BNL-113790-2017-JA

File # 94747

Recent advances in the understanding of the influence of electric and magnetic fields on protein crystal growth

C. Pareja-Rivera, V. Stojanoff

Submitted to Journal Crystal Growth & Design

January 1, 2017

Photon Sciences Department

Brookhaven National Laboratory

**U.S. Department of Energy
USDOE Office of Science (SC),
Basic Energy Sciences (BES) (SC-22)**

Notice: This manuscript has been authored by employees of Brookhaven Science Associates, LLC under Contract No. DE- SC0012704 with the U.S. Department of Energy. The publisher by accepting the manuscript for publication acknowledges that the United States Government retains a non-exclusive, paid-up, irrevocable, world-wide license to publish or reproduce the published form of this manuscript, or allow others to do so, for United States Government purposes.

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or any third party's use or the results of such use of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof or its contractors or subcontractors. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

RECENT ADVANCES IN THE UNDERSTANDING OF THE INFLUENCE OF ELECTRIC AND MAGNETIC FIELDS ON PROTEIN CRYSTAL GROWTH

Carina Pareja-Rivera¹, Mayra Cuéllar-Cruz², Nuria Esturau-Escofet¹, Nicola Demitri³,
Maurizio Polentarutti³, Vivian Stojanoff⁴ and Abel Moreno^{1,*}

¹ Instituto de Química, Universidad Nacional Autónoma de México, Av. Universidad 3000, Ciudad Universitaria, C.P. 04510, México, D.F., México.

² Departamento de Biología, División de Ciencias Naturales y Exactas, Campus Guanajuato, Universidad de Guanajuato, Noria Alta S/N. C.P. 36050 Guanajuato, Guanajuato, México.

³ Elettra – Sincrotrone Trieste, S.S. 14 km 163.5 in Area Science Park, 34149 Basovizza-Trieste, Italy.

⁴ NSLS- Brookhaven National Laboratory, Upton New York 11973 United States.

*Corresponding Author: Prof. Dr. Abel Moreno. Instituto de Química, Universidad Nacional Autónoma de México, Av. Universidad 3000, Ciudad Universitaria, Cd.Mx. 04510, México. E-mail address: carcamo@unam.mx.

Abstract. In this contribution we use non-conventional methods that help to increase the success rate of a protein crystal growth, and consequently of structural projects using X-ray diffraction techniques. In order to achieve this purpose, this contribution presents new approaches involving more sophisticated techniques of protein crystallization, not just for growing protein crystals of different sizes by using electric fields, but also for controlling crystal size and orientation. This latter was possible through the use of magnetic fields that allow to obtain protein crystals suitable for both high-resolution X-ray and neutron diffraction crystallography where big crystals are required. This contribution discusses some pros, cons and realities of the role of electromagnetic fields in protein crystallization research, and their effect on protein crystal contacts. Additionally, we discuss the importance of room and low temperatures during data collection. Finally, we also discuss the effect of applying a rather strong magnetic field of 16.5 Tesla, for shorts and long periods of time, on protein crystal growth, and on the 3D structure of two model proteins.

Key words electric fields, magnetic fields, lysozyme, glucose isomerase, crystal growth, in situ data collection, protein crystallization.

1. Introduction

X-ray crystallography is a powerful technique for determining the 3D structure of proteins and other biomolecules at high resolution.¹ Nowadays, X-ray diffraction of single crystals (by means of rotating anode generator or synchrotron radiation facility) is still the most important technique for obtaining the 3D structure, at atomic resolution, of small and large biological macromolecules. Additionally, a technique that provides suitable structural data (not at atomic level) is small angle X-ray scattering (SAXS) though it depends on the complexity of the protein structure. In the case of glycoproteins, for instance, the approach will be limited due to the large conformational freedom of sugars surrounding the structure and other flexible biomacromolecular complexes.²⁻⁴ However, X-ray diffraction crystallography is not the only technique available for obtaining the 3D structure of biological macromolecules. Other techniques such as Nuclear Magnetic Resonance (NMR) allow structural characterization of proteins in solution. This technique works well with proteins of molecular weight (MW) below 100 kDa with a high precision of the assignments of the residues.⁵ However, the recent and the most sophisticated technique, exclusively applied to large biomacromolecular complexes is the cryo-Electron Microscopy (cryo-EM).⁶⁻⁸ Another way of predicting the 3D structure of biomolecules is through computational approaches that allow to model biomolecules considering their homology and similarities (either using NMR or X-ray data), though not at a very high resolution.^{9, 10} We now count on a wide range of techniques (from classic to advanced methods) that focus on obtaining crystals for a variety of applications.¹¹ However, as obvious as all these methods seem for obtaining the 3D structure, they still require the production of suitable crystals for high resolution crystallographic analyses, as some molecules will not crystallize properly (we have not yet fully identified why this happens). Most of these limitations could be due either to intrinsically disordered protein sequences or to the low crystallizability propensity, possibly induced by the intrinsic physico-chemical properties of the protein itself.^{3, 12} Therefore, obtaining good protein crystals can be very tricky because of the multifactorial parameters involved into the crystallization process, and to the lack of knowledge of some of the aspects involved in it, such as nucleation details. More advanced methods for obtaining very high crystal quality have been

recently developed. Among these methods we find electric,^{13, 14} magnetic fields,¹⁵⁻¹⁷ crystal growth in gels,¹⁸ microfluidic chips,¹⁹ and many others. Experiments carried out through these methods proved, in general, that higher crystal quality is usually obtained. Additionally, these crystals diffracted at higher resolution and were much better compared to samples obtained with standard crystallization methods.^{17, 20}

The detailed enzymatic mechanisms have been studied at molecular and atomic levels via neutron crystallography, which is gaining popularity. Neutron crystallography is in fact, a powerful complementary technique to X-ray structural studies, which explicitly determines the location and orientation of deuterium atoms in proteins.²¹⁻²³ However, this technique requires protein crystals larger than those used for conventional X-ray crystallography, due to the low flux of neutron sources (requiring longer data collection time, varying from days to weeks). For this reason only a few 3D structures have been deposited in the PDB (24 unique, 10 perdeuterated, and only 10 at very high resolution), where neutron diffraction has been used. Nonetheless, despite the apparent heavy restrictions on sample dimensions, recent significant advances, not only in the beamlines and the detectors technology, but also in the sample preparation (perdeuteration) allowed to obtain high resolution structures from neutron diffraction.²³

In this work, we have used advanced techniques for protein crystallization focused on crystal's size control through electric fields (AC/DC), and crystal's orientation by strong magnetic fields. These techniques helped to obtain large and suitable crystals not just for high-resolution X-ray crystallography, but also for neutron diffraction crystallography. This contribution also discusses some pros, cons, and realities on the use of electromagnetic fields for protein crystal growth through studying two model proteins (lysozyme and glucose isomerase). This work also highlights the importance of room and low temperatures during data collection, therefore we show the data collected into two synchrotron facilities (Elettra in Trieste, Italy and SLAC-SSRL in California, USA). Finally, we also discuss the effect of applying a rather strong magnetic field of 16.5 Tesla, for short and long periods of time, on protein crystal growth, and on the 3D structure of the two model proteins.

2. Materials and methods

2.1 Protein crystallization conditions and cryoprotection

One of the proteins used in these experiments was glucose isomerase (Hampton Research Corporation Cat. No. HR7-102). The crystallization conditions consisted of a typical protein concentration of 30 mg/mL and a precipitating agent consisting in a 0.2 M ammonium acetate buffer pH 7.0, 30% (w/v) PEG 6000. The second model protein was lysozyme (Seikagaku, Co. Code 100940 six times recrystallized). A stock protein solution of 80 mg/mL and 1.4 M NaCl as precipitating agent, both were prepared in 0.1 M sodium acetate buffer solution pH 4.5. For batch crystallization experiments, the same volumes of both solutions, protein and precipitant, are carefully mixed, so that the final concentrations are half of the initial concentration. The data collection at low temperature by synchrotron radiation was performed under a flux of nitrogen gas kept at 100 K (as well as under conventional loops from 100 to 300 microns in size to fish the crystals). The precipitating agent of glucose isomerase perfectly worked as a cryo-protectant. For lysozyme, a mixture of 30% (v/v) PEG-1000 with mother liquor of NaCl (precipitating agent) has been used as cryo-protectant. We found that higher concentrations of PEG-1000 were not efficient since crystals were dissolved and damaged, whereas ice rings appeared, when using lower concentrations. For experiments at room temperature, the data collection was performed mounting protein crystals in capillary tubes²⁴ or directly mounting the crystallization apparatus on the diffractometer (in situ data collection). It is important to remark that room temperature experiments for data collection were possible due to the high velocity of the Pilatus 2M hybrid-pixel area detector (described later in the X-ray part).²⁵

2.2. Experimental setup for constructing a growth cell for applying electric fields

The crystal growth cell consists of two polished float conductive ITO (Indium Tin Oxide Electrodes) glasses of 3.0 x 2.5 cm², with a resistance ranging from 4 to 8 Ohms (Delta Technologies, Minnesota, USA). The conductive ITO-coated glass surfaces were placed inwards, facing each other. Each of the ITO electrodes is displaced by 0.5 cm from one another, in order to provide safe connection of electric alligators to the electrodes (anode/cathode) when applying a direct (DC). The cell is prepared using a double well frame (for vapor diffusion set up) as shown in Figure 1a, made of elastic black rubber material, sealed with vacuum grease so the rubber frame is perfectly

attached to the ITO glasses to avoid leakage. The growth cell can be closed afterwards by using a gun for silicone bar melting. This was constructed following the procedure of Flores-Hernandez et al., with some modifications and improvements into the original design.¹³

Figure 1. (a) the growth cell using an electric field applying a DC (vapor diffusion set-up). The right-side (b) shows the cell mounted on the goniometer of the XRD1 diffraction beamline (Elettra, Trieste) for in situ data collection at room temperature.

The DC apparatus was a Potentiostat/Galvanostat (Vimar, Mexico) that supplied direct current ranging from 2-6 μA . During the nucleation the DC applied current was first turned on, and off after 48 h for the nuclei to be fixed on the surface of the ITO electrodes. After that, the DC growth cell was left at constant temperature for the rest of the experiment, allowing the crystal to grow by the conventional vapor diffusion setup (Figure 1a). Let's just point out that crystal growth cells can be put directly to the X-ray beam (as shown in Figure 1b) when collecting at room temperature only if the ITO electrodes are made on thin PET (polyethylene) layers (windows with a thickness of 0.05 mm have been used), as PET is almost transparent to the X-ray energies commonly used (above 12 keV).

Figure 2. The growth cell for a batch crystallization experiment using an electric field that can be applied to four samples at the same time.

For alternating current (AC) ranging from 2 to 8 Hz, a device fixed in dense-disperse wave mode (pulse wave) was used for these experiments (KWD-808 I Multi-purpose health device, usually recommended for acupuncture treatment). The amplitude of these frequencies was in the range of $40\text{V} \pm 10\text{V}$. Figure 2 shows the experimental set-up for this type of experiment (in AC mode), where protein and precipitating agent are carefully mixed and introduced into the cell helped by insulin syringes. Needles are used to expel the air when introducing the mixture of protein and precipitating agent. At the beginning of the experiment, during the nucleation the AC applied current was first turned on, and off after 48 h. The crystals grew by the classic batch crystallization process. Afterwards, it was put directly to the X-ray beam, when collecting at room temperature only if the ITO electrodes are made on thin PET (polyethylene) layers as described previously.

2.3. Experimental setup for the crystallization of proteins under the influence of magnetic field

The mixture of protein stock plus precipitant (prepared in small Eppendorf tubes of 0.5 mL) is introduced in a disposable capillary pipette of 50 μ L (Sigma-Aldrich Cod. Z-543292 of 1.0 mm inner diameter) by capillarity forces. Green mounting clay from Hampton research (Cod. HR4-326) can be used to seal both ends of the capillary pipettes (Figure 3). Once sealed, the capillary pipettes are introduced into an NMR glass tube (5 mm in diameter) and left for at least 48 hours under the influence of a magnetic field of 16.5 Tesla by using an NMR instrument of 700 MHz usually common in Chemistry labs.

Figure 3. The experimental set up to prepare the mix is shown on the left-side of the scheme in order to grow protein crystals under the influence of a strong magnetic force of 16.5 Tesla.

All experiments are performed at the temperature of the control unit of the NMR probe head, that usually goes from 291 to 293 K. The sample is left in the magnetic field

during the whole process of the crystal growth, which may take two or more days. The longer the time, the higher the size and quality of the crystals. The lower part of the NMR magnetic tube (which is more or less 2.5 cm from the bottom of the NMR tube) is the area where the magnetic force is homogeneous and the best crystals are obtained. Once the experiment is over, the NMR tube is recovered from the magnet and the capillary pipettes are then carefully extracted from it. After being extracted, the capillary pipettes are cut at both sides in order to harvest the crystals. This cut is performed with a glass-capillary cutting stone (Hampton Research Cod. HR4-334). Once both ends of the capillary pipettes are opened, we applied a little air pressure using either a plastic latex tubing attached to 1 mL syringe or a pipette bulb. The little air pressure applied is enough to expel the solution with the crystals into a few microliters of a mother liquor or cryoprotectant, on either a two-well or a nine-well glass plate. If crystals would be attached to the walls of the capillary, we could then cut the capillary with microtools to recover them. There are two ways in which crystals data can be collected directly into the X-ray beam, one is mounting them in borosilicate capillary tubes at room temperature, and the other is by mounting them (previously cryo-protected) in plastics loops, followed by flash-cooling at low temperature.

2.4. Design of a novel device® for transportation of protein crystals to the synchrotron facilities

One of the main problems that protein crystals face when being transported to the synchrotrons, is airports custom revision, if the Dewar is opened the crystals inside are usually damaged. As we have encounter this last situation quite a few times, we decided that it was best to design a new device made of a patented polymer (polyolefin: US patent US 7,971,744 with a density between 0.064 to 0.13 g/cm³, that could resist changes in temperatures from -100 °C up to 50 °C without altering the quality of the protein crystals transported inside it. The heat transfer (heat capacity) is minimized both inside and out of the fluid. This is because the polyolefin foam have a high density and does not allow a proper heat exchange. Figure 4 shows a sketch of this new device, which consisted of a rectangular piece of this porous polyolefin with some pinholes in it. The procedure to store samples in this support is as follows: first both sides of the

---

® Patent submitted.

glass capillary pipettes, with the proteins crystal grown inside, have to be perfectly sealed. The following step, is to introduce the glass capillary pipettes containing the protein crystals, inside the NMR tubes, but before we do that, and in order to make the protein crystals extra safe, the NMR tubes must be filled with a hydrogel made by an acidic neutralization of sodium metasilicate solutions,²⁶ (this hydrogel is both inert and also has high softness that permits the insertion of the capillary pipettes). It is after filling the NMR tubes with the hydrogel that the glass capillary pipettes are introduced inside them. Once this is done, the NMR tubes carrying the pipettes, must be inserted inside the pinholes of the polyolefin device (as shown in Figure 4). From here on, the protein crystals can be safely transported even in a normal suitcase, through any airport, to synchrotron facilities. The thermal protection efficiency of the device, for protein crystals, was tested in the two synchrotrons where the X-ray data collection was performed.

Figure 4. Device constructed in polyolefin used for thermal-safe storage and transportation of fresh room temperature grown protein crystals to the synchrotrons. Details of the composition are described in the text.

The same type of experiments were carried out for lipase used as thermosensor for testing the efficiency of the thermal protection of the polyolefin device, because lipase crystals are extremely sensitive to small changes in temperature. In order to do this, we used the facilities of two different synchrotrons. One of the synchrotrons was Elettra in

Trieste, Italy, and the other was SLAC-SSRL in Stanford California, United States. The lipase crystals arrived without being dissolved when transported to the two synchrotrons, where data collections were performed.

2.5. X-ray diffraction analyses

X-Ray diffraction (XRD) analysis was performed at the X-ray diffraction beamline (XRD1) of the Elettra Synchrotron, Trieste (Italy).²⁵ All datasets of protein single crystals grown under the presence of a strong magnetic force of 16.5 Tesla were collected at room temperature (298 K), with a monochromatic wavelength of 0.77491 Å (16 keV) and 100x100 μm² spot size, using a Pilatus 2M hybrid-pixel area detector.²⁵ The crystals grown at room temperature were prepared in the same growth cell or glass capillary tubes in which they grew. In order for the crystal to undergo X-ray in a capillary tube, crystals have to be separated from the mother liquor. This is done using small filter paper sticks that help to keep the mother liquor at both sides of the capillary, so the crystal maintains its humidity without being directly in contact with the mother liquor. A second experiment was carried out using the electrocrystallization growth cell (as shown in Figure 1 and 2) fitted on a standard goniometer head with a custom built frame. Data were collected at room temperature, using a monochromatic wavelength of 0.700 Å to limit absorption due to ITO glasses or ITO PET. The third experiment, focused on collecting X-ray diffraction of big protein crystals grown under the presence of strong magnetic force, allowed us to directly collect three or four datasets with only one crystal due to its big size, and exploiting small tunable beam dimensions available on modern MX synchrotrons beamlines.

We duplicated the data collection of the experiment carried out for protein crystals grown in a magnetic force of 16.5 Tesla. This was performed at the X-ray beamline BL 14-1 of the synchrotron SLAC-SSRL (Stanford California, USA), with a monochromatic wavelength of 0.95 Å (6 keV) and 50x80 μm² spot size using a MARmosaic CCD 325 detector. The crystals were properly cryo-protected using either the reservoir solution or the cryoprotector solution, depending on the needs. The crystals were then plunged into liquid nitrogen prior irradiation. Data processing was performed using either HKL 2000²⁷ or XDS²⁸ combined with POINTLESS²⁹ and scaled using SCALA from the CCP4 suite v.6.1.2 (Collaborative Computational Project 4,

Number 4, 1994).³⁰ The 3D structures were solved by the molecular replacement method with PHASER-MR³¹ in PHENIX³² and map inspection was performed with COOT.³³ Subsequent rounds of model building and refinement were performed using PHENIX-REFINE.³⁴

3. Results and discussions

3.1. Effect of the electric field upon the number, size, and crystal quality for crystals of glucose isomerase and lysozyme.

In some previous publications from our group, and based on the first observations done by Nanev and Penkova,³⁵ we demonstrated, in some cases, that the use of this electrically-assisted crystallization growth-cell applied to proteins by using DC (in the range from 2 to 4 μ A), revealed that crystals grew better on the cathode (when the protein molecule was positively charged).^{13, 36} The combination of the electromigration process for fixing the nuclei on the walls of the ITO electrodes, together with crystal growth by vapor diffusion setup, allowed us to grow crystals of glucose isomerase applying a direct current (Figure 1a).³⁷ Even by using this electro-assisted crystallization technique permitted to grow protein crystals as a function of temperature under the influence of DC electric field. It also permitted to isolate one of the polymorphs of glucose isomerase as published elsewhere.³⁷ However, the batch method used to grow crystals applying either DC³⁸⁻⁴⁰ or AC^{14, 41-43} currents is the most widely used so far. The results on the crystal quality are shown in Tables 1S and 2S (Supplementary material), where the effect of electric, magnetic fields, temperature of data collection are compared with the crystal quality of lysozyme and glucose isomerase. We noticed that the electric field (DC) did not affect the crystal structure. The application of this type of electric field was aimed first to nucleate lysozyme and glucose isomerase on the ITO electrodes and later to allow the crystals to grow by vapor diffusion setup as shown in our previous publications.^{13, 37} However, it was interesting for us to test the effect of alternate current (AC) on the crystallization of these two proteins. According to our results, AC current could affect not only the number of crystals, but also their size, depending on its frequency. The trend in crystal growth for lysozyme and glucose isomerase applying an AC of 2 Hz and 8 Hz to the crystal growth experiment can be described as follows: The higher the frequency (AC) the

higher the number of crystals, this trend is experimentally shown in Figure 5. The amplitude of these frequencies was in the range of $40\text{V} \pm 10\text{V}$.

Figure 5. The effect of AC applied for 24h on lysozyme and glucose isomerase crystals grown is shown in the presence of this alternating current compared to controls. The scale bar in yellow corresponds to 0.1 mm in size.

3.2. Effect of the magnetic field upon the number, orientation and quality of crystals of glucose isomerase and lysozyme

A number of new advanced methods for obtaining very high crystalline quality crystals, have been developed. These methods can be applied, not only to grow crystals controlling the diffusion transport, but also growing them in the presence of strong magnetic fields. The impact of the magnetic field on protein crystallization was found for the first time by Japanese scientists in 1997.^{44, 45} The main characteristic of magnetic fields, whether homogeneous or non-homogeneous, is that they always act differently on protein samples. Non-homogeneous magnetic fields are responsible for the reduction of the gravity forces on the solution through the action of the magnetic

force.⁴⁶⁻⁴⁸ By applying a vertical magnetic field gradient, a magnetizing force is generated on the sample. If this force is opposite to the gravitational force, the result will be a reduction in the vertical acceleration (effective gravity) with the subsequent diminution in natural convection.⁴⁹ The convection is practically nullified, generating a situation similar to that found under the conditions of microgravity.⁵⁰ Experimentally, the crystals of higher quality obtained diffracted to higher resolution.⁵¹ In previous experiments, we had observed that when applying strong homogenous magnetic fields (above 10 Tesla), better quality crystals were obtained.¹⁵ Nonetheless, the effect of the magnetic fields on the crystals will differ depending on the crystallographic space group in which the protein is being crystallized.¹⁷ All these observations are inter-related and are due to the orienting effect of the magnetic field at a microscopic level. In a supersaturated solution, protein nuclei are in suspension in the solution, but they sediment when reaching an adequate size, which depends on the value of the field.

Bearing in mind this approach, a novel experiment using the batch method under the presence of a rather strong magnetic field of 16.5 Tesla in capillary tubes was performed for lysozyme and glucose isomerase crystals. We studied the different crystal growth behavior of all proteins at two different period of times. In the first experiment, the crystal growth behavior was studied for 48h. In the second experiment for three weeks time (See Tables 2S to 4S of supplementary material). The idea was to see whether this magnetic force was affecting the crystallization and the crystal growth processes. Our previous works on glucose isomerase showed the existence of different polymorphs obtained at different temperatures.³⁷ Based on these results, we were able to select the right temperature only for one of the polymorphs. Additionally, we had to choose the best crystallization conditions for lysozyme and lipase in order to apply a strong magnetic force of 16.5 Tesla. For this, we used a conventional NMR apparatus commonly found in chemical laboratories. We observed that all protein crystals were significantly affected on their orientation and size. Modifications on the orientation and size of the crystal growth behavior were noticeable in all the experiments, but the longer the experiment, the more remarkable these modifications were. The crystals were greatly improved in size and substantially reduced in number, compared to the controls. In all cases crystals grown under this strong magnetic force were 4 times bigger than those in the controls, as shown in Figure 6.

Figure 6. Crystals of lysozyme, and glucose isomerase grown under the presence of strong magnetic field of 16.5 Tesla compared to the controls. The size was improved more or less 4 times as can be seen. The scale bar in red corresponds to 0.2 mm in size.

For all these crystals, data collection (performed at room temperature) and refinement statistics of the different data sets are summarized in the following X-ray data statistics shown in supplementary material on Tables 1S to 4S. Most of the crystals grown under the influence of electromagnetic field compared to controls, showed higher crystal quality (lower mosaicity, lower B-factors, higher resolution values).

In order to check whether the intra- or inter- crystal-contacts are affected in their packing, which are involved in the main or side backbone (chain), we calculated all changes in the residues detected along the main backbone (MC) and on the side backbone (SC) for these model proteins (PDBs: 5avh for glucose isomerase and 4a7d for lysozyme) with the help of the crystallographic data and the software Lsqkab (superpose) of the Suite CCP4.³⁰ According to the results shown in Figures 7 and 8, the side-chain conformations can slightly be altered when applying AC in contrast to strong magnetic fields. The effect caused by an 8 Hz AC, produced higher number of protein-protein interactions, which substantially increased the number of nuclei. However, when applying strong magnetic fields, neither the MC nor the SC were substantially affected. It means that crystals grown in these conditions by using a strong magnetic field, grew within the normal ranges expected for crystallographic structural data obtained from

two crystals, even when they are grown under identical conditions. Consequently, we decided to check carefully by synchrotron radiation, how the crystal quality was affected for these two model proteins when the electric fields (AC or DC) and the strong magnetic field were applied in the growth processes.

Figure 7. The residue number (I res) versus their deviation involved in the crystal contacts of lysozyme, affected by the electric field (AC/DC), but hardly by the magnetic field. Here SC and MC on the right-side plot represent the side-chain and the main-chain respectively.

Figure 8. The residue number (I res) versus their deviation involved in the crystal contacts of glucose isomerase affected by the electric field (AC/DC), but hardly affected

by the magnetic field. Here SC and MC on the right-side plot represent the side-chain and the main-chain respectively.

An additional experiment of glucose isomerase was carried out for one week under the presence of this magnetic force. On this experiment, the mixture of glucose isomerase and the precipitating agent was shaken strongly, in order to induce the extra nucleation from the beginning. Figure 9 shows the positive effect on glucose isomerase crystals grown inside the magnetic field (see the front plane of the picture). However, those nuclei deliberately produced at the beginning of the experiment when mixing the protein and the precipitating agent, were not affected by the magnetic field (see the shadow of non-oriented crystals in the back plane of the picture). These best crystals were easily detected when extracted and analyzed by X-ray, because they had, generally speaking, the same size. additionally, as previously mentioned, the lower part of the capillary tube is the area with the highest magnetic intensity and where the magnetic force is much more homogeneous.

From the PDB's of the crystal structures we calculated the anisotropy of the diamagnetic susceptibility for these two proteins. This calculation was based on the approach of Worcester⁵² and the corrections suggested by Pauling.⁵³ When replacing the aminoacids of the structure of the α helices and β sheets contents we obtained ΔX_α and ΔX_β . From the Worcester's equation: N_α and N_β represent the number of amino acids in the alpha helices and beta sheets respectively. ΔX_α and ΔX_β are added to give the total anisotropy of the diamagnetic susceptibility per molecule, which is dimensionless in the SI system of units. Table 1 shows all needed values to complete these calculations.

Table 1. Calculation of the anisotropy of the diamagnetic susceptibility for these two proteins.

Protein	PDB code	α	N_α	ΔX_α	β	N_β	ΔX_β	$\Delta X_{\alpha+\beta}$
GI	5AVH	20	191	5.12×10^{-6}	10	35	2.35×10^{-6}	7.47×10^{-6}
HEWL	4A7D	7	53	1.42×10^{-6}	4	10	6.7×10^{-6}	8.12×10^{-6}

In order to avoid any influence on the crystal quality due to the cryo-protection, data have been collected twice; the first data collection was performed at room temperature on the XRD1 beamline of the synchrotron Elettra in Trieste (Italy). Additionally, in order to further investigate this, glucose isomerase crystals were tested (as a duplicated experiment) in a second synchrotron facility, in this case SLAC-SSRL located in Stanford California (USA).

This experiment shows that if the mixture is carefully done at the very beginning, the effect of magnetic field will produced bigger size crystals that are better oriented to the magnetic field. These crystals of Figure 9 were analyzed by X-ray diffraction (synchrotron radiation) at low temperature. Most of the crystals grown under the influence of magnetic field compared to controls, showed lower mosaicity, lower B-factors, higher resolution values, see Table 2. This table shows the high reproducibility in obtaining high-quality single crystals when a strong magnetic force is applied during crystallization process.

Figure 9. These crystals perfectly orientated to the direction of the magnetic field were grown for one week. However, some crystals at the back of the picture were not oriented to the magnetic field, as they were deliberately induced from the very beginning of the experiment, when shaking strongly the mixture of the protein and the precipitating agent.

Table 2. Crystallographic X-ray data statistics for glucose isomerase of three crystals grown (shown on Figure 9) under the influence of the magnetic field of 16.5 Tesla for one week collected at low temperature.

Condition	Control	Glucose Isomerase Crystal-1	Glucose Isomerase Crystal-2	Glucose Isomerase Crystal-3
Wavelength (Å)	0.950	0.950	0.950	0.950
Angular step (°)	0.25	0.25	0.25	0.25
No. of images	1440	1440	842	720
Space group	I222	I222	I222	I222
Unit Cell (Å)	a	93.18	93.36	92.75
	b	99.96	98.55	98.73
	c	102.23	102.72	102.39
	alpha=beta=gamma	90	90	90
Total reflections	820681	1439930	1120810	2676369
Resolution range	40-1.60(1.63-1.60)	40-1.25(1.27-1.25)	40-1.18(1.20-1.18)	40-1.15(1.17-1.15)
Completeness (%)	94.5(99-.5)	96.6(72.6)	97.9(84.2)	99.1(85.2)
R _{merge}	0.139	0.076	0.071	0.069
R _{meas}	0.145	0.08	0.076	0.071
I/sigma (I)	28.19(1.5)	42.4(0.63)	35.41(1.58)	54.27(1.23)
Multiplicity	13.7(13.7)	11.5(6.1)	7.5(5.2)	16.3(5.4)
Wilson B-factor (Å ²)	22.3	13.9	11.0	10.8
CC1/2 last shell	0.821	0.457	0.753	0.577
Mosaicity (°)	0.901	0.552	0.557	0.502

Finally, we compared the best of the best results and the statistics for crystals grown under the presence of this strong magnetic field to those crystals grown in the presence of electric fields (DC) and controls (at room and low temperature of data collection), these results are shown in Table 3. Crystal quality indicators like $I/\sigma(I)$, resolution limits, mosaicities and B-factors for crystals grown under the influence of this magnetic field, at larger period, were better than for those grown under the influence of DC/AC electric fields. This internal structural order (in terms of crystal contacts) was substantially improved when this magnetic field was applied for at least 48h up to three weeks. It is clear that crystals of glucose isomerase collected at room temperature or at

lower temperature showed a substantially improved crystal quality. However, the most remarkable result was observed when the crystals were properly cryo-protected into the precipitating agent before being plunged into liquid nitrogen prior irradiation. The fact that the cryo-protector of glucose isomerase was exactly the same as the one used as precipitating agent, made all the difference.

Table 3. Crystallographic X-ray data statistics for glucose isomerase the best of the best of crystal grown in all these methods described in this contribution.

	Cryo-control SLAC-SSRL	Magnetic Field (TR) Elettra Synchrotron At Room Temperature	E-crystallization (DC) Elettra Synchrotron At room temperature	Magnetic Field SLAC-SSRL cryo-protected
Wavelength (Å)	0.950	0.77491	0.77491	0.950
Space group	<i>I</i> 2 2 2	<i>I</i> 2 2 2	<i>P</i> 2 ₁ 2 ₁ 2	<i>I</i> 2 2 2
Unit cell (Å, °)	<i>a</i> = 93.18	<i>a</i> = 94.003	<i>a</i> = 82.091	<i>a</i> = 92.83
	<i>b</i> = 99.96	<i>b</i> = 99.492	<i>b</i> = 93.893	<i>b</i> = 98.33
	<i>c</i> = 102.23	<i>c</i> = 103.066	<i>c</i> = 97.688	<i>c</i> = 102.63
	$\alpha = \beta = \gamma = 90^\circ$	$\alpha = \beta = \gamma = 90^\circ$	$\alpha = \beta = \gamma = 90^\circ$	$\alpha = \beta = \gamma = 90^\circ$
Total reflections	820681	314375 (24152)	535276 (39520)	2676369
Unique reflections	60107	154948 (11736)	275861 (20643)	163993
Resolution range (Å)	40 - 1.60	50 - 1.46	50 - 1.40	40–1.15
	(1.63 - 1.60)	(1.50 - 1.46)	(1.44 - 1.40)	(1.17 - 1.15)
Completeness (%)	94.5 (99.5)	0.96 (0.97)	0.96 (0.97)	99.1 (85.2)
<i>R</i>_{merge}	0.139	0.040 (0.541)	0.061 (0.491)	0.069 (0.430)
<i>R</i>_{meas}	0.145	0.051 (0.699)	0.081 (0.659)	0.071
<i>I</i>/σ(<i>I</i>)	28.19 (1.5)	14.03 (1.89)	8.47 (1.75)	54.27 (1.23)
Multiplicity	13.7 (13.7)	2.0 (2.1)	1.9 (1.9)	16.3 (5.4)
Wilson <i>B</i>-factor (Å²)	24	24.8	17.7	16.9
<i>CC</i>_{1/2}	0.821	0.999 (0.649)	0.997 (0.669)	0.577
Mosaicity	0.901	0.077	0.173	0.502

Figures 10 and 11 show the most important surface of crystal contacts found by crystallographic data, for lysozyme and glucose isomerase respectively. These surface contacts can be affected when AC electric fields are applied. However, the crystal structure remains unchanged (in terms of side-chain conformations of aminoacids) after applying strong magnetic fields. These crystal surface contacts are particularly relevant to the electromagnetic fields mentioned in this contribution. In the case of glucose isomerase, Figure 11 shows the most important residues for the crystal contacts of the main chain, which are susceptible to changes depending on the electric field applied AC or DC. From now on we will be focused on applying all this knowledge on more

challenging biomolecules that diffract poorly to X-ray, such as membrane proteins or macromolecular complexes. It has been suggested that in the case of membrane proteins, they should display better crystal quality when grown under the presence of strong magnetic fields. This happens because these membrane proteins have a large anisotropy of the diamagnetic susceptibility due to the high content of alpha helices. The higher the alpha helice content, the higher the anisotropy of the diamagnetic susceptibility.⁵⁴ Finally, the combination of electric and magnetic fields using this experimental setup should be evaluated in the near future for soluble and membrane proteins, the preliminary investigations combining both electric and magnetic fields applied to lysozyme crystallization were done by Sazaki et al., in 2004.⁵⁵

Figure 10. (a) an overview of the crystal structure contacts area, where the most important surface contacts are observed (b) a close-up of the main residues contacts of lysozyme obtained with the best X-ray data for the controls.

Figure 11. (a) an overview of the crystal structure contacts area for glucose isomerase, where the most important surface contacts are observed (b) a close-up of the main residues contacts of glucose isomerase obtained with the best X-ray data of the controls.

4. Conclusively remarks and perspectives

From these results, we conclude that when growing crystals in the presence of electromagnetic fields, beneficial effects are obtained also when applying electric field in DC configuration combined with vapor diffusion setup. The most remarkable results in terms of crystal quality (lower mosaicity, lower B-factors, higher resolution values) were obtained when applying strong magnetic fields to the crystals for at least 48h (the longer the experiment, the better the crystal size and quality). The crystal contacts (side chains conformational changes) are sparingly affected when applying an alternating AC field ranging from 2 to 8 Hz. The effect is basically observed on the number and on the size of crystals grown. All this knowledge will be applied in the near future on more challenging biomolecules that diffract poorly, such as membrane proteins or biomacromolecular complexes.

Acknowledgements

The authors (M.C.C. and A.M.) acknowledge the XRD1-Hard X-ray Diffraction Beamline and Structural Biology Laboratory of the Elettra Synchrotron, in Italy for the

support and beamtime awarded to collect data from the different protein crystals at room temperature. Research reported herein was performed in part at the BL 14-1 at the SSRL and supported by NSLSII scientist under NIGMS contracts P41GM103393 and P41 GM111244-01 and DOE contracts DE-AC02-76SF00515 and DE-SC0012704. One of the authors (A.M.) acknowledges to Prof. Dr. Antonio Romero-Garrido from the CIB-C.S.I.C. Madrid (Spain) for the crystallographic support in solving the 3D structures, and data analyses during the intership of Carina Pareja-Rivera sponsored by CONACYT Mexico (Scholarship holder No. 576597). The same author (A.M.) thanks to DGAPA UNAM for the support of the project No. IT200215. The authors acknowledge to Ms. Antonia Sanchez-Marín for the English style correction of this manuscript.

References

- (1) Giege, R.; Sauter, C., Biocrystallography: past, present, future. *Hfsp J* **2010**, 4, (3-4), 109-121.
- (2) De la Mora, E.; Flores-Hernandez, E.; Jakoncic, J.; Stojanoff, V.; Siliqi, D.; Sanchez-Puig, N.; Moreno, A., SdsA polymorph isolation and improvement of their crystal quality using nonconventional crystallization techniques. *J Appl Crystallogr* **2015**, 48, 1551-1559.
- (3) Bernado, P.; Mylonas, E.; Petoukhov, M. V.; Blackledge, M.; Svergun, D. I., Structural characterization of flexible proteins using small-angle X-ray scattering. *J Am Chem Soc* **2007**, 129, (17), 5656-5664.
- (4) Mertens, H. D. T.; Svergun, D. I., Structural characterization of proteins and complexes using small-angle X-ray solution scattering. *J Struct Biol* **2010**, 172, (1), 128-141.
- (5) Hudaky, P.; Perczel, A., Toward direct determination of conformations of protein building units from multidimensional NMR experiments VI. Chemical shift analysis of his to gain 3D structure and protonation state information. *J Comput Chem* **2005**, 26, (13), 1307-1317.
- (6) Baxter, E. L.; Aguila, L.; Alonso-Mori, R.; Barnes, C. O.; Bonagura, C. A.; Brehmer, W.; Brunger, A. T.; Calero, G.; Caradoc-Davies, T. T.; Chatterjee, R.; Degrado, W. F.; Fraser, J. S.; Ibrahim, M.; Kern, J.; Kobilka, B. K.; Kruse, A. C.; Larsson, K. M.; Lemke, H. T.; Lyubimov, A. Y.; Manglik, A.; McPhillips, S. E.; Norgren, E.; Pang, S. S.; Soltis, S. M.; Song, J. H.; Thomaston, J.; Tsai, Y.; Weis, W. I.; Woldeyes, R. A.; Yachandra, V.; Yano, J.; Zouni, A.; Cohen, A. E., High-density grids for efficient data collection from multiple crystals. *Acta Crystallogr D* **2016**, 72, 2-11.
- (7) Boutet, S.; Lomb, L.; Williams, G. J.; Barends, T. R. M.; Aquila, A.; Doak, R. B.; Weierstall, U.; DePonte, D. P.; Steinbrener, J.; Shoeman, R. L.; Messerschmidt, M.; Barty, A.; White, T. A.; Kassemeyer, S.; Kirian, R. A.; Seibert, M. M.; Montanez, P. A.; Kenney, C.; Herbst, R.; Hart, P.; Pines, J.; Haller, G.; Gruner, S. M.; Philipp, H. T.; Tate, M. W.; Hromalik, M.; Koerner, L. J.; van Bakel, N.; Morse, J.; Ghonsalves, W.; Arnlund, D.; Bogan, M. J.; Coleman, C.; Fromme, R.; Hampton, C. Y.; Hunter, M. S.;

- Johansson, L. C.; Katona, G.; Kupitz, C.; Liang, M. N.; Martin, A. V.; Nass, K.; Redecke, L.; Stellato, F.; Timneanu, N.; Wang, D. J.; Zatsepin, N. A.; Schafer, D.; Defever, J.; Neutze, R.; Fromme, P.; Spence, J. C. H.; Chapman, H. N.; Schlichting, I., High-Resolution Protein Structure Determination by Serial Femtosecond Crystallography. *Science* **2012**, 337, (6092), 362-364.
- (8) Hunter, M. S.; Fromme, P., Toward structure determination using membrane-protein nanocrystals and microcrystals. *Methods* **2011**, 55, (4), 387-404.
- (9) Vranken, W. F.; Vuister, G. W.; Bonvin, A. M. J. J., NMR-Based Modeling and Refinement of Protein 3D Structures. *Methods Mol Biol* **2015**, 1215, 351-380.
- (10) Galindo-Murillo, R.; Ruiz-Azuara, L.; Moreno-Esparza, R.; Cortes-Guzman, F., Molecular recognition between DNA and a copper-based anticancer complex. *Phys Chem Chem Phys* **2012**, 14, (44), 15539-15546.
- (11) Moreno, A., Focused issue on: Novel approaches to protein crystallisation and crystal quality enhancement. *Progress in Biophysics & Molecular Biology* **2009**, 101, (1-3), 1-2.
- (12) Sanchez-Puig, N.; Sauter, C.; Lorber, B.; Giege, R.; Moreno, A., Predicting Protein Crystallizability and Nucleation. *Protein Peptide Lett* **2012**, 19, (7), 725-731.
- (13) Flores-Hernandez, E.; Stojanoff, V.; Arreguin-Espinosa, R.; Moreno, A.; Sanchez-Puig, N., An electrically assisted device for protein crystallization in a vapor-diffusion setup. *J Appl Crystallogr* **2013**, 46, 832-834.
- (14) Koizumi, H.; Uda, S.; Fujiwara, K.; Tachibana, M.; Kojima, K.; Nozawa, J., Crystallization of high-quality protein crystals using an external electric field. *J Appl Crystallogr* **2015**, 48, 1507-1513.
- (15) Moreno, A.; Quiroz-García, B.; Yokaichiya, F.; Stojanoff, V.; Rudolph, P., Protein crystal growth in gels and stationary magnetic fields. *Crystal Research and Technology* **2007**, 42, (3), 231-236.
- (16) Sazaki, G., Crystal quality enhancement by magnetic fields. *Progress in Biophysics & Molecular Biology* **2009**, 101, (1-3), 45-55.
- (17) Surade, S.; Ochi, T.; Nietlispach, D.; Chirgadze, D.; Moreno, A., Investigations into Protein Crystallization in the Presence of a Strong Magnetic Field. *Crystal Growth & Design* **2010**, 10, (2), 691-699.
- (18) Lorber, B.; Sauter, C.; Theobald-Dietrich, A.; Moreno, A.; Schellenberger, P.; Robert, M. C.; Capelle, B.; Sanglier, S.; Potier, N.; Giege, R., Crystal growth of proteins, nucleic acids, and viruses in gels. *Progress in Biophysics & Molecular Biology* **2009**, 101, (1-3), 13-25.
- (19) Pawate, A. S.; Srajer, V.; Schieferstein, J.; Guha, S.; Henning, R.; Kosheleva, I.; Schmidt, M.; Ren, Z.; Kenis, P. J. A.; Perry, S. L., Towards time-resolved serial crystallography in a microfluidic device. *Acta Crystallogr F* **2015**, 71, 823-830.
- (20) Yin, D. C., Protein crystallization in a magnetic field. *Prog Cryst Growth Ch* **2015**, 61, (1), 1-26.
- (21) Myles, D. A. A.; Dauvergne, F.; Blakeley, M. P.; Meilleur, F., Neutron protein crystallography at ultra-low (< 15 K) temperatures. *J Appl Crystallogr* **2012**, 45, 686-692.
- (22) Niimura, N.; Bau, R., Neutron protein crystallography: beyond the folding structure of biological macromolecules. *Acta Crystallogr A* **2008**, 64, 12-22.
- (23) Blakeley, M. P.; Hasnain, S. S.; Antonyuk, S. V., Sub-atomic resolution X-ray crystallography and neutron crystallography: promise, challenges and potential. *Iucrj* **2015**, 2, 464-474.
- (24) Baba, S.; Hoshino, T.; Ito, L.; Kumasaka, T., Humidity control and hydrophilic glue coating applied to mounted protein crystals improves X-ray diffraction

experiments. *Acta Crystallographica Section D-Biological Crystallography* **2013**, 69, 1839-1849.

(25) Lausi, A.; Polentarutti, M.; Onesti, S.; Plaisier, J. R.; Busetto, E.; Bais, G.; Barba, L.; Cassetta, A.; Campi, G.; Lamba, D.; Pifferi, A.; Mande, S. C.; Sarma, D. D.; Sharma, S. M.; Paolucci, G., Status of the crystallography beamlines at Elettra. *Eur Phys J Plus* **2015**, 130, (3).

(26) Moreno, A.; Juarez-Martinez, G.; Hernandez-Perez, T.; Batina, N.; Mundo, M.; McPherson, A., Physical and chemical properties of gels - Application to protein nucleation control in the gel acupuncture technique. *Journal of Crystal Growth* **1999**, 205, (3), 375-381.

(27) Otwinowski, Z.; Minor, W., Processing of X-ray diffraction data collected in oscillation mode. *Method Enzymol* **1997**, 276, 307-326.

(28) Kabsch, W., Xds. *Acta Crystallographica Section D-Biological Crystallography* **2010**, 66, 125-132.

(29) Evans, P., Scaling and assessment of data quality. *Acta Crystallographica Section D-Biological Crystallography* **2006**, 62, 72-82.

(30) Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G. W.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S., Overview of the CCP4 suite and current developments. *Acta Crystallographica Section D-Biological Crystallography* **2011**, 67, 235-242.

(31) McCoy, A. J., Solving structures of protein complexes by molecular replacement with Phaser. *Acta Crystallographica Section D-Biological Crystallography* **2007**, 63, 32-41.

(32) Adams, P. D.; Afonine, P. V.; Bunkoczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H., PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallographica Section D-Biological Crystallography* **2010**, 66, 213-221.

(33) Emsley, P.; Cowtan, K., Coot: model-building tools for molecular graphics. *Acta Crystallographica Section D-Biological Crystallography* **2004**, 60, 2126-2132.

(34) Afonine, P. V.; Grosse-Kunstleve, R. W.; Echols, N.; Headd, J. J.; Moriarty, N. W.; Mustyakimov, M.; Terwilliger, T. C.; Urzhumtsev, A.; Zwart, P. H.; Adams, P. D., Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallographica Section D-Biological Crystallography* **2012**, 68, 352-367.

(35) Nanev, C. N.; Penkova, A., Nucleation of lysozyme crystals under external electric and ultrasonic fields. *Journal of Crystal Growth* **2001**, 232, (1-4), 285-293.

(36) Pérez, Y.; Eid, D.; Acosta, F.; Marín-García, L.; Jakoncic, J.; Stojanoff, V.; Frontana-Urbe, B. A.; Moreno, A., Electrochemically Assisted Protein Crystallization of Commercial Cytochrome c without Previous Purification. *Crystal Growth & Design* **2008**, 8, (7), 2493-2496.

(37) Martinez-Caballero, S.; Cuellar-Cruz, M.; Demitri, N.; Polentarutti, M.; Rodriguez-Romero, A.; Moreno, A., Glucose Isomerase Polymorphs Obtained Using an Ad Hoc Protein Crystallization Temperature Device and a Growth Cell Applying an Electric Field. *Crystal Growth & Design* **2016**, 16, (3), 1679-1686.

(38) Gil-Alvaradejo, G.; Ruiz-Arellano, R. R.; Owen, C.; Rodriguez-Romero, A.; Rudino-Pinera, E.; Antwi, M. K.; Stojanoff, V.; Moreno, A., Novel Protein Crystal Growth Electrochemical Cell For Applications In X-ray Diffraction and Atomic Force Microscopy. *Crystal Growth & Design* **2011**, 11, (9), 3917-3922.

- (39) Koizumi, H.; Uda, S.; Fujiwara, K.; Nozawa, J., Control of Effect on the Nucleation Rate for Hen Egg White Lysozyme Crystals under Application of an External ac Electric Field. *Langmuir* **2011**, 27, (13), 8333-8338.
- (40) Nieto-Mendoza, E.; Frontana-Uribe, B. A.; Sazaki, G.; Moreno, A., Investigations on electromigration phenomena for protein crystallization using crystal growth cells with multiple electrodes: effect of the potential control. *Journal of Crystal Growth* **2005**, 275, (1-2), e1437-e1446.
- (41) Frontana-Uribe, B. A.; Moreno, A., On Electrochemically Assisted Protein Crystallization and Related Methods. *Crystal Growth & Design* **2008**, 8, (12), 4194-4199.
- (42) Uda, S.; Koizumi, H.; Nozawa, J.; Fujiwara, K., Crystal Growth under External Electric Fields. *Aip Conf Proc* **2014**, 1618, 261-264.
- (43) Koizumi, H.; Uda, S.; Fujiwara, K.; Tachibana, M.; Kojima, K.; Nozawa, J., Improvement of crystal quality for tetragonal hen egg white lysozyme crystals under application of an external alternating current electric field. *J Appl Crystallogr* **2013**, 46, 25-29.
- (44) Sazaki, G.; Yoshida, E.; Komatsu, H.; Nakada, T.; Miyashita, S.; Watanabe, K., Effects of a magnetic field on the nucleation and growth of protein crystals. *Journal of Crystal Growth* **1997**, 173, (1-2), 231-234.
- (45) Ataka, M.; Katoh, E.; Wakayama, N. I., Magnetic orientation as a tool to study the initial stage of crystallization of lysozyme. *Journal of Crystal Growth* **1997**, 173, (3-4), 592-596.
- (46) Adachi, H.; Takano, K.; Yoshimura, M.; Mori, Y.; Sasaki, T., Effective protein crystallization using crystal hysteresis. *Jpn J Appl Phys* **2003**, 42, (4a), L384-L385.
- (47) Numoto, N.; Shimizu, K.-i.; Matsumoto, K.; Miki, K.; Kita, A., Observation of the orientation of membrane protein crystals grown in high magnetic force fields. *Journal of Crystal Growth* **2013**, 367, 53-56.
- (48) Heijna, M. C. R.; Poodt, P. W. G.; Tsukamoto, K.; de Grip, W. J.; Christianen, P. C. M.; Maan, J. C.; Hendrix, J. L. A.; van Enkevort, W. J. P.; Vlieg, E., Magnetically controlled gravity for protein crystal growth. *Appl Phys Lett* **2007**, 90, (26).
- (49) Wada, H.; Hirota, N.; Matsumoto, S.; Okada, H.; Kiyohara, M.; Ode, T.; Tanokura, M.; Nakamura, A.; Ohtsuka, J.; Kita, A.; Numoto, N.; Kashiwagi, T.; Suzuki, E.-i., Application of High-Field Superconducting Magnet to Protein Crystallization. *Physics Procedia* **2012**, 36, 953-957.
- (50) Wakayama, N. I., Effects of a strong magnetic field on protein crystal growth. *Crystal Growth & Design* **2003**, 3, (1), 17-24.
- (51) Yin, D.-C., Protein crystallization in a magnetic field. *Prog Cryst Growth Ch* **2015**, 61, (1), 1-26.
- (52) Worcester, D. L., STRUCTURAL ORIGINS OF DIAMAGNETIC ANISOTROPY IN PROTEINS. *P Natl Acad Sci USA* **1978**, 75, (11), 5475-5477.
- (53) Cameron, E.; Pauling, L., ASCORBATE AND CANCER. *Proceedings of the American Philosophical Society* **1979**, 123, (2), 117-123.
- (54) Wakayama, N. I.; Wang, L. B.; Ataka, M., The effect of a strong magnetic field on protein crystal growth. *P Soc Photo-Opt Ins* **2002**, 4813, 1-11.
- (55) Sazaki, G.; Moreno, A.; Nakajima, K., Novel coupling effects of the magnetic and electric fields on protein crystallization. *Journal of Crystal Growth* **2004**, 262, (1-4), 499-502.

Table of Contents Graphic and Synopsis

The effect of electromagnetic fields on protein crystal growth is shown. From our results, the crystal quality is higher when a strong magnetic field is applied for a long time, while this effect is different when DC or AC are applied to the crystal growth process.