from Phycocyanin LB multilayered films

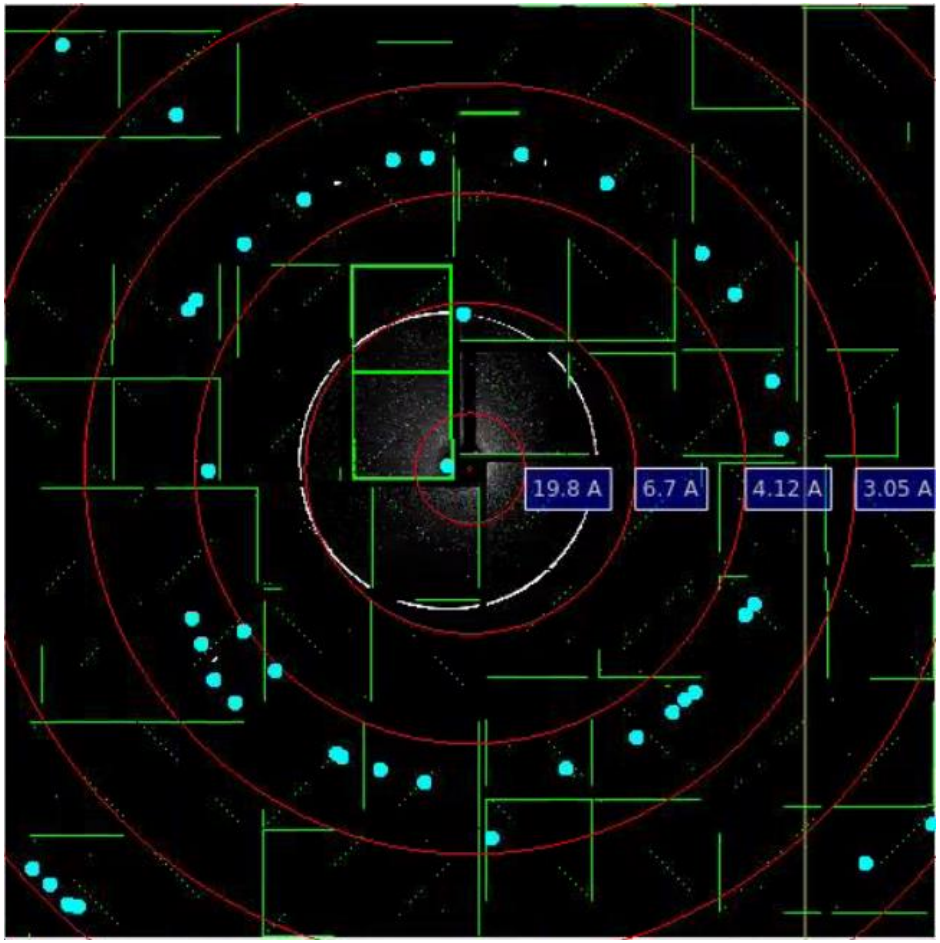


Duration (sec)	Sample	X ray transmission	T°C	Humidification (%) Sens 1	Sens 2	Sens 3	Sens 4	Det.pos. (mm)	Energy (eV)	Freq (Hz)	Puls E (mJ)	Puls Duration
	1 empty SiN4 window 200 nm, no sample	0.5-100%	24.2	24.9	25.52	24.61	25.67	313	9456	120	1.6	20 fs
2:55												
3:11	Phyco 20 rt	5-100%	24.2	24.9	25.52	24.61	25.67	313	9456	120	1.6	20 fs
2:27	Phyco 20 150	5-40%	24.2	24.9	25.52	24.61	25.67	313	9456	120	1.6	20 fs
37	Phyco 20 150	62	24.2	24.9	25.52	24.61	25.67	313	9456	120	1.6	20 fs

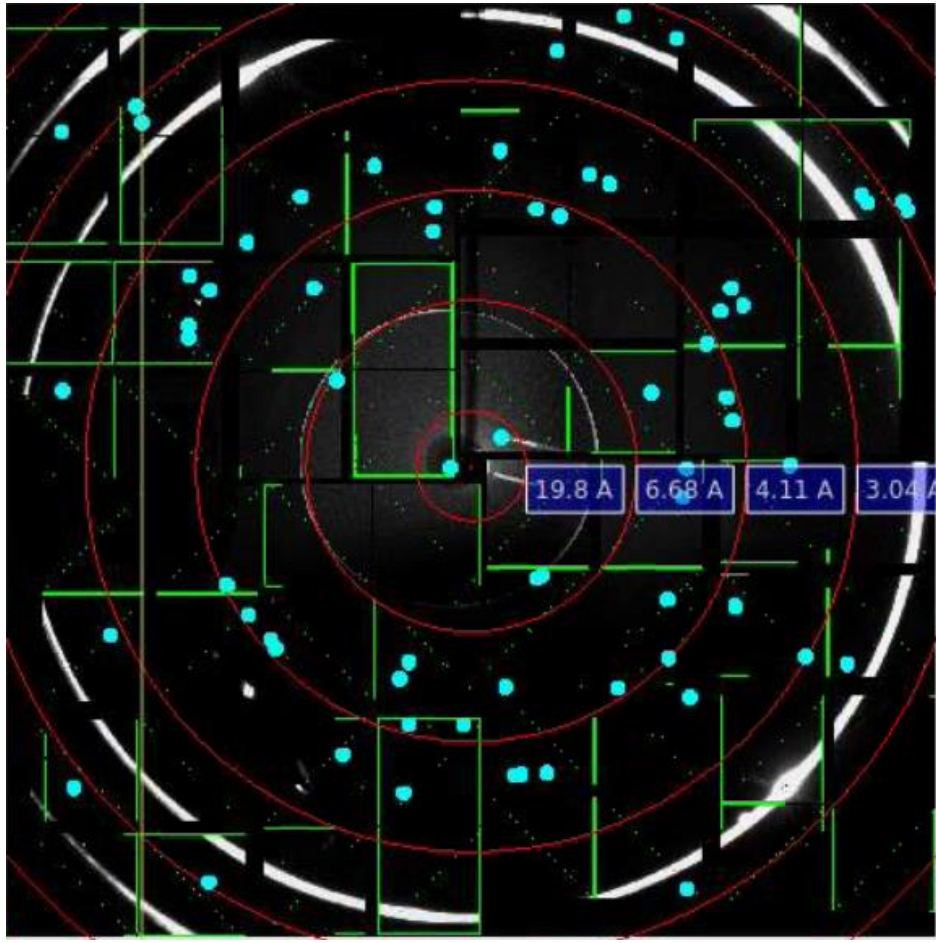
22920 images were collected from room temperature LB film sample and 22080 images from heated up to 150°C and cooled LB film sample, which remained stable after this experiment

Phycocyanin LB MLs studied by XFEL, SLAC, Stanford University

r.t.

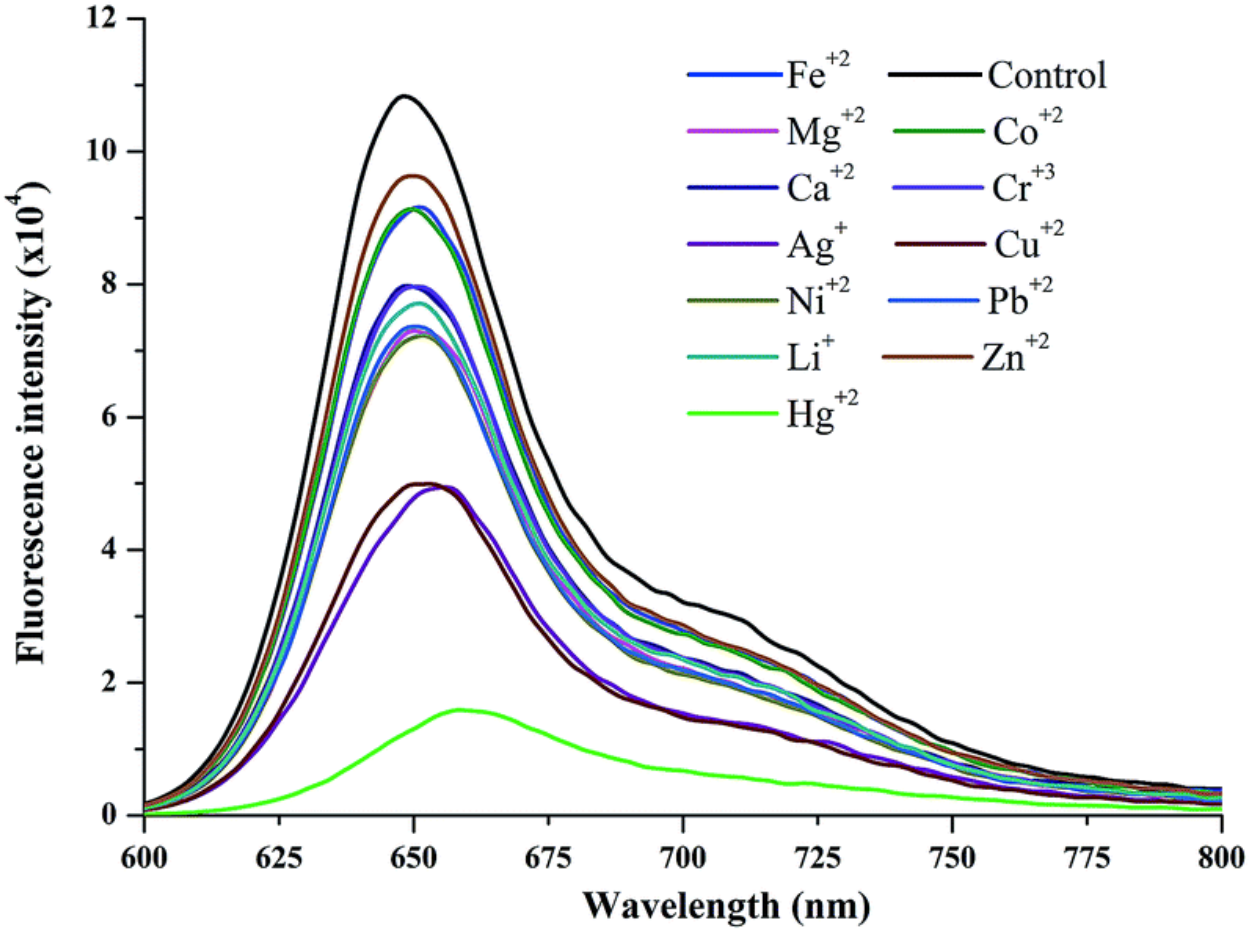


150° C



Effect of different metal cations on the fluorescence emission of PC.

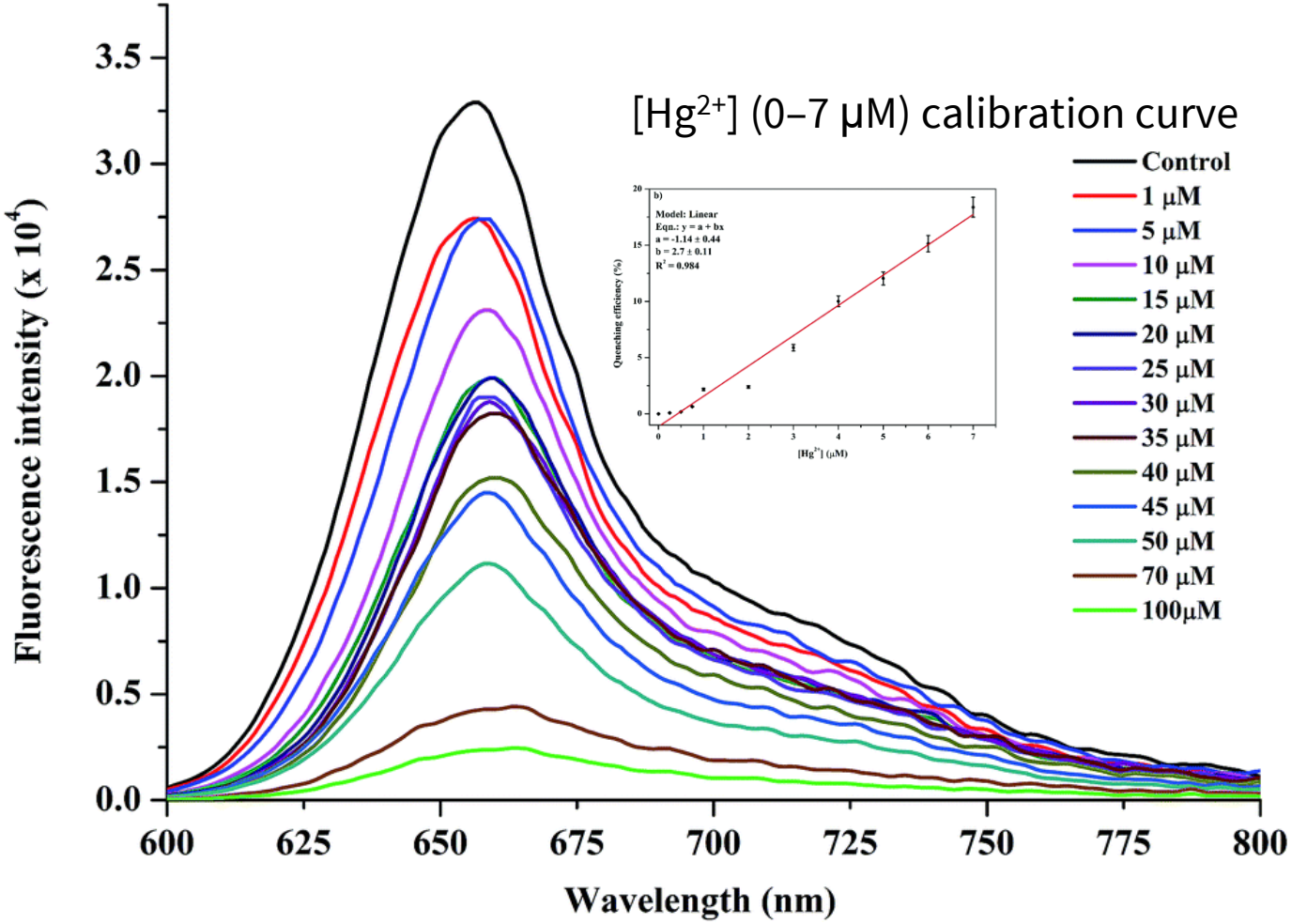
Hg^{2+} associated with major quenched response from PC



Bhayani et al, ***RSC Advances.***, 2016,**6**, 111599-111605

The fluorescence titration of CPC with increasing Hg^{2+} concentrations (1–100 µM)

PC fluorescence emission shows a consistent decthe fluorescence intensity with increasing concentrations of Hg^{2}rease in $^{+}$



Bhayani et al, ***RSC Advances.***, 2016, **6**, 111599-111605

Bellamy-Carter, ***The FEBS Journal*** 2022, **289,** 4646–4656

Conclusions:

- **Phycocyanin molecules**, which are part of light-harvesting complexes in cyanobacteria, can be assembled into mesoscale multilayer nanofilms by the Langmuir− Blodgett (LB) technique.

- Results obtained by quartz crystal microbalance (QCM) and atomic force microscopy (AFM) confirm the homogeneity and reproducibility of phycocyanin LB multilayer deposition.

- Cryo-electron microdiffraction, X-ray nanodiffraction and XFEL show that amorphous phycocyanin LB multilayers form, after annealing at 150 ° C and cooling to room temperature, a layered nanofibrillar lattice.

- **Hierarchical macromolecular architectures of LB multilayers can find their application in the development of PC-based biosensor for heavy metals in aqueous systems.**

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