Chapter

Advanced Biocrystallogenesis

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Abstract

Nowadays, X-ray crystallography is one of the most popular structural biology methods. Successful crystallization depends not only on the quality of the protein sample, precipitant composition, pH or other biophysical and biochemical parameters, but also largely on the use of crystallization technique. Some proteins are difficult to be crystallized using basic crystallization methods; therefore, several advanced methods for macromolecular crystallization have been developed. This chapter briefly reviews the most promising advanced crystallization techniques and strategies as one of the efficient tools for crystallization of macromolecules. Crystallization in capillaries, gels, microfluidic chips, electric and magnetic fields as well as crystallization under microgravity condition and crystallization in living cells are briefly described.

Keywords: protein crystallization, protein crystal, advanced methods, crystallization strategies, biocrystallogenesis

1. Introduction

Macromolecular crystallization was invented accidentally in the late 19th century. The initial goal of crystallization processes was to purify and to prove the chemical purity of the examined chemical compound. One of the first achievements in macromolecular crystallization were crystals of hen-egg albumin at the end of the 19th century and crystals of insulin in the 1920s [1, 2]. Since then, macromolecular crystallization has developed into a powerful tool for the three-dimensional structure determination of nucleic acids, proteins as well as for larger macromolecular complexes.

A good-quality macromolecular crystal is needed in X-ray crystallography for obtaining a 3D model of corresponding nucleic acids, proteins, macromolecular complexes or larger biological assemblies, for instance, viruses or ribosomes. Nevertheless, macromolecular crystallization is a highly unpredictable process, which relies on a large number of chemical and biochemical parameters, such as the purity and concentration of the protein, type of precipitant, pH of the buffer, as well as on physical parameters, as temperature, time, pressure or vibrations, etc.

In order to crystallize the protein from the solution, the supersaturated state has to be reached using various crystallization techniques. Crystallization process can be divided into three stages - nucleation, crystal growth and termination. So-called phase diagrams typically illustrate these steps. For the investigation of the best conditions for crystal growth of the individual macromolecule, two general strategies are usually applied. Firstly, initial crystallization screening using commercially

available screening kits is performed to explore the suitable conditions for crystal growth. Secondly, conditions yielding macromolecular crystals are systematically modified to allow the growth of the best-quality crystals adequate for X-ray diffraction analysis. Basic crystallization techniques, namely batch, vapour diffusion and free-interface diffusion are used for initial crystallization screening generally [3]. In addition, these techniques can be further modified to obtain as good-quality crystals as possible during the optimization of protein crystallization. However, some proteins cannot be easily crystallized and produced crystals in diffraction quality and thus initial hits need to be further improved. It is important to note that different crystallization methods screen the phase diagram from different points of view and hence affect the properties of the resulting crystals. Therefore, advanced biocrystallogenesis methods are recommended to be applied for screening or optimization of crystallization conditions.

This chapter reviews different advanced methods and strategies as efficient tools for crystallization of macromolecules. During the past few years, the field of protein crystallography has significantly developed regarding instrumentation. Different technologies require different sizes of crystals, for instance homogeneous nanocrystals for XFEL or big size crystals for neutron diffraction [4, 5]. This also drives the necessity to develop new and convenient methods for growing suitable high-quality protein crystals. For this purpose advanced crystallization methods and strategies have been developed.

2. Advanced biocrystallogenesis methods

2.1 Counter-diffusion

Counter-diffusion belongs to the non-equilibrium crystallization approaches. The principle of this method is based on the use of one-dimensional experimental chambers, including protein and precipitant solutions, resulting in slow mutual diffusion, in other words the slow mixing of the molecules of the solutions and therefore slow reaching of the supersaturation state [6]. During the diffusion, the solubility of the macromolecule is significantly reduced (**Figure 1**–(3)) [7].

The ideal concentration of the protein solution is advised to be higher (more than 4 mg/ml) and the concentration of the precipitant solution should be very high, nearly reaching the supersaturation state [8]. At the beginning of the experiment, the diffusion is faster and thus the amorphous precipitate is formed. Eventually, the protein concentration decreases at the liquid-liquid interface, which leads to the nucleation followed by the protein crystal growth [9].

Counter-diffusion method for protein crystallization and screening is popular since it screens a wide range of the phase diagram while carrying out only one experiment and increases the chance of hits at the same time.

2.2 Crystallization in capillaries

Crystallization in thin and long capillaries is one of the most used advanced crystallization technique, frequently applying counter-diffusion. Furthermore, capillaries can be used also in batch crystallization or vapour diffusion (**Figure 1**–(2)). This technique does not require further optimization as it precisely screens many crystallization conditions for one protein solution and one precipitant solution. Another advantage of thin capillaries is that a small amount of the protein solution is required for the experiment. Moreover, the protein crystals in capillaries can be also directly measured at the synchrotrons [10].

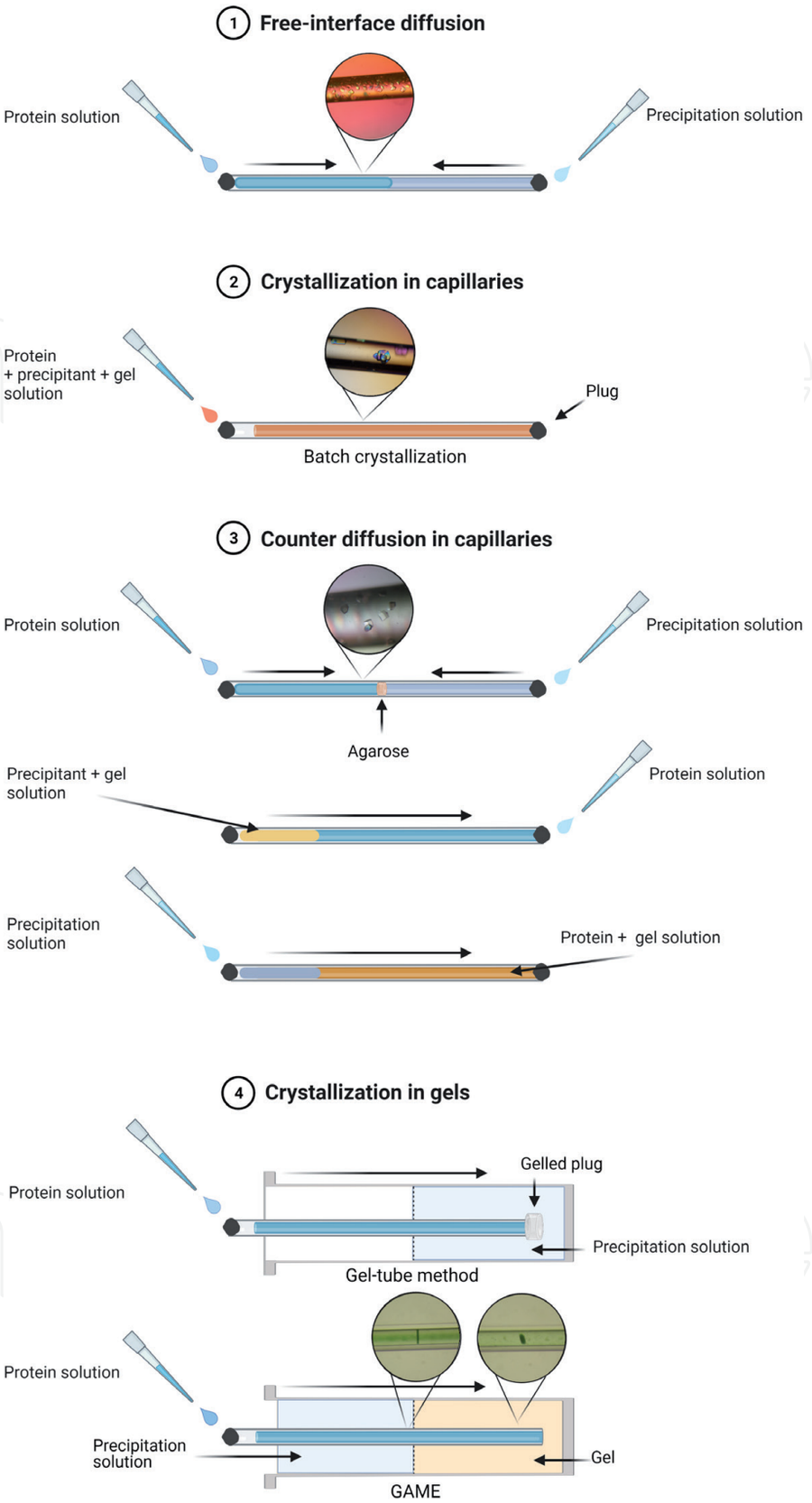

Figure 1.
Advanced biocrystallogenesis methods. 1. Free-interface diffusion, 2. Crystallization in capillaries, 3. Counter-diffusion in capillaries, 4. Crystallization in gels and GAME (prepared by author Barbora Kascakova).

Three different organizations of capillaries can be applied for counter-diffusion experiments: (i) simple one chamber organization composed of the protein and precipitant solutions, (ii) two chamber single capillary organization is formed by the precipitant solution and the protein solution mixed with the gel and (iii) three chamber

single capillary organisation that blocks fast diffusion of the protein and precipitant solutions by a physical barrier, typically formed by agarose or silica gel, known as gel acupuncture method (GAME) (**Figure 1**–(1, 3, 4)). Modifications (ii) and (iii) have been successfully used for the crystallization of not only globular proteins, but also RNA and DNA protein complexes and larger membrane protein complexes [10–12].

2.3 Crystallization in gels

Crystallization in gels is a suitable method for growth of high-quality macromolecular crystals. This method is effective due to the invariable environment enabling crystal growth, preventing the production of protein aggregates or sedimentation and slows diffusion of molecules in the crystallization chamber [13]. The most used gels for macromolecular crystallization are hydrophilic gels, namely agarose and silica gels, even though they have different properties triggering the crystal growth [14].

Regarding nucleation, agarose gels significantly promote it, whereas, silica gels inhibit this process, but the exact mechanism behind it remains unknown. The benefits of the use of agarose gels are (i) the concentration of agarose gel can be very low, (ii) agarose gel polymerises faster than silica gels, (iii) agarose gel can be employed in any crystallization technique, (iv) cofactors, ligands and even cryoprotectants are reported to be easily soaked into the crystal in the presence of the agarose gel, (v) agarose gels help to produce bigger crystals for neutron diffraction analysis and also (vi) serves as delivery medium for nanocrystals for X-ray free-electron laser analysis (**Figure 1**–(3, 4)) [14–17].

2.4 Microgravity

The first attempts to confirm the substantial role of microgravity for crystal growth were done more than 35 years ago [18]. There was an assumption that absence of gravity can lead to growing of top quality crystals, thus crystallization experiments in the microgravity environment in the space shuttle were carried out. First experiments were performed by vapour diffusion method, later the counter diffusion and dialysis crystallization were applied as well [18, 19]. The main advantage of growing protein crystals in space is that there is very diffusive mass transport and reduction of formation of possible crystal defects.

There are different views on the success of this method. In many cases, it was proven that space provides good conditions for growing better diffractive crystals against the results of crystals grown on Earth [20, 21]. Many positive examples of growing the crystals in space show improved diffraction resolution, better diffraction intensity, reduction of crystal disordering and clustering, prolonged nucleation process, excluded crystal sedimentation and in some cases growing of larger crystals, sometimes also with different space groups [20, 22]. Of course, the microgravity experiments are connected with some limitations that are mainly bureaucracy for flight approval of protein samples, delays of mission and with this connected possible degradation of protein or crystal samples [22].

2.5 Microfluidic chips

Recently, screening and crystallization by counter diffusion method has been improved by the application of microfluidic chips. Typically, the chips are composed of channels connected by a cross section. This organization of the crystallization chamber facilitates the screening of the wide range of the protein and precipitant concentrations; the supersaturation state is achieved by diffusion of the molecules [9]. Another advantage of this approach is the requirement of a small

amount of the protein and thus is suitable for proteins that are demanding to purify in larger volumes [23]. In practice, the protein sample is loaded into the eight channels at once by micropipettes and precipitant solutions are placed in the reservoirs [24]. The crystal growth in microfluidic chips is much faster than in other crystallization systems and the results are highly reproducible [25]. Microfluidic chips are also applicable for the microseeding strategy or soaking with the ligands [26]. The resulting crystals can be analysed by *in situ* serial crystallography [24, 27].

2.6 Electric and magnetic field

The principle of the method benefiting from electric fields relies on the separation of the nucleation and crystal growth; it can be applied in the electric as well as the electromagnetic field. The nucleation process takes place while the current is switched off [28]. In order to perform crystallization experiments in the electric field, the specially designed crystal growth cell has to be used. The system contains two parallel electrodes and is connected to a direct current source that provides direct or alternating current. Nevertheless, the experiment has to be set up before the application of the current. It was reported that the current positively influences the size of protein crystals. Specifically, lower frequencies of alternating current provide less larger crystals compared to higher frequencies [28]. For the purposes of using electric current the e-crystallization cells based on vapour diffusion or batch method were made [29].

In the case of using magnetic fields, there is a similar outcome as carrying out an experiment in microgravity. This can be achieved when using a vertical magnetic field with use of anti-gravitational force. The crystals treated by magnetic fields show better diffraction quality as was revealed for microgravity experiments [30, 31]. In addition, the crystallization conditions need to be known before initiating the experiment. Moreno 2017 mentions the use of gels for obtaining large well-arranged crystals together with use of magnetic forces for at best two days and more days. For this type of experiment, the capillary glass pipettes were reported to be suitable for use of homogeneous or nonhomogeneous magnetic fields [28]. The best influence of magnetic field use is regulation of quality and crystal size [32].

3. Advanced biocrystallogenesis strategies

3.1 Seeding

The supersaturation level needed for the nucleation and the growth of best diffracting crystals is often different. Nucleation takes place when the supersaturation level is very high, while crystal-growing process appears when the conditions are slightly changing to lower supersaturation level [33]. Therefore, the seeding strategy has been developed as one of the most powerful tools for the optimization of the size and diffractive quality of single crystal. Another benefit is that it reduces the amount of the used protein and saves time needed for the spontaneous nucleation. This is also suitable for the examination of the slightly changing crystallization conditions, such as testing different pH ranges, adding new additives or even new precipitants. Several seeding strategies such as macroseeding, microseeding and/or their modifications can be applied.

In order to perform macroseeding, a single seed crystal has to be selected; its size is not relevant. The solubility of the crystal has to be investigated by testing of different precipitant concentrations to establish the conditions where the crystal is stable. This method is more demanding as multiple washes of the crystal are needed before the crystal is put into the suitable equilibration condition. The washes are

aimed to eliminate additional nuclei. The main disadvantage of the method is that during this handling, the crystal seeds can be damaged and can form only the microcrystals in the new equilibration condition, which are not suitable for the X-ray diffraction analysis [33].

Microseeding can be described as a delivery of microseeds into a new equilibration condition. Before the experiment, the seed stock has to be prepared by crushing crystals using vortex, sonication or seed beads [34]. Once the seed stock is prepared, the dilution series have to be done in order to stabilize the nuclei presented in the seed stock and enable them to form suitable crystals in the new crystallization drop.

Another way of seeding strategy is the streak seeding. The drop containing the protein crystal is opened and the crystal is touched using an animal (cat, horse, rabbit or chinchilla) whisker. The nuclei can be attached to the whisker and transfer into a new crystallization drop. The sitting drop technique is recommended for this experiment, as it does not dry as quickly as the drops in hanging drop technique [33].

Cross seeding is specifically designed for crystallization of related proteins. These related proteins are different recombinantly modified protein variants, homologous proteins, and chemically modified proteins, macromolecular complexes with ligand or even misfolded proteins that are crystallizing difficult [35]. Some of the related proteins do not crystallize in the same conditions; however, sometimes this method can help and save the protein solution and time.

Random microseeding is a modified microseeding strategy using random screening kits. It is reported to create extra additional hits as well as produce better quality protein crystals for diffraction experiments [36].

3.2 Co-crystallization with ligands and crystallization chaperones

The co-crystallization of macromolecules with ligands have been developed because some proteins crystallize efficiently with ligands considering its structure stabilization role. The use of ligands has benefits not only for improving crystallization but also for obtaining stable and functional protein during purification because they enhance thermal stability of molecules. The study of small ligands stabilization roles for proteins in aqueous solution is still in progress. There exist online libraries of ligands as compounds used for crystallization purposes to prepare the best condition for protein stabilization [37]. The use of ligands for crystallization promotes the possibility of obtaining good-quality protein crystals. Ligands are regularly used to expand the screening capacity of commercially available screens [38]. On the contrary, it can be challenging to crystalize protein-ligand complex and so, the use of stabilizing additives is necessary. The process of co-crystallization can be further affected by temperature used for the complex formation prior its crystallization and by concentration of protein or used ligand [39].

Another "compounds" used for stabilization of proteins are amino acids and their derivatives such as glycine ethyl ester or glycine amide. There is noted considerable benefit of the stabilization role of amino acids for protein purification as well as crystallization [40, 41]. Hence, small molecule additives such as coenzymes, prosthetic groups, inhibitors and small molecules, mentioned in [42], are used for successful crystallization of macromolecules. Usage of these additives helps forming hydrogen bonds and crosslinks between molecules that helps formation of stable crystals with regular lattice order of molecules [38].

Additionally, the antibodies and nanobodies are used to improve protein crystallization. Antibodies such as conventional IgG, Fab-fragments (fragment antigen binding), scFv (single-chain variable fragments) and Camelid hcAb (heavy chain IgG) were used to reinforce protein crystallization, mainly for membrane protein

co-crystallization. Latterly, nanobodies replaced the use of antibodies because production of antibodies is limited and obtained yield tends to be conformationally heterogeneous with limited solubility [43, 44]. On the contrary, nanobodies are extremely stable and soluble antibody single-domain fragments that stabilize unstructured proteins by binding to them, form crystal contacts and thus speed up crystal growth. For crystallization of selected protein, many conformationally different nanobodies can be used to enhance the probability of best quality crystal hits [45].

3.3 Protein engineering

Protein engineering is one of the most used strategies in molecular biology to produce sufficient amounts of soluble protein. Protein biocrystallogenesis is likewise using protein engineering for crystallization of proteins that are hard to crystallize owing to have highly flexible regions, high surface entropy or less common high surface hydrophobicity or proteins are not able to be produced soluble [46]. To conquer these issues some strategies such as designing modified proteins, mutation of surface residues or designing fusion proteins are used [47].

The modifications of proteins include designing only some parts of protein such as conserved domains, and removing flexible parts or internal loops that prevent crystal formation. This can be fulfilled by creation of genetic constructs based on e.g. computation selection of expression clone libraries to find the clones with fragments that would encode the concerned part of the protein [47]. In the literature, several strategies for protein modification have been mentioned such as colony filtration blot technique [48], the open reading frame selection through the ESPRIT automation to find soluble complexes [49] and fluorescence screening based on protein fusion with GFP protein [47, 50].

Next strategy to enhance crystal formation is design of protein mutants by site-specific mutation or chemical modification. Mutagenesis is ordinarily used to produce soluble protein assuming that wild type is not soluble. The substitution of Lys, Gln or Glu that are frequently located on the molecule surface [51] with Ala that has lower surface entropy is a commonly used strategy [52]. There are plenty of protein structures that were solved mainly due to mutagenesis [47]. In addition, many chemical modifications as reductive methylation of Lys that reduce protein solubility and reductive carboxymethylation or substitution of Cys that on the contrary enhance solubility, stability and have benefits for protein function and oligomerization [52]. Different strategy is to introduce Cys at the surface to strengthen formation of symmetrical dimers that seem to crystallize more easily [52, 53].

Design of fusion proteins or fusion tags is another approach to produce soluble proteins and subsequently solve protein structures. In many cases the fusion partner such as short *Strep*-tag or poly-histidine tag and some larger tags as glutathione S-transferase (GST) and Maltose-binding protein (MBP) are removed before crystallization [54–56]. This is done to remove possible highly flexible regions that can block formation of protein contacts during crystallization. However, it was shown that in two different approaches fusion proteins manage to crystalize target proteins. First, one is based on increasing protein surface area by incorporation of fused protein such as MBP, Green fluorescent protein (GFP), Barnase or T4 lysozyme (T4L) [56]. Second is aimed to merge interacting proteins or protein and peptide with linker, mostly to stabilize one of fused proteins or to support their interaction [56, 57].

3.4 Crystallization in living cells

The formation of native protein crystals in living cells has been detected over the last few decades as a natural process. This approach is ideal for proteins that cannot

be crystallized applying basic crystallization techniques. Recently, X-ray sources have been exceedingly improved, such as microfocus beamlines, X-ray free-electron lasers, or even serial crystallography as a new data collection strategy [58, 59]. These advances in the field of X-ray crystallography enable data collection from smaller crystals and thus enable the development of *in cellulo* crystallization as a powerful advanced crystallization approach.

Recombinant or fusion recombinant proteins can be crystallized in a number of living cells, including plant cells, mammalian cells or insect cells [60]. The bottleneck of this strategy is the detection of the protein crystals inside the cells. Crystals can occur in different compartments of the cell, such as cytosol, endoplasmic reticulum (ER) or peroxisomes [60, 61]. The crystals can be detected using various methods, e.g. bright-field microscopy techniques or transmission electron microscopy (TEM), which is also used for the observation of crystal growth and subsequent optimization. The crystal can be isolated from the cell before the diffraction experiment or directly measured in the cell [60, 62].

3.5 Automation

The expansion of the crystallographic field and running extensive research led to need for facilitation of performing crystallization experiments. This was achieved by automation that supported faster progress of experiments thanks to development of a variety of robots [63]. There are robots adjusted to establishment of crystallization conditions, arrangement of drops, for carrying out the vapour diffusion experiments specifically hanging drop, microbatch, free-interface diffusion and random micro-seeding seeding methods with sitting drop method as the most preferred method. Most widely used crystallization robots are from the Oryx series (Douglas Instruments Ltd.), the Mosquito (TTP Labtech, Royston, UK) and TOPAZ system (Fluidigm Corp., San Francisco CA, USA) [64]. Automation devices allow crystallization of nanovolumes into an array of plates for 96 or more conditions [28]. Each robot has its own specification, from which can be chosen preferred experimental method suitable for protein of interest.

4. Conclusion

In conclusion, a growth the good-quality macromolecular crystal is crucial for the determination of its atomic structure using of X-ray crystallography. Several effective advanced methods leading to the formation of crystal in diffraction quality are available. Nowadays, the extensive improvement of X-ray facilities enables data collection from smaller crystals and allows using the new trends in crystallization strategies that are developing as a powerful advanced crystallization approach.

The protein crystallization manufacturers such as Hampton Research, Molecular Dimensions Ltd., PerkinElmer, GE Healthcare, Danaher, Bruker, Agilent, Jena Bioscience, Rigaku, Formulatrix and MiTeGen are basic companies responsible for systematic invention of new products and technologies that constantly improve protein crystallization methods and are looking for crystallization techniques for hardly crystallisable proteins such as membrane proteins.

Next essentials that have an effort to improve crystallization techniques are attempts to use crystals in different non-conventional ways. One of them is sending biosubstances (protein-based medicines) into space to try to crystallize them for finding new uses of crystallized proteins as pharmacotherapeutics. These protein crystals can be supposedly thereafter used for long-term storage or for stabilization of biosubstances by transferring them into solid state (Bristol-Myers

Squibb Company). Another use of crystallization was reported in material sciences, more precisely for improvement of electronic materials developed on the base of biomolecules arrangement in crystals [65].

These and many more applications of protein crystallization are reasons for development of new methods and strategies for faster crystallization and obtaining of well diffractive crystals and for reduction of protein volume. However, there is still a necessity for improvement and new methods and strategies for biomolecules crystallization awaits to be discovered. Although this can be achieved more easily because biochemistry, biotechnology, physics, chemistry and applied nanosciences are extra overlapped and allow to improve and explain in depth the process of protein crystallization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

[1] Hofmeister, T. (1890). *Z. Physiol. Chem.* **14**, 165.

[2] Abel, J. (1927). Chemistry in Relation to Biology and Medicine with Especial Reference to Insulin and Other Hormones. *Science, 66*(1710), 307-319. Retrieved February 22, 2021, from http://www.jstor.org/stable/1651597

[3] McPherson A, Gavira JA. Introduction to protein crystallization. Acta Crystallogr F Struct Biol Commun. 2014 Jan;70(Pt 1):2-20. doi: 10.1107/S2053230X13033141. Epub 2013 Dec 24. PMID: 24419610; PMCID: PMC3943105.

[4] Boudes M, Garriga D, Coulibaly FJ. Reflections on the Many Facets of Protein Microcrystallography. Australian Journal of Chemistry. 2014;67(12):1793-1806. https://doi.org/10.1071/CH14455

[5] Nannenga BL, Gonen T. Protein structure determination by MicroED. Curr Opin Struct Biol. 2014 Aug;27:24-31. doi: 10.1016/j.sbi.2014.03.004. Epub 2014 Apr 5. PMID: 24709395; PMCID: PMC5656570.

[6] Manuel García-Ruiz J. Nucleation of protein crystals. J Struct Biol. 2003 Apr;142(1):22-31. doi: 10.1016/s1047-8477(03)00035-2. PMID: 12718916.

[7] Otálora F, Gavira JA,Ng JD, García-Ruiza JM. Counter diffusion methods applied to protein crystallization. Progress in Biophysics and Molecular Biology. 2009;101(1-3):26-37. DOI: 10.1016/j.pbiomolbio.2009.12.004.

[8] Henisch HK, García-Ruiz JM. Crystal growth in gels and Liesegang ring formation: I. Diffusion relationships. Journal of Crystal Growth. 1986;75(2):195-202. https://doi.org/10.1016/0022-0248(86)90028-X.

[9] García-Ruiza JM, Novella ML, Moreno R, Gavira JA. Agarose as crystallization media for proteins: I: Transport processes. Journal of Crystal Growth. 2001;232(1-4):165-172. ISSN 0022-0248. https://doi.org/10.1016/S0022-0248(01)01146-0.

[10] López-Jaramillo FJ, García-Ruiz JM, Gavira JA and F. Otálora. Crystallization and cryocrystallography inside X-ray capillaries. J. Appl. Cryst. 2001;34:365-370. https://doi.org/10.1107/S0021889801003697

[11] Biertümpfel C, Basquin J, Suck D, Sauter C. Crystallization of biological macromolecules using agarose gel. Acta Crystallogr D Biol Crystallogr. 2002 Oct;58(Pt 10 Pt 1):1657-9. doi: 10.1107/s0907444902012738. Epub 2002 Sep 26. PMID: 12351881.

[12] Kutá Smatanová, I., Gavira, J.A., Řezáčová, P. *et al.* New techniques for membrane protein crystallization tested on photosystem II core complex of *Pisum sativum* . *Photosynth Res* 90, 255-259 (2006). https://doi.org/10.1007/s11120-007-9131-y

[13] Gavira JA, de Jesus W, Camara-Artigas A, López-Garriga J, García-Ruiz JM. Capillary crystallization and molecular-replacement solution of haemoglobin II from the clam Lucina pectinata. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2006 Mar 1;62(Pt 3):196-9. doi: 10.1107/S1744309106002648. Epub 2006 Feb 10. PMID: 16511300; PMCID: PMC2197196.

[14] Artusio F, Castellví A, Sacristán A, Pisano R, and JA Gavira. Agarose Gel as a Medium for Growing and Tailoring Protein Crystals. Cryst. Growth Des. 2020;20(8):5564-5571. https://doi.org/10.1021/acs.cgd.0c00736

[15] Gavira JA, García-Ruiz JM. Agarose as crystallisation media for proteins II: trapping of gel fibres into the crystals. Acta Crystallogr D Biol Crystallogr.

2002 Oct;58(Pt 10 Pt 1):1653-6. doi: 10.1107/s0907444902014609. Epub 2002 Sep 26. PMID: 12351880.

[16] Sugiyama Y, Ikarugi S, Oura K, Ikeda A, Matsuyama E, Ono R, Nomura M, Tawarayama H, Saito T, Kuwahara K. MFI zeolite membranes prepared on novel silica substrates. Journal of Chemical Engineering of Japan. 2015 ;48(11):891-896. https://doi.org/10.1252/jcej.15we014

[17] Conrad CE, Basu S, James D, Wang D, Schaffer A, Roy-Chowdhury S, Zatsepin NA, Aquila A, Coe J, Gati C, Hunter MS, Koglin JE, Kupitz C, Nelson G, Subramanian G, White TA, Zhao Y, Zook J, Boutet S, Cherezov V, Spence JC, Fromme R, Weierstall U, Fromme P. A novel inert crystal delivery medium for serial femtosecond crystallography. IUCrJ. 2015 Jun 30;2(Pt 4):421-30. doi: 10.1107/S2052252515009811. PMID: 26177184; PMCID: PMC4491314.

[18] Littke W, John C. Materials: protein single crystal growth under microgravity. Science. 1984 Jul 13;225(4658):203-4. doi: 10.1126/science.225.4658.203. PMID: 17837944.

[19] DeLucas LJ, Suddath FL, Snyder R, Naumann R, Broom MB, Pusey M, Yost V, Herren B, Carter D, Nelson B, Meehan EJ, McPherson A, Bugg ChE. Preliminary investigations of protein crystal growth using the space shuttle. Journal of Crystal Growth. 1986;76(3):681-693. ISSN 0022-0248, https://doi.org/10.1016/0022-0248(86)90185-5.

[20] Ng JD. Space-grown protein crystals are more useful for structure determination. Ann N Y Acad Sci. 2002 Oct;974:598-609. doi: 10.1111/j.1749-6632.2002.tb05934.x. PMID: 12446351.

[21] Vergara, A., Lorber, B., Sauter, C., Giegé, R., & Zagari, A. (2005). Lessons from crystals grown in the Advanced Protein Crystallisation Facility for conventional crystallisation applied to structural biology. Biophysical chemistry, 118(2-3), 102-112. https://doi.org/10.1016/j.bpc.2005.06.014

[22] McPherson A, DeLucas LJ. Microgravity protein crystallization. NPJ Microgravity. 2015 Sep 3;1:15010. doi: 10.1038/npjmgrav.2015.10. PMID: 28725714; PMCID: PMC5515504.

[23] van der Woerd M, Ferree D, Pusey M. The promise of macromolecular crystallization in microfluidic chips. J Struct Biol. 2003 Apr;142(1):180-7. doi: 10.1016/s1047-8477(03)00049-2. PMID: 12718930.

[24] de Wijn R, Hennig O, Roche J, Engilberge S, Rollet K, Fernandez-Millan P, Brillet K, Betat H, Mörl M, Roussel A, Girard E, Mueller-Dieckmann C, Fox GC, Olieric V, Gavira JA, Lorber B, Sauter C. A simple and versatile microfluidic device for efficient biomacromolecule crystallization and structural analysis by serial crystallography. IUCrJ. 2019 Apr 19;6(Pt 3):454-464. doi: 10.1107/S2052252519003622. PMID: 31098026; PMCID: PMC6503916.

[25] Abdalla M, Ali Eltayb W, Samad A, Elshareef SHM, Dafaalla TIM. Important Factors Influencing Protein Crystallization. Glob J Biotechnol Biomater Sci. 2016;2(1):025-028 DOI: 10.17352/gjbbs.000008

[26] Martin-Garcia JM, Conrad CE, Coe J, Roy-Chowdhury S, Fromme P. Serial femtosecond crystallography: A revolution in structural biology. Arch Biochem Biophys. 2016 Jul 15;602:32-47. doi: 10.1016/j.abb.2016.03.036. Epub 2016 Apr 30. PMID: 27143509; PMCID: PMC4909539.

[27] Johansson LC, Stauch B, Ishchenko A, Cherezov V. A Bright Future for Serial Femtosecond Crystallography with XFELs. Trends

Biochem Sci. 2017 Sep;42(9):749-762. doi: 10.1016/j.tibs.2017.06.007. Epub 2017 Jul 18. PMID: 28733116; PMCID: PMC5600683.

[28] Moreno A. Advanced Methods of Protein Crystallization. Methods Mol Biol. 2017;1607:51-76. doi: 10.1007/978-1-4939-7000-1_3. PMID: 28573569.

[29] Flores-Hernández E, Stojanoff V, Arreguín-Espinosa R, Moreno A, Sánchez-Puig N. An electrically assisted device for protein crystallization in a vapor-diffusion setup. J Appl Crystallogr. 2013 Jun 1;46(Pt 3):832-834. doi: 10.1107/S0021889813010558. Epub 2013 May 15. PMID: 23682197; PMCID: PMC3654317.

[30] Wakayama NI, Yin DC, Harata K, Kiyoshi T, Fujiwara M, Tanimoto Y. Macromolecular crystallization in microgravity generated by a superconducting magnet. Ann N Y Acad Sci. 2006 Sep;1077:184-93. doi: 10.1196/annals.1362.024. PMID: 17124123.

[31] Wada H, Hirota N, Matsumoto S, Okada H, Kiyohara M, Ode T, Tanokura M, Nakamura A, Ohtsuka J, Kita A, Numoto N, Kashiwagi T, Suzuki E. Application of High-Field Superconducting Magnet to Protein Crystallization. Physics Procedia. 2012;36:953-957. ISSN 1875-3892. https://doi.org/10.1016/j.phpro.2012.06.236.

[32] Sazaki G, Moreno A, Nakajima K. Novel coupling effects of the magnetic and electric fields on protein crystallization. Journal of Crystal Growth. 2004; 262(1-4):499-502. ISSN 0022-0248. https://doi.org/10.1016/j.jcrysgro.2003.09.050.

[33] Bergfors T. Seeds to crystals. J Struct Biol. 2003 Apr;142(1):66-76. doi: 10.1016/s1047-8477(03)00039-x. PMID: 12718920.

[34] Luft JR, DeTitta GT. A method to produce microseed stock for use in the crystallization of biological macromolecules. Acta Crystallogr D Biol Crystallogr. 1999 May;55(Pt 5):988-93. doi: 10.1107/s0907444999002085. PMID: 10216295.

[35] Abuhammad A, McDonough MA, Brem J, Makena A, Johnson S, Schofield ChJ, and EF Garman. "To Cross-Seed or Not To Cross-Seed": A Pilot Study Using Metallo-β-lactamases. Crystal Growth & Design. 2017;17(2):913-924. DOI: 10.1021/acs.cgd.6b01805

[36] Stewart SPD, Kolek SA, Briggs RA, Chayen NE, and PFM Baldock. Random Microseeding: A Theoretical and Practical Exploration of Seed Stability and Seeding Techniques for Successful Protein Crystallization. Cryst. Growth Des. 2011;11(8):3432-3441. https://doi.org/10.1021/cg2001442

[37] Bolanos-Garcia VM, Chayen NE. New directions in conventional methods of protein crystallization. Prog Biophys Mol Biol. 2009 Nov;101(1-3):3-12. doi: 10.1016/j.pbiomolbio.2009.12.006. Epub 2009 Dec 16. PMID: 20018205.

[38] McPherson A, Cudney B. Searching for silver bullets: an alternative strategy for crystallizing macromolecules. J Struct Biol. 2006 Dec;156(3):387-406. doi: 10.1016/j.jsb.2006.09.006. Epub 2006 Oct 11. PMID: 17101277.

[39] Hassell AM, An G, Bledsoe RK, Bynum JM, Carter HL 3rd, Deng SJ, Gampe RT, Grisard TE, Madauss KP, Nolte RT, Rocque WJ, Wang L, Weaver KL, Williams SP, Wisely GB, Xu R, Shewchuk LM. Crystallization of protein-ligand complexes. Acta Crystallogr D Biol Crystallogr. 2007 Jan;63(Pt 1):72-9. doi: 10.1107/S0907444906047020. Epub 2006 Dec 13. PMID: 17164529; PMCID: PMC2483499.

[40] Ito L, Kobayashi T, Shiraki K, Yamaguchi H. Effect of amino acids and amino acid derivatives on crystallization of hemoglobin and ribonuclease A. J Synchrotron Radiat. 2008 May;15(Pt 3):

316-8. doi: 10.1107/S0909049507068598. Epub 2008 Apr 18. PMID: 18421168; PMCID: PMC2394812.

[41] Uyen DT, Huy NT, Trang DT, Nhien NT, Oida T, Hirayama K, Harada S, Kamei K. Effects of amino acids on malarial heme crystallization. Biol Pharm Bull. 2008 Aug;31(8):1483-8. doi: 10.1248/bpb.31.1483. PMID: 18670076.

[42] Manjasetty BA, Turnbull AP, Panjikar S, Büssow K, Chance MR. Automated technologies and novel techniques to accelerate protein crystallography for structural genomics. Proteomics. 2008 Feb;8(4):612-25. doi: 10.1002/pmic.200700687. PMID: 18210369.

[43] Lieberman RL, Culver JA, Entzminger KC, Pai JC, Maynard JA. Crystallization chaperone strategies for membrane proteins. Methods. 2011 Dec;55(4):293-302. doi: 10.1016/j.ymeth.2011.08.004. Epub 2011 Aug 11. PMID: 21854852; PMCID: PMC6186007.

[44] Mir SH, Escher C, Kao WC, Birth D, Wirth C, Hunte C. Generation of recombinant antibody fragments for membrane protein crystallization. Methods Enzymol. 2015;557:201-18. doi: 10.1016/bs.mie.2014.12.029. Epub 2015 Mar 24. PMID: 25950966.

[45] Traenkle B, Kaiser PD, Rothbauer U. Nanobody Platform for Determination of Protein Structure, Application of. Encyclopedia of Analytical Chemistry: Applications, Theory and Instrumentation. Peptides and Proteins. 2016. https://doi.org/10.1002/9780470027318.a9548

[46] Derewenda ZS. Application of protein engineering to enhance crystallizability and improve crystal properties. Acta Crystallogr D Biol Crystallogr. 2010 May;66(Pt 5):604-15. doi: 10.1107/S090744491000644X. Epub 2010 Apr 21. PMID: 20445236; PMCID: PMC3089013.

[47] Manjasetty BA, Büssow K, Panjikar S, Turnbull A. Current methods in structural proteomics and its applications in biological sciences. 3 Biotech. 2012; 2:89-113. https://doi.org/10.1007/s13205-011-0037-1

[48] Cornvik T, Dahlroth SL, Magnusdottir A, Herman MD, Knaust R, Ekberg M, Nordlund P. Colony filtration blot: a new screening method for soluble protein expression in Escherichia coli. Nat Methods. 2005 Jul;2(7):507-9. doi: 10.1038/nmeth767. PMID: 15973420.

[49] An Y, Yumerefendi H, Mas PJ, Chesneau A, Hart DJ. ORF-selector ESPRIT: a second generation library screen for soluble protein expression employing precise open reading frame selection. J Struct Biol. 2011 Aug; 175(2):189-97. doi: 10.1016/j.jsb.2011.04.004. Epub 2011 Apr 16. PMID: 21515383.

[50] Pedelacq JD, Nguyen HB, Cabantous S, Mark BL, Listwan P, Bell C, Friedland N, Lockard M, Faille A, Mourey L, Terwilliger TC, Waldo GS. Experimental mapping of soluble protein domains using a hierarchical approach. Nucleic Acids Res. 2011 Oct;39(18):e125. doi: 10.1093/nar/gkr548. Epub 2011 Jul 19. PMID: 21771856; PMCID: PMC3185438.

[51] Baud F, Karlin S. Measures of residue density in protein structures. Proc Natl Acad Sci U S A. 1999 Oct 26;96(22):12494-9. doi: 10.1073/pnas.96.22.12494. PMID: 10535950; PMCID: PMC22960.

[52] Bolanos-Garcia VM, Chayen NE. New directions in conventional methods of protein crystallization. Prog Biophys Mol Biol. 2009 Nov;101(1-3):3-12. doi: 10.1016/j.pbiomolbio.2009.12.006. Epub 2009 Dec 16. PMID: 20018205.

[53] Banatao DR, Cascio D, Crowley CS, Fleissner MR, Tienson HL, Yeates TO. An approach to crystallizing proteins by synthetic symmetrization. Proc Natl Acad Sci U S A. 2006 Oct 31;103(44): 16230-5. doi: 10.1073/pnas.0607674103. Epub 2006 Oct 18. PMID: 17050682; PMCID: PMC1637565.

[54] Derewenda ZS. The use of recombinant methods and molecular engineering in protein crystallization. Methods. 2004 Nov;34(3):354-63. doi: 10.1016/j.ymeth.2004.03.024. PMID: 15325653.

[55] Waugh DS. Making the most of affinity tags. Trends Biotechnol. 2005 Jun;23(6):316-20. doi: 10.1016/j.tibtech.2005.03.012. PMID: 15922084.

[56] Kobe B, Ve T, Williams SJ. Fusion-protein-assisted protein crystallization. Acta Crystallogr F Struct Biol Commun. 2015 Jul;71(Pt 7):861-9. doi: 10.1107/S2053230X15011061. Epub 2015 Jun 27. PMID: 26144231; PMCID: PMC4498707.

[57] Reddy Chichili VP, Kumar V, Sivaraman J. Linkers in the structural biology of protein-protein interactions. Protein Sci. 2013 Feb;22(2):153-67. doi: 10.1002/pro.2206. Epub 2013 Jan 8. PMID: 23225024; PMCID: PMC3588912.

[58] Yabashi M, Tanaka H. The next ten years of X-ray science. Nature Photon. 2017;11:12-14. https://doi.org/10.1038/nphoton.2016.251

[59] Chapman HN, Fromme P, Barty A, White TA, Kirian RA, Aquila A, Hunter MS, Schulz J, DePonte DP, Weierstall U, Doak RB, Maia FR, Martin AV, Schlichting I, Lomb L, Coppola N, Shoeman RL, Epp SW, Hartmann R, Rolles D, Rudenko A, Foucar L, Kimmel N, Weidenspointner G, Holl P, Liang M, Barthelmess M, Caleman C, Boutet S, Bogan MJ, Krzywinski J, Bostedt C, Bajt S, Gumprecht L, Rudek B, Erk B, Schmidt C, Hömke A, Reich C, Pietschner D, Strüder L, Hauser G, Gorke H, Ullrich J, Herrmann S, Schaller G, Schopper F, Soltau H, Kühnel KU, Messerschmidt M, Bozek JD, Hau-Riege SP, Frank M, Hampton CY, Sierra RG, Starodub D, Williams GJ, Hajdu J, Timneanu N, Seibert MM, Andreasson J, Rocker A, Jönsson O, Svenda M, Stern S, Nass K, Andritschke R, Schröter CD, Krasniqi F, Bott M, Schmidt KE, Wang X, Grotjohann I, Holton JM, Barends TR, Neutze R, Marchesini S, Fromme R, Schorb S, Rupp D, Adolph M, Gorkhover T, Andersson I, Hirsemann H, Potdevin G, Graafsma H, Nilsson B, Spence JC. Femtosecond X-ray protein nanocrystallography. Nature. 2011 Feb 3;470(7332):73-7. doi: 10.1038/nature09750. PMID: 21293373; PMCID: PMC3429598.

[60] Schönherr R, Rudolph JM, Redecke L. Protein crystallization in living cells. Biol Chem. 2018 Jun 27;399(7):751-772. doi: 10.1515/hsz-2018-0158. PMID: 29894295.

[61] Koopmann R, Cupelli K, Redecke L, Nass K, Deponte DP, White TA, Stellato F, Rehders D, Liang M, Andreasson J, Aquila A, Bajt S, Barthelmess M, Barty A, Bogan MJ, Bostedt C, Boutet S, Bozek JD, Caleman C, Coppola N, Davidsson J, Doak RB, Ekeberg T, Epp SW, Erk B, Fleckenstein H, Foucar L, Graafsma H, Gumprecht L, Hajdu J, Hampton CY, Hartmann A, Hartmann R, Hauser G, Hirsemann H, Holl P, Hunter MS, Kassemeyer S, Kirian RA, Lomb L, Maia FR, Kimmel N, Martin AV, Messerschmidt M, Reich C, Rolles D, Rudek B, Rudenko A, Schlichting I, Schulz J, Seibert MM, Shoeman RL, Sierra RG, Soltau H, Stern S, Strüder L, Timneanu N, Ullrich J, Wang X, Weidenspointner G, Weierstall U, Williams GJ, Wunderer CB, Fromme P, Spence JC, Stehle T, Chapman HN, Betzel C, Duszenko M. In vivo protein crystallization opens new routes in structural biology. Nat Methods. 2012

Jan 29;9(3):259-62. doi: 10.1038/nmeth.1859. PMID: 22286384; PMCID: PMC3429599.

[62] Stevenson HP, Lin G, Barnes CO, Sutkeviciute I, Krzysiak T, Weiss SC, Reynolds S, Wu Y, Nagarajan V, Makhov AM, Lawrence R, Lamm E, Clark L, Gardella TJ, Hogue BG, Ogata CM, Ahn J, Gronenborn AM, Conway JF, Vilardaga JP, Cohen AE, Calero G. Transmission electron microscopy for the evaluation and optimization of crystal growth. Acta Crystallogr D Struct Biol. 2016 May;72(Pt 5):603-15. doi: 10.1107/S2059798316001546. Epub 2016 Apr 26. PMID: 27139624; PMCID: PMC4854312.

[63] Stewart PS, Mueller-Dieckmann J. Automation in biological crystallization. Acta Crystallogr F Struct Biol Commun. 2014 Jun;70(Pt 6):686-96. doi: 10.1107/S2053230X14011601. Epub 2014 May 28. PMID: 24915074; PMCID: PMC4051518.

[64] Saridakis E. Perspectives on high-throughput technologies applied to protein crystallization. Protein Pept Lett. 2012 Jul;19(7):778-83. doi: 10.2174/092986612800793154. PMID: 22489783.

[65] Byrappa, K.; Klapper, H.; Ohachi, T.; Fornari, R. Crystal Growth of Technologically Important Electronic Materials; Allied Publishers, Mysore: India, 2003; ISBN 81-7764-375-4.