Advances in Crystal Growth Techniques of Biological Macromolecules

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Received January 26, 2005; accepted March 3, 2005

Abstract. The structural knowledge of some biological macromolecules helps to understand their mechanisms of working and their role on health sciences, food science and even their effect on the economy. In this work, some recent solutions to crystallogenesis for structural analysis (convective transport, counter-diffusion, the challenge of membrane protein crystallization, and high throughput techniques) are described. Finally, investigations on microgravity, crystal growth under magnetic and electric fields, as well as crystal growth in mesophases (lipid membranes) and microfluidics are carefully revised.

Keywords: protein crystallization, transport phenomena, crystal growth methods, proteins.

Introduction

The progress in molecular biology during the last twenty-five years has been deeply dependent on the structural knowledge at atomic resolution of biological macromolecules. Many efforts have been done around the world to stimulate the structural study of proteins and of the different conformations they adopt in nature. The final aim is to understand the diversity of protein structural families, their folding and the relation that exists between structure and function. Up to date, the redundancy in the motives and structural elements found in nature suggests that the number of different conformations is finite and manageable. Once most of them will be known, it will be possible to predict the function of new unknown protein domains.

Structural biology has also influenced notoriously the field of protein-engineering. While recombinant DNA technologies are the synthetic tools, structure determination conform the analytical tool. This leads the introduction of changes in an intelligent and intentional way in opposition to random modifier techniques. The advances in genomics have allowed the expression of proteins in alive systems, study their activities and based on their structure modify them genetically for any practical purpose, like increasing their stability or affinity for a substrate, inhibitor, etc. All this will revolutionize human life in different aspects: economics, health, nutrition.

Beyond the impact of structural biology in biochemistry and biology, the three-dimensional structures of macromolecules have demonstrated to be a formidable value in biotechnology. Structural biology has promoted the pharmacology field, through the rational drug design based on the structure of target macromolecules. This will make a great impact on such diverse problems like curing human diseases, solving veterinary problems or attacking crop affections [1].

The advances in genomics, as well as in proteomics, have produced thousands of new proteins for their study in structural biology and drug design projects. The complete sequencing of vertebrate and invertebrate genomes [2] have sped up the international efforts for developing high throughput methods and technologies that allow the fast three-dimension protein structure determination [3]. Since the number of new proteins will continue to increase, as well as the number of scientist that studies them, the necessity for new efficient and effective methods for structure determination comes up [4]. Up to date and in the near future, X-ray diffraction of unique macromolecule crystals is the only technique that can 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 the purposes expressed previously [1].

Some public and private projects emerge under the name of Structural Genomics. These efforts need fast and efficient methods and techniques for three-dimensional structure elucidation. They are focused on high-throughput crystal growth for X-ray diffraction, pondering new strategies for reducing the amount of raw materials used, accelerating the work and increasing the success-rates. These efforts are multidisciplinary and they interrelate between biochemists, biophysicists, microbiologists with molecular biologists, as well as physicists and engineers for developing new strategies and equipment. Here, some of the problems that they fence and their plans for solving them will be reviewed.

For X-ray crystallography to be applied crystals of adequate size and quality must be required for precise data collect-
tion, since the fidelity of the final structure depends directly on the perfection, size and physical properties of the crystals. This converts the crystals in the key of the whole process and its production in the bottleneck. The problem of growing adequate crystals involves diverse aspects and in this article some of them will be commented, in addition to some novel and ingenious approaches to solve them.

One of the inconveniences for obtaining quality crystals is due to the natural convection that exists in every experiment performed under normal earth gravity. In addition, the problems involved in membrane protein crystallization, so important in the well-functioning of all living organisms and so difficult to crystallized, will be commented. Their amphipathic nature allows them to mediate the passage of metabolites, to sense the presence or absence of nutrients, to transfer signals into and out of the cell and to produce the energy essential for life. This characteristic is at the same time the reason that makes their crystallization so difficult to us, because of our misunderstanding of many of their physicochemical properties.

**Transport Phenomena and Crystallization**

Transport processes, and in particular mass transport, are very important for crystal growth from aqueous solutions [5-7]. As a matter of fact, mass and heat transport processes are critical for the final quality and characteristics of the crystals [8]. Many crystallogenesis techniques have been explicitly developed for controlling the relative contributions of convective and diffusive transport in crystal growth [9]. During the active incorporation of ions or molecules to 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 [10-12]. Convective transport of molecules competes with pure diffusive transport and the interaction between them will determine the way and the kinetics of nutrients presentation to the growing crystal.

Transport phenomena not only do affect 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 [13]. On the other hand, convective transport only occurs in the presence of earth gravity. Only then heavier fluids can go down and lighter fluids will go up, letting convective currents to emerge in the bulk of the solution. Other types of convection do exist, as the convection due to surface tension [14] but they are not significant for the crystallization process from solutions.

**Crystallization in Microgravity Environments**

Many years of experimentation with diverse crystals have confirmed the notion that minimizing the convective mass transport better quality crystals can be obtained, with improved mechanical and optical properties, reduced defects density and larger sizes.

How is it possible to suppress the natural convection in crystallogenesis? Nowadays, different approaches arise for deleting or at least reducing it. One of them is the crystallization of macromolecules in space, where in the absence of gravity the convection disappears. In the last decade, a new approach that involves the use of magnetic fields has materialized. Magnetic forces opposed to gravity can reduce natural convection inside solutions [15-16]. Also, the methods for crystallizing macromolecules in gels are good and well-accepted alternatives for eliminating natural convection [17].

It’s natural to think that at zero gravity, or at a reduced one, crystals with superior properties can be grown [13]. How can that happen? Observations and experimental data support the hypothesis that convective fluxes introduce statistical disorder, defects and dislocations in the growing crystal surfaces [18-20]. Convective transport tends to be variable and random, producing variations of the supersaturation levels in the environment of the developing faces, exposing them permanently to high levels of nutrients, similar to those of the bulk. On the contrary, under microgravity the convection is suppressed and the concentration of nutrients in the interface of the crystal is reduced (Fig. 1). The mass transport is purely diffusive, which for proteins is very slow, and a region of depletion of nutrients is established around the nucleus. Thanks to the absence of gravity, this zone is quasi-stable. On the right part of figure 1a, nutrient molecules are shown diffusing very slowly because of their size, lengthening the effect. On the left, impurity molecules diffuse more slowly than monomers do. As a consequence, the depletion zone acts as a “diffusive filter” avoiding the incorporation of impurities to the growing crystal. Apparently, this is the principal mechanism for the improvement in crystal quality under microgravity.

This hypothesis is not only supported by the experimental data but also with a mathematical model that explains the mass transport process involved [21]. Figure 1b shows that up to 40% reduction of nutrient molecules near the nucleus can be achieved in the absence of gravity, related to the bulk concentration.

The problem of suppression of the convection was the inspiration of many scientists who devoted their work to develop new techniques and devices. The first serious experiment of crystallizing macromolecules under microgravity was made by a German team under the direction of Professor Walter Littke in 1978. In that occasion, lysozyme and β-galactosidase were crystallized successfully by the liquid-liquid diffusion method within a series of reactors [22-25].

The progression made in this crystal growth area was hard and slowly owing to little documentation of the experiments performed in microgravity, during the 70s and 80s. Moreover, many results were unavailable to the research community because they belong to private companies or because of the little communication between oriental and occidental scientists [13]. It was until 1989 that the first formal scientific paper was published in Science, in which X-ray diffraction analysis from many crystals grown in microgravity were reported. They presented higher $I/\sigma(I)$ rates through the
whole range of resolution, and higher R values [26]. The article stated a tangible proof that crystals formed under microgravity environments generate more data of higher quality so as to obtain more precise structures.

**How are the experiments in microgravity performed?**

Nowadays many devices do exist based primarily on two techniques: vapor diffusion and liquid-liquid diffusion [27], even though the thermal-induced batch technique has also been successful in growing some large crystals that gave very high resolution [28]. The experiments are performed by governmental space agencies or private consortia, such as Payload Systems (USA), Intospace (European consortium, recently dissolved) and Bioserved (a centre for the commercial development sponsor by NASA). Each consortium has developed its own devices for its experiments [27]. Among the vapor diffusion devices, VDA (vapor diffusion apparatus), designed and built by Dr. C. Bugg and collaborators from Alabama University at Birmingham, USA [29-30] is the most used device, with more than 25 missions. Nevertheless originally it was a very simple one; it was possible to crystallize many diverse proteins, like lysozyme, canavaline, bovine serum albumin, among others. Presently, newer and more complex versions exist, which offer major advantages and possibilities for experimentation.

As mentioned above, the experiments made in space do not course in the same manner as the ones performed on earth. For example, for vapor diffusion experiments made under both environments, the equilibrium kinetic is different, and this difference is more notorious for those liquid-liquid diffusion experiments. In spite of this, the superiority of the crystals grown in space respect those grown under earth gravity, is established by comparison, following 4 well set up criteria. First, a Visual Exam is fulfilled (this is a subjective analysis: observation of the crystal at the microscope). Then, the sizes and the distribution of those values in the experiment are analyzed. Morphology is another criterion to evaluate, as many protein crystals grow with different crystalline habits, depending if the process was on earth or in space [26]. Finally, the properties of the X-ray diffraction pattern generated by a crystal must be considered (the internal order of a crystal rely on the kinetics of growth).

Following the mentioned criteria, a number of advantages found for the microgravity crystals can be summed up [26, 31-32].

- Visual superiority.
- Larger crystals, many orders of magnitude higher than the biggest crystal grown on earth [13].
- Higher resolutions achieved in x-ray diffraction patterns.
- Better signals I/σ(I) (Wilson Plot). In the whole range of R, higher values of I/σ(I) for microgravity crystals were found. However, the physical properties responsible for this observation is yet unknown. Apparently, the reason is a lower defect density.
- Sharper x-ray diffraction intensity peaks, showing quantitatively the better internal order and perfection of space protein crystals.
- Not only proteins can be crystallize in the absence of gravity, but also other macromolecules, difficult to crystallize on earth, like viruses (Satellite Tobacco Mosaic Virus), DNA, pharmaceutical targets (reverse transcriptase of HIV) or membrane proteins (bacteriorhodopsin).
- Under microgravity, the sedimentation effect disappears. In space, the crystals keep their defined and stable positions over long periods of time. This is a favourable environment for multiple crystal growth, minimizing the superposition of diffusive fields and assuring a more or less uniform access of nutrients to all of the faces [33]. Besides, the incorporation of microcrystals or three-dimensional nucleus by sedimentation over the growing faces is avoided.
Thanks to the reasons previously expressed and the advantages numbered, the crystallization of macromolecules in microgravity is very convenient, particularly for those macromolecules hard to crystallize on earth, or in case of knowing the optimal crystallizing conditions it's possible to study some aspects of its growth process. On the other hand, among its disadvantages we have to mention the cost, it is an expensive method and requires much time since the missions are not daily and they last many days, specially when consecutive experiments are desired.

**Crystallization Using Magnetic Fields**

It is possible to reduce natural convection on earth with the help of magnetic fields. Counting if they are homogenous or inhomogeneous, they act upon a sample in different ways. Inhomogeneous magnetic fields are responsible for reducing the effective gravity that a solution feels through the action of a magnetization force [16]. If a magnetic field gradient is applied vertically, a magnetization force will be generated. When this force opposes to gravitational force, a reduction of vertical acceleration (effective gravity) is obtained. Hence, a decrease in natural convection is accomplished.

With a mathematical model of a crystallization system under a magnetic field, the concentrations of macromolecules in the surroundings of a growing crystal were estimated (Fig. 2), verifying that a magnetic gradient of -685 T²/m reduces 50% the convection, while a magnetic gradient of -1370 T²/m practically eliminates it, producing a similar background to that of microgravity [34]. Experimentally, high-quality high-resolution crystals were obtained, in agreement with the mathematical model [35]. Moreover, Wakayama and colleagues found that in the presence of a magnetizing force opposite to g, fewer lysozyme crystals were obtained than in absence of magnetic force [16].

When a homogeneous magnetic field is applied, high quality crystals are also observed [36], even though the mechanism involved is different. An increase of viscosity near the growing crystal was observed when a magnetic field of 10 T was applied [37-38]. The increase in viscosity means a reduction of natural convection inside the solution. Furthermore, an orientation effect was observed upon the crystals formed under high magnetic fields [15-16].

More recently, in another study, the diminution of the diffusion coefficient of lysozyme inside a crystallization solution under a homogeneous magnetic field of 6 and 10 T was evaluated [39]. All these observations are interrelated and are the consequence of the orientation effect by the magnetic field at a microscopic level. In a supersaturated solution, proteinaceous nuclei are suspended in the solution bulk and sediment upon reaching the adequate size, which depends on the magnitude of magnetic field applied. These nuclei act as blocks avoiding the free diffusion of monomers, turning the solution more viscous and as a result with less convection [39].

![Fig. 2. Isoconcentration lines (O) for the crystal growing at the centre of the bottom under (a) microgravity, (b) normal gravity, (c) a magnetic field gradient, $\mu_0 (dH/dz) = -685$ T²/m and (d) $\mu_0 (dH/dz) = -1370$ T²/m. Reprinted from [34], with permission from Elsevier.](image)

The research area of crystal growth under magnetic fields is relatively new and needs more study. A lot of things still remain to be understood about the effects of magnetic fields (homogeneous and inhomogeneous) over macromolecular solutions. Evidently, an external strong magnetic field induces a magnetizing force, increases the viscosity of the protein solution, orients the growing crystals and affects the growing process in a complex manner. All these phenomena seem to favor the resulting crystal quality, although a more complete investigation is needed to understand the mechanism [38].

**Crystallization with Counter-Diffusion Methods**

Another way to reduce the natural convection under earth gravity is to incorporate gelled media inside the solutions. In 1968, Vepczauzer and coworkers described the use of microdialysis cells, made of capillary tubes sealed with gel stoppers (polyacrylamide), to reduce the convection from crystallization solutions so as to obtain better crystals [40]. Then, in 1972, Salemne also intended to crystallize proteins inside a glass capillary tube. He put into contact a protein solution with...
a precipitant agent and let the system reach the equilibrium by counter-diffusion [22]. Some years later, ribosomal subunits were crystallized successfully with the same set-up [41].

After many years of investigation, Dr. García Ruiz J.M proposed the use of gelled media for crystallizing macromolecules by counter-diffusion. This technique puts together the principle that reduces the convection and the advantage of having a wide range of conditions in a single experiment [42]. All these progress allowed Dr. J.M. García-Ruiz and colleagues in 1983 the development of a new technique called gel acupuncture [43-44]. This novel technique consists in the permeation of the precipitating agent solution through the gel and the penetration by capillary force into the capillary tube, filled with a protein solution, allowing the crystallization [44]. This technique is well known today, and different types of gels, capillary tubes, additives and precipitating agents have been evaluated for their use [44-45]. A difference with other methods is that inside de capillary tube there is not an only one supersaturation level, thus precipitation zones will be found in regions of very high supersaturation, nucleation will happen at high supersaturation levels and the growth of those nuclei will be found in regions of lower supersaturation levels. This raises the probabilities of finding the right conditions for crystallization [46]. Other advantages are the possibility of crystallizing proteins inside capillary tubes ready for direct x-ray diffraction data collection, keeping from the usual physical manipulation of the crystals, reducing the risks of breaks at the moment of mounting them or transporting to the synchrotron [47], or the use of cryoprotectors and/or heavy metals inside the crystallization solutions [48].

Through the gel acupuncture method it was possible to crystallize diverse proteins of different molecular weight and wide range of isoelectric point, viruses and protein-DNA complexes [49]. In addition, thanks to the advances in structural genomics, a new device was developed for executing multiple and independent experiments, which is proper for effective screening of crystallization conditions of biological macromolecules [47]. It combines the benefits of multiple conditions in one capillary, increasing the chances of finding the optimal ones, with the possibility of direct x-ray diffraction analysis from the device. All this converts the device into the first totally canalized system since the initial steps to data collection for structural analysis [47].

One modification of this technique is the protein crystallization by the gel acupuncture under the presence of an internal electric field [50]. The study of the effect of electric fields over crystallization solutions has not been explored till a few years ago. Recent studies performed by Dr. Aubry and co-workers, with electric fields externals to the crystallization solution, showed that it is possible to reduce lysozyme nucleation and increase the crystal growth rate [51]. Then, the same group evaluated the crystal growth kinetics and found an increase in the protein concentration near the drops that were close to the cathode [52]. Nanev and Penkova [53] came up with similar results when crystallizing lysozyme by the batch method, in the presence of an external electric field. They reported that lysozyme crystals grew with a definite orientation towards the cathode.

Biological macromolecule crystallization in the presence of an internal electric field uses a similar set-up to that used by the gel acupuncture method, except that an inert electrode (Pt) is introduced inside the capillary tube, in contact with the protein solution, and another electrode is set collinear to it, in the gel. Protein molecules are charged since the pH solution is far from the protein’s isoelectric point. When a small direct and constant current is imposed to the system, a potential difference is established between the electrodes, provoking an orientation effect over the macro-ions (protein molecules). Lysozyme and thaumatin crystals were found to be firmly attached to the anode, during the crystallization process.

With the purpose of understanding what is going on when the potential difference is established, is important to comprehend how the solution structures and the effects of the electric field over it. The proposed hypothesis for the nucleation inside the capillary tube, in contact with an anode, considers the presence of an electric double layer in the surroundings of the electrode. When an electrode is put into contact with an electrolyte solution, the ions feel asymmetric forces and order themselves forming an electric double layer. The first layer is composed of water molecules with their dipoles oriented, and the second layer is composed of counter-ions [54]. Positively charged protein molecules need a negative ion for favorable protein-protein interactions (for example Cl⁻ for lysozyme [55], tartrate for thaumatin). The potential difference established in the cell allows the migration of anions of the precipitating agent into the capillary tube, encouraging the interaction with protein molecules in solution, at the first instance. Then the counter-ions of the electric double layer will act as support for positively charged protein molecules or nuclei, letting the crystal growth over the anode. This behavior was not found when a cathode is placed inside de capillary tube [50].

This technique is very new and was only evaluated with model proteins, like lysozyme and thaumatin). However, it seems very promising as crystals with similar quality than the ones grown with gel acupuncture method were obtained, but with shorter induction of nucleation times, without affecting the three-dimensional growth rates [50, 56]. This is very interesting from a biotechnology point of view, since the necessity for shortening the crystalline production times is desired.

The reduction of the induction time in the crystallization had also been observed by Moreno and Sazaki [57]. Lately, these scientists have studied the effect of the electric field with a different set-up, using the batch method and with parallel electrodes. Although these variations, they noticed an induction time three times shorter in the presence of an internal electric field, corroborating the results found with the gel acupuncture method plus the internal electric field. Besides, in the same work the benefit of the nucleation control is remarked, since they obtained fewer lysozyme crystals with a homogeneous size distribution.

The simultaneous effect of magnetic and electric fields is a new research field. Depending on the configuration of the
system, great advantages can be acquired, such as the homogeneity in crystal size, thanks to an apparent suppression of secondary nucleation events that permits the continuous growth of previously formed nuclei [58]. Also, an orientation effect is commented when the magnetic field is parallel to the electrodes, the same effect as described in previous works [39, 59].

Membrane Protein Crystallization

It’s amazing how few membrane proteins have been crystalized and their structures have been reported, when minimally one third of the genome codes for proteins with at least one transmembrane domain. Up to the year 2004 least than 70 membrane integral proteins have been published [60].

Why do membrane proteins, in opposition to soluble proteins, represent such a challenge to crystallize? The answer can be found if we analyze the steps involved in crystal production. The difficult for obtaining high quality crystals of membrane proteins is related to our limited knowledge to manipulate proteins that have hydrophobic/amphipathic surfaces, usually covered with lipids from the membrane of origin. Moreover, proteins aggregate without a defined order during the aqueous transition from membranes to crystals, stopping us from knowing their structures and activities [61-62].

The first membrane protein structure elucidated by x-ray diffraction to atomic resolution was a photosynthetic reaction center in 1985, by Drs. Michel, Diesenhofer and Huber [63].

Even though there has been a great advance since the 80s thanks to the “in surfo” method, this has been slow and continuous, with a rate of one structure per year. The “in surfo” method is based on the use of mixed micelles made of detergent, designed to solubilized membrane proteins from native membranes. Then, this aqueous dispersion is treated like a solution of soluble proteins, whose crystallization can be achieved by vapor diffusion or microdialysis techniques. This method proved to be very useful in many cases, and several revisions about it were written [64-65], so it will no be commented in this work.

There are some situations where the “In Surfo” method is not able to crystallize some proteins and other alternatives have arisen. Six or eight years ago, two completely new methods have been developed; one based on bilayer micelles [66] and the other on lipidic mesophases [67]. Recently a new approach has emerged based on the use of lipids and detergents, called “bicelle method”. Even though only bacteriorhodopsin has been crystallized, giving crystals adequate for x-ray diffraction, the method is in its initial stage [68]. All these three methods mentioned are based on the formation of extended lipid bilayers, composed of lipids, detergents and proteins.

Cubic Phase Method

This method is also known as “in cube” or under the more general term “in meso”, since it involves the formation of a liquid crystal or mesophase, as a support for the crystallization process. Very little is known about how and why the method works [69-70], as well as how is the phase or the microscopic structure that supply nutrients to the growing faces of the crystals.

The proposed hypothesis explains how the protein is reconstituted inside a cubic phase from the original membrane and then how the protein molecules aggregate slowly in the presence of precipitating agents and additives. Initially the protein is embedded in an aqueous micellar solution, formed by residues of the starting membrane and the solubilizing detergent (usually an alkyl glucoside). The dispersion is mixed with another lipid, in solid state, giving rise to the different phases. For example, for monoolein, a frequently used lipid, a stable cubic phase is formed by adding 40% w/v at 20°C [71].

With the first contact, water molecules will migrate from the protein solution towards the new added lipid, which can be hydrated to some extend, establishing a gradient of activity for water molecules. Throughout this gradient different phases will appear: at low hydration level a lamellar phase (liquid crystal) will be formed and with higher hydration levels the diverse cubic phases will appear (Fig. 3). Thanks to water dis-
placement, protein and detergent molecules will concentrate, favoring the formation of another lamellar phase [72]: a continuous lipid bilayer will be formed where protein molecules will be reconstituted, under equilibrium conditions. Membrane protein and the detergent will be homogeneously distributed all over the phase and the membrane protein will recover some physicochemical properties of lipidic bilayer molecules, such as lateral pressure and hydrophobic matching, similar to those of native membranes. During the crystallization steps, precipitating agents are added in proper amounts to induce the formation of nuclei, which will get together in a lipidic yard, where they will associate and eventually they will be organized into a three dimensional lattice. The process is favored by the high ionic strength, masking the protein charges so as to facilitate the contact between protein molecules, their nucleation and crystal growth [73].

During mesophases formation, lipidic molecules self assemble spontaneously in the aqueous media because of their amphipathic nature. Depending on the hydration level of the lipid molecule, different cubic phases will be formed; being the less hydrated the liquid crystalline lamellar phase (Lά), where plane lipidic bilayers can be seen (figure 3).

The conventional protocol designed by Drs. Landau and Rosenbusch [67] suggests the mix of two parts of protein dispersion with three parts of lipids (usually monoolein). The cubic phase is formed almost immediately. Then, salts or precipitating agents are added and after the incubation of the mix at 20ºC, crystals will appear in hours or weeks. The lipids evaluated for this technique are monoolein (C18:c9), monopalmitolein (C16:c9) [67] and monovacenin (C18:c7) [75].

This technique has been proved for soluble and membrane proteins [67, 76] and it has been slightly modified for its application in high throughput crystallization. Among the proteins successfully crystallized we can find bacteriorhodopsin, which diffracted at 1.55 Å [77], and halorhodopsin [78].

For using this technique, some considerations must be put in mind. The choice of the detergent is very important since it will be present in the mix through all the crystallization process. Non ionic detergents are preferred, like alkyl gluco-sides [79]. Salts are responsible for the generation of the dri-

The second method called “vesicle fusion”, has been success-

High Throughput Crystallization

In the last years, genomic advances have encouraged high through put structural biology studies in such a way that it received the name of Structural Genomics and many huge public grants were given to academic laboratories and private enterprises, like pharmaceutical industries, around the world. The projects are diverse and cover from the study of the relation structure-function of proteins, passing through the mechanisms involved in protein folding [87], to a more pragmatic approach, the rational drug design based on the structure of target molecules [88]. X-ray diffraction crystallography is critical in these studies, being the battle-horse in such initiatives. In this way, the crystallogenesis of biological macromolecules arise as vital step in the whole process, although it’s the more complicate and less understood step in structural biology. In this section some aspects involved in high throughput crystal growth of biological macromolecules will be reviewed.

The growing of crystals of biological macromolecules in large quantities takes in several steps: protein production in large amounts (by heterologous expression or from its natural source), purification, crystallization trials and their corresponding inspections. Since many proteins are evaluated at the same time it’s mandatory to have automated systems that accelerate the work but at the same time they should be trustable since the efficiency of each step affects the following one.
Heterologous protein production is almost completely automated for massive aims. It includes cloning, transformation and gene expression. These stages involved DNA molecules and thanks to their high stability they could be automated easily [89-90]. Nevertheless, there isn’t yet a totally automated system for protein purification in large scale with applications in structural biology. Among proteins, they differ in their expression levels, solubilities and physicochemical properties, so that most scientists prefer to adopt a combination of manual techniques for obtaining pure proteins. Despite this, many biotechnological companies have developed more integral solutions for large scale purification. For example, Syrrx (San Diego, Ca., USA) uses a purification system developed in the Genomics Institute of Novartis Research Foundation, which combines the centrifugation and robotized sonication with a system of column chromatography arranged in parallel, able to purify 96-162 proteins per day [90]. Affinium Pharmaceuticals (Toronto, Canada) has also developed an integral purification system. Proteomax® covers all the steps, since the cellular extract processing till the pure, concentrated sample, ready for analysis. This equipment can clarify the lysate, perform column chromatographies, desalt and concentrate samples, giving in the best cases a pure protein, ready for structural studies. So many automated steps results in a very useful purification equipment for large scale studies [88].

Structural biology laboratories have the capability to handle more than 1000 different proteins in a month. In consequence they required the maximum possible automation of every stage, included crystallogenesis. This is not such a big problem, particularly if considering the crystallization by vapor diffusion or microbatch techniques. As a matter of fact, diverse robots that can perform those functions mentioned do exist in the market. Decode Biostructures produces ROBOHTC, composed of a robot that prepare all different conditions (Matrix Maker) and another robot that dispense the drops. Douglas Instruments is responsible of ORIX 6, which performs vapor diffusion with sitting drops or microbatch essays. This robot can bring about 240 cells per hour. Another very used robot is Mosquito, from Molecular Dimensions. Mosquito is built by TTP LabTech, of TTP Group p.l.c. one of the most successful technology companies of the world. This robot contains a set of precision micropipettes mounted on a continuous band, which emits small volumes, from 50 nl to 1.2 μl. Moreover, the micropipettes are disposable, avoiding cross contamination problems and long time exhaustive washing. The robot dispenses drops for microbatch or vapor diffusion, hanging or sitting drops crystallization experiments. It can be used with 96, 384 o 1536 well. Also, it comes with a software system easy to use and that can be programmed.

Once the essays are set for incubation, a regular inspection is required so as to find the adequate conditions for obtaining high quality crystals. This is the most arduous part of the high throughput crystallization. For a certain protein 1000 experiments are needed, in average, for obtaining an appropriate crystal for x-ray diffraction analysis [88]. Many companies use human inspection, a very tedious and laborious step, so the idea of designing an automated inspection system is very tempting. An ideal inspection system should have the following characteristics:

1. Identification and elimination of clear drops, with 0% of error (without risk of losing any crystal).
2. capture of kinetic data (of the growing process or precipitation vs. time)
3. Ability to distinguish crystals from precipitates and to find crystals inside precipitates most of the cases.
4. Determination of size and form of the crystal
5. Ability to improve points 3 and 4 based on an “internal learning” (data bases).

Some promissory advances in the image technology area are made by Decode BioStructures, which offers “Crystal Monitor Workstation”. This equipment has a stereoscopic microscope, digital camera, voice control, a database interface and can be coupled to ROBOHTC (from the same company). There also exists “Crystal Score” by Diversified Scientific, which provides a microscope over a motorized plate. It comes with a device that count and size the crystals. RoboDesign has in the market two options, RoboMicroscope II, that can localize the drops, focus them, capture a color image and store them automatically, while CPXO can classify the drops into clear, precipitated, with crystals or other options.

Some of the advantages of automated image capture are the high frequency with which images are registered, at precise times and the possibility of being evaluated with diverse computer softwares, applying artificial intelligence. Although the equipment doesn’t have human experience it can be trained for developing its own database. Anyway, there is no system that totally ignores human inspection. Crystals are very difficult to obtain and meantime an automated system with 0% of false negatives doesn’t exist, the human eye will be indispensable [88].

Up to here it was mentioned in a general way the advances and problems of high throughput structural biology. However, most of the works are on soluble proteins. Membrane proteins entail other kinds of problems, previously mentioned (in the membrane crystallization section).

Which are the specific challenges of membrane proteins structural biology? Diverse stages can be mentioned, like protein production, purification and crystallogenesis. Unlike soluble proteins, where more than 90% of the new protein structures come from recombinant samples, membrane proteins obtained from molecular biology techniques are less than 50% (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). This is in part because the strategies developed for overexpressing proteins are designed for soluble ones and they don’t favor integral membrane proteins [89]. The synthesis of membrane proteins makes use of the cell’s secretory system, certain directionality and the insertion of them inside the membranes [90-91]. Besides, many cells are not equipped for standing such a flux of new membrane proteins, saturating their secretory pathways and generating inclusion bodies in
the cytosol or toxic intermediates for them. So, the election of the expression system is an important issue in the production of membrane proteins.

Prokaryotic integral proteins can be expressed in prokaryotic systems with hopeful results. To date, some examples demonstrate that, like the structures of ionic channels [92] and certain proteins from the outer membrane [93] among others. On the contrary, eukaryotic membrane proteins over-expressed in prokaryotic organisms have been harder to achieved [94] since prokaryotic membranes have a different lipidic composition and can be a hostile background for heterologous proteins. Besides, post-translational modifications are necessary for the correct folding of eukaryotic proteins or their insertion into membranes, but they are absent in prokaryotic cells [95]. Despite this, some isolated successful examples do exist, such as the case of an enzyme bound to mammal membranes over-expressed in E. coli, when was crystallized [96] and its structure solved [97], or the over-expression of a eukaryotic receptor coupled to G-protein in Halobacterium salinarum [98]. On the other hand yeasts are good over-expression systems for eukaryotic proteins since they are easy to handle and powerful genetics tools [99-100]. Mammal cells are the best choice for preserving structural and functional integrity of mammal membrane proteins. However they are expensive and very complex to use, as a result they are the last choice.

With regard to purification, detergents are usually used for solubilizing membrane proteins. So, the election of the detergent is essential, especially when designing studies in large scale. Ideally a detergent should solubilize the membrane protein without forming aggregates [101]. An appropriate detergent is one that can selectively stabilize the native structure of proteins [102]. Each protein behaves in a particular way and this individuality is responsible for the need of a specific purification protocol for each one. This concept is in opposition to “a unique measure for all”, motto of en masse experiments. Diverse strategies have been thought up for resolving this, like to fuse a protein, which has certain affinity for a ligand, to the extreme of a membrane protein, which is desired to over-express, so as to facilitate its purification [103-104].

Once pure, integral proteins are ready for crystallizing. Two alternative paths can be applied:

1. To crystallize the complex protein-detergent directly.
2. To incorporate once again the protein into a lipidic bilayer environment, previously to its crystallization.

Most of the structures solved by x-ray diffraction analysis come from crystals formed by the first path, by vapor diffusion or microbatch techniques. The method is similar to that used for soluble proteins just that in this occasion the solute is the complex detergent-protein [101]. The use of robots simplifies the handle of these mixtures. The other path consists on restoring the membrane proteins to a lipidic bilayer environment, before setting the crystallization experiments. This approach has its major model in the lipidic cubic phases method, as previously explained, so that it won’t be commented again [67]. Automated equipment that can use this criterion for high throughput membrane protein crystallization is under construction [84].

Finally, we can comprehend how the advances in the processes involved and the automation achieved in the last years have influenced the development of structural biology throughout the world. Many laboratories can successfully clone, express, purify and crystallize soluble proteins in an unthinkable proportion years ago. Nevertheless, there is yet much to control and predict in many different stages of the general process. Relating to integral membrane proteins, the required management of the whole process has not been achieved, even less than the one required for soluble proteins. In the near future, the problem of the amount of protein available for crystallization must be overcome by increasing the protein production levels and improving the systems of purification, specially the detergent election. All this will help to obtain more successful crystallization trials. Besides, studies of the mechanisms that rule the process will aid to its scaling up and automation. Moreover, so many crystallization experiments will enrich databases and in consequence it will be possible to extract the tendencies and crystallization patrons, been beneficial for structural genomics, particularly with the aim of crystallizing new proteins.

New Tendencies in Protein Crystallization, Microfluidic Devices

In the year 2001, a rough copy of the human genome was announced [105] and approximately 30000 different proteins were estimated to exist, coded on it [106]. Many of those proteins have unknown functions and structures; furthermore, of those proteins that have reported structures, the details of their mechanism of action or function continuous to be a mystery. In many cases, to know the function of a macromolecule requires the interpretation of precise structural data, which is obtained in a reliable way from x-ray diffraction analysis [107]. Thus, thanks to the post-genomic era the need for a better understanding of the mechanisms involved and the optimization of the crystallization methods has increased significantly.

With the aim of exerting a more effective control of the process and obtaining more successful essays, diverse techniques and novel instruments have been developed that extend from mixer robots that can dispense thousands of drops in very short times to new devices for microgravity experiments or for the use under magnetic fields.

Automated production of crystals of macromolecules is a priority for proteomics, even though it’s not the only problem. The availability of proteins is a crucial issue [108], so it’s necessary to have systems that use very little amounts of proteins in the crystallization essay. The restriction of substrate doesn’t solely involve the volume used on the trials but also to the
emission systems. The high throughput macromolecular crystallization methods depend on a very good formulation and the ability to emit solutions with a defined composition. This is easy when working with liters or milliliters, but when going down the scale 5 or 6 orders it turns to be more complicated [109].

On the other hand, the experiments in microgravity, that gave impressive results thanks to the lack of convection, have evolved and now it's possible to send experiments to space with a high density of simultaneous essays. However, for optimizing the crystallization conditions two criteria can be used. One of them consists in evaluating a wide range of conditions (although narrower than the initial screening experiments) consuming a huge quantity of raw material (macromolecules). The other criterion proposes the adjustment of the conditions found in future spatial missions. Nevertheless, the possibility of consecutives voyages can be delayed months or years, marrying the acquired benefits of the method [109].

As a result, microfluidics devices arise as a potential solution, thanks to their system of encapsulated solutions and the capacity for high density experiments in a reduced space. The development of these devices, also known as "microfluidics chips", can be achieved thanks to the experimental results of many scientists who showed the compatibility of these chips with proteins. Among the observed characteristics, the injection of very exact volumes of protein solution and a considerable reproducibility of the results can be mentioned [110]. Moreover, enzymatic reactions can be optimized thanks to the reduced space where they course [111] and to the fewer amounts of reactants used [110]. Its functioning won't be discussed in detailed here, except some of it novel traits.

First, volume must be considered. Microfluidic chips, as their name points out, are little instruments that can emit and handle very little volumes, of the picoliter order [112] thank to a system of cross-linked channels that regulate the driving forces of the fluids. Those volumes are smaller than the ones used in crystallization test in conventional laboratories (μl) or those used for automated high throughput crystallization equipments (nl) [108, 113-114].

Besides, the physical characteristics of the device can be useful for protein crystallization. On one hand, they have a small Reynolds’s number, or absence of turbulence, that permits the emission of only laminar fluxes and the ultra-fast diffusive mixing, more efficient than the manual mixing [112,115]. On the other hand, microfluidics systems have a small Grashof’s number, or absence of convection (due to a density gradient). This property has shown the possibility of protein crystallization with very effective kinetics [116]. In that work, 144 parallel reactions were set. Solutions were introduced in 48 wells manually or with the help of a robot. The protein solution and the precipitating agent were placed in individual chambers, separated by a barrier which is eliminated upon the crystallization essay. The total volume of both chambers was 25 nl. With this kind of device it was possible to crystallize 11 different macromolecules and at least one structure was solved by x-ray diffraction. The advantages of this chip, mentioned by Hansen and co-workers [117] were the great precision in the measure of the solutions, the lost of viscosity effects that affects the diffusion of molecules, the ease for collecting the crystals grown, the possibility to apply the liquid-liquid diffusion method in gravity (before this it was a method used in microgravity), thanks to the absence of convection. In addition, the authors [116] observed shorter equilibration times and faster crystal growth.

Nevertheless, microfluidics chips have yet some disadvantages that must be overcome for their successful implementation in high throughput crystallization laboratories. Among those drawbacks, the permeability of the elastic connections owing to incapacity of sealing or isolating certain areas of the device when it’s not under function can be mentioned. Also, it’s difficult to implement for optimizing the crystallization conditions, since the equipment starts from stocks solutions. For the future, it would be advisable to include another chip that prepares the solutions and be connected in series to the other chip [109].

The design of this kind of devices has been possible thanks to the advances in engineering, although, from the point of view of costs, they are very expensive compared to the current systems. The chip’s manufacture, like the integrated circuits, required a very tight control of the cleanness of the process, as micrometric lines are done on it. The associated equipment, for its control, generally is very sophisticated and expensive. All this things for only one experiment, since the devices as are known nowadays are disposable. On the contrary, in manual systems the highest cost is the scientist’s labor. Robots have come to do this work. Despite the high costs, they can set up the experiments faster than humans do and give freedom to the scientist to do other activities. In consequence, the costs of microfluidics systems always will be higher than current technologies. For their general acceptance and implementation, their advantages should be reinforced, such as the experiments density, probability of success and flexibility [109].

Conclusions

- Natural convection is one of the principal problems that affect crystal quality. It allows a major adsorption of impurities and a higher defect density, generating poor diffracting crystals (high resolution). Microgravity experiments give high quality crystals due to the diffusive transport domination that allows a good arrangement of the monomers in the crystal lattice, diminishing the mosaicism and increasing the final quality (lower resolution, better I/σ(I) data).

- Microgravity experiments use diverse equipment specially designed by the companies or organizations involved. The most used techniques are vapor diffusion and liquid-liquid diffusion.

- Crystallization experiments under high magnetic fields can also reduce natural convection on earth. Those crystals grown in this way also showed a high quality, reaching
lower resolutions in the diffraction pattern, when exposed to x-rays. A high, uniform magnetic field induces an internal organization of the crystallization solution, increasing the viscosity and decreasing the diffusion coefficients of the molecules present. On the other hand, the magnetic field gradients induce a magnetizing force opposite to the gravitational force, reducing the effective gravity around the growing nuclei and consequently annihilating the natural convection. So, with both possible situations better quality crystals can be obtained.

- Gel acupuncture crystal growth experiments offer the advantage of reducing crystals manipulation, since they grow inside x-ray capillary tubes. They can even be grown in the presence of cryoprotecting agents or heavy metals, which will be helpful for the x-ray data collection or interpretation of the data, respectively. However, the material of the capillary tube and the presence of mother liquor inside it affect negatively in the resolution.

- Protein crystallization in the presence of an internal electric field doesn’t affect significantly the crystal quality and offers the possibility of shortening the nucleation time giving rise to crystals of adequate sizes.

- Up to date, very few membrane proteins have reported structures and its reason is the little knowledge to manipulate them, due to their amphiphatic nature. In the last decade, three new methods have been created, based on extended lipidic bilayers. Of them, the lipidic cubic phase method is the most developed, and many proteins were crystallized with it. All three methods present lamellar structures and crystallize membrane proteins in the presence of the original lipids.

- Advances in genome’s sequencing of diverse organisms, encourage the structural study of proteins with practical aims such as the discovery of metabolic paths, studies of the relation structure-function of pharmaceutical targets or protein engineering. For successful large scale protein crystallization, many inconvenient in protein production and crystallization are yet to be overcome especially for membrane proteins.

- Many devices for high throughput crystallization do exist in the market, especially for screening the crystallization conditions. Of them, those that are based on sitting drops and microbatch methods are the most known. Recently a cassette for protein crystallization by the gel acupuncture method in large scale was proposed.

- Microfluidic systems, even though they are in their initial phase, offer a crystallizing system for a huge number of essays in a reduced physical media, using very little amount of substrates, and with a very good control of the emission systems. In addition, they work without convection, promising high quality crystals. Despite some actual disadvantages due to their high sophistication, in the future they will bring great benefits to pharmaceutical or structural genomics industries.

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