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Promising approaches to crystallization of macromolecules suppressing the convective mass transport to the growing crystal

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Conditions of mass transport to growing crystals are important factors that have an impact on the size and quality of macromolecular crystals. The mass transport occurs *via* two mechanisms — by diffusion and convection. The crystal quality can be influenced by changing (either suppressing or enhancing) the convective mass transport. The review gives an overview and analysis of the published data on different methods of macromolecular crystallization providing the suppression of convective mass transport to growing crystals in order to improve the crystal quality.
The bibliography includes 91 references.

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I. Introduction

A knowledge of the three-dimensional structures of biological macromolecules is necessary for investigation of molecular mechanisms of cell functions.[1] The determination of the three-dimensional structures of protein targets is very important also in the search for new promising drug candidates.[2, 3]

As of July 2015, the Protein Data Bank, a crystallographic database of three-dimensional structures of proteins and nucleic acids (www.rcsb.org), contained more than 100 000 different structures, almost 90% of which were determined by X-ray diffraction. In recent years, considerable technological progress in the solution of problems of structural biology (the development of robotic crystallization systems, the advent of a new generation of synchrotron radiation sources, *etc.*) has led to a sharp rise in the number of known three-dimensional structures of biological macromolecules. Getting a protein to crystallize is still the major bottleneck in X-ray crystallography.

The general principle of crystallization of macromolecules from solution is to decrease the solubility of these samples until the supersaturation reaches a critical value by varying such parameters, as pH, temperature, precipitant

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concentration, *etc*.[4,5] However, due to specific properties of macromolecules (conformational flexibility, the presence of several solubility minima, the tendency to aggregate and denature), they are difficult or impossible to crystallize. Therefore, the crystallization is a difficult task in structural studies[1,4–6] and it is still very much an empirical and least predictable process.

It is known that during the crystal growth, the mass transport to the growing crystal occurs by diffusion and convection,[7,8] and the crystal size and quality depend on the ratio between these two processes. In the published reviews on macromolecular crystallization,[4–6,9–13] little attention was paid to the problem of the suppression of the convective mass transport. The present review helps to close this gap and summarizes modern methods of crystallization of biological macromolecules that suppress the convection in order to improve the quality of growing crystals.

II. Protein crystallization. Standard crystallization methods. Factors influencing the crystal quality

The crystallization is a phase transition from the dissolved state to the solid crystalline state. The main driving force for the crystallization is the supersaturation, *i.e*., the state of the crystallization system, in which the concentration of the dissolved compound (protein, nucleic acid and so on) is higher than its concentration in the saturated solution. When the supersaturation is established, the crystallization system goes to the equilibrium, at which the compound is distributed between the solution and the solid phase.[5] The attendant decrease in the free energy of the system due to the formation of new stable bonds between macromolecules can induce their crystallization. Therefore, the crystallization can take place only from supersaturated solutions.[1,4,6,10]

The supersaturated state can be achieved in different ways — by varying the temperature, pH, the concentration of the precipitating agent (salt, polymer), *etc*. Figure 1 presents a schematic phase diagram, which shows the dependence of the solubility of macromolecules on their concentration in solution and the precipitant concentration. The solubility curve separates the regions, where the nucleation and crystal growth can take place, from the undersaturation region, where such processes are impossible.

The macromolecular crystallization involves three interconnected processes: the nucleation (the appearance of crystalline nuclei), the post-nucleation growth, and the cessation of growth.[4,5,10] The nucleation is a transition from macromolecules disordered in solution to the ordered state that occurs mainly due to the formation of small molecular aggregates (nuclei) having a short-range order. This process was studied in detail (for example, see Refs 14 and 15). This step is followed by the growth of the nuclei by different mechanisms (spiral dislocation, 2D-nucleation growth, *etc*.)[10,16,17] and the subsequent cessation of growth.

The crystallization of macromolecules for structural studies requires the solution of two main problems: the attainment of the conditions providing the nucleation and the growing of crystals of sizes suitable for X-ray diffraction.[18] As can be seen in Fig. 1, the region of supersaturation can be divided into three zones: metastable and labile zones and the precipitation zone, where macromolecules

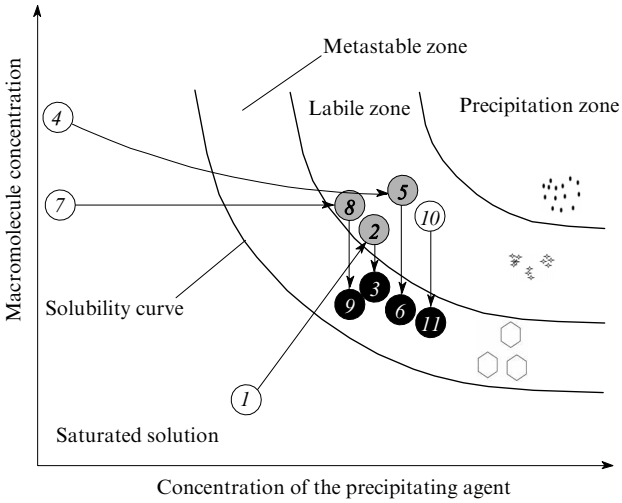


Figure 1. Schematic representation of the phase diagram illustrating the dependence of the solubility of macromolecules on their concentration in solution and the precipitant concentration.
In the undersaturation region (the region below the solubility curve), the protein is in a dissolved state. In the metastable zone, the crystal growth can occur, but the nucleation is impossible. In the precipitation zone, the level of supersaturation is so high that the protein precipitates. The states of the crystallization system attained by different crystallization methods: (*1–3*) vapour diffusion, (*4–6*) counter-diffusion, (*7–9*) dialysis, (*10* and *11*) batch crystallization. Empty circles represent the initial state of the system; shaded circles, the intermediate stale, solid circles, the final state. The directions of the transitions of the system between the states are indicated by arrows. The notations are given in the text.

precipitate due to a very high level of supersaturation. The nucleation requires a higher level of supersaturation than that sufficient for the crystal growth. Therefore, the nucleation can occur only in the labile zone and does not take place in the metastable one. The crystal growth occurs in both these zones,[4–6,10] but the growth in the labile zone competes with the nucleation. Due to a high nucleation rate, a large number of nuclei appear under these conditions, resulting in that the crystal growth is substantially limited and the labile zone becomes non-optimal for this process.[5]

The macromolecular crystallization is usually a multistep process, which involves the initial screening that is performed in order to find initial crystallization conditions followed by the optimization of these conditions with the aim of producing good-quality crystals.[19] Below we briefly describe modern methods most commonly used for the crystallization of soluble proteins.[9]

Vapour diffusion. The essence of this method is that the solvent and volatile components of the precipitating agent are slowly evaporated, resulting in an increase in the precipitant and protein concentrations until the supersaturation is achieved.[13] In this method, a certain volume of the crystallization solution (the so-called reservouir solution, in which the precipitant concentration is slightly lower than that required for the precipitation of the protein) is separated from a drop containing a protein mixed with the reservouir solution (state *1* in Fig. 1). Due to a lower precipitant concentration in the drop, the solvent evaporates and equilibrates with the reservouir solution, which

leads to the supersaturation of the protein solution and brings the crystallization system to the labile zone (state *2* in Fig. 1). In the course of nucleation, the protein is partially transformed into the crystalline state. This leads to a decrease in the concentration of the dissolved form and brings the system into a metastable state, in which the further crystal growth takes place (state *3* in Fig. 1).

Counter-diffusion is a crystallization technique developed in the 1970s.[20] According to this technique, protein and precipitant solutions are poured in a small well or a capillary one above another.[12] At the beginning, the protein solution is in the zone below the solubility curve (state *4* in Fig. 1). Then the solutions are allowed to diffuse across an interface one against another giving rise to a gradient of the precipitant concentration along the capillary. At some points in the capillary, the precipitant concentration is sufficient to bring the protein to the labile zone, where the nucleation occurs (state *5* in Fig. 1). An increase in the concentration of the protein that transforms into the crystalline state drives the crystallization system into the metastable zone (state *6* in Fig. 1).

Researchers from the University of Granada (Spain) modified this technique and designed the Granada Crystallization Box to perform counter-diffusion using gels in capillaries.[21] In this device, the gel serves as the boundary between the protein and precipitant solutions, which hinders the diffusion of the protein but allows solute molecules to pass accross this barrier. The presence of the gel induces slow diffusion of the precipitating agent into the protein solution, and the protein solution is more smoothly brought to a supersaturated state. In experiments with the Granada Crystallization Box, very high supersaturation conditions, which impair the crystal quality, are excluded for the growing crystal that sink to the bottom of the capillary under gravity.[10]

Dialysis. The crystallization by dialysis employs a semipermeable membrane, which allows solute molecules to freely pass but prevents protein molecules from crossing the membrane.[13] A protein solution is poured into a dialysis bag made of a semipermeable membrane, which is placed in a reservoir solution (state *7* in Fig. 1). By gradually varying the composition of the reservoir solution, the protein is brought to the supersaturation zone, where the nucleation (state *8* in Fig. 1) and subsequent crystal growth (state *9* in Fig. 1) begin. The advantage of this method is that the degree of saturation of the protein solution can be easily controlled and changed. The crystals that precipitated can be dissolved by changing the composition of the solution outside of the membrane. This allows researchers to perform several crystallization trials.

Batch crystallization is the oldest method used for crystallizing proteins based on the direct mixing of the precipitating agent and the protein solution.[13] In this method, the protein solution is rapidly brought to a supersaturated state (state *10* in Fig. 1). The resulting nuclei are rapidly removed by centrifugation, thus driving the crystallization system into the metastable zone (state *11* in Fig. 1), where the crystal growth takes place.

Chayen *et al.*[22] proposed a modification of this method, called microbatch crystallization. This method was developed to reduce the sample consumption, because it uses small drops of a protein solution ($\sim$10 μl), which are coated with paraffin oil to reduce the evaporation.

Various modifications of the above-described methods were developed, which differ in the mechanism by which the supersaturation is achieved, technical details of experiments, the volume of the compound under study and other parameters.[4, 6] Robotic crystallization systems based on the above-considered techniques are increasingly used for initial screening of crystallization conditions, since they consume less proteins, are capable of rapidly performing crystallization trials and provide better reproducibility of the results.[9, 11, 23, 24]

III. Mass transport to the growing crystal

Conditions of the mass transport to growing crystals are important factors influencing the crystal size and quality. The mass transport occurs *via* two mechanisms — diffusion and convection.[7, 8, 25, 26] During the crystal growth, a region with a low concentration of the substance and, correspondingly, with a lower density is formed around the growing crystal. Under the effects of gravity, a region of the solution with a lower density is lifted up and a region with a higher density is drawn down, resulting in the formation of convection currents.The ratio between the diffusive and convective mass transport is responsible for the way and rate of delivery of molecules to the growing crystals.[5] Exclusively diffusive mass transport makes it possible to avoid the adverse effect of an inhomogeneous concentration of the substance around the crystal on its growth.[5] Both theoretical[27, 28] and experimental[29–33] studies showed that the quality of growing crystals is higher if the diffusive transport dominates. Rosenberger *et al.*[26] hypothesized that the competition between diffusion and convection leads to deterioration of the crystal quality and proposed a model, according to which the crystal quality can be improved by changing (either decreasing or increasing) the level of convection.

Another factor influencing the crystal quality is the inclusion of impurities (isoforms of the sample being crystallized or other macromolecules) into the crystal. This factor can have a substantial effect on the crystal growth and interfere with the preparation of high-quality crystals. This was demonstrated for lysozyme[34] and some other proteins.[25, 35–37] A decrease in the convection currents was shown[38] to reduce the degree of inclusion of impurities into the growing crystal.

IV. Crystallization methods suppressing the convective mass transport to the growing crystal

The convective mass transport to the crystal is associated with effects of gravity.[39] According to the results of calculations, 10^{-6} g (microgravity) is sufficient to suppress the influence of convection, *i.e.*, for the diffusive mass transport to dominate.[28] Microgravity conditions can be achieved in outer space. Meanwhile, different methods were developed, which allow the convection to be influenced in experiments performed on Earth. The most promising, in our opinion, methods are considered below. It should also be noted that such methods have an impact not only on the convection but also on other factors (the number of nuclei that are formed, the separation of the nucleation and crystal growth processes, sedimentation and so on), which influence the crystal quality. However, the detailed consideration of the

mechanisms of these effects is beyond the scope of the present review.

IV.1. Crystallization in hydrogel

The crystallization in hydrogel has been known since the 1980s. This method gained renewed interest in the late 1990s,[40] when the effect of hydrogel on the crystallization was studied in more detail. The main advantages of this method are as follows:

— the absence of sedimentation of growing crystals due to the viscosity of the gel (agarose gel at a concentration of not lower than 0.04%);[41]

— a significant limitation of convection and a weak effect of the gel structure on the level of diffusive transport;[40]

— resistance of the grown crystals to drying, osmotic shock[42,43] and changes in the temperature[44,45] due to the dense structure of the gel surrounding the growing crystal.

The crystallization in hydrogel can be performed employing different techniques (batch crystallization, counter-diffusion); agarose, acrylamide and silica gel are most commonly used as the gel.[45] This is associated with both mechanical characteristics of agarose and the fact that this compound is widely employed in other areas of biochemistry. Until recently, the necessity of heating the sample to be crystallized to a temperature above 35 °C before mixing with the gel was considered as the main drawback of this method.[46] Evidently, not all of the proteins can withstand such heating. In 2006 a new water-soluble synthetic thermoreversible gelation polymer (TGP) was produced.[47] This is a block copolymer of poly(*N*-isopropylacrylamide-co-n-butyl methacrylate) and the hydrophilic polymer polyethylene glycol.[48] Unlike the agarose gel, TGP exists in the liquid state at 4–10 °C and is transformed into a gel-like state with an increase in the temperature. This polymer was used[42] for the crystallization of the following three proteins: elastase, glucose isomerase and lysozyme. In these experiments, the protein to be crystallized was mixed with the gel at a temperature not higher than 10 °C.

The drawback of this method is that it is difficult to extract crystals from the gel for X-ray diffraction studies.[42]

It should be noted that the crystallization in hydrogel can be implemented under microgravity conditions.[49] This approach makes it possible to avoid convection currents and the sinking of the crystals to the bottom during the descent and landing of a spacecraft.

IV.2. Ceiling crystallization

The ceiling crystallization is a modification of the batch crystallization. This method was developed[50] in 2008 and was later improved.[51] The schematic representation of ceiling crystallization is shown in Fig. 2. A crystallization unit (vial) completely filled with a supersaturated protein solution is sealed with a glass cover slip, on which the heterogeneous nucleation occurs. The nucleus that is formed on the glass cover slip gradually adsorbs the protein from the neighbouring region, resulting in the formation of a depletion zone around the crystal. Since the growing crystal lies in the upper part of the crystallization unit, a vertical concentration gradient is established under gravity and the mass transport occurs only by diffusion,[50–52] due to which the microcrystals that are formed do not sediment on the growing crystal and, consequently, do not perturb its structure.[51]

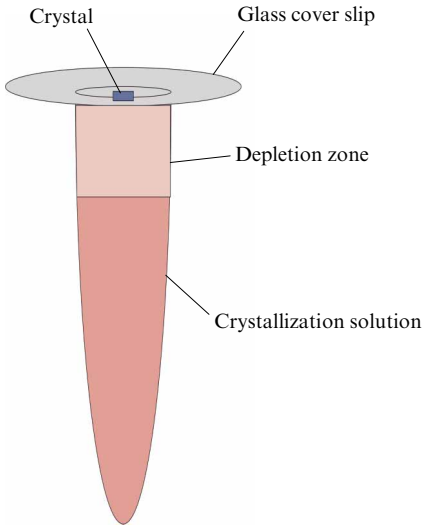


Figure 2. Schematic representation of a ceiling crystallization experiment.

In recent years, this method was shown to improve the quality of growing crystals of model proteins — lysozyme and bovine insulin.[51,52] The crystals grown by this method have a larger size and show a higher diffraction limit than the crystals of these proteins grown on the bottom of the crystallization unit in the same experiment.

IV.3. Microfluidics

Microfluidics is an interdisciplinary field dealing with the behaviour of small (micro- and nanolitre) volumes of fluids.[53] It is widely used in different areas of biology, particularly when a high-throughput approach and a considerable reduction of sample consumption are required. As applied to macromolecular crystallization, microfluidics is a rather new method, which has found use due to technological possibility to perform crystallization in extremely thin capillaries. It was demonstrated[54] that in crystallization experiments by the capillary counter-diffusion technique at a certain ratio between the capillary parameters and the solution viscosity, the mass transfer will occur only by diffusion, while the convection currents will be absent.

The first commercially available device for microfluidic crystallization was manufactured in 2002 and was based on the counter-diffusion technique.[55] The device was composed of several reservouirs with different volumes containing a protein and a crystallization solution (Fig. 3). The reservouirs connected by long thin capillaries were closed before the onset of the experiment. After the opening of reservouirs, the fluids slowly move towards each other along the capillaries by diffusion, thus generating the concentration gradient. This techniques allows for the simultaneous examination of a certain set of crystallization conditions. Using this device, 144 different conditions can be tested, the total amount of the sample to be crystallized being <2 μl (10 nl of a sample per experimental point).[55]

Modifications of available and the design of new microfluidic devices provide opportunities to reduce the sample consumption for crystallization,[56] *i.e.*, these devices allow researchers to probe the crystallization phase space with small amounts of samples at hand. Devices for batch crystallization were designed[53,57] and substantially simplified.[58] Devices were constructed for vapour-diffusion[53] and

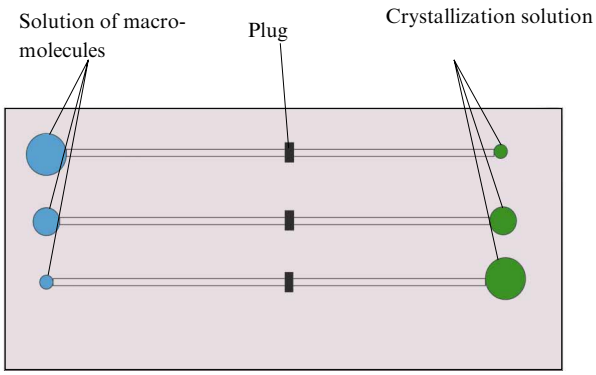


Figure 3. Schematic representation of a microfluidic device for macromolecular crystallization.

counter-diffusion [53, 57, 59, 60] crystallization. In some devices, the screening of crystallization conditions is combined with preliminary X-ray diffraction study at room temperature, which makes it possible to estimate the diffraction quality, the unit cell parameters and the space group.[59, 60] Devices enabling the control of evaporation in order to influence the crystallization process are available.[61] Besides, the materials of the devices were improved — they became more transparent to visible light and X-rays.[59]

Microfluidic technique holds promise due to the availability of commercial equipment, low sample consumption and the possibility of controlling the composition of the microvolume with high accuracy.[56] The drawback of microfluidics is only the labour-consuming extraction of crystals if they have to be frozen before the X-ray experiment. Meanwhile, a technique of *in situ* X-ray data collection was developed,[12, 53] which enables the freezing of the grown crystals without their extraction (in this case, a cryoprotectant solution should be added to the precipitant solution before the onset of crystallization). Besides, the serial crystallography approach was designed. This method does not require the extraction of crystals, and diffraction patterns are measured not from one crystal but from numerous randomly oriented crystals at room temperature. At this temperature, crystals undergo rapid degradation when exposed to X-rays, but the merging of diffraction data sets from differently oriented crystals provides information sufficient for the determination of the three-dimensional structure.[62] Due to the above-mentioned advantages, the microfluidics is considered as one of the most promising techniques of protein crystallization.

IV.4. True microgravity

By true microgravity are meant macromolecular crystallization experiments in outer space aboard a spacecraft using different methods. In the absence of gravity, convection currents in the crystallization unit disappear, and the mass transport to the growing crystal occurs only by diffusion.

Pioneering experiments under microgravity conditions were carried out in 1984 for lysozyme and β-galactosidase.[63] The crystals grown in these experiments were larger than earth-grown crystals. A strong interest in this area gave impetus to experiments in outer space.[29, 64] Such countries as Russia, the United States, EU countries, China, Canada, Japan, *etc.*, perform their own experiments and participate in international collaborative projects on protein crystallization under microgravity conditions.

The results of these experiments showed that under microgravity conditions crystals are able to grow larger, in many cases have a more ordered structure, reduced twinning and lower mosaicity and, consequently, demonstrate improved diffraction compared with earth-grown crystals of the same proteins.[39, 65–69] The improvement of diffraction characteristics of the crystals was observed in ~30% of cases.[70] However, microgravity does not lead to a desired increase in the crystal quality of some proteins.[39]

The crystallization in outer space is performed using such well-known methods as vapour diffusion,[71, 72] counter-diffusion [12, 73, 74] and dialysis.[75, 76] The equipment for such experiments is modified (and sometimes specially designed) taking into account the specific features of outer-space experiments.[64] In 2002 the European Space Agency successfully tested a device for crystallization by the counter-diffusion crystallization technique (Granada Crystallization Box).[77] Later on, this technique was modified to simplify the preparation of experiments and reduce the sample consumption.[74] In 2013 an equipment for crystallization by microfluidic technique (to reduce the sample consumption) was elaborated and successfully tested.[78]

At the present time, macromolecular crystallization experiments in outer space are performed at the International Space Station (ISS). In Russia, such experiments are carried out under the guidance of the Russian Federal Space Agency. In the framework of this long-term project, studies are performed by Russian scientific institutions, including the National Research Centre 'Kurchatov Institute', the A V Shubnikov Institute of Crystallography of the Russian

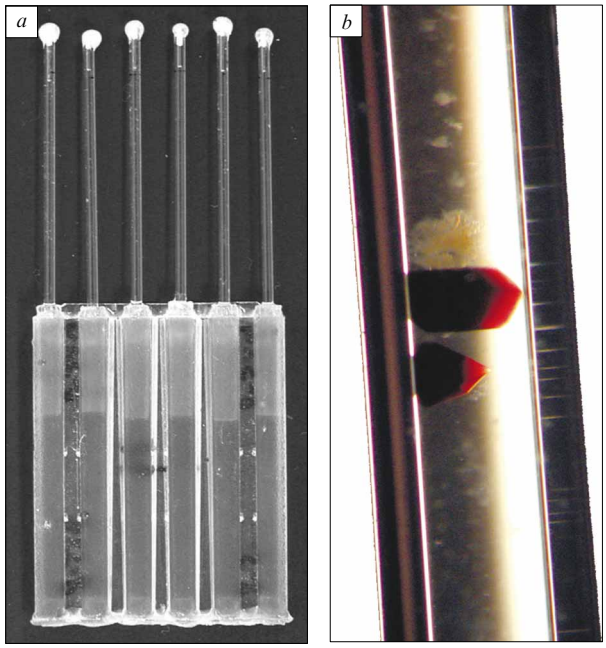


Figure 4. JAXA crystallization box (JCB) for microgravity crystallization experiments.
(*a*) The device contains six assembled cells (capillaries); six different crystallization conditions can be tried in one JCB. Each capillary contains a protein solution connected to the reservouir solution (lower reservouir) through a gel tube.
(*b*) Crystals of *T. nitratireducens* cytochrome *c*-nitrite reductase, which grow in a crystallization box under microgravity (one capillary with an inner diameter of 0.3 mm is shown).

Academy of Sciences, the A N Bach Institute of Biochemistry of the Russian Academy of Sciences, *etc*. From 2005 to 2009, a specialized Modul-1 protein crystallization apparatus was used for this purpose.[79] At the present time, the Japanese crystallization device (JAXA Crystallization Box)[80] (Fig. 4) is employed in the framework of the Russian–Japanese collaborative project.

The JCB device is based on counter-diffusion using the gel-tube method.[81] The advantages of this technique over vapour diffusion are the absence of Marangoni convection[82] and the possibility of examining a spectrum of crystallization conditions in one capillary due to a gradual change in the precipitant concentration as the precipitant solution moves along the capillary containing the protein solution. The results of experiments using both devices showed that in most cases, crystals grown under microgravity conditions are characterized by reduced twinning, lower mosaicity,[83] higher resolution[81,84–89] and larger sizes.[79,83,90] In some cases, this made it possible to reveal structural details for these crystals, which have not been determined using earth-grown crystals.[24,86,91] New published studies, which demonstrated higher quality of protein crystals grown under true microgravity conditions over crystals grown on Earth in control experiments,[78] confirm the prospects of this area of research.

The drawbacks of crystallization under true microgravity conditions are high cost and technical complexity of experiments. In particular, it is necessary to control all stages of the experiment from the loading of the device with protein samples to their opening after the landing.

* * *

The macromolecular crystallization is an important and one of the least predictable steps in the solution of problems of structural biology. Different crystallization techniques were developed since it is impossible to *a priori* specify crystallization conditions for a particular macromolecule. The type of mass transport to a growing crystal — by diffusion or convection induced by gravity — has a high impact on the crystal quality. The competition of diffusion or convection is least favourable for the crystal growth. The crystal quality of protein macromolecules can be improved by influencing convection currents using different approaches covered in this review.

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