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Synchrotron Diffraction of Multilayered LS PGA Films after Heating and Cooling

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Abstract

X-ray diffraction patterns of multilayered Langmuir-Schaefer (LS) film of penicillin G acylase (PGA) enzyme were acquired at the ID11 of Synchrotron Radiation at ESRF (Grenoble, France). In addition to what shown by GISAXS at ID13 and by AFM, the ID11 beamline appears capable to monitor the diffraction and structural properties of the Langmuir-Shaefer multilayered PGA enzyme film similar to what apparent in the corresponding PGA crystals. The dramatic increase of long-range order in the LB multi-layered enzyme films after heating and cooling, made previously apparent by grazing incidence small angle X-ray scattering using ID13 microbeam, was here utilized at the ID11 beamline to yield unique diffraction patterns of the PGA LB linked to the enzyme atomic structure. This could open the way to bypass the bottleneck of protein crystallization which is leaving still unsolved large part of important proteins, like the membrane ones.

Keywords

Penicillin G acylase, Synchrotron diffraction patterns, Multi-layered LB films

Introduction

Microbeam grazing-incidence small-angle X-ray scattering (μGISAX) is a powerful technique of investigating locally thin films and surfaces [1-3], giving access to length scales of up to several hundred nanometres. The unique combination of a micrometer-sized beam with the reflection geometry allows us to gain, in principle, two orders of magnitude in spatial resolution compared with conventional GISAXS experiments, and thereby represents a very promising tool for study the biomolecular ordered systems, such as Langmuir-Schaefer (LS) thin protein films and crystals [2, 3]. It was shown that this LB method of deposition of protein monolayers onto solid substrates preserve protein structure and function, providing new useful properties, such as protein thermal and temporal stability and film anisotropy [4, 5]. The secondary structure of all studied proteins in LS films is heat-proofed up to 200°C [4, 5]. Moreover, it was shown for LB films of photosynthetic reaction centers that special thermal treatment can improve the film ordering [6]. In order to further understand this phenomenon it is necessary to study the changes taking place in the protein orientation and long-range order in thin films after heating. Though non-contact "tapping" mode AFM can be used as a topography-sensitive method to provide the surface image of LS protein sample [7], but scattering analysis can give better in-depth insight. Ten years ago, synchrotron study was originally performed using Elettra Synchrotron in Trieste [8]. Since then, much progress in X-ray scattering technique have been

done, namely at ID13 beamline at European Synchrotron Facility (ESRF) in Grenoble [9], where the microGISAX set up has been developed [1, 10]. Now it is possible to investigate laterally inhomogeneous surfaces and interfaces with a two order-of-magnitude increase in spatial resolution compared to standard reflection set-ups and a ten-fold lower q_{min} compared to transmission geometry. Using ID11 beamline at ESRF in this report we finally were able to study closely the phenomenon of the increased order of the protein LB film after thermal treatment. For this investigation we choose a classical protein, namely Penicillin G Acylase (PGA) recently optimally immobilized [11, 12]. In contrast to previous attempts with the long range goal to probe the crystal-like structure of protein LS film, we used a very high number of protein monolayers and the ID11 beamline exploiting the possibility to study the structures embedded in the films as well as morphology and distribution of the structures in the film. A 3D highly ordered arrangement of structures in the film can play an important additional role in protein nanotemplate based crystallization process [13, 14], namely could help to avoid the bottleneck of protein crystallization in protein structure determination at the atomic level with the recent progress in X-ray micro- and nanodiffraction techniques. In this manuscript X-ray diffraction patterns of multilayered Langmuir-Schaefer (LS) film of penicillin G acylase (PGA) enzyme were acquired at the ID11 of Synchrotron Radiation at ESRF (Grenoble, France). As previously shown by GISAXS at ID13 [10, 14, 15] and Atomic Force Microscopy [14, 16] the Langmuir-multilayered enzyme film appears quite reproducible for the PGA here utilized to monitor their diffraction and structural properties in comparison of those apparent in the same PGA after crystallization by classical methods. Our study do show indeed that the enzyme structures are becoming quite more organized due to the heating and cooling process, which leads to the establishment of long-range order and unique diffraction properties.

Materials and Methods

Protein thin LS film preparation

PGA (Penicillin G Acylase) has been obtained from Antibioticos [11, 12]. This enzyme thin films were prepared at the air-water interface utilizing a Langmuir Teflon trough, with a subphase volume of 800 ml and a surface area of 44 × 11 cm^2. The surface pressure was measured with an accuracy of 20 mNm^{-1} by means of a Willhelmy balance. The procedures adopted to form protein films of urease were as described in [4, 17], while for PGA the thin films were prepared by the "protective-plate" method [11, 12, 18].

The Langmuir-Schaefer (LS) modification of the LB technique (horizontal lift) was used and the floating monolayer is transferred over the substrate surface by horizontally touching the monolayer and the layer transfers onto the substrate. In such a way the 60 monolayers were deposited subsequently on over another using the silica wafer as a substrate.

Atomic force microscopy

Atomic Force Microscopy characterization (shown in Figure 1 or PGA) and the routine assays discussed and referenced above [4, 11, 12, 16], namely Brewster microscopy, isotherms, absorbance, transmission infrared spectroscopy and nanogravimetry versus number of layers, confirm the accurate LB film formation as indicated up to exactly 60 monolayers. Every silica wafer of dimensions 5 × 5 mm^2 was properly cleaned by sulfuric acid solution and dried before thin film deposition. Every deposited monolayer was dried by the nitrogen flux. The fresh film for each enzyme was divided in two sample groups: the first group was incubated at 20°C, while the second group was placed in the oven pre-heated up to 150°C for 10 minutes, and then cooled down to the room temperature.

Images of the protein thin LS films have been obtained in tapping mode with a cantilever I type NSC14/Cr-Au MikroMash in a dry atmosphere utilizing an instrument based on the SPMagic controller built in house [7, 19]. The freeware WSxM (http://www.nanotec.es) was utilized for the processing of the acquired images. The typical resonance frequency of the cantilever tip is between 110 and 220 kHz, and the proper positioning of the cantilever on the AFM tip holder has been found at a frequency of 92 kHz, with intensity 0.6 V. The set point for loop control was at 0.2 V. The integral gain value during image grabbing has been set at 4.4 (I gain) and the proportional gain value during image acquisition has been set at 8.03 (P gain). Figures 1A and 1B show AFM images of the superposed 60 layers of PGA protein at room temperature in 2D (Figure 1A) and in 3D (Figure 1B) arrangement, pointing to an ordered protein organization quite distinct in enzyme size and overall morphology.

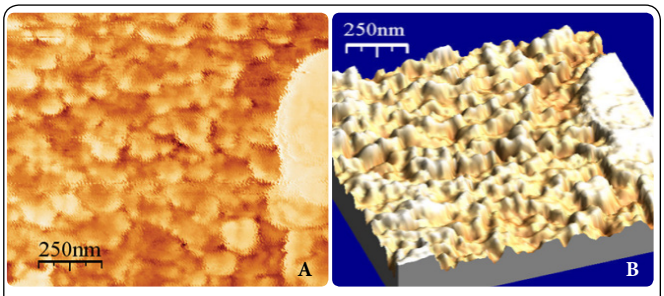


Figure 1: Representative AFM image PGA (60 monolayers LB film) at room temperature in 2D (A) and 3D (B). The dramatic increase in long-range order in the Langmuir-Blodgett multilayered enzyme films after heating and cooling was best made apparent time ago by the grazing-incidence small-angle X-ray scattering using a microbeam [14] and was this evidence that suggested to carry the experimentation reported in the following figures. The out-of-plane cut suggested that structures in LB are getting closer and are becoming more organized owing to the heating effect.

Synchrotron Radiation at ID11 beamline

X-ray diffraction patterns of multilayered Langmuir-Schaefer (LS) film of penicillin G acylase (PGA) enzyme were acquired at the ID11 (Figure 2) of Synchrotron Radiation at ESRF (Grenoble, France). We were in the EH1 hutch so the ID11 beam size is between 50 and 200 microns. The beam size at the sample position was defined by the X-ray source and focussing optics and was about 40 microns [20]. As previously shown by GISAXS at ID13 and AFM [14] the Langmuir-multilayered enzyme film appears quite reproducible for the PGA here utilized to monitor their diffraction and structural properties in comparison of those apparent in the same PGA after crystallization by classical methods. Our study do show indeed that the enzyme structures are becoming quite more

organized due to the heating and cooling process, which leads to the establishment of long-range order and unique diffraction properties.

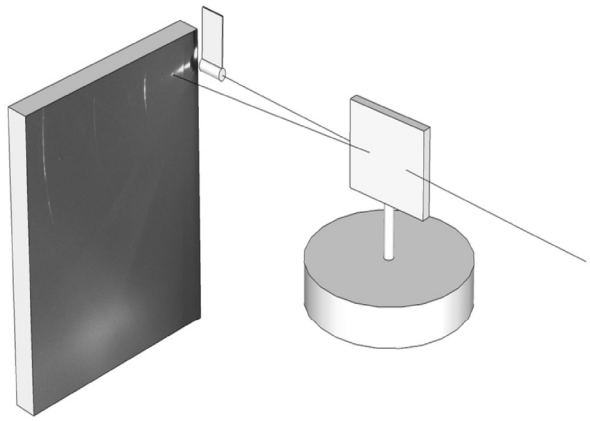


Figure 2: 2D X-ray diffraction images were recorded at the ID11 beamline using the setup shown above and wavelength of 0.3444 Å. The sample was rotated to vary the grazing angle.

μGISAXS of LS film are apparent in image 28 of Figure 3 (below right) where the scattered beams are collected in both the directions i.e. in plane and out of plane. A characteristic feature of a GISAXS pattern is the Yoneda peak (Y) and the possible physical interpretations for the phenomenon is the decreasing of film thickness and its re-ordering upon heating up to 150˚C and cooling to room temperature [14, 21]. The step size was about 0.027 degrees at both 150˚C and 22˚C.

Results and Discussions

From the AFM analysis (Figure 1) the clear difference in the size and morphology for PGA enzyme (Figure 1A and 1B, respectively in 2D and 3D). After heating at 150˚C and cooling down to 20˚C the PGA LB films appear quite better organized in both the 2D and 3D space, confirming previous STM analysis of *photosynthetic reaction center* [6] and in the same PGA enzyme [14] by both earlier GISAXS at ID13 and by AFM. From the systematic AFM and GISAXS analysis with ID11 beamline acquisition at ESRF (Figure 2) of treated and untreated samples of the enzyme patterns are clearly distinguishable in the case of PGA LB films for heat-treated versus untreated samples, with uniquely reproducible out of plane and detector scans whereby in the two dimensional map of the Yoneda region two peaks from the sample, which was heated up to 150˚C and cooled down to the room temperature are observed also in the ID11 beamline (Figure 3 below right). In the case of PGA LB films, the GISAXS patterns is also different for treated and untreated samples pointing to a long range order induced by heat treatment.

In order to study the LB film structure perpendicular to the surface, the detector scan was performed by making a vertical cut at q_y = 0. This cut shows the locally ordered films along the vertical direction. Different interfaces scatter the X-rays individually leading to interaction of the scattered rays. If the interfaces are correlated then interference leads to resonance diffused scattering, while in the opposite case (interfaces are not correlated) individual scatterings superpose [22]. This correlation could occur in the case of protein multilayers as the roughness correlation between interfaces of different layers. The partial phase coherence of the waves scattered from the interfaces concentrates the intensity in narrow sheets. These sheets of resonant diffused scattering are parallel to q_x and at the center satisfies the Bragg condition $\Delta q_z = 2\pi/dcorr$.

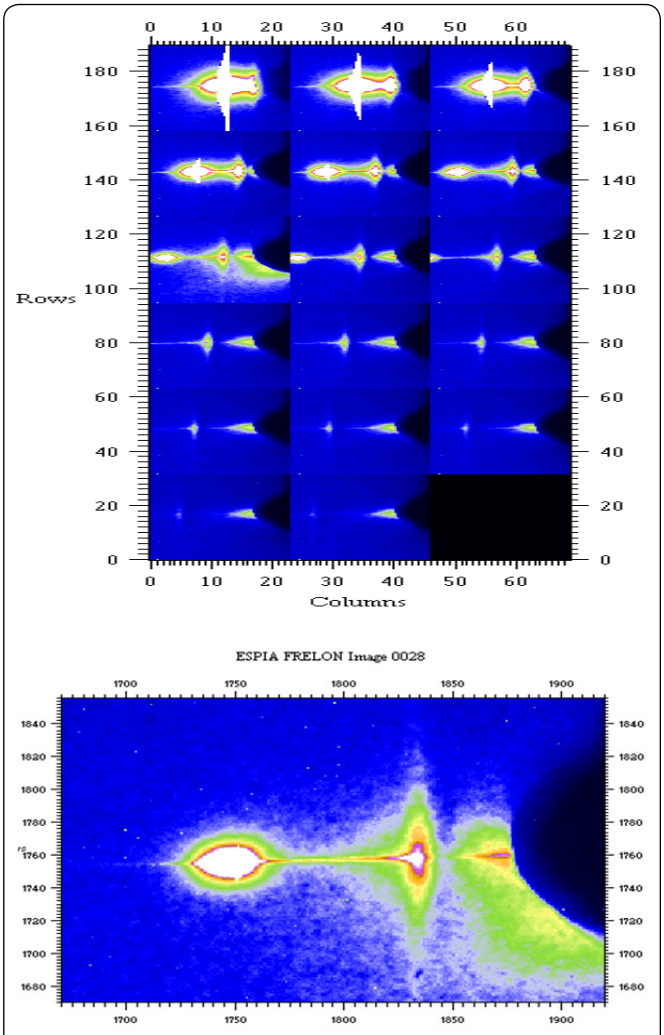


Figure 3: Data regarding **Pga at 22C**. Hereafter the Yoneda Region **changing between 22 and 38 images**. Data from PGA at 22˚C in the Yoneda Region for images 22 to 38. The peak is particularly evident in image 28 (below)
The series of images were collected while the silicon substrate was rotated.
Images are collected in order:
1 2 3
4 5 6
7 8 9
. . .
. . .
, .
Top left: reflection of the direct beam causes detector saturation.
As we progress and we reach the critical angle the reflection becomes weaker.
The diffuse scattering around the main reflection appears to be the signal from the thin film (Yoneda region)

In conclusion, from the detector cut along the vertical q_z cut at q_y = 0 more Yoneda peaks are apparent in the heated than in the unheated urease LB film, pointing to a significant reorganization of PGA in the multilayers LS film associated with heating up to 150 ˚C and cooling.

Figures 4 above and below point to the clear appearance in 2D and 3D space respectively of the diffraction pattern

typical of the corresponding crystal structure of homologous PGA enzyme crystal. The edge of the detector is (roughly) 95 mm from the centre, so two theta is about 7.5 degrees and this corresponds to 2.6 Angstrom d-spacing. The peaks are therefore somewhere between 3 and 10 Angstrom in d-spacing. Figure 5 finally makes evident that with the incident angle of 1 degree the reflection appears similarly epitaxial with the surface for both PGA samples , respectively at 22°C (top) and at 150°C (bottom).

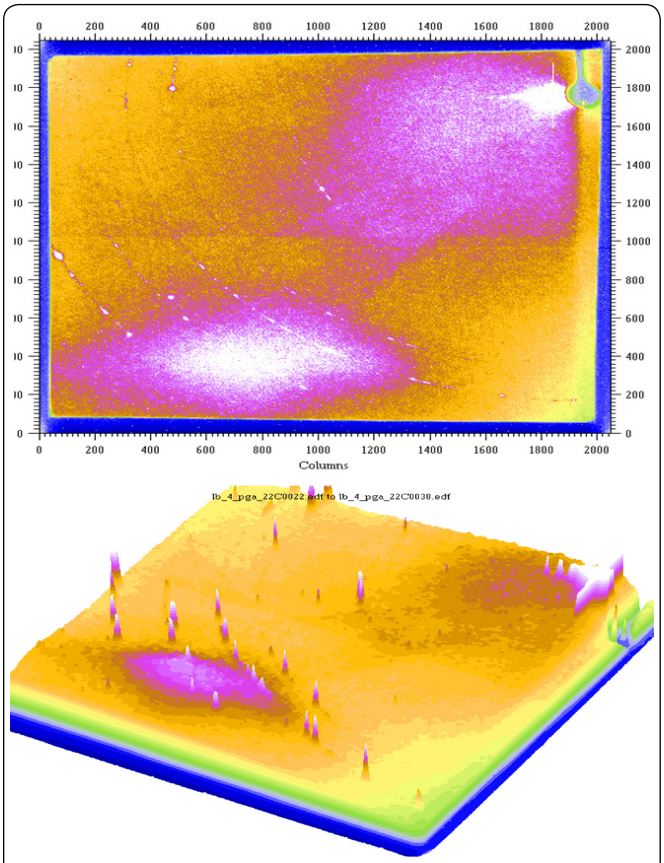


Figure 4: (Above) Summation of the same images . This is the full area detected during the measurement. The green box shows the region of interest on figure 3; (Below) The image of the surface in 3D. 3D representation of the area detector data for Pga at 22°C showing clear diffraction peaks 3D representation of the area detector data for Pga at 22°C showing clear diffraction. The spots visible in Figures 4 above and below are due to the reorganized multilayer film which shows now long-range order. The diffuse peak in the lower left of the Figure is likely scattering from silicon

Conclusions

The present ID11 synchrotron diffraction study of protein LB multilayers film of PGA supports the previous synchrotron radiation [8, 14] and STM [6] studies and confirms that significant heating the protein LB film and subsequently cooling down leads to a quite more ordered arrangement. Moreover, the μGISAX method [14] appears to yield new insights on the long range order previously unforeseeable and on the type of reorganization taking place within the multilayers LB, suggesting a possible model for the reorganization of the structures in the film, in terms of an higher packing occurring when the film is subjected to high temperature and subsequently cooled down. As shown previously [14] and confirmed here by ID11 GISAXS evaluation the in plane structure distance is indeed decreasing with heat compatibly with the merging of layers macromolecular structures, with the decrease in the plane length being likely due to the loss of water caused by LB formation and heating to 150°C.

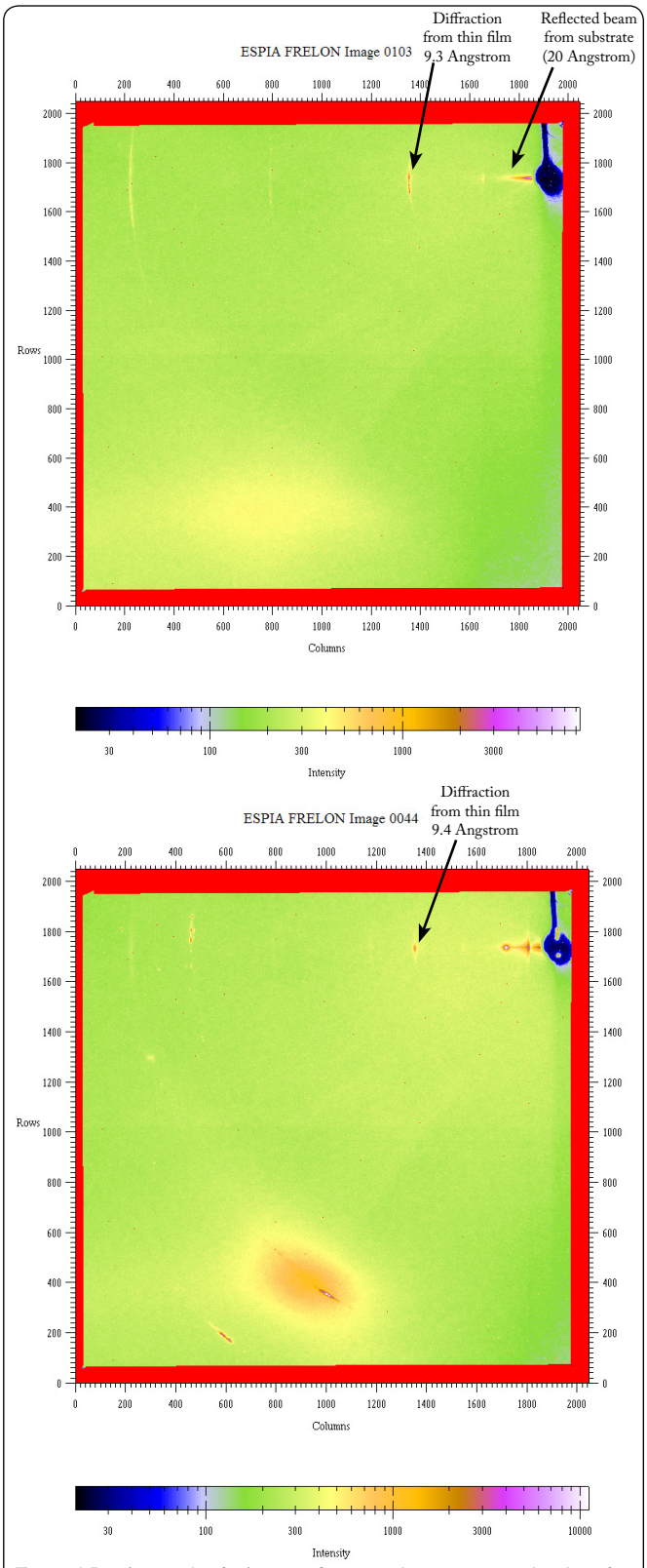


Figure 5: Incident angle of 1 degree, reflection is the same, epitaxial with surface
Top: Sample is PGA 22 C **Bottom**: Sample is PGA 150 C

Chromosoma 118: 179-191, 2009.

The comparative atomic structure characterization of thermophilic versus mesophilic proteins by X-ray crystallographic diffraction and nanogravimetric analysis [19] in protein solution, thin film and crystal has recently allowed to draw a coherent explanation for the origin and the molecular

mechanisms of heat stability, namely pointing to the role inner bound water in determining protein thermostability as suggested in this study.

In conclusion, the change of long-range order in multilayered LB enzyme films after heating and cooling could open the way to avoid the bottleneck of protein crystallization in protein structure determination at the atomic level. In addition to what shown [14] by GISAXS at ID13 and by AFM, X-ray diffraction patterns of multilayered Langmuir-Schaefer (LS) film of penicillin G acylase (PGA) enzyme acquired at the ID11 of Synchrotron Radiation at ESRF appears capable to uniquely monitor the diffraction patterns of the Langmuir- multilayered PGA enzyme film, linked to the enzyme atomic structure apparent in the corresponding PGA crystals. This could open the way to bypass the bottleneck of protein crystallization which is leaving still unsolved large part of important proteins, like the membrane ones.

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