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Editorial

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Emerging Advanced Techniques for the Protein Nanofilms Characterization

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With the new generation of synchrotrons and micro- and nano-focussed beamlines a great progress is achieved in the area of X-ray protein crystallography resulting in new protein 3D atomic structures of high interest to pharmaceutical industry and life science. Recently, cryo-electron microscopy in microbeam electron diffraction mode (microED) [1], X-ray nanodiffraction (nanoXRD) [2, 3] and serial femtosecond X-ray nanocrystallography (SFX) at X-ray free electron lasers (XFELs) [4] have opened a new way to diffraction data collection. However, production of the protein crystals, as well as their quality remain open problems. Since this field is rapidly evolving, the novel methods of macromolecule organization into the diffracting arrays (nanocrystals, 2D crystals, etc.) come to the forefront. At this stage, nanotechnology could offer great potential in structural, functional proteomics and medicine, aiming to *ab initio* construction of the solid protein-based materials for these studies.

The Langmuir-Blodgett (LB) nanotemplate method [5], applicable to any protein (including membrane proteins) allows highly ordered 2D protein nanofilms formation on the air-water interface and their deposition onto the solid supports. These nanofilms can be applied as a 2D nanotemplate for triggering of 3D protein crystals. Generally, LB protein nanotemplate approach includes the diffraction data collection from nanocrystal grown by LB nanotemplate, but it can be also applied to LB protein multilayers (MLs), aiming the protein structural data collection.

So far, interesting phenomena of the multilayers internal re-ordering have been observed after heating and cooling procedures. Indeed, surface ordering of the multilayered nanofilms and an improvement of the correlation between the layers during thermal annealing have been revealed by atomic force microscopy and grazing-incidence small-angle X-ray scattering (GISAX) [6]. However, information on the structural changes in the bulk of nanofilms as well as limits to thermal stability of protein-based materials and the mechanism and size-scale of processes occurring at high temperatures [7, 8], require more advanced techniques and sophisticated experiments now available for the scientific community.

For this reason, number of experiments have been performed on the protein LB MLs deposited onto Si_3N_4 chips or TEM grids and annealed, by means of emerging advanced techniques as nanoXRD, microED and SFX at XFEL.

In 2018, NanoWorld Journal has published the pioneering experiment on protein (phycocyanin) LB MLs study by SFX at XFEL [9]. In the following years, scanning X-ray nanodiffraction experiments on penicillin-G-acylase LB MLs deposited on Si_3N_4 membranes and annealed at 150 °C resulted in observation of locally globular aggregates and filamentous spherulites based on

nanofibrillar subunits with cross-ß amyloidic motifs [10, 11]. Finally, this year it was shown by microED that amorphous phycocyanin LB MLs, after annealing at 150 ° C and cooling to room temperature, form a layered nanofibrillar lattice with rotational disorder. Scanning X-ray nanodiffraction suggests that structural transformation is not homogeneous through the film but limited to patches of up to about 10 µm diameter [12].

Serial femtosecond X-ray nanocrystallography experiments at XFEL often require large amounts of sample, specialized experts and equipment available at only few X-ray light sources and complicated optimization of sample-delivery systems. However, XFEL measurements, performed at ambient temperature can reveal physiological conformation and dynamics of the molecules, and in case of fix-target MLs analysis, the protein amount required is very small. From the other hand, microED can overcome some of the obstacles encountered by an XFEL while maintaining many of advantages. The quantity of crystalline material in a MicroED experiment can be much smaller than in an XFEL experiment (e.g. single crystal of 50 nm thickness for full data collection with relatively small radiation damage). The equipment needed for a MicroED experiment is relatively cheap and readily available, and one single nanocrystal is sufficient for an entire data set to be collected and determined by MicroED [13].

MicroED provides access to surface and near-surface structural features for a few tens of layers of protein film. Scanning nanoXRD was used for 5 times thicker samples, providing data on the homogeneity of transformation with a more extended SAXS range than microED [14]. Moreover, the European Synchrotron Radiation Facility (ESRF) is currently being upgraded in the context of the "Extremely Bright Source" (EBS) project from a 3rd to a 4th generation synchrotron radiation source with increase in brightness by more than an order in magnitude [15]. This should allow scanning nanoXRD techniques to provide enhanced reciprocal space (SAXS) resolution for focal spots down to around 100 nm [2].

In conclusion, the specific properties of LB protein thin films (long range order, thermal stability, ability of trigger protein crystallization [5] can be exploited in new procedures both for microED, nanoXRD and SFX at XFEL. Combining these advanced techniques with the ability to measure structural parameters for large MLs area, the LB MLs could in future become a powerful tool for biophysical studies, considering the possibility of the dynamics parameters observation (catalytic reactions, etc.) in MLs. Remains the challenge of the protein structural data collection from LB MLs at crystallographic resolution.

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