Chromatography and Separation Techniques Journal

SCIENTIFIC LITERATURE

Special Issue Article" Crystallization" Review Article

Crystallization of Membrane and Soluble Proteins: Difference in Approach

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ARTICLE INFO

Received Date: January 07, 2019
Accepted Date: February 25, 2019
Published Date: February 27, 2019

KEYWORDS

Membrane proteins
Detergents; Nucleation
Crystal growth
Thermodynamics
Optimization
Additives

Citation for this article: Neha Upmanyu and Viveka Nand Malviya. Crystallization of Membrane and Soluble Proteins: Difference in Approach. Chromatography and Separation Techniques Journal. 2019; 2(1):118

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ABSTRACT

X-ray crystallography is the most efficient technique to decipher 3D structure of a protein at atomic resolution with high accuracy. Obtaining protein crystals is often considered the bottleneck of this technique. Crystallization involve high precision of protein-protein contacts in the crystals which is governed by the prevailing solution conditions and many other factors. The equilibrium between various factors lead to different stages of protein crystallization; aggregation to nucleation to crystal formation. Since crystallization is a process which is governed mainly by surface chemistry, any factor which will affecting the surface property of protein molecule (pH, ionic strength, detergents, additives) will ultimately affect the crystal growth. Protein crystallography is highly successful in 3D structure elucidation of soluble proteins, but same is not true for membrane proteins. Though, the basic principle of crystallization is same for both types of proteins, but due to extreme hydrophobic characteristic of membrane proteins, they require special methodology to purify and crystallize. Presence of hydrophobic detergents in the working solution poses additional challenges at every step. This manuscript reviews major highlights in setting up crystallization for both proteins in addition to recent advancement in the field of crystallization, particularly for membrane proteins.

INTRODUCTION

To understand the molecular mechanism of protein function, it is prerequisite to have knowledge about 3D structure of that particular protein. Since the discovery of first protein structure myoglobin by X-ray crystallography, this particular technique gained huge importance for protein structure determination. In last five decades, thousands of protein structures have been determined and their atomic coordinates have been deposited in the Protein Data Bank (PDB) (http://www.pdb.org) [1]. Over the years, the field grew exponentially but still the basics of the technique still remain same. The first and foremost requirement is a good quality crystal. Despite the advancement in the field, obtaining good diffraction quality crystal still remain very cumbersome and often rate limiting. Various approach have been adopted to improve the success rate of obtaining crystals in recent years. A term has been coined called "crystallization space" to mention collectively all the variables present in the crystallization condition of the protein like; temperature, pH, salt concentration etc. These variables should be optimized one by one or simultaneously in order to obtain

good quality crystals. The crystallization space vary from protein to protein and so far it couldn't be rationalized and hence a general protocol to obtain good crystal for every protein is lacking. Two members from the same family proteins and having similar physico-chemical properties can be crystallized in different conditions and two completely different proteins might require similar conditions to crystallize. Because of the presence of various factor which can affect the crystallization process, a careful observation of the crystallization condition on regular basis is very important [2-8]. The feedback from every observation time and its implementation into the experiment is key to increase the success rate in obtaining good quality crystals. In recent years enormous progress have been made in structural biology filed with the development of NMR [9-11] and Cryo-Electron Microscopy. But these techniques have some limitations of size of the protein. NMR is useful in studying smaller proteins (<50 kDa) where as Cryo-EM is useful to study protein bigger than 200 kDa. Even then Cryo-EM proved to be very successful to study membrane protein and complexes [12-14]. Nevertheless, crystallography still remains the most successful technique to study 3D structure of the protein. Though there are enormous amounts of literature in the form of research articles and book chapters are available on this topic, our aim of this review is to update the readers about recent advancements in this field, keeping some basic information intact.

HETEROLOGOUS PROTEIN PRODUCTION

For crystallization, large amount of pure protein is required. Therefore it is advisable to optimize at the protein production stage in the beginning. This is extremely important particularly in case of membrane protein which has relatively poor yield compared to soluble proteins when produced heterologously. In addition to that, purification of membrane proteins require some additional steps and at every step some loss of protein is inevitable. Therefore, to manage good amount of high quality membrane proteins for downstream experiments, yield optimization is recommended.

Bacterial expression system is still favored for overproducing proteins. However, when the system cannot provide the folding machinery and posttranslational modifications, especially for eukaryotic protein, other production systems are adapted like *Saccharomyces cerevisiae* yeast [15,16], *Pichia pastoris* yeast [17-19], Sf9 insect cells [20-22] and human embryonic kidney (HEK293S) cells [23,24]. Over-expressed membrane proteins are often toxic to host cells. In such cases tight promoter system should be opted to prevent leaky expression. Induction at mid-log phase is helpful to keep the cells growing after induction. For membrane protein production, it is important to ensure that the protein is properly folded in the lipid-bilayer membrane. To ensure this, protein should be produced at a slow rate to allow them to fold and inserted into the membrane properly. Overnight production at 16-25 °C is recommended for membrane proteins. In addition, slow induction (lower concentration of inducer) is also favored though in many cases auto induction works better. In order to facilitate purification, these proteins are always overproduced with specific tags (Histidine , Streptavidin, FLAG, GST etc). Some membrane proteins are sensitive to the N- or C- terminus tags. To ensure better yield, tag position should be optimized. Mostly, membrane proteins have N-terminus signal sequence, which should be included in the protein sequence and hence in such cases, C-terminus tag would be more suitable [25-34]. For soluble proteins these factors are not as critical as for membrane proteins, nevertheless, in some cases particularly for soluble complexes, these factors might play a role. Different host-vector combinations should be screened in order to obtain optimum condition for protein production [29,35]. Since membrane proteins are purified in the presence of detergents, binding affinity of the tags are lower compared to soluble protein tags. Therefore, wherever possible high-affinity tags should be used for them e.g. 10-His tag instead of 6-His tag which is widely used for purification of soluble proteins. In many cases even after optimization, some membrane proteins are very difficult to produce heterologously. The main reason is the toxicity of foreign protein for host cells as they are inserted into the membrane [32-35]. Such proteins can be overproduced by cell-free protein production system. This technique is gaining importance for expressing mammalian genes as they are difficult to express in prokaryotic or even in some cases, eukaryotic expression system. It is also easy to manipulate proteins produced in cell-free system as modified amino acids can be easily inserted into their sequences. This is very helpful in case of sample preparation for NMR studies as isotopically labeled amino acids can be directly inserted into

the protein. This technique is applicable to both membrane and soluble proteins [36-40].

In order to obtain stable protein sample to increase the crystallization propensity of some membrane protein targets, use of stabilizing mutants have a huge impact. Additional approach is to use truncated version of target proteins where flexible region of the protein is selectively removed. Often, membrane proteins are produced in fusion with soluble protein like maltose binding protein (MBP) [41,42], glutathione-*S*-transferase (GST) [43], the PelB leader sequence [44], green fluorescence protein (GFP) [26] or mistic tag [45] in order to increase their production and facilitate proper insertion into the membrane. The most recent development in this field is to produce membrane protein in soluble form in the cytoplasm by fusion with 200-residue C-terminal lipid binding domain of apolipoprotein from *E. coli*. Termed as SIMPLE x (solubilization of IMPs with high levels of expression) method, the fusion partner ApoAI, being amphipathic, act as a solubilizing partner [46]. In recent years several new strategies are adopted to purify membrane proteins. Usage of amphipoles [47,48], bicelles [49], nanodisc [50] and SMA polymer [51,52] are the most noticeable ones. Except for SMA polymer, others require membrane protein to be isolated in detergents prior to incorporation into the new system. Mostly the samples prepared by these alternative methods are useful in structure determination via cryo-EM.

Protein purification

Immobilized metal affinity chromatography (IMAC) and ion exchange chromatography (IEC) are two most commonly used technique to purify proteins [53-57]. Basic principle is same for purification of either soluble or membrane proteins, but in later case, extra steps and precautions are required. Soluble proteins are purified directly from the aqueous cytoplasmic soup whereas, membrane proteins are purified from membrane vesicles which are highly enriched in lipids. Membrane protein production consists of 3 basic steps: solubilization, purification & size exclusion chromatography, and concentration [58-67].

The target protein is solubilized form membrane vesicles, then purified to a stable and homogenous state. The homogeneous sample is concentrated while minimizing the detergent concentration and maintaining homogeneity before entering crystallization trials. In order to purify these proteins, they are extracted from the membrane into the solution form with the help of detergents. After solubilization, hydrophobic regions of membrane protein which are embedded in the lipid bilayer membrane are surrounded by detergent molecules and the hydrophilic portions are exposed to the aqueous solution. The choice and amount of detergents vary from protein to protein [58]. Since detergent environment is far away from natural one for the proteins, they tend to misfold very fast. Therefore, extraction of membrane proteins should be done in milder condition, like 4 °C for 30 min to 1 hour in the presence of 1-2 % of detergent. Key to purify membrane protein is to have fast protocol having only necessary steps, and as little amount of constituents in the buffer possible [28-30]. Membrane proteins from thermophilic organisms are mostly more stable. Therefore, more stringent steps can be adopted for their purification, like membrane solubilization at room temperature or even at 37 °C for overnight. After extraction from membrane vesicles, treatment for membrane and soluble proteins are similar. The only difference is the presence of detergent in the buffer solution for membrane proteins. The choice of detergent is protein dependent. The amount of detergent in the buffer during purification and other downstream process should be 2-3X CMC of the detergent [66,67].

Purified protein (IMAC/IEC or other methods) should be subjected to size exclusion chromatography immediately to separate different forms of protein present in the purified sample. This is necessary as for crystallization purpose homogeneous protein sample is required. In case of membrane proteins there are three key parameters (Detergent, pH, and ionic strength) which should be iteratively examined to find a condition which maintains a homogeneous population of protein-detergent-complexes or protein-detergent-lipid-complexes [68-70]. Mixture of detergents and/or lipids can be used in order to obtain a well folded population of target protein. Selection of optimum detergent or detergent-mixture should be done at purification step followed by SEC in order to avoid incomplete exchange if only one step is adopted. Other parameters like ionic strength or pH of buffer can be altered at SEC directly. Sometimes, dialysis of purified protein is required in case of soluble proteins, but such steps should be avoided in case of membrane proteins as this might remove

some naturally bound lipids which are important for structural stability or function of the protein. Nevertheless, in some cases dialysis is performed to reduced the detergent concentration before crystallization [66,67,71].

Third step in sample preparation for crystallization is to concentrate the protein. For soluble proteins relatively higher concentration (up to 50 mg/ml) is easily achieved, but for membrane proteins it is advisable not to concentrate higher than 15 mg/ml as they tends to precipitate. One major reason is the detergent which is also concentrated with the protein. Try to keep a check on final detergent concentration in the sample by thin layer chromatography before heading for crystallization. High concentration of detergents not only affect the protein quality, but also the property of crystallization drops [72].

Crystallization (Table 1-4)

Table 1: Biochemical characteristics of commonly used detergents.

Detergents	CMC (%)	CMC (mM)	Conc. needed for extraction (%)	Conc. during purification	Average mol wt of micelle (kDa)
DDM	0.0087	0.17	0.5-2.0	0.002-0.003	60-75
UM	0.029	0.58	1-2	0.07-0.1	50
DM	0.087	1.8	1-2	0.02-0.03	40
LDAO	0.023	2.0	1-2	0.05-0.075	22
OG	0.53	20	2	0.6-1	25
NG	0.20	5.5	2	0.5-0.6	40
Cymal5	0.12	2.5	1-2	0.25-0.4	28
Cymal6	0.028	0.56	1-2	0.07-0.1	45
C12E8	0.005	0.09	0.5-1	0.01-0.015	65
C12E5	0.003	0.05	0.5-1	0.007-0.1	82
TritonX	0.01	0.2	1-2	0.02-0.03	70-90
Digitonin	0.002	0.5	0.5-1	0.004-0.006	72

*Data adapted from Anatrace website.

Crystallization is a process which is governed by both kinetic and thermodynamic parameters. In this process molecules arrange themselves in three-dimensional space in a repetitive manner. Though the basic principles of soluble and membrane protein crystallization are same but there are noticeable differences exist when handling membrane protein crystals. Apart from traditional approach of crystallizing soluble proteins, some new methods have been developed, dedicated only to membrane protein crystallization. In many cases these new strategies worked better than the conventional approach.

Table 2: Common biological buffers used in crystallization.

Buffers	Useful pH range	pKa at 25°C
Acetate	3.6-5.6	4.76
MES	5.5-6.7	6.1
Bis-Tris	5.8-7.2	6.5
Carbonate	6.0-8.0	6.35
MOPS	6.5-7.9	7.2
HEPES	6.8-8.2	7.5
Tricin	7.4-8.8	8.05
Tris	7.0-9.0	8.06
Bicine	7.6-9.0	8.26

Data adapted from TEKNOVA and Applichem website.

Crystallization is a well defined process of precipitation, which is governed through many variables present in the crystallization solvent. Macromolecular crystallization, which includes crystallization of proteins is far more complex and there is no comprehensive theory is available to understand the fundamental properties of the process. So far, macromolecular crystallization is fundamentally dependent on trial-and-error approach. Protein crystallization involves largely two steps: a) systematic screening of range of individual parameters that affect crystal formation, b) fine tuning of condition to improve the quality of the crystal. Since, there are several variables involved in the process, both steps are time demanding. But there are several commercial screening kits are available to assist these process. The user should make careful assessment of the results at every stage which will ultimately help in narrowing down the search space to find out the ideal crystallization condition for a particular protein [73-80].

Table 3: common additives used during fine optimization of crystals.

Chromium salt	Copper salt	Proline	Spermidine	Ethanol
Berium salt	Cobalt salt	Phenol	Sarcosine	Methanol
Mangenese salt	Nickel salt	Sodium bromide	Urea	Butanol
Zinc salt	Iron salt	Glycine	Sucrose	Propanol
Yttrium salt	Magnesium salt	DMSO	Xylitol	Formamide
Strontium salt	Cesium salt	Taurine	Glycerol	Acetone

Data adapted from Hampton research website.

Protein crystals are composed of approximately 50% solvent and 50% of protein molecules. They contain large solvent channels through which big organic and inorganic molecules can diffuse easily. In contrary to conventional crystals, protein crystals are smaller in size, soft and easy to break. They are extremely sensitive to temperature, hydration state and exposure to radiation. Due to high solvent content they have poor resolution of diffraction pattern. Protein crystals are largely characterized by very few number of molecular interactions (hydrophobic interaction, salt bridges, hydrogen bonds) if compared to relative molecular mass. This results in poor lattice interactions which also affect diffraction resolution. In addition to this, conformational flexibility of the polypeptide chains within the protein is also a major factor in limiting the diffraction resolution. Because of above mentioned reasons, protein crystals are often polymorphic which is of limited use [78,81].

Table 4: Common Precipitants used in crystallization.

PEG	Ammonium sulfate	Ammonium nitrate	2-Methyl-2, 4-pentanediol
Jeffamine T	Sodium Sulfate	Sodium formate	PEG Mono Methly ether
Polyamine	Lithium sulfate	Magnesium sulfate	Dioxane
Isopropanol	Lithium chloride	Calcium sulfate	Acetone
Acetonitrile	$Na^+/K^+/(NH4)^+$/ chloride	Citrate/acetate salt	Hexanediol
Malic acid	Malonic acid	Succinic acid	Ethylene glycol

Data adapted from various publications.

Different stages of protein crystallization [81-116]

Crystallization of macromolecules can be divided into four major stages; a) supersaturation of protein molecules, b) onset of nucleation process, c) crystal growth, d) cessation of crystal growth. Supersaturation is an unstable condition of protein molecules which are still in solution state under given conditions. This can be achieved by varying parameters like protein concentration, salt concentration, temperature and pressure. Supersaturation is the key driving stage of crystallization. Under this condition nucleation stage is obtained which is essentially the step where solute molecules start to come together into clusters on the nanometer scale, resulting into formation of particles with interface. In this cluster, atoms are arranged in a defined manner to form crystal lattice. However, sometimes these clusters are not stable and the tiny crystals redissolve in to the solution. Stability of the nucleation stage is governed by temperature, concentration and ionic strength of the solution. The crystal growth is the next stage of nucleation. When the cluster is stable and reached to a critical size, growth of crystals continue within supersaturation condition. Crystal quality is affected by the growth rate. Too fast growth rate may result in amorphous crystals with poor diffraction quality. Growth of crystals will come to halt if the protein concentration drops in the solution below supersaturation level. At this stage, crystals should be harvested immediately and stored for further analysis [81-90,94,95].

Factors governing protein crystallization

As mentioned above there are many factors which affect crystallization process; a) protein purity and homogeneity, b) stability of the protein, c) flexible region of the protein, d) soluble domain of the protein, e) precipitant in the crystallization drop, f) pH and ionic strength of the solution and incubation temperature of the crystallization plate, g) importance of additives.

Good protein sample

A stable, pure and homogeneous form of protein sample at high concentration is must for crystallization. To achieve this condition for soluble protein is not difficult, but it can be extremely cumbersome for membrane protein. To achieve stability, different detergents and lipid combinations should be examined. Membrane proteins are often more soluble and stable in detergents with long alkyl chains like DDM or Triton X, because they mimic cell membrane architecture better. But the crystals produced with these detergents are of poor quality because of their big micelle size. Since proteins are mostly covered with such detergent molecules, poor crystals contacts are obtained resulting into non diffracting crystals [97,112,113]. Therefore, it is wise to use such detergents in the initial stages of purification and then should be exchanged with shorter chain detergents during crystallization. Since protein is not so stable with shorter chain detergent, a compromise should be done in making detergent choice. Common detergents used in crystallography: OG (0.5-1%), DDM (0.02%), DM (0.2%), C12E9 (0.03%) and LDAO (0.1%) Membrane protein concentration should be kept low compared to soluble protein in order to avoid detergent getting concentrated and protein getting precipitated. Mutagenesis and truncation of flexible

region and N- and C-termini are two common approaches to achieve stable form of protein. Ligands (agonists, antagonists) also provide stabiliy to proteins.Therefore, they can also be included during crystallization. Some membrane proteins are shielded by detergent molecules, resulting in poor crystal contact. In order to increase the solvent exposed area on the protein surface, they are often produced with soluble fusion partners like T4 lysozyme (in case of GPCRs). Some recently reported specific binding partners of proteins like DARPins, Nanobodies, Sybodies or antibody fragments can be used during cocrystallization. They are not only helpful in increasing the soluble area of the protein but also provide additional stabiliy to the protein [76,95,108-110,115,116].

Role of precipitant

A precipitant specifically competes with the protein for water molecule and hence reducing water availability for the protein, which mimics higher protein concentration. The most common precipitant used for crystallization is polyethylene glycols (PEGS) of different molecular weights (200–20000). These PEGs can be classified into four groups; PEGs (200-600), PEGs (600-1500), PEGs (3350-8000) and PEGs (10000-20000). Experience shows that protein tends to crystallize within same group of PEGs, until it is a promiscuous crystallizer. Low molecular weight PEGs (200-600) are often preferred to crystallize membrane proteins as they can also serve as nice cryo-protectant, and the crystals can be directly harvested from the crystallization drops and frozen immediately. This is very useful since membrane protein crystals are more fragile, and to look for proper cryo-protectant is often cumbersome. Other commonly used precipitants are ammonium sulfate and high concentration of multivalent, organic or inorganic salt ions. The effects of these ions on proteins are different from each other. So, optimal concentration should be achieved by systematic trial and error screening. The same hold true for selection of PEGs too [79,80,82,99,103,104].

Effect of temperature, salt concentration and pH on crystallization

Temperature has huge effect on not only protein's stability but also its solubility in the crystallization drop. Higher the temperature, faster in the vapor diffusion, and hence faster attainment of supersaturation in the drop and vice versa. Therefore, careful observation of temperature effect on protein in the crystallization drop is required to select optimum condition favoring nucleation. Protein can be crystallized in the range of 4-30 °C [101,105,106].

Salts provide ionic strength to the solution and affect intermolecular electrostatic interaction by shielding surface charge on the protein molecule. As a result, higher salt concentration decreases protein's solubility and favor its precipitation. Different salts will have different effect on the protein. Though most proteins are purified in the presence of 100-400 mM of NaCl concentration which is suitable for setting up crystallization plates, but in some cases, optimization is required. Protein solubility in PEG solution is increased in higher salt concentration which can slow down the crystallization process. This strategy is also exploited in some cases [104,107,112,114].

pH of the solution affects protonation and deprotonation states of the charged surface of the molecule. Hence it also affects the stability of the protein. Surface charge interaction affects crystal packing more than the hydrophobic interaction. Therefore, role of pH is more than mere maintenance of buffer. Some proteins particularly membrane proteins are stable between narrow range of pH. In such cases higher concentration (100-200 mM) of buffering agent should be included in the purification buffer. This will help in keeping protein stable for longer time in the different crystallization drop. Earlier it was believed that same pH achieved with differ buffer constituents have no effect on the crystallization. But recently it has been shown that individual buffer can alter crystallization property. pH has more significant effect in case of low ionic strength mother liquor compared to high ionic strength. Therefore, pH screening between pH 4.0-8.0 under different salt concentration is necessary to examine protein's stability and crystallization property [104,107,112,114].

Usage of additives

Additives are referred to some small molecules or ions which if present in mother liquor, are supposed to affect crystallization property. Potentially, the number could go beyond thousands, but some commercially available additive kits can be used to improve the crystal quality and hence diffraction resolution of some proteins. Sometimes, ligands or metal ions are also used as additives. These ligands are used in high concentrations to saturate the protein completely as they do not have much

negative effect on crystal growth even in higher concentration [99,103,104].

For membrane proteins, choice of detergents and their handling are protein dependent and already extensively studied. In most cases non-ionic detergents with long alkyl chains are automatically selected as starting detergent as they are mild in nature and less denaturating to proteins. However, detergents can be used as an additive too. After protein concentration, 0.2-3X CMC of another detergent can be added directly to the protein before setting up crystallization plates. Mostly, non ionic, small alkyl chain detergents like (OG, NG, LDAO etc.) are used as additives. The choice of detergent is completely protein dependent. Therefore, crystallization screening should be performed with as many as detergents possible as an additive [76,77,108].

Different methods of crystallization

There are many different ways of obtaining protein crystals; a) vapor diffusion method [86,103], b) counter diffusion [91,93], c) ion pairing [100], d) microbatch method [84,97], e) lipidic cubic phase method [109,113,115,116], f) bicelle method [109,113]. The last two methods are specifically dedicated to membrane protein crystallization. These methods are extensively studied and are well documented. Still so far, vapor diffusion method is the most common method to crystallize both soluble and membrane proteins. Both sitting drop and hanging drop methods are equally effective. In recent times lipidic cubic phase method provide outstanding success in obtaining crystals for proteins which were hard to crystallize by conventional method. Lipidic cubic phase method is particularly helpful in maintaining complex structures since they mimic bilayer membrane much more than the detergent environment.

Initial screening conditions should cover large part of chemical space to increase the chance of a successful hit. Though past experience can be very handy in this case. There are three strategies for initial screening; a) sparse matrix method [79] which is based on the published data and designed in such a way to cover all crystallization space which was successful in obtaining crystals in the past. Specific sparse matrix screens for membrane protein are commercially available. b) Grid screens are used in case of availability of prior information of protein's crystallization property [114]. In this screen, systematically two factors are varied by keeping other variables constant. c) Ionic screens are also used to optimize the crystals by varying salt concentration at different pH in the presence of different precipitant [98,99].

Interpretation of results

While the setup of a crystallization experiment is straightforward, interpretation of the results on the crystal plate requires more expertise. There are different possibilities exist, a) clear drop, b) precipitate, c) phase separation, d) microcrystals and needles formation, e) crystals. In case of clear drops, protein concentration or incubation temperature should be increased to enhance supersaturation. In case of precipitate, protein concentration and incubation temperature should be decreased. Some other measures can be taken to increase the solubility and stability of the protein. Phase separation is a good sign to obtain crystals. In this case, there are areas in the drop where protein concentration is increased. However, further optimization is needed in order to get crystals. Microcrystals or needle crystals are good sign for successful crystallization. Fine optimization with additives or ligands are necessary to improve the crystal quality. In case where initial crystals were obtained in smaller drops and data collection is not possible with small crystals, upscaling to a larger drop size can be attempted. Switching from smaller to bigger volume changes kinetics of the drop since surface to volume ration is altered. Therefore, in some cases further optimization is required to reproduce same quality of crystals.

CONCLUSION

In this review we have tried to outline a general strategy for crystallization by providing a concise overview for both membrane and soluble proteins. The difference in approach for membrane and soluble protein are highlighted in every section. Since, protein crystallization still depends largely on trial and error approach, the selection of specific strategy for a particular target is user dependent. Since, this topic is enormously large, the review cannot cover all the topics in detail. But we referred to most of the published articles in the field in our article, so that if anyone wants to enlighten themselves in detail on some specific aspect, they can refer to the original articles. To conclude the article, it must be admitted that the optimization of crystallization condition will remain a challenging area for structural biologist because of the vast

diversity in the field. Few important constituents for crystallization are mentioned in table 1-4.

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