Chapter 3

Advanced Methods of Protein Crystallization

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Abstract

This chapter provides a review of different advanced methods that help to increase the success rate of a crystallization project, by producing larger and higher quality single crystals for determination of macromolecular structures by crystallographic methods. For this purpose, the chapter is divided into three parts. The first part deals with the fundamentals for understanding the crystallization process through different strategies based on physical and chemical approaches. The second part presents new approaches involved in more sophisticated methods not only for growing protein crystals but also for controlling the size and orientation of crystals through utilization of electromagnetic fields and other advanced techniques. The last section deals with three different aspects: the importance of microgravity, the use of ligands to stabilize proteins, and the use of microfluidics to obtain protein crystals. All these advanced methods will allow the readers to obtain suitable crystalline samples for high-resolution X-ray and neutron crystallography.

Key words Electric fields, Magnetic fields, Counter-diffusion techniques, Crystal growth in gels, Protein crystallization

1 Introduction

Proteins, nucleic acids, polysaccharides, and lipids are regarded as the most important molecules of life. The function of these molecules in sustaining life depends on their three-dimensional structure and on their highly specific mutual interactions, dictated by their structure and bonding properties [1]. Getting to know the structures of macromolecules and of their complexes will enhance our understanding of biological processes of life. It will also hint at novel ways to treat a wide range of diseases, from congenital anomalies through bacterial and viral infections to autoimmunity diseases [2], or even many different types of cancers [3, 4]. X-ray crystallography is the hallmark of this search (it is the most powerful technique for structure elucidation of macromolecules), as it reaches near-atomic resolution in the most favorable cases, without a priori limitation on the size or on the complexity of the studied molecules. X-ray crystallography requires the growth of large and well-diffracting crystals (for conventional crystallography) or

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nanocrystals (for free electron lasers, XFELs). The production of such crystals is the most intractable stage in the process of structure determination [5, 6].

There are a number of strategies, from classical techniques to advanced methods, that focus on obtaining high quality single crystals (Fig. 1) for high resolution crystallographic analyses. Despite the existence of a large variety of conventional crystallization techniques (*see* Chapters 2 and 4 by McPherson and Derewenda) and the automation of high-throughput screening systems, statistics from various structural programs indicate that only fewer than 20% of de novo overexpressed proteins yield diffracting crystals [7]. This represents a very low success rate considering the cumulative difficulties of cloning, expressing, and purifying proteins. Although we cannot fully identify why some proteins do not crystallize, this may be due to the intrinsic physico-chemical properties of the protein per se. For this reason, it will be useful to have user-friendly tools that allow the experimenter to a priori select successful protein targets for crystallization and for identifying problematic proteins. The proteins that are recalcitrant to crystallization can be highly flexible as well as completely unstructured. They will not nucleate properly for different reasons, such as propensity to aggregate in an amorphous phase or difficulty to form stable crystal contacts. Therefore, obtaining good crystals can be very tricky and often needs a combination of strategies such as protein engineering, sophisticated crystallization techniques, and a good understanding of the nucleation and crystal growth processes [8–10].

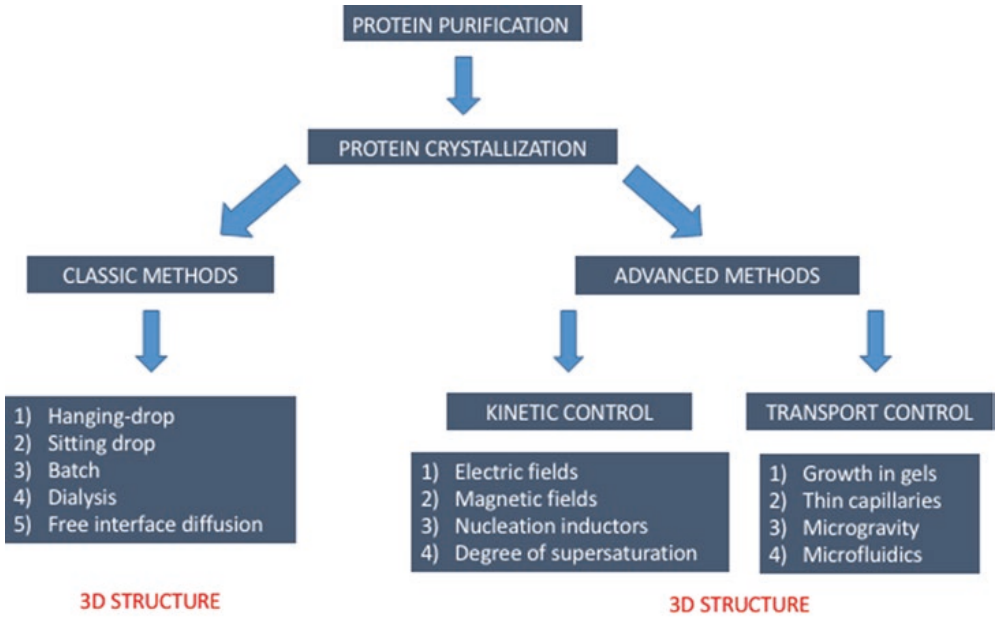

Fig. 1 A scheme representing different methods used to crystallize proteins. The classical methods are shown on the *left*, and the advanced methods, usually called nonconventional methods of protein crystallization, are shown on the *right*

In this chapter different advanced methods that help to increase the success rate of a crystallization project in order to obtain high quality single crystals for crystallographic research are discussed. The first part presents the body of knowledge regarding the crystallization process from physical and chemical perspectives. The second part introduces the reader to new approaches related to more sophisticated methods, not only for growing protein crystals but also for controlling the crystal size and orientation by electromagnetic fields, as well as through other advanced methods. Additional information including the importance of microgravity, the use of ligands to stabilize proteins, and the use of microfluidics to obtain suitable protein crystals for high-resolution X-ray crystallography, is also presented.

2 Technical Approaches

2.1 Fundamentals of Protein Crystallization Process Applied to Advanced Methods of Protein Crystal Growth

The solubility diagrams and the energetics of nucleation (Fig. 2a, b, respectively) provide vital and necessary information for the optimization of crystal growth [11–13]. In most cases, their use will lead to a reasonable strategy for obtaining protein crystals and for assuring high reproducibility. Crystal nucleation occurs in two stages: nucleation of new crystal embryos, and growth of a few nuclei into full-size diffracting crystals (Fig. 2b). It has been shown that the optimal conditions for growing high-quality crystals (large size, and minimum of imperfections) involve lower macromolecule supersaturation levels than those required for initial nucleation [14, 15]. Nucleation cannot take place at these lower supersaturations because an energy barrier of kinetic origin

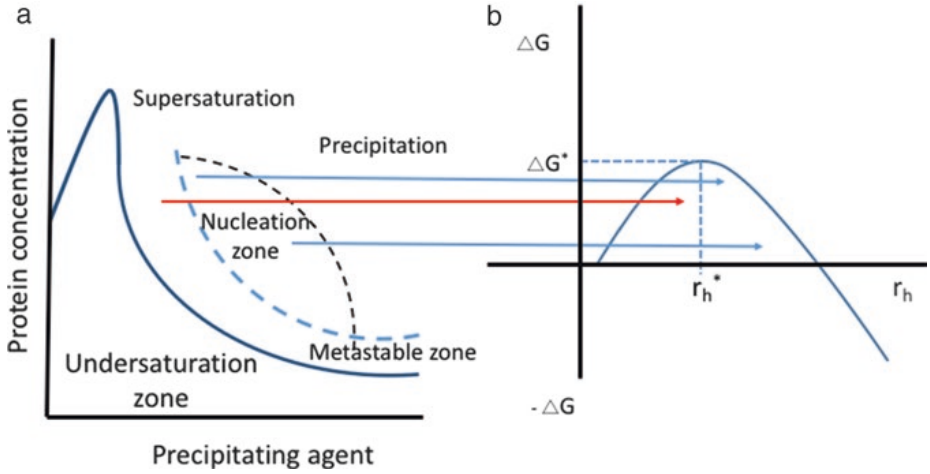

Fig. 2 (**a**) The solubility phase diagram (also known as Oswald-Miers diagram) is divided into different zones: undersaturated, supersaturated, metastable, nucleation, and precipitation. (**b**) The energetics of the system is very important to understand; it expresses the kinetics of the crystallization and allows to predict the critical size of the nucleus to be converted into crystal

(due to the energetically expensive formation of the crystal–solution interface) is involved [16, 17]. The establishment of crystallization solubility phase diagrams allows precise identification of the limits between the spontaneous nucleation and the optimal growth (often called "metastable") zones [18, 19]. That information can subsequently be used for growing crystals as close as possible to the metastable zone or for incubating the trials at nucleation conditions for a time sufficient for the formation of a few nuclei before transiting to metastable conditions for optimal growth (by changing the concentration of the precipitating agent, pH, or temperature) [18, 20–22].

There are alternative setup techniques such as microbatch under oil [23] or crystallization in capillaries [24, 25]. Often, these alternatives produce crystals under screening conditions that will be difficult to produce with other setups (e.g., standard vapor diffusion). These alternative techniques can also produce higher-quality crystals. Each technique relies on a different geometry and different way to reach supersaturation, therefore they present a kinetically different situation. These subtle differences frequently lead to different results in an unpredictable way. Tiny crystals of the same protein can start the nucleation process. There are various crystal seeding techniques, including the standard microseeding and streak-seeding into metastable conditions using microcrystals as sources of crystalline seeds [15, 26–28]. A new method called "Random Microseed Matrix Screening" and related techniques [29–32] that have been recently developed, involve crushing and preparing a seed-stock from microcrystalline material of any quality present in one or more droplets of the initial crystallization screen. This method can also dispense nano-volumes of seed stock into all the conditions of the same or other screens. This procedure allows crystals to appear in screen conditions that are adequate for crystal growth, but not for nucleation. There is also a recently published new technique that combines the results of moderately successful initial screenings based on Genetic Algorithms [33].

In order to initiate nucleation, nucleation-inducing particles or glass-based nucleants [34, 35], ultrasonic fields [36], or electromagnetic fields [37–46] have been applied, leading to conditions impossible to obtain by classical approaches. Subsequently, the growth of crystals can proceed by varying the temperature (either reducing or increasing it). Temperature can be modified to grow single crystals or to dissolve tiny crystals around a growing crystal. It is also possible to avoid the formation of long, thin needles [22, 47] by moving to higher or lower temperatures. In the crystallization of proteins, temperature and mainly pressure have been poorly explored [22, 48–50]. There are usually two temperatures available (most commonly 4 and 18 °C) for growing protein crystals. The existence of different polymorphs has been recently reported, after carefully testing a wide range of temperatures as well as other physicochemical parameters of the crystallization experiment [47, 51–54].

2.2 Protein Concentration

All concentrations should be measured in triplicate with an UV-VIS spectrophotometer, following the calibration procedures provided by the supplier. A calibration concentration plot can be obtained for each new protein, even if its extinction coefficient is not reported in the literature, for the calculation of protein concentration [55].

2.3 Gel Preparation

Agarose gel 0.6% (w/v) stock solution of low melting point agarose (T_{gel} = 297–298 K, Hampton Research HR8-092) can be prepared by dissolving 0.06 g agarose in 10 mL of water heated at 363 K up to a transparent solution with constant stirring. The solution is passed through a 0.22 μm porosity membrane filter for removing all dust particles or insoluble fibers of agarose. The gel-solution can be stored in 1.0 mL aliquots in Eppendorf tubes in the refrigerator. Prior to crystallization in agarose gels, an Eppendorf tube of 1.0 mL is heated at 363 K in order to melt the gel. Most proteins are damaged when exposed to high temperature, so it is best to mix only the precipitant agent with agarose to allow reaching the proper temperature without damaging the protein. Although in the last decade agarose has been the most popular gel for protein crystallization [56–58], there are other types of gels that have also been used for the same purpose [59–62].

3 Advanced Crystallization Methods in Practice

3.1 Experimental Setup for Constructing a Growth-Cell for Applying Electric Fields

As mentioned in Subheading 2.1, it is important to separate the nucleation and crystal growth phenomena. This can be also accomplished using electromagnetic fields. In particular, the use of electric fields has been shown to be useful for successful crystallization of proteins.

For that purpose, one can use a crystal growth cell that consists of two polished float conductive ITO (Indium Tin Oxide Electrode) glass plates, 3.0 × 2.5 cm^2, with a resistance ranging from 4 to 8 Ω (Delta Technologies, Minnesota, USA). The two electrodes are placed parallel to each other. The cell is prepared using a U-like or double well frame (for vapor diffusion set up) as shown in Fig. 3, made of elastic black rubber material, sealed with vacuum grease. Closure of the growth cell can be done by using a gun for melting silicone. The conductive ITO-coated surfaces are placed inwards, at 0.5 cm from each other, to provide appropriate connection area when applying direct (DC) or alternating current (AC) (Fig. 4a, b respectively). Each cell has a volume capacity of approximately 100 μL for precipitant (larger well) and 50 μL for protein plus precipitant (smaller well, as shown in Fig. 3 on the right), or a full volume of 200 μL when a batch configuration is used (Fig. 3, left). The sitting-drop vapor diffusion or batch crystallization conditions for each protein have to be properly established before applying the current. After closing the cell with a cover of melted silicone,

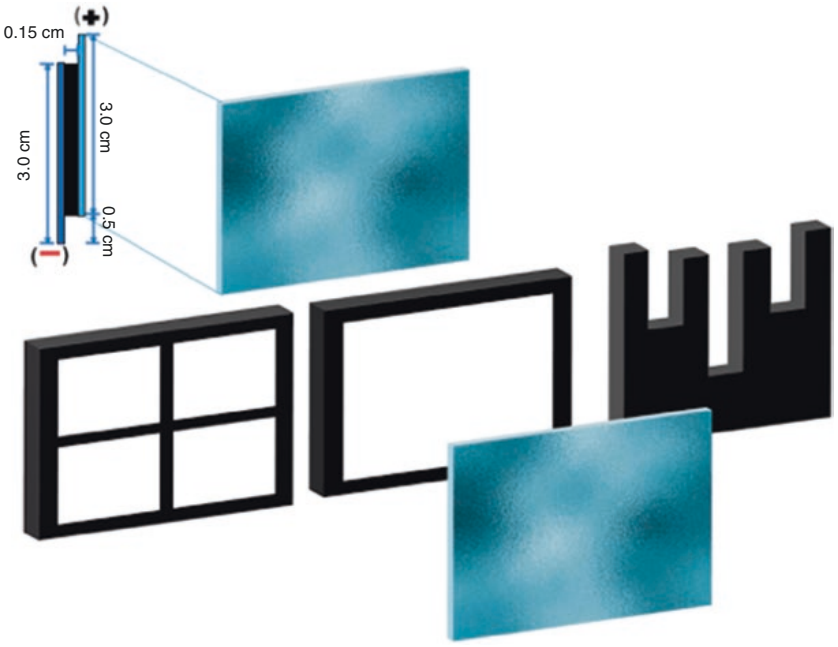

Fig. 3 Different designs of e-crystallization growth-cell for applying electric field to the crystallization process of biological macromolecules. The two frames on the *left* are useful for batch crystallization setup, and the one on the *right* is for a vapor diffusion setup

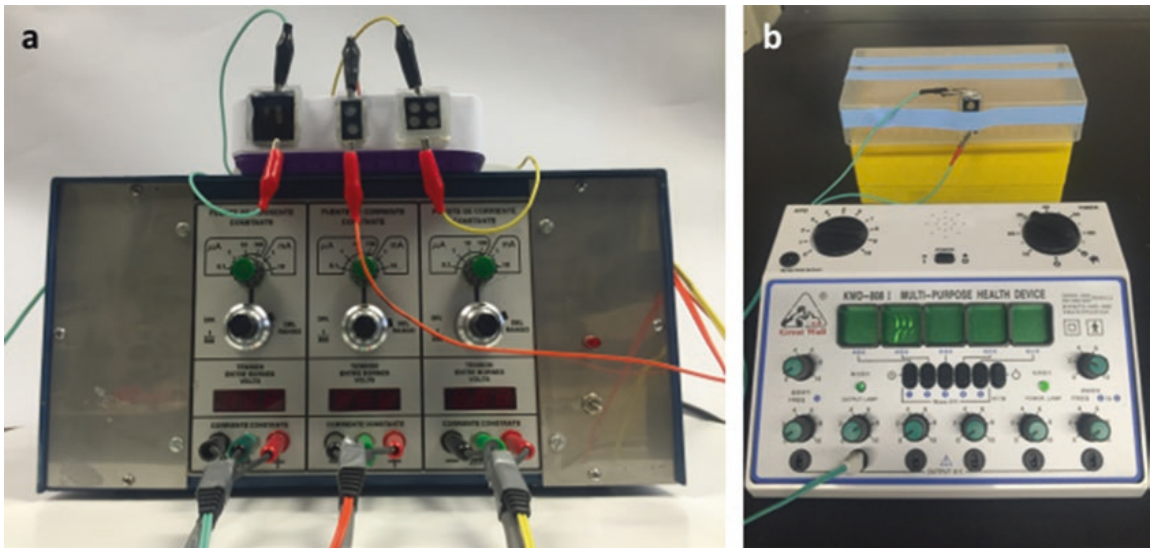

Fig. 4 Two pieces of apparatus used for e-crystallization of proteins: (**a**) for applying DC ranging from 2–6 μA, (**b**) for applying AC during the crystallization of proteins. First, nucleation is induced and then the crystal growth proceeds via vapor diffusion

the system is connected to a DC source (Fig. 4a) that supplies direct current (ranging from 2 to 6 μA) or alternating current (ranging from 2 to 8 Hz), as shown in Fig. 4b. During nucleation, the AC or DC current is turned off after 48 h, so the nuclei are

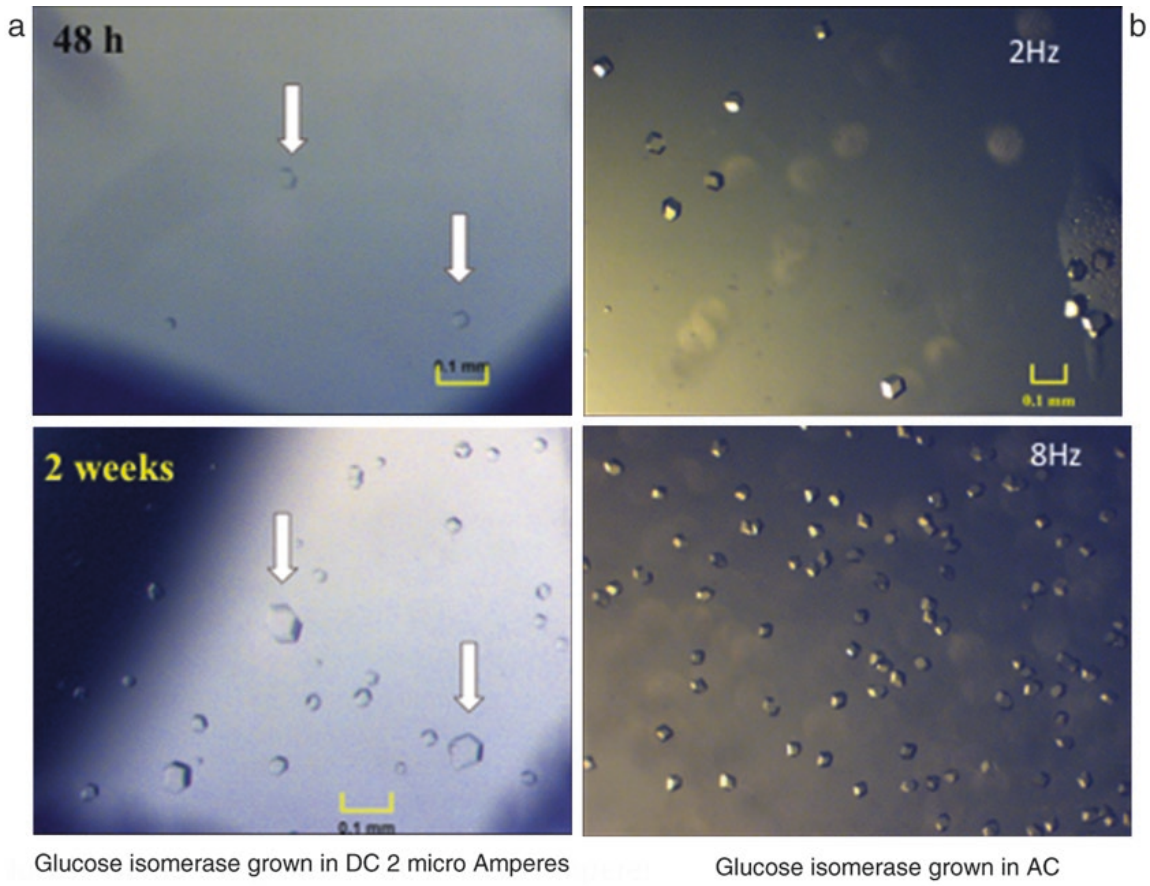

Fig. 5 Crystals of glucose isomerase grown: (**a**) when a direct current (DC) of 2 μA is applied for 48 h and subsequently the crystal growth proceeds in 2 weeks by the sitting-drop setup, and (**b**) when an alternant current (AC) of 2 and 8 Hz is applied for 48 h. The bar scale for (**b**) is the same at 2 and 8 Hz

fixed on the surface of the ITO electrodes. After that the DC growth cell is left at a constant temperature to allow crystals to grow by vapor diffusion (Fig. 5a). In the case of AC, a current of 2 Hz will produce fewer crystals and at 8 Hz will produce a higher number of crystals, although smaller in size (Fig. 5b). Thus AC of 8 Hz or higher values could be used to prepare protein nanocrystals for XFEL experiments.

3.2 The Influence of Electric Fields in the Control of Nucleation

New devices and novel methodologies to control nucleation and the size of crystals (utilizing glass beads for fragmentation of protein crystals to be analyzed in a fine mesh grid via cryo-EM) have been recently described [5, 63]. Magnetic [64, 65] or electric fields [41, 66–68] have been applied in order to obtain larger and higher quality protein single crystals either for conventional X-ray crystallography or for neutron diffraction [69]. The use of AC currents has demonstrated that there is an effect on crystal size (see above). Higher frequencies (between 10 and 50 Hz) have produced tiny crystals for seeding purposes and for crystal growth research.

There are other strategies that use specific electromagnetic fields to control transport phenomena [67, 70–73].

In the particular case of ultrasonic and electric fields, one of the pioneering contributions to study the positive effect on the nucleation processes was the proposal by Nanev and Penkova [36] in 2001. The results of these experiments in which a 25 kHz ultrasonic field (thermal double pulse technique) was applied to the crystallization process of lysozyme, demonstrated that the length of time required to obtain crystals, compared to the usual length, is reduced in half. However, the intensity of the ultrasonic field is a parameter to be considered, as the crystals broke mechanically, leading to excess of nucleation and less time for the induction of growing crystals. Along this line, other idea about using femtosecond lasers was developed in order to control nucleation [74–76]. Their use permitted to observe the area where the laser strike led to formation of only a few crystalline nuclei (this can be explained by the formation of small assemblies of protein that serve as seeds for growing nucleation centers, produced by the focalized laser radiation).

It has been shown that a growth-cell that utilizes electric fields (called e-crystallization cell with transparent electrodes), when applied to proteins, results in crystals that grow better oriented to the cathode (if the protein molecule was positively charged), compared to the crystals grown on the anode (negatively charged protein molecules) [42]. The batch method used to grow crystals applying either AC current [77–79] or DC [80–82] has been most widely used. However, in most cases, these batch crystallization conditions are not experimentally feasible to apply AC current to other proteins more than lysozyme [83]. A reengineered e-crystallization growth cell adapted to a sitting-drop setup has recently been described [42]. Another advantage has been reported for the experimental e-crystallization growth-cell, where after applying DC (to fix the nuclei on the electrode), the crystal growth process proceeds by vapor diffusion. Such a device has been used for crystallization and to search for different polymorphs of glucose isomerase [51] and lysozyme [70] at different temperatures. Along the crystal growth process, we usually obtain four different regimes: (1) induction/equilibration, (2) transient nucleation, (3) steady state nucleation and crystal growth, and (4) depletion [14]. During induction/equilibration, the sitting drop is equilibrating against the reservoir solution and becomes supersaturated when the electric field is applied; there were no nuclei visible in the light passing through the glasses of the ITO transparent electrodes. Eventually, no new crystals were formed and the existing protein crystal nuclei just continued to grow until completion of the process, reaching sizes from 100 to 300 μm, thus becoming suitable for X-ray crystallography. The crystals can be even used for diffraction experiments in situ, if the commercially available ITO electrodes made of plastic material (polyethylene) are used.

3.3 Experimental Setup for Crystallization of Proteins Under the Influence of Magnetic Fields

A majority of the advanced methods mentioned in this chapter are based on the solubility diagram, such as that shown in Fig. 2a [11, 12, 21] or that phase diagram obtained from the physical and chemistry approaches [84]. Recently, advanced methods have been developed for obtaining very high quality crystals not only by growth in gels but also in the presence of strong magnetic fields. In the particular case of magnetic fields, whether they are homogeneous or nonhomogeneous, they always act differently on samples. Nonhomogeneous magnetic fields are responsible for the reduction of gravity forces on the solution through the action of the magnetic force [46, 64, 85]. 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 subsequent decrease of natural convection [86]. Convection is practically nullified, generating a situation similar to that found under microgravity conditions [45]. Furthermore, Wakayama et al. found that, in the presence of a magnetizing force opposite to "g" (gravitational vector), fewer lysozyme crystals were obtained than in its absence [87]. The crystals that were obtained diffracted to a higher resolution, in agreement with the mathematical model [46].

For experiments of protein crystallization under the influence of magnetic forces, all proteins and precipitating agents have to be mixed according to the known batch crystallization conditions. It is important to emphasize that the preparation of the batch solution for crystallization must follow the rule that the most viscous solution must be added first, followed by the less viscous ones. Additionally, in order to guarantee highly ordered crystals, a gel can be introduced into the crystallization droplets. This must be done by mixing 1:1:1 (e.g., 5 μL + 5 μL + 5 μL) in the following order: precipitant, agar (0.60% w/v), and the protein. In the cases of standard solution, the gel might be replaced by water to preserve the same crystallization conditions as in the classic crystal growth methods. One must bear in mind that all concentrations from the stock solutions will be reduced to 1/3. Once mixed, the solution or the gelled mix is ready for the magnetic field experiments, as shown in Fig. 6. The mixture (prepared in 0.5 mL Eppendorf tubes) is drawn into a disposable 50 μL glass pipette of (Sigma-Aldrich Z-543292, 1.0-mm inner diameter), using capillarity forces. Green mounting clay from Hampton Research (HR4-326) can be used to seal both ends of the capillary tubes. Once sealed, the capillary pipettes are introduced into an NMR glass tube (8 mm in diameter) and left for at least 48 h in the presence of a magnetic field generated in a 500–700 MHz (11.7–16.5 T) NMR instrument (Fig. 6). All experiments are performed at the temperature of the control unit of the NMR probe head, usually ranging from 291 to 293 K. The sample is left in the magnetic field for at least 2 days or more. Crystals will be better and larger if the time is longer.

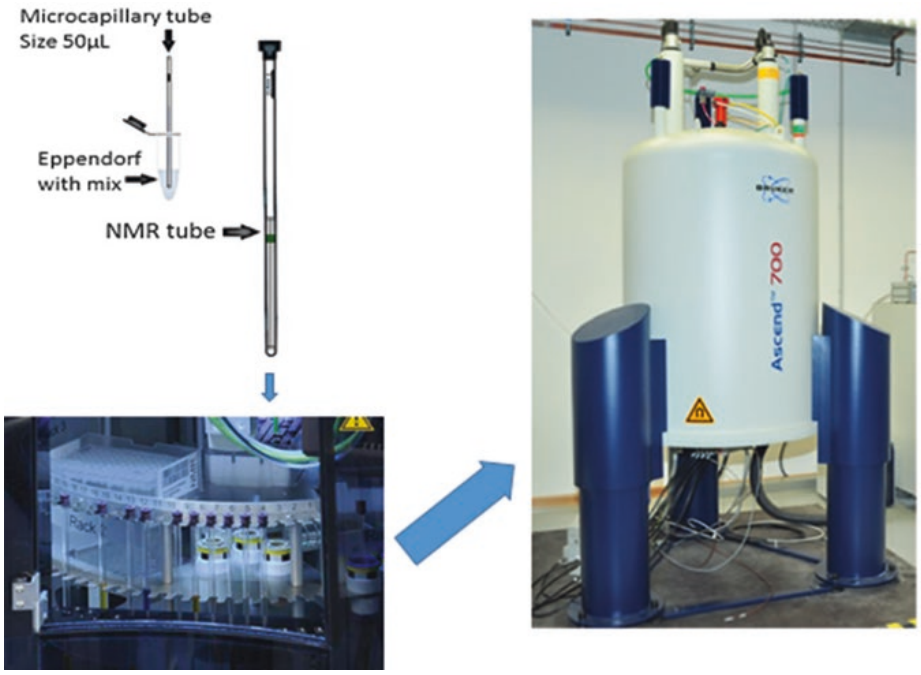

Fig. 6 A setup for experiments performed in the presence of strong magnetic field. Two types of capillaries are used: glass pipettes and NMR tubes. The magnetic field should be applied from 500 to 700 MHz (11.7–16.5 T) by using an NMR apparatus, this is that commonly used in analytical chemistry laboratories

After the end of the experiment, the NMR tube is recovered from the magnet and the capillary pipettes are extracted from it. Then, the capillary pipettes are cut at both ends in order to harvest the crystals. The cut in the capillary pipettes can be done with a glass-capillary cutting stone (Hampton Research Cod. HR4-334). Once both ends of the capillary pipettes are opened, a little air pressure (applied by using plastic latex tubing attached to a 1 mL syringe for blowing it out, or by using a pipette bulb) is sufficient to expel the solution or gel with the crystals into a few microliters of a mother liquor or cryoprotectant on either a two-well or a nine-well glass plate. When necessary, the gel can be dissected with microtools in order to release the crystals. A small incision will open the gel and liberate the crystal to permit the cryoprotectant to enter and to replace the water molecules. All crystals should be immediately mounted and flash-cooled for X-ray data collection.

3.4 The Influence of Magnetic Force to Orient and to Grow Large Protein Crystals

We could observe better quality crystals when applying strong homogeneous magnetic fields, although the field effect was different depending on the space group in which the protein crystallized. The most remarkable effect of this strong magnetic force for the growth of lysozyme crystals was when they grew in the polar space group $P2_1$ [65]. The viscosity of the solution increased when magnetic fields of 10 T were applied [88, 89]. The increase in viscosity was translated into reduced convection. In addition, an

orientation effect was observed in the crystals formed under high magnetic fields. In a more recent study, decreasing the diffusion coefficient of lysozyme was assessed in a crystallization solution exposed to a homogeneous magnetic field of 10 T [44, 65, 90]. All these observations are interrelated 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 and sediment when reaching an adequate size, which depends on the value of the field. These nuclei would act as blocks that hinder free diffusion of monomers, making the solution more viscous and, hence, lowering convection. Additionally, paramagnetic salts will produce multiple orientation responses to the application of strong magnetic fields [91].

Figure 7 shows the results of growing lysozyme and glucose isomerase crystals for 1 week inside a 700 MHz (16.5 Tesla) NMR magnet. To achieve this, it is necessary to know the conditions of batch crystallization of the protein under study. Once these conditions are known, the time needed to induce nucleation must be known and, finally, access to an NMR equipment of at least 500 MHz (11.7 MHz) or higher is needed to grow large protein crystals. The equipment must be available for the duration of the experiment (at least 3 consecutive days).

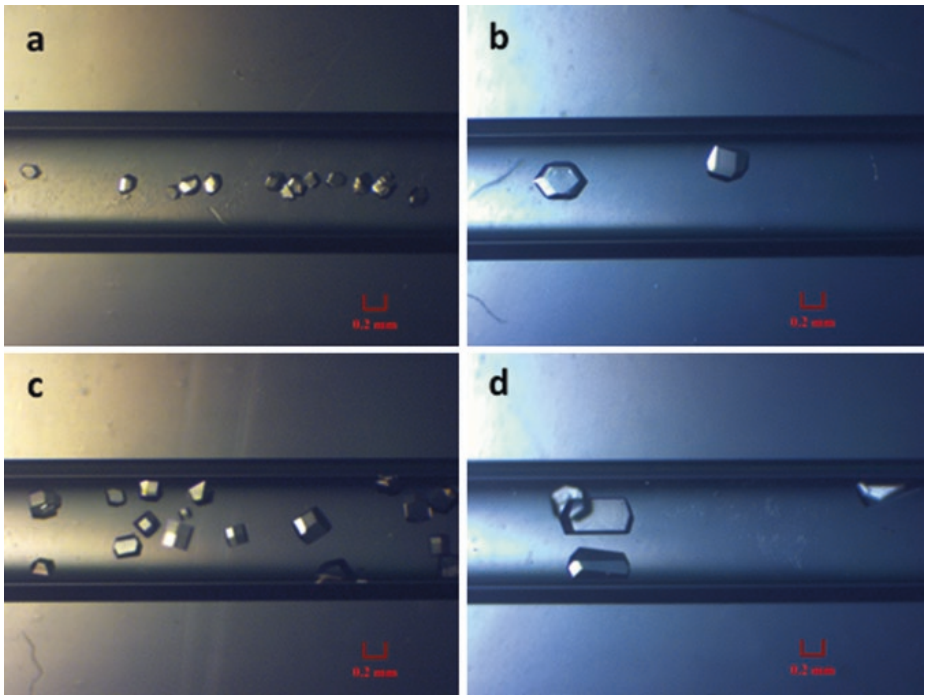

Fig. 7 Crystals of glucose isomerase: Control (**a**) and (**b**) grown in the presence of a magnetic field of 700 MHz (16.5 T). Crystals of lysozyme used as control (**c**), and (**d**) grown in the presence of a magnetic field of 700 MHz (16.5 T). The control crystals are four times smaller than those obtained inside the magnet

Studies of the influence of magnetic fields on crystal growth have been conducted during the last 15 years and they are still being continued [44, 46, 87]. There is still much to be learned about the effect of homogeneous and nonhomogeneous magnetic fields in solutions on a variety of biological macromolecules [89, 92, 93]. All these phenomena apparently favor the quality of the resulting crystals, although we still need more detailed research to understand the underlying mechanisms [64, 94]. There have been a few efforts in this respect, such as combining the positive effect of crystal growth in gels and strong magnetic fields to prove that the crystal growth kinetics is quite close to that obtained in microgravity conditions [43, 65, 86]. The effects of many physical parameters, such as electrical [66, 67, 77, 78] and magnetic fields [45, 46, 64, 65] on the control of nucleation and growth of protein crystals have been assessed. On the other hand, combining the electric and magnetic fields in order to influence crystal orientation can also benefit its homogeneous size in average of many crystals at the same time [68]. One of the main advantages of growing crystals under magnetic fields for a long time (1–3 weeks) is the ability to control their size. The large crystals obtained by applying magnetic force could be suitable not only for neutron diffraction experiments, but also for conventional X-ray crystallography, since one large crystal could yield several data sets of high quality.

3.5 Crystallization by Counter-Diffusion

Recently, several reviews demonstrated the potential of growing protein crystals in gels, which produce crystals of high quality for high-resolution X-ray crystallography compared to the crystals obtained in solution (Fig. 8a) [25, 59, 62, 95, 96]. Another way of reducing the natural convection of solutions under earth gravity is to incorporate jellified media into the solutions. Already in 1968, Zeppezauer et al. [97] described the use of micro-dialysis cells formed by capillary tubes sealed with gel caps (polyacrylamide) for reducing convection in crystallization solutions, and obtaining better crystals. In 1972, Salemme also applied crystallization inside a glass capillary tube [98], placing a protein solution in contact with the precipitating agent solution and reaching equilibrium through counter-diffusion. That technique was subsequently used to crystallize the ribosomal subunits [99]. The combination of gel-growth and the use of capillary tubes have led to the production of a considerable number of protein crystals by counter-diffusion methods [25, 95]. The historical journey of counter-diffusion methods and its fundamentals and experimental development are described below. These counter-diffusion techniques, based on diffusion-control transport processes [100–104], can also be considered as advanced methods for protein crystallization. The gel-growth technique has been used for the crystallization of inorganic salts and it was first applied for protein crystallization at the beginning of 1990s [105–107]. The counter-diffusion methods have proved efficient

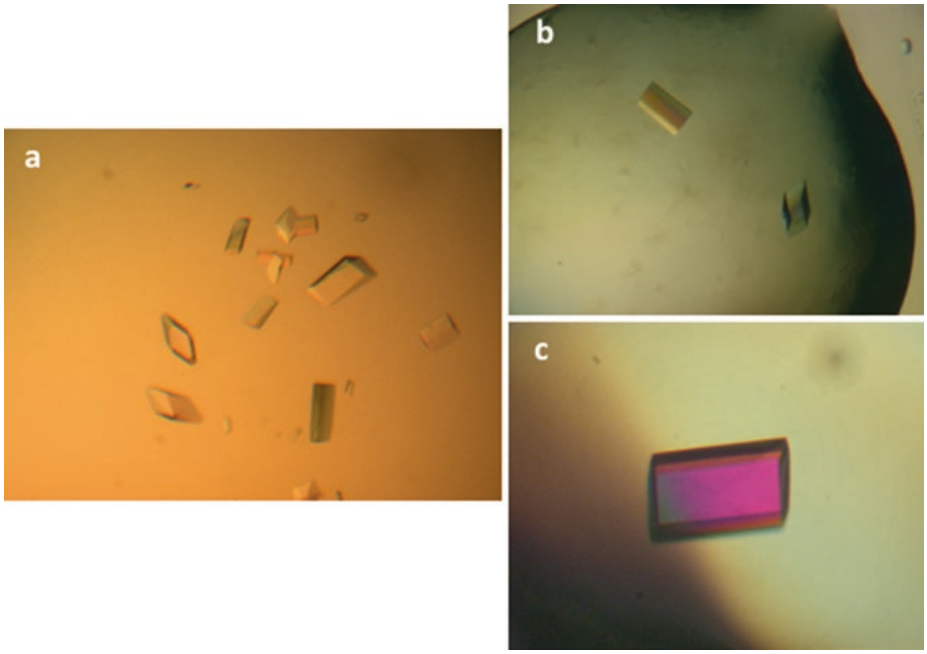

Fig. 8 Crystals of the enzyme aspartyl t-RNA synthetase grown: (**a**) in solution, (**b**) in an agarose gel (0.2% w/v), and (**c**) in a silica gel obtained by the neutralization of sodium metasilicate. As reference, the size of the larger crystal in (**a**) is 100 μm. In (**b**) both crystals are 200–250 μm, and in (**c**) ~400 μm

and effective in crystallizing a certain number of proteins, which could not be crystallized by conventional approaches [25, 95, 96].

García-Ruiz and Henisch theoretically proposed in the middle of 1980s the use jellified media to crystallize biological macromolecules by the gel-growth method (Fig. 8b, c) [102, 103]. This technique, based on the principles of reduced convection and diffusion transport, also offers an advantage of including a wide range of consecutive conditions in a single experiment [108, 109]. These advances permitted García-Ruiz and his team to develop, in 1993, the first variant of the counter-diffusion methods, called the gel acupuncture technique (Fig. 9a) [24]. This novel technique utilizes a precipitating agent that diffuses through the gel support by the capillary force inside a capillary tube filled with a protein solution, thus enabling crystallization [110]. Nowadays, this technique is better known thanks to the assessment of the different types of gels, capillaries, additives, as well as the type of precipitating agents that can be used [24, 59, 62, 111, 112]. In contrast to other techniques that use capillaries, different levels of supersaturation can exist, allowing precipitation to occur in very high supersaturation zones (nucleation occurs when supersaturation is high, and the growth of the nuclei when supersaturation diminishes), increasing the probability of finding adequate crystallization conditions [100, 108]. Another advantage includes a possibility of crystallizing proteins in capillaries smaller than 0.5 mm in diameter

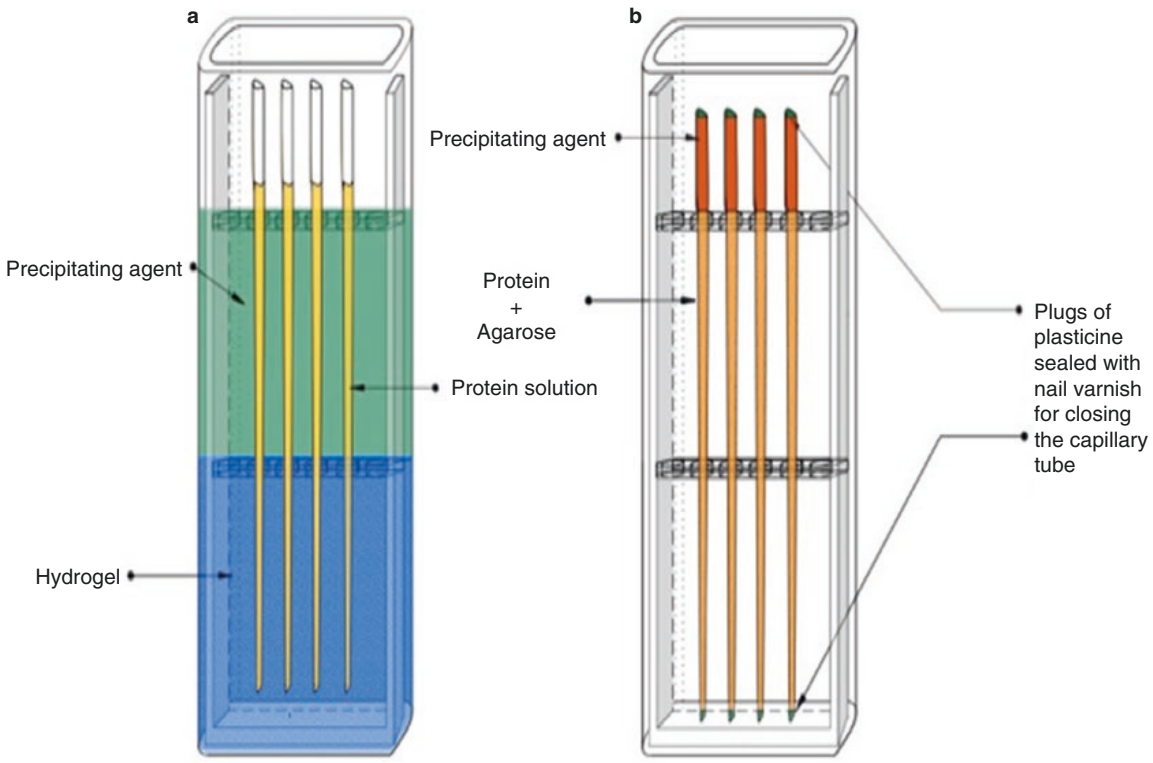

Fig. 9 Two basic experimental setups of the counter-diffusion methods. (**a**) The gel acupuncture method (known as GAME) is shown on the *left*. The capillary tubes are inserted into the gel, the protein is inside the capillaries and the precipitant on top of the gel. (**b**) The *right panel* illustrates counter diffusion in Lindemann capillary tubes, where the protein is mixed with agarose (or any other gel) and the precipitant is applied on top of the gel

(sometimes this allows to obtain cylinders of protein crystals). This helps to avoid their later physical manipulation and the risk of breakage when collecting X-ray diffraction data [56, 113]. Additionally, this method allows crystallization of macromolecules in the presence of cryoprotectant agents and/or heavy metals.

By means of crystallization strategies utilizing the counter-diffusion method (Fig. 9b) it has been possible to crystallize a variety of proteins with different molecular weights and with a wide range of isoelectric points, as well as viruses and protein-nucleic acid complexes [25, 95, 96]. In addition, as demanded by the advances in structural proteomics, there is a device that allows multiple simultaneous and independent crystallization assays suitable for an effective screening of crystallization conditions [113]. It combines the advantages of multiple conditions inside a capillary, increasing the chances of finding the optimal conditions and the possibility to obtain diffraction data directly from the crystallization device. This would turn this method into the first fully automated process, leading from the initial stages until data acquisition for structural analysis.

4 Other Practical Approaches

4.1 Crystallization in Microgravity

Years of experimenting with different crystals have confirmed that by minimizing the convective transport of mass, it is generally possible to obtain higher quality crystals, with improved mechanical and optical properties, with reduced density of defects, and larger in size.

It is natural to think that reduction or absence of gravity will lead to superior quality crystals [114–117]. Experimental observations and data support the hypothesis that convective flow introduces statistical disorder, defects, and surface dislocations into growing crystals [118–123]. Convective transport tends to be variable and erratic, generates variations in supersaturation levels around the crystal faces that are being formed, and exposes them to permanently high nutrient concentrations, equal to those inside the solution. However, in microgravity, where convection is suppressed, a reduction in nutrients concentration is produced in the crystal interphase. Transport is then purely diffusive, which is very slow for proteins. This gives rise to a "nutrients diminution zone" around the nucleus and, due to the absence of gravity, the nucleus is quasi-stable. Generally speaking, the nutrient molecules diffuse very slowly due to their size, which lengthens and extends the nucleation. Large aggregates diffuse even more slowly than the monomers that form the crystal. Hence, the vacuum zone acts as a "diffusive filter," preventing their incorporation into the growing crystal. Apparently, this is the main mechanism responsible for the improvement in the crystal quality due to microgravity. This hypothesis is not only supported by experimentation but also by a mathematical model that explains the transport process [124].

Microgravity experiments, in which the lack of convection leads to impressive results, have been evolving. It is now possible to perform a multitude of simultaneous experiments. However, there are still two criteria that can be applied for optimizing crystallization conditions: namely (1) one performs several assays that assess a wide range of conditions, consuming a large amount of material, or (2) one adjusts beforehand the preliminary conditions for future space missions. However, the consecutive missions may be delayed for months or years, which will counter the advantages of the microgravity method [125].

Utilization of microgravity has been reborn at the beginning of the twenty-first century, but only for crystallization of macromolecular complexes that have never been observed in crystalline form on Earth [126–128]. It is not surprising that most of the effort put in this new trend will offer interesting results that were difficult to get in the past due to uncontrolled experimental conditions in the rockets or during space missions (temperature variations, pressure issues, inadequate containers, etc.). We should expect specific

missions for specific problems in protein crystallization in the near future (perhaps the intrinsically disordered proteins would give us some structures that are hard to obtain on Earth or never seen in a crystalline state) [129, 130].

4.2 The Use of Ligands to Stabilize and Crystallize Proteins

When we do not know the crystallization conditions for a protein, bioinformatics analysis to predict if the protein is not intrinsically disordered should be performed first. Next, one should first make simple solubility tests (precipitation with ammonium sulfate (AMS), polyethylene glycols (PEGs), to try different temperatures for crystallization experiments as well as different pH values). Nowadays, there are commercial kits available; these are tools that allow investigating many crystallization conditions based on statistical analysis of protein crystallization. They are based on sets of conditions published at the beginning of the 1990s and even more recently [131, 132]. Crystallization robots have been developed to facilitate screening of hundreds of conditions in a short time. Once an adequate crystallization condition has been found, it can be refined by screening around it. However, there is an additional limitation if the protein under investigation is intrinsically disordered. Many proteins require ligands to stabilize their fold and to allow them to crystallize more readily. The main characteristics of the strategies and limitations of how to stabilize a protein were reviewed elsewhere [133, 134]. It was shown that 100 out of 200 proteins had been crystallized thanks to the use of specific ligands, although not all of them crystallized favorably. The use of amino acids and their analogs has been widely studied and yielded promising results [134]. Details on how to use specific ligands and nucleants in order to crystallize any protein were reviewed elsewhere [135, 136]. Pharmaceutical companies have used these strategies to investigate drugs targeted for diverse diseases. The system MAESTRO (http://www.schrodinger.com) is a suite of programs based on computational chemistry, enabling the prediction of the most probable molecules and bonds that can be used to stabilize protein, RNA, DNA, or macromolecular complexes.

4.3 Application of Microfluidics to Protein Crystallization

The limited availability of many proteins is often the key impediment in crystallographic research [137], emphasizing the need for systems that require minimal amounts of protein for crystallization. This is easy when working on the scale of liters or milliliters, but the process gets complicated as we lower the scale by 5 or 6 orders of magnitude [138]. In this way, devices that use microfluidics arise as potential tools for protein crystallization due to their ability to perform many experiments in reduced volume.

Among the desired features of these "microfluidic chips," we can highlight injection of very exact solution volumes and high reproducibility of the results [139–141]. They are characterized by either a low Reynold's number, or a lack of turbulence, which

allows only laminar flows, and ultra-fast diffusive mixtures [142]. Due to a density gradient, the microfluidic systems present either a low Grashof number or the absence of convection. This property demonstrates that it is possible to crystallize proteins with very effective kinetics [143, 144]. In the work of Hansen et al., [143] many parallel reactions were performed. The necessary solutions were introduced either manually or with the help of a robot, into 48 wells. The protein and the precipitating agents were placed in individual chambers that were later connected by eliminating the separating barrier. The total volume of the two chambers was 25 nL, and the relations between both species were set when designing the chip (in this case, they were 1:4, 1:1, and 4:1). Thanks to this device, 11 different macromolecules were successfully crystallized and one was used for diffraction experiments. Among the advantages of these devices [143] are the very precise measurement of the amount of solutions, the absence of the effects of viscosity that affect diffusion of molecules, the ease of harvesting the grown crystals, and the fact that liquid-liquid diffusion methods can be applied in the presence of gravity due to the absence of convection. With the use of microfluidics, equilibrium is achieved faster and the time to grow crystals is reduced. The plan for the future is to enable time-resolved serial crystallography using smaller size chips suitable for collecting X-ray data in situ [139].

However, the microfluidic chips still pose some disadvantages that must be addressed before they can be implemented for large-scale crystallization. Among the disadvantages is the permeability of the elastic connections. Another disadvantage is that it is hard to implement optimization stages, as the experiment starts with premixed solutions (stocks). In the future, it would be advisable to incorporate a chip of this type that can prepare solutions and to couple it in a series [145, 146]. On the other hand, harvesting of crystals is a manual process, in which the whole device is opened, increasing the risk of losing the remaining crystals in order to extract just one.

The design of these devices has been possible thanks to advances in engineering; however, the cost is still very high compared to the traditional systems. Fabrication of chips, which are similar to integrated circuits, requires strict control of cleanliness in the process because micrometric lines are being manufactured. The equipment used for their manipulation is usually very sophisticated and costly and can be used for just one experiment. The advantages of microfluidics have been recently demonstrated for different applications using graphene and a variety of materials in the fabrication of the chips, even when applied for the crystallization of membrane proteins [142, 147–157].

4.4 Automation of Mass Crystallization (High-Throughput)

Recent advances in genomics have led to large-scale efforts in structural biology in a variety of biological samples [157], culminating in Structural Proteomics Consortia and in granting large

public subsidies to scientific laboratories as well as to private enterprises, particularly pharmaceutical companies [158, 159]. Projects on metabolomics are diverse and range from studies of structure–function relationships, through mechanisms involved in protein folding and applications to biomedical research [160], to a more pragmatic focus, involving rational design of drugs based on the structure of their target molecules. The use of X-ray crystallography is critical in these studies [161].

Laboratories specializing in structural biology have, in theory, the capacity for handling large-scale projects that require maximal automation at all stages, including crystallographic research [5, 162, 163]. This is not a big issue, particularly if we consider crystallization through microfluidic techniques. In fact, there are already different types of robots on the market that perform these functions. For example, Decode Biostructures produces ROBOHTC, comprising a robot that prepares different crystallization solutions (Matrix Maker) and another robot that arranges the drops. Douglas Instruments is responsible for ORYX 6, which can be used to perform vapor diffusion assays through sitting drops, as well as microbatch assays. This company has also developed a random micro-seeding matrix screening for high throughput hints for protein crystallization conditions [30]. This robot can set up 240 cells per hour. Another commonly used robot is the TTP LabTech Mosquito. This robot contains a set of precision micropipettes mounted on a continuous band, which dispense drop volumes from 50 nL to 1.2 μL. Discarding the disposable micropipettes avoids cross-contamination and eliminates exhaustive washings time. The equipment permits sitting drop or microbatch crystallization tests, as well as hanging drop experiments. It can dispense drops into plates with 96, 384, or 1536 wells.

Up to now we have generally mentioned the advances and drawbacks of large-scale structural biology. Although most experiments have dealt with soluble proteins rather than membrane proteins, high-throughput methodologies have nonetheless been implemented also for membrane protein crystallization [149, 153, 164]. Many strategies and techniques known as in meso crystallization [165, 166] (including crystallization in lipid cubic and sponge phases) have allowed the determination of several hundred membrane protein structures [167, 168].

Finally, we can understand that the advances in the processes and automation made in the past have allowed structural biology to be developed worldwide [169]. Many laboratories are able to successfully clone, express, purify, and crystallize soluble proteins at a rate that was unthinkable some years ago. However, there is still much to be done to control and predict each of the different stages of the general process. A summary of all types of possibilities provided by the high-throughput equipment related to proteins crystallization, has been reviewed and published elsewhere [170].

5 Criteria to Analyze Crystal Quality

Beautiful crystals do not necessarily diffract X-rays to high resolution, but only a few publications have dealt with the strategies for increasing crystal quality [171]. A majority of publications were focused on proteins, though the principle can be applied to other biological macromolecules (DNA, RNA, polysaccharides, macromolecular complexes) [136, 172, 173].

The most adequate techniques to estimate the quality of a crystal are those that employ X-ray topography [174–178]. Here the diffraction equipment is placed in a very characteristic way and the crystal oriented in a preferred direction. Once this has been achieved, the diffraction of a spot is followed through the Ewald sphere (around the crystal), and its quality is characterized through rocking curves. The obtained curves are processed with specific programs that allow us to determine the crystal quality very accurately. If the curve is very fine or pointed, we can confirm that the quality of the crystal is very good; and on the contrary, when the curve is Gaussian-shaped, we can confirm that the quality of the crystal is not very good.

All the advanced methods for protein crystallization mentioned here are the result of the developments in biological crystallogenesis. The name "protein crystallogenesis" was coined by Richard Giegé in the middle of 1990s [179]. It is an outstanding science that studies all the physicochemical processes that govern the growth of crystals of biological macromolecules [180]. The methods, strategies, and devices used to obtain high quality crystals for X-ray crystallography are also part of this fascinating science.

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