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Perspectives

On Electrochemically Assisted Protein Crystallization and Related Methods[†]

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ABSTRACT: In this communication, the basic strategies to control the kinetics and transport phenomena of the crystal growth process, as well as novel methods to induce either nucleation or protein crystal growth via electrochemical processes, are reviewed. Additionally, recent advances, where low direct/alternant current or voltages are applied to the crystal growth cell to obtain high-quality single crystals for X-ray diffraction, are also discussed.

1. Introduction

The advances in genomics, as well as in proteomics, have produced thousands of new proteins to be studied through structural biology and eventually to be used in drug design projects. The complete sequencing of vertebrate and invertebrate genomes[1] have sped up international efforts in developing high throughput methods and technologies to allow the rapid determination of fast three-dimensional protein structure.[2] Since the number of new proteins, as well as the number of scientists who study them, will continue to increase the need for new efficient and effective methods of structure determination has arrived.[3] Until now and in the near future, X-ray diffraction of single crystals of specific macromolecules has been the only technique able to provide structural data at atomic resolution for the purposes mentioned. Other techniques that generate structural and molecular dynamic data do exist, but they are not used for these specific purposes.[4]

Precise data collection is required for X-ray crystallography to be applied to crystals of adequate size and quality, since the fidelity of the final structure depends directly on the perfection, size, and physical properties of these crystals. Thus, crystals are the key of the entire process and their production can be a bottleneck. The problem of growing adequate crystals involves many facets and some are discussed in this contribution, such as model biomolecules, in addition to some novel and ingenious approaches that use electrochemical methods to overcome this problem, as well as to grow them in a high quality crystalline state.

2. Transport Phenomena and Crystallization

One of the inconveniences of obtaining high quality crystals is the natural convection that exists in every experiment performed under normal earth gravity conditions. In addition, the problems involved in protein crystallization from solutions related to transport phenomena must be solved and the methods of crystal growth need to be carefully performed. This characteristic is the reason why their crystallization is so difficult, since many of their physicochemical properties are not well understood yet.

Nucleation is a major step in the crystallization process. It is primarily defined in terms of nuclei formation and size distribution. As soon as crystallites are detectable, the phenomenon is called crystal growth. During nucleation several events occur simultaneously at various time scales, namely, molecular conformational changes that take place in ~0.01 ns, surface structure and defect displacements occurring within 1.0 ns, surface step displacement in the time frame of μs, growth of one atomic layer in 1.0 ms, hydrodynamic transport in about a second, and finally homogeneous nucleation that needs not more than a few minutes.[5] Chemical and physical interactions between different molecules can be monitored, but only some methods provide sufficient resolution in terms of particle size and time scale. Static and dynamic light scattering methods have been employed to verify protein homogeneity and to measure protein−protein interactions under precrystallization conditions. They have also been applied without a sophisticated data reduction scheme to predict protein solubility and crystallizability.[6,7] As a consequence, the combination of spectroscopic, physicochemical, and crystallographic data may provide insight into the energetics of nucleation.

Many years of experimentation with diverse crystals types have confirmed the notion that, by minimizing the convective

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mass transport, a better quality crystal can be obtained, with improved mechanical and optical properties, reduced defects density, and larger size. Transport processes and, in particular, mass transport, are very important for crystal growth from aqueous solutions.[8−10] Mass and heat transport processes are critical to the final quality and characteristics of the crystals.[11] Many crystallogenesis techniques have been explicitly developed for controlling the relative contributions of convective and diffusive transport in crystal growth.[12] During the active incorporation of ions or molecules in the three-dimensional lattice, density differences are generated in the proximal area of the developing faces, leading to a convective flux in the surroundings of the crystal.[13−15] Convective transport of molecules competes with pure diffusive transport and the interaction between them will determine the way and the kinetics of nutrient presentation to the growing crystal.

Transport phenomena not only affect the nutrients of the crystals but also the rate of adsorption and incorporation of impurities, which affects the size, morphology development, and perfection of the crystal.[16] On the other hand, convective transport only occurs in the presence of Earth's gravitational field. Only then can heavier fluids sink and lighter fluids rise, leading to convective currents in the bulk of the solution. Other types of convection do exist, such as the convection due to surface tension,[17] but they are not significant in the crystallization process of solutions.

How is it possible to suppress the natural convection in crystallogenesis? Nowadays, different approaches have arisen for eliminating or at least reducing it. One of them is the crystallization of macromolecules in outer space, where, in the absence of gravity, the convection disappears.[18−21] In the past decade, a new approach that involves the use of magnetic fields has materialized. Magnetic forces opposed to gravity can reduce natural convection inside solutions.[22−24] Also, the methods for crystallizing macromolecules in gels are good and well-accepted alternatives to eliminating natural convection.[25] Despite the discovery of these benefits, gels are largely underexploited by protein crystal growers. The same holds for counter diffusion,[26,27] a crystallization method in which capillary forces exerted in cylindrical tubes of small diameter strongly reduce convection and stabilize the concentration gradients that exist around growing crystals.[28−30] Besides these three possibilities to control the transport processes, minimizing the convective transport, there are a few contributions devoted to control the kinetics from nucleation to crystal growth of biological macromolecules.

3. New Trends in Crystal Growth (Crystal Quality Enhancement)

Nowadays, several current structural projects have been devoted to the solving of diseases based on the knowledge of the three-dimensional structure of specific biological targets; the problem has been to obtain high quality single biocrystals to be investigated by X-ray diffraction. There are several and novel approaches to overcome the poor quality that is usually obtained in biological crystals for high resolution X-ray crystallography.[31] Among them are the application of an internal electric field in the crystal growth process,[32−35] the use of strong external electric fields in protein crystallization,[36−39] the strong magnetic fields and high pressure,[40−42] combining electric and magnetic fields,[35] the use of ultrasonic fields,[38] and the use of femtosecond laser irradiation (FSLI), and the solution-stirring (SS) method[43] as well as nucleants' addition to the crystallization droplets.[44−49] The basic idea behind all of them is related to locating the system in the nucleation area of the solubility plot

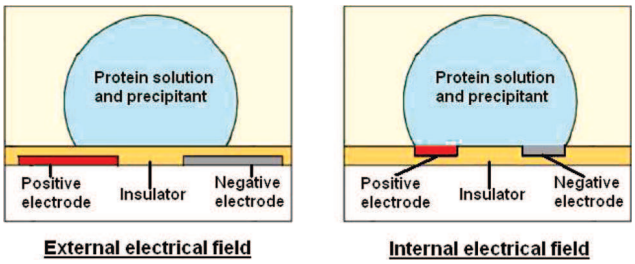

Figure 1. Experimental set-ups of a crystallization cell using an external and an internal electrical field, respectively.

and to give the system energy to spontaneously produce the first nucleation. In order to separate the nucleation phenomenon from the crystal growth process, it is necessary to look for a precise technique to investigate the limits of these two processes. In this regard, dynamic light scattering methods usually help to define those areas where nucleation is taking place[50−54] while atomic force microscopy[55,56] or video-microscopy is appropriate to investigate mechanisms of crystal growth.

4. Protein Crystallization Using an Internal Electric Field

Two different approaches have been used to crystallize proteins using an electrical field (EF) generated by a direct current (dc): (a) internal electrical field and (b) external electrical field (Figure 1). The references in this topic until 2006 have been previously reviewed, covering several features observed during those years.[57]

External EF experiments require high fields of up to 7.5 kV/cm;[36−38,58] in consequence, the electrical devices to carry out these experiments are not readily available in a typical biochemistry laboratory. In this technique, the electrodes (generally parallel plates or printed electrodes) are not in contact with the solution containing the protein, and the electric field affects the crystallization solution without electrochemical reactions occurring on the electrodes. The main effect observed in these experiments is an induced orientation of the crystals and the limitation of the number of nuclei observed. These observations have been explained by suggesting large protein concentration gradients inside the mother liquor, provoked by the external EF, leading to local supersaturation regions in the crystallization solution.

In contrast to the external EF, the internal EF requires the electrodes to be in contact with the crystallizing solution (Figure 1). This methodology needs precise control in applying the adequate current or potential due to the possibility of inducing electrochemical reactions on the electrodes. In order to succeed, these variables should be in the appropriate range to produce only capacitive current. This current can be observed in a cyclic voltammetry experiment as a large current plateau, located just before the current associated with electrochemical reactions (Faradaic current) becomes important (Figure 2). The current and potential values used are in the range of $1-20\ \mu$A or ± 1 V.[32−34,60] Capacitive current is associated with ion migration that charges the electrical double layer of the electrodes with ions of opposite polarity (Figure 3).[59] The ion transport phenomenon provokes a controlled flux of mass directed toward the electrodes. In the case of proteins solutions, these macromolecules could be seen as giant ions, due to the net charge that they have at the pH of crystallization. Using these current or voltage values, electrolysis reactions that can affect the pH of the solution are limited, and any eventual change of pH is controlled by the buffer system used to stabilize the protein.[33,34]

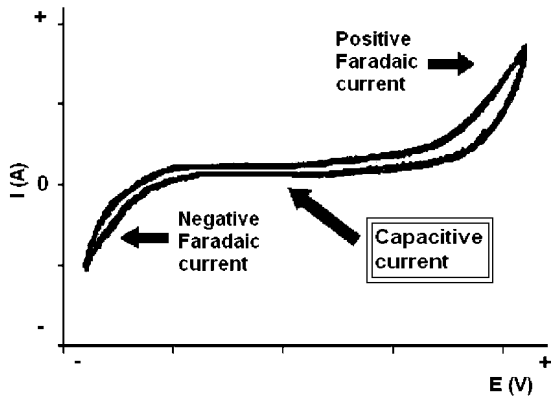

Figure 2. Cyclic voltammogram showing the faradaic current and capacitive current regions.

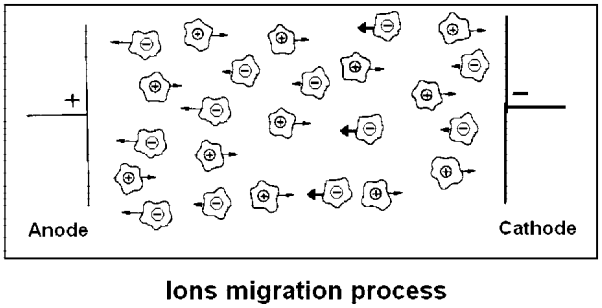

Figure 3. Scheme of ions migration process promoted by an internal electric field.

Several experiments have demonstrated that either solutions or gels containing the protein can be successfully used to more rapidly crystallize the proteins.[33,34,60,63] The results obtained with both systems are different in several aspects related to the mechanisms involved in crystal growth. For instance, crystallization experiments in solution for bovine pancreatic trypsin inhibitor protein[60] or lysozyme show a higher adsorption on the electrodes and, in some cases, crystals can cover the electrodes.[61] In gel experiments, this adsorption is less marked and the crystals grow mainly between the electrodes in the gel.[33,34] This observation can be associated with the ionic mobility of the protein under the electrical field, a property that can be described in terms of Stokes' law.[62] Thus, it is possible to propose that the change in the medium's viscosity affects the ionic velocity, decreasing the amount of protein near the electrodes when a gel is used. To see clearly the internal EF effect, protein supersaturation must be controlled to observe the crystallization process in the control cell (without current) at times longer than 12 h.[33,34,60,63]

The main features of the internal EF protein crystallization technique described in the literature are a faster crystallization process due to an accelerated kinetics of crystal growth,[32] crystallization being favored near one of the electrodes,[33,60] crystal adsorption process occurring on the electrodes,[33,60] and the excellent quality of the crystals that make them suitable for X-ray studies.[32,63] Using this methodology, cytochrome c was recently crystallized inside a dynamic light scattering (DLS) cell equipped with parallel Pt electrodes (Figure 4).[63] Using this setup it was possible to follow in situ the crystallization process using an internal EF. Three features of these experiments are remarkable: (a) the extremely faster crystallization process that allowed one to obtain crystals in one week (vs. 60 days in microseeding[64]), (b) the higher resolution of the obtained crystals (internal EF 1 Å vs microseeding 1.5 Å), and (c) protein electroseparation, favoring solely the crystallization of one of the isoforms present in the sample (Figure 4, chromatograms). This is a case where the system autodiscriminates between two of the protein isoforms present during crystallization. The cytochrome c redox properties observed in solution are observed in the solid state, as demonstrated with the cyclic voltammogram of the cytochrome c crystal.[65] Internal EF methodology has been applied to several different proteins, including the redox and nonredox proteins, such as lysozyme,[32−35,60] thaumatin,[32] bovine pancreatic trypsin inhibitor (BPTI),[60] catalase,[33] and cytochrome c.[63]

The easy way to obtain protein crystals more rapidly when an internal EF is used can be associated with a higher flux of mass from the bulk of the cell to the region near one of the electrodes, where the crystallization is being favored. This process is well-known in electrochemistry and corresponds to a diffusion process stimulated by a constant current that generates the migration of charged species (proteins, in this case) toward the electrode.[66] A mathematical model that describes accurately the crystallization process using an internal EF is not yet available, and should be matter of interest for physical chemists. This type of research will give rise to more insights about the details of the process. Thanks to the migration process, which is faster than the pure diffusion-control, the velocity of crystal growth is speeded up, but the higher resolution obtained for the crystals indicates that this mass transfer process is through an extremely controlled mechanism, and does not generate convective forces. It is clear from the published photographs of the cells that were used that the regions where the current is higher (tips of the electrodes) generally do not present crystals. This means that a high current value is not beneficial for the crystallization,[33,60] probably due to the presence of a convective mass-transfer pattern in these regions. According to the migration process, most proteins crystallize near the electrode of opposite polarity to the net charge of the protein. There are some cases, already published, where the protein crystallizes near the electrode with the same polarity;[32,60] here the protein counterion mobility has been proposed to favor the crystallization process (Cl^- for the case of lysozyme).

The effect of the current in the internal EF experiments on the crystal quality has been scarcely studied. It is clear that there is a lower threshold of the electrical current effect; below this limit the behavior of the crystallization process is the same as the control (without electrical current).[33] The upper limit is fixed by the electrochemical reactions, such as water or proton electrolysis that generates gases.[34,60] This gas production disturbs the crystallization process due to a source of convection forces and of electrical resistance via electrolytic processes of water. Between these two limits, there is a region of optimal crystallization. Preliminary studies focused on the trend of crystalline parameters, like resolution, mosaicity, and the obtained crystal error, have been carried out by our group for lysozyme and have demonstrated the effect of current on crystal quality.[67]

A variant of the previous methodology was recently reported; it is called the alternant current (ac) enhanced protein crystallization process.[68] An ac electric field at high frequencies can minimize the Faradaic reactions frequently found when direct current is used, allowing for higher applied voltages. The ac internal EF was applied to the crystallization of lysozyme using parallel and diagonal electrodes using frequency values between 0.1 and 100 MHz and voltages from 5 to 24 V (Figure 5). As in the previous dc current studies, an important decrease in the

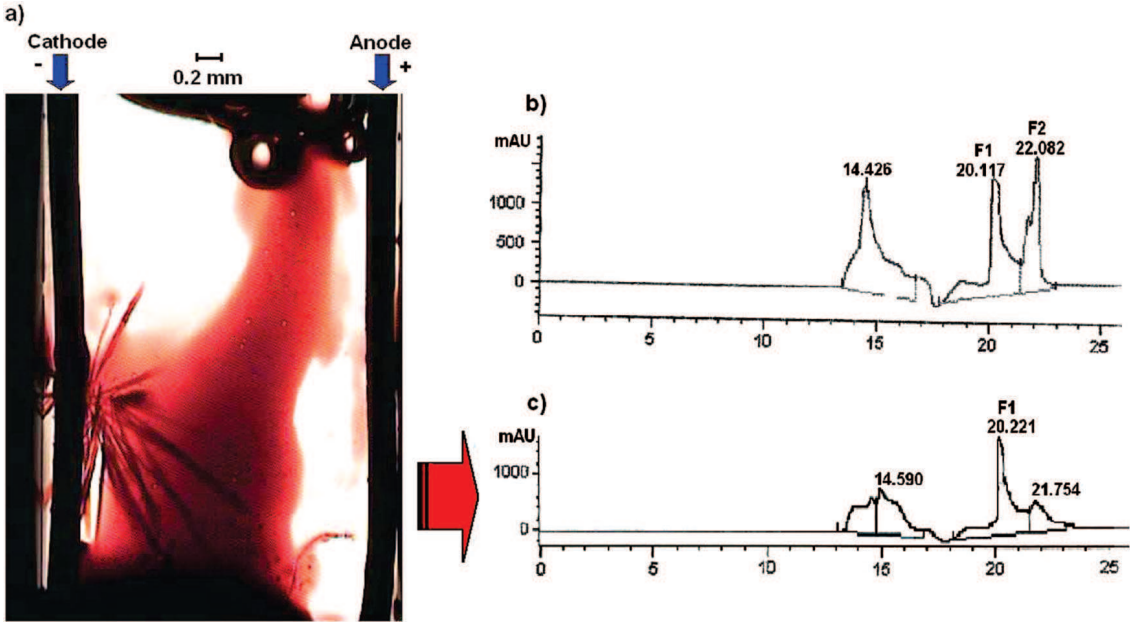

Figure 4. (a) Electrochemically assisted crystallization of cytochrome c (cyt c) in a DLS modified cell at 10 °C after 8 days. (b) Chromatogram (215 nm) of commercial cyt-c from bovine heart in the $K_3Fe(CN)_6$ solution; peak at 14.5 min corresponds to the matrix of the sample. (c) Chromatogram of cyt-c from crystals (needles) obtained by electrochemically assisted crystallization dissolved in the same matrix and conditions. Adapted with permission from ref 63. Copyright 2008 American Chemical Society.

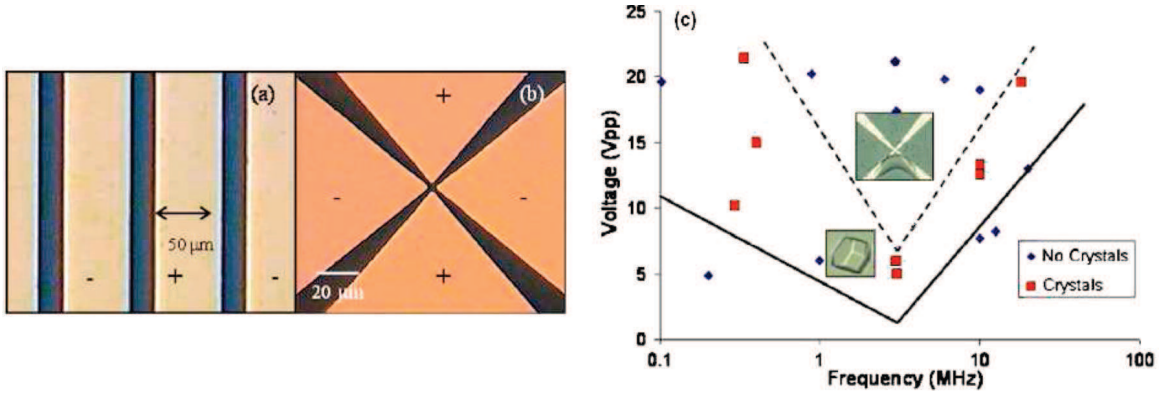

Figure 5. (a) Parallel electrodes cell with opposite polarities; (b) quadrupole electrode cell with opposite polarities; and (c) lysozyme solutions under different ac fields that were monitored for 24 h for indications of nucleation. Conditions where crystals were seen are marked by the red squares and conditions where no nucleation sites were seen are marked with a blue diamond. Adapted from ref 68 with permission. Copyright 2008 American Institute of Physics.

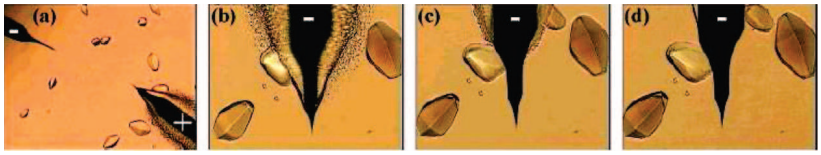

Figure 6. In situ observations under optical microscopy of BPTI crystallization at 20 °C with a direct voltage of 0.785 V at (a) $t = 18$ h and after inverting the electrode polarity at times of (b) 0, (c) 3.5, and (d) 9 h. As reference, the W-electrode wire diameter is 125 μm (the + sign indicates the anode and − sign indicates the cathode). Reprinted with permission from ref 60. Copyright 2007 American Chemical Society.

number of nuclei and a faster growth rate of crystals was observed. There was also a defined region where the nucleation process was enhanced.

During the ac internal EF experiments, a gel-phase was detected that, when the current was discontinued, led to the formation of high-quality large crystals (>100 μm).[68] Something equivalent has been recently reported with BPTI and lysozyme using dc internal electrical field;[60] the researchers succeeded in extracting a part of this gel-like layer from the electrode, demonstrating that this layer is a protein-rich phase (Figure 6). The authors propose that this layer is a liquid−liquid phase separation (LLPS) that contains a large amount of protein, generated by the electrical field. Once the polarity was inverted, at the site where the LLPS was deposited, the protein was dissolved but at the same time the crystals in the proximity increased their size. This is an interesting way to localize and concentrate protein molecules favoring the crystallization process.

The internal electrical field crystallization process cannot be described as an electrocrystallization process, because this concept has another meaning in the electrochemical terminology. Electrocrystallization processes deal with the phase formation and crystal growth phenomena involved in cathodic or anodic electrochemical reactions (with electron transfer). The most frequently studied electrocrystallization process is cathodic metal deposition on foreign and native substrates from electrolytes containing simple and/or complex metal ions.[69] It is important not to forget that the electron transfer in the internal electrical field crystallization process is minimized in the capacitive current region in order to favor ion migration. This last phenomenon is a fundamental part of electrochemistry, and in order to clarify the name of the technique we propose that it should be called *ac/dc* electrochemically assisted protein crystallization.[63] By using this name, the difference with the external electrical field methodology is clear, because *electrochemistry* can only be carried out when the electrodes are in contact with the solution. The term *assisted* is included in the name because the techniques that involve electrical fields require one to know previously the protein crystallization conditions. The electric field has effect only on the orientation, nucleation velocity, or induction time required to obtain these crystals, but does not generate the crystallization process. In order to avoid irreproducibility of the experiments the current value should be expressed in terms of current density (j), obtained dividing the applied current (I) by the electrode area (A) ($j = I/A$). In this way, even if the size of the electrode changes, when it is subjected to the same current density the results should be the same. This is a normal practice in synthetic electrochemistry, where the electrodes size changes from one laboratory to another.[70]

5. Perspectives in the Future of Electrochemically Assisted Proteins Crystallization

One of the major challenges of this technique is to find suitable cell arrays in which to conduct several experiments at the same time and, if possible, with disposable materials. This requires low cost instrumentation (low current galvanostat) and materials. Graphite has all the required characteristics to reach this objective and future work should be focused on this evaluation. Coupling physicochemical techniques, such as DLS, with the electrochemically assisted protein crystallization method[63] can give rise to an important insight about the crystallization process, and would lead us to identify intermediates of the crystallization process, optimizing the crystallization media. Finally, interdisciplinary research with mathematicians, physical chemists, and electrochemists would be an interesting way to find the kinetic models that could give the answers to all the questions that still remain. It is important to point out that our work on biomolecule crystallization and characterization is a multidisciplinary research.

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