Research Journal of Chemical Sciences ISSN 2231-606X
Vol. **13(1),** 65-78, February **(2023)** *Res. J. Chem. Sci.*

Review paper

Crystallization approach for purification of intact monoclonal antibodies: A review

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Available online at: www.isca.in, www.isca.me
Received 15th June 2022, revised 30th October 2022, accepted 3rd February 2023

Abstract

Over the 20th century, protein crystallization had been accepted and developed as a powerful purification tool before chromatography. It has also been applied for various biologically important macromolecules for efficacious durability and contracted dosage in the field of drug formulation. Right from the evolution, intact monoclonal antibodies (mAbs) have gained prime importance in the field of therapeutic drug applications, especially in immunotherapies. The fragments of mAbs have also been used for different applications. The purification strategies researched and established for these molecules during the last 20 years are predominantly chromatographies. But considering the process cost limitations, crystallization was found to be effective to purify the intact monoclonal antibodies from a massive number of proteins in culture broth and feasible to use as an alternative platform. This review presents the success rate of crystallization in intact monoclonal antibody purification. Further, the importance of phase behavior studies, the effects of additives on monoclonal antibody crystallization is discussed with the help of case studies. Also, the comparison of different batch versus continuous crystallization methods applied is discussed. In the end, the requirement and prospects of large-scale crystallization studies of intact monoclonal antibodies invoking the accomplishment of high throughput demand are discussed.

Keywords: Purification; crystallization; intact monoclonal antibody; crystallization additives; continuous crystallization.

Introduction

The thousands of proteins had been successfully discovered over almost a hundred years having the collective information on protein data bank (PDB). For the identification of the functional role of numerous proteins, attempts were made to purify and bring the proteins in a crystallized form. The necessity of structural analysis of these proteins stimulated the crystallographic community making the protein crystallization technique a common platform for structure determination. Almost 85% of these proteins have been studied with the help of X-ray crystallography[1]. These crystallized proteins show more benefits enabling greater purity and longer shelf life than the dissolved ones. This advantage offers numerous applications for the industrial crystallization of pharmaceutical proteins[2,3]. Besides the use as structure determination, crystallization works as an alternative technique for conventional chromatography purification giving satisfactory purity and cost-effectiveness. In addition to this, it can be used as a substitute for methods, such as lyophilization in the product finishing step. On a commercial basis therapeutically important proteins, including Proteinase and Concanavalin have been crystallized.

Though the formation of crystalline proteins is a complex procedure, various trials and errors have been carried out to ease the process. To achieve successful crystals, certain variations in parameters e.g., pH, temperature, concentration, and purity of protein, crystallization agents, etc. played a vital role[4]. Along with these, the process of crystallization is affected equivalently by the use of different techniques of Vapor diffusion, micro-batch, and micro dialysis[5,6]. The micro-batch method gives rapid screening of crystallization conditions with high accuracy[7], while, vapor diffusion gives larger crystals as compared to conventional methods of crystallization[8]. The provided process conditions lead to super saturation, as a result, it gives nucleation of molecules giving the crystals yield[9]. After the process reached an equilibrium, the crystals are harvested and analyzed for purity and yield. The process of intact monoclonal antibody (mAb) crystallization is highly sensitive and can be affected easily by various provided conditions. The generally accepted procedure for mAb crystallization is depicted in Figure-1.

Very few experimentations have been reported within the last two decades giving evidence of successful crystallization of intact monoclonal antibodies. Therefore, this work aims to highlight the crystallization techniques applied by various researchers as a preparative purification stage for monoclonal antibodies along with some simple and inexpensive agents stimulating the process of crystallization. Certain future perspectives also have been added that described the scope of crystallization in the purification and formulation of intact monoclonal antibodies.

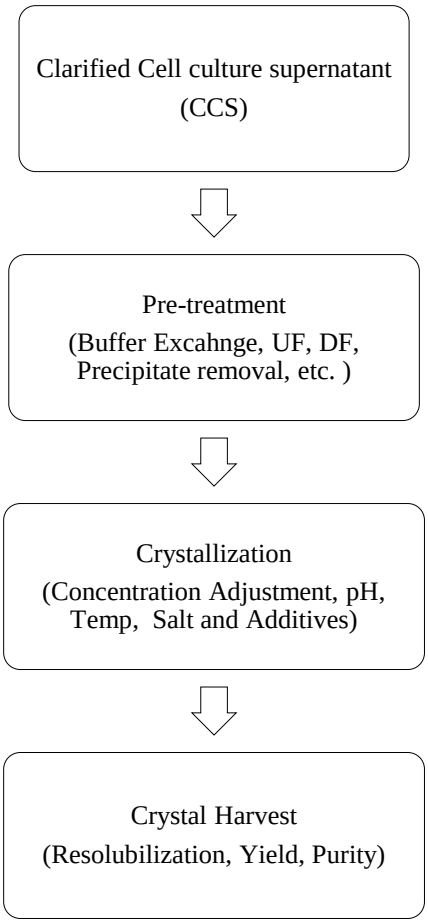

Figure-1: Process flow for monoclonal antibody crystallization.

Comparison of crystallization to chromatography for monoclonal antibody purification

In the recent developments of the medicinal field, the intact monoclonal antibodies mainly obtained from the CHO cell line played a significant role as therapeutics for different cancers and immunotherapies[10,11]. According to the Compound Annual Growth Rate (CAGR), the monoclonal antibodies have vast growth potential indicating more than 30% of all biopharmaceutical products[12]. The downstream processes involved in bio-separation of monoclonal antibodies are filtration (UF, DF), dialysis, electro-dialysis, extraction, centrifugation, distillation, chromatography, precipitation, crystallization, etc.[13]. In the formulation of therapeutic drugs, the applied methods for purification are of great importance and have a significant impact on commercialization. Among these methods, crystallization and chromatography are widely accepted and work as bottle-neck for protein purification at the industrial scale. In the case of intact monoclonal antibodies, preparative chromatography is a conventionally employed technique. The typical process established for mAb purification is protein-A affinity chromatography followed by subsequent polishing steps which may include crystallization. However, it has limitations on mass transfer to generate optimum yield, also requires multiple sub-processes for finishing, which creates the step of chromatography as a major cost center in purification processing[14].

On the other hand, crystallization was adapted to be a powerful substitute for chromatography, which eliminates other required stages and offers work feasibility with low-cost utilities and less buffer solution requirements give high productivity in less investment. Also, the crystallized form of monoclonal antibody products is convenient for the subcutaneous delivery of extremely concentrated doses of therapeutic agents[15]. However, the process of intact monoclonal antibody crystallization may face certain complications due to provided environmental conditions. During the process of crystallization, the solution may remain as it is in a homogeneous state, or liquid phases may appear separated without crystals or crystals may form but unsuitable for determination[16]. Because of this, crystallization has never been used as a simultaneous purification cum polishing step. Nevertheless, the several modifications in process parameters, and the use of large-scale production vessels, along with modernized mechatronic equipment, such as robotic hands may outweigh these disadvantages with enhancing purity and productivity[17]. The stability obtained from the crystallized monoclonal antibody is high to sustain in presence of several organic solvents, heat, and pressure, which act as denaturants[18]. Along with these circumstances, the three main ways to achieve good quality crystals by implementing alterations in the process of crystallization, which includes (1) Nucleation zone bypassing (2) controlling the formation of nuclei during the experiment (3) Prompting the crystallization kinetics[19]. Considering all the factors related to purification, yield, purity, and cost of the product, crystallization can be an alternative chromatography and subsequent polishing steps. The imminent sections give an account of successfully crystallized intact monoclonal antibodies.

Phase behavior fundamentals

Crystallization is a phenomenon where the transport of molecules takes place from liquid to solid aggregated state in a super-saturated solution due to a decrease in solubility. An understanding of the fundamentals of protein crystallization is essential to produce protein crystals. The aggregation may give rise to precipitate which is amorphous in nature or crystalline in structure. The success routes of the crystallization process lie in the structural nature and concentration of the protein as well as the other parameters. The problems evolving with an optimization related to basic features of crystallization, including some aggregation aiding agents (Salts and Polymers) and process conditions (e.g., temperature, pH, volume, and concentration) can be mapped easily with the help of a phase diagram[16]. It can also determine the interaction between protein phases in a crystallization solution. Various parameters are utilized for obtaining the phase diagram, where it shows the

stability of three distinct phases: liquid phase, precipitate phase, and crystalline solid phase[20].

The phase diagram (Figure-2) is studied by experimentation on adjustable parameters, keeping the parameter of protein concentration constant. To shows the probable zones of protein aggregation, which briefs about the four areas of crystallization according to the concentration of protein. In the zone of high super-saturation, the precipitation of protein will occur. While the moderate super-saturation zone leads to nucleation in the vicinity of a gas-liquid critical point. In the area of lower super-saturation, the nucleated crystals will reach stability and begins to grow; it is also referred to as a metastable zone that provides the best conditions for large and well-ordered crystals. An area of under-saturation below the metastable zone, where protein dissolves[19]. Along with protein, the phase diagram also aids monoclonal antibody crystallization.

The difficulty associated with the crystallization of monoclonal antibodies is due to its complexity and variations in structure. In the case of monoclonal antibodies, the metastable zone is extremely narrow, hence there is minimal scope in the optimization of crystallization conditions, but few studies on β alanine represent the widening of the metastable zone via oiling out at high temperature[21]. And hence, to attain crystallization successfully, it is necessary to understand the phase behavior of mAbs. However, due to the long nucleation interval associated with crystallization, equilibrium may be attained slowly. Various researchers used different types of additives to study the phase behavior for reducing nucleation time. Similarly, to diminish the barriers in nucleation, the understanding of spinodal decomposition helps.

The spinodal measurements performed using B22 give insights into protein-protein interaction and play an important role in the prediction of crystallization conditions. Rachael A. Lewus, et. al,[22] demonstrated the phase behavior measurements of mAb IDEC 152 over a period of 2 months by altering the concentration of salts and/ or PEG. According to the phase behavior of each of the salt, lithium sulfate was found to be most efficient for crystallization. On the other hand, Egor Trilisky, et. al[23] studied the phase separation of monoclonal antibody IgG2 with the help of PEG. The generated two-dimensional diagrams (Phase diagrams) aids to map the phase behavior of mAbs, which can be referred to prepare crystal seed stock by mixing the mAb solution with PEG at a specific pH. Knowing the phase separation and aggregation boundaries is useful to compare the effectiveness of different additives that shows a specific point, where crystallization occurs for particular additives.

Phase behavior study is complex and can vary with salt and additives added but it is very useful in determining monoclonal antibody crystallization zone. The process of crystallizing macromolecules and interrelations can be understood, easily through phase diagrams. So, this technique is frequently applied to scrutinize formulation and production in the case of several monoclonal antibodies.

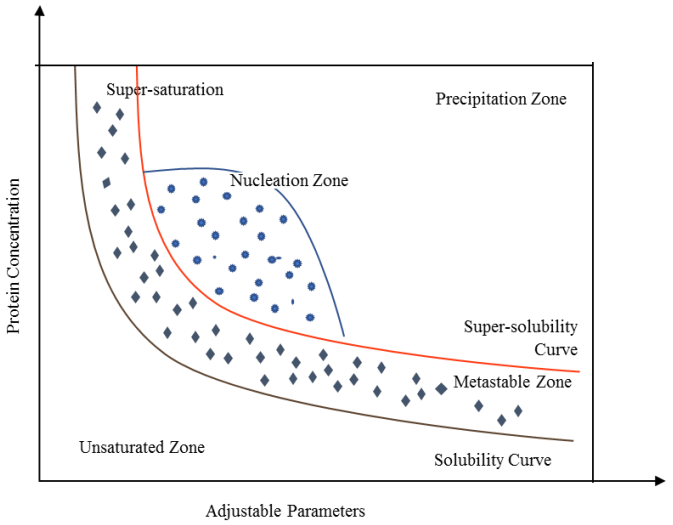

Figure-2: Representation of a protein (mAb) crystallization phase diagram. Adjustable parameters include precipitant or additive concentration, pH, and temperature. According to the concentration of the protein, the solubility and super-solubility curves were obtained at equilibrium with crystals (Modified from[19]).

Additives utilized during monoclonal antibody crystallization

In therapeutic drug production, control of the crystallization process is indispensable, and therefore, to achieve desired quality crystals concerning purity and productivity it is necessary to introduce some external substrates or additives. Generally, type additives, such as detergents, reducing agents (e.g. DTT, DTE, and β-ME), salts, polymers, etc. are widely used to gain crystal yield. In monoclonal antibody crystallization, salts and polymers are two main components that play a crucial role. A wide range of salts are used in precipitation and crystallization, however, only a few can promote nucleation and propels better yield. Certainly, a high concentration of salt is not feasible to commence probable crystallization and hence, PEG is employed in combination[24]. The following sections would brief the scenario of the use of additives in intact monoclonal antibody crystallization.

Effects of Salts: The precipitation years back to the 1850s and the relationship between the salt concentration and protein solubility was described by Hofmeister in late 1888. In successful crystallization, it is important to maintain the optimum salt concentration which leads to giving crystals in the protein solution. Hofmeister series assisted some attempts carried out to determine a specific range of anions and cations concerning the propensity of protein crystallization[25,26]. The salts for crystallization are preferred based on the chaotropic and kosmotropic nature. Normally, the kosmotropes are utilized

for protein crystallization, which has the ability to improve the stability of the proteins by developing the structure of water molecules surrounding by ions, which leads to the hydrophobic effect[27]. Several salts, including ammonium sulfate, sodium sulfate, sodium chloride, etc. recommended by the Hofmeister series act as the most efficacious additives, which plays a vital role in driving protein crystallization. It also enables to reduction of the repulsive electrostatic interaction owing to electrostatic screening effects[28].

The solubility of therapeutic protein increases with a rise in salt concentration and the phenomenon is known as salting-in which ultimately increases the ionic strength of the solution. Beyond the certain limit of salt concentration, the therapeutic proteins in the solution begin to associate resulting in salting-out and start forming crystals due to an increase in protein concentration through dehydration. Figure-3 represents the effect of salt concentration and its solubility in the mixture, which briefs about the salting-in and salting-out effects on protein aggregation[29]. Crystallization in salting-out regions is more likely to result in a crystal suspension with high osmolality. Within the low range of osmolality, desired crystallization can be achieved by adding excipients giving rise to the spherulite-like particles of mAbs. Various salts have various effects on the process of crystallization, with varying concentrations that affect crystallization yield and aid to achieve super-saturation point in the least time which enhances nucleation. Crystallization can also be accelerated by chemical parameters e.g., buffer systems, tonicity of agents, that influence the protein solution following the conformational, thermal, and colloidal stability, which affect the protein precipitation and may change the behavior of crystal formation[30].

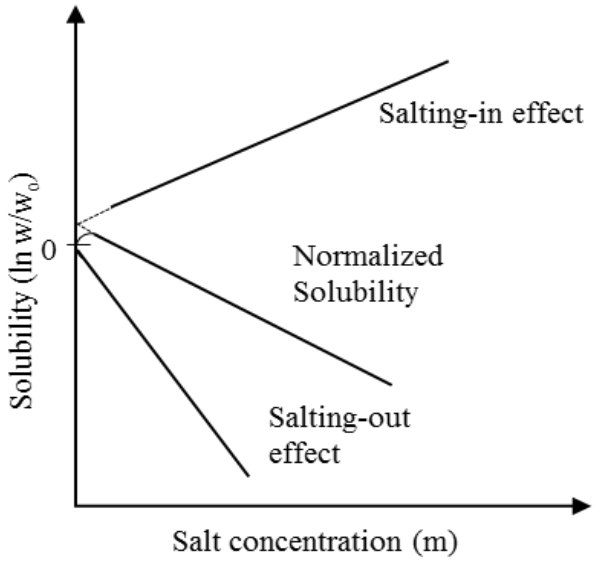

Figure-3: Graphical relation between protein solubility and salt concentration (Modified from[29]).

The work of Rachael A. Lewus, et al, [22], is focused on the use of different ammonium, lithium, and magnesium sulfate salts to obtain maximum crystal growth for mAb IDEC-152. The nucleation occurs above 25mg/mL in the case of 0.7M concentrations of ammonium sulfate and 5 mg/ml in the case of 1.0 M concentrations of lithium sulfate in respective solution at pH 5.0. The increased concentration of antibody results in enlarging the size of crystals obtained from solution at a fixed concentration of salt as shown with ammonium sulfate. Lithium sulfate gives more promising results for protein-protein interaction as compared to magnesium and ammonium sulfate at the same concentration due to the downwards shift of phase boundaries of the IDEC-152. The interactive suitability of lithium sulfate is confirmed by the osmotic second virial coefficient (B22).

The more negative shift of B22 values for salts represents stronger and attractive interaction within the protein molecules and influences the selection of salts. The different types of salts, such as lithium sulfate, ammonium sulfate, sodium chloride, sodium sulfate, etc. showed a successful crystallization in the case of mAbs. Overall, the effects of salts have a major impact on crystal formation in the process of crystallization. Use of salts in adequate proportion aid to dehydrate the solution forming a solvation layer and thereby accumulating the intact monoclonal antibodies followed by crystallization. Sulfate salts are widely accepted and possess extreme importance in process of crystallization, because of their ability to exclude away from the surface of the monoclonal antibody to avoid denaturation. Although the concentration is different for each salt for stabilizing the monoclonal antibody, crystallization occurs at a specific point. Therefore, it seems that in the case of monoclonal antibody crystallization, salts do not give any specific pattern of concentration in terms of molarity.

Significance of Polymer: There are many key elements to be added while protein crystallization, used for highly supersaturated formulations. Among these, polymers, including polyethylene glycol (PEG), have been contemplated as an efficacious crystallizing agent due to their non-toxic and non-absorbing nature[31]. Nowadays, PEGs are part of a vast amount of screening kits as dehydrating agents for protein crystallization. The extensive structure of PEG and its high molecular weight strongly affects the morphology of precipitants in a mixture, which enables to exclusion of protein molecules from the hydrodynamic volume of PEG[32,33]. The PEG increases attractive forces between particles of protein in solution, resulting in high protein aggregation which is represented by the depletion mechanism[34]. Especially, it has been found that the molecular weight of polymer from 400 to 20000 is applied widely for macromolecular crystallization. The volume of the polymer has a greater impact on the solvent, due to its increasing molecular weight (Figure-4). As the molecular weight of polymer increases, the volume required for protein aggregation decreases, enhancing the solubility of polymer[35].

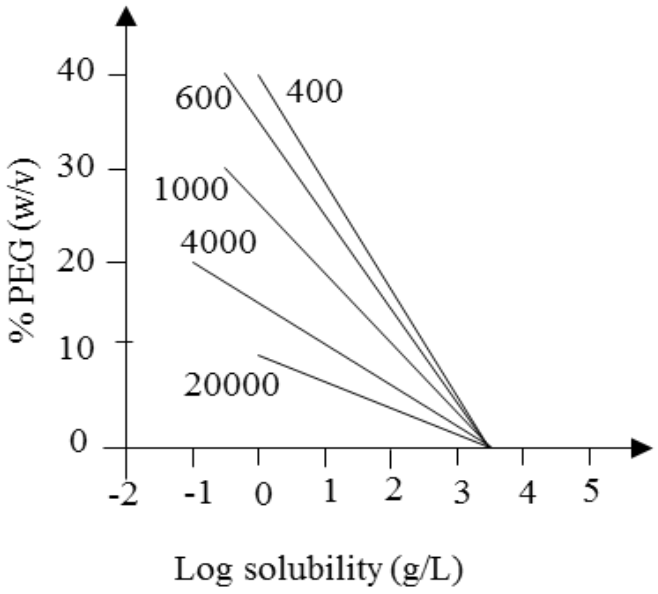

Figure-4: The semi-log graph of protein solubility v/s polymer concentration. The size of the polymer is inversely proportional to the solubility of the protein, giving decreasing slop with increasing molecular weight (Modified from[35]).

Yet, there are some problems associated with the control of the concentration and molecular weight of PEG in mAb crystallization. Therefore, some alternate additives have been used to ameliorate crystallization. Yurii G. Kuznetsov, et. al[36] examined the effect of two different polymers on the crystallization of intact monoclonal antibodies IDEC-151. The study showed higher concentrations of Jeamine ED-2000 resulted in considerable phase separation and can give a high yield of crystal formation compared to PEG. The size range within 2000–4000 of Jeamine ED and PEG was found to promote nucleation rapidly. It also has been postulated that the presence of impurities in such polymers affects the aggregation of mAb. The auto-oxidation and development of impurities in PEG affects the formation of aggregates, protein degradation as well as drug stability, however, the completely pure PEG prevents the mAb crystallization. While the presence of a minor amount of impurities aids the process of crystallization. To clarify this Christian, et. al[37] examined the effects of impurities in PEG for mAb crystallization and aggregate formation mechanisms using the IgG1 antibody. mAb1 crystallization was investigated by freshly supplied PEG and aged PEG, using different concentrations of peroxide and formaldehyde residues as impurities.

Generally, the polymer entities added to the crystallizing mAb solution exist as the strongest agent prompting the water removal due to its structure and molecular weight. A wide range of molecular weight of polymer has been suggested to affect the solubility of monoclonal antibodies and the quantity of polymer to be included. Among the different types of polymers, PEG has been accepted widely, while Jeffamine ED can also be used as a replacement. An occurrence of impurities in PEG also assists the crystallization of monoclonal antibodies.

The success of additives in monoclonal antibody crystallization: Only a few studies can be found out describing the effect of salts and polymers on intact monoclonal antibody crystallization. The main factors, including type and amount of salts used as well as protein concentration, affect the process of crystallization. According to the literature, studies of crystallization represented the concentrations of various salts in combination with different types of polymers utilized for the crystallization of intact monoclonal antibodies, which are also have represented in Table-1.

In which, IDEC-151 with 9mg/ml concentration is crystallized using Lithium Sulfate as a precipitating agent used in combination with polymers such as different molecular weights of Jeffamine ED or PEG to give rise to effective crystallization. The reduction in time of crystallization from 2 weeks to 6hr. by accelerating the crystal growth in the presence of PEG 3350 was obtained. The effect of pH showed little influence and the best temperature of 19°C was found for crystal formation in the sample of LiSO4 and HEPES[36]. AntiCD-20 antibody was crystallized entailing the sodium sulfate and PEG as crystallizing agents, which gives an idea of conditions offered to get successful crystallization of antibodies and to obtain maximum crystal growth. The buffer solution with dissolved salt and PEG400 works as a crystallization solution. The crystallization results varying the mAb concentration almost double and a slight decrease in salt concentration keeping PEG constant were compared to optimize the yield. The provided conditions produced the largest crystals in a period of 2-3 days with high crystal density for 30 mg/ml anti-CD 20 with 1.1 M sodium sulfate. While a significant amount of crystals were obtained for 60 mg/ml anti-CD 20 with o.7 M sodium sulfate[38].

Therefore, as the concentration of monoclonal antibody in the crystallization solution increases, the requirement of salts may reduce. In the case of mAb01, the crystallization prone zone was found to be between pH 6.0 to 7.0 and at lower sodium chloride and TRIS base concentrations. Showing that, even the least concentration of sodium chloride is sufficient for effective crystallization of 25 mg/ml mAb01 and high concentration may lead to an inhibitory effect on crystallization[39]. The HuCC49CH2 antibody crystallized in presence of detergents, such as Triton X-100 and Anapoe X-405 with 4.0 M sodium format at 290 K and 7.0 pH. The thick laths and plates shaped crystals grown in the presence of Anapoe X-405[40]. The examined effect detergent also shows a similar kind of activity as in salt and polymer. The formation of the size and shapes of crystals depends on the morphology and concentration of monoclonal antibodies, which can also stimulate by the concentration of several additives. Along with salts, co-solvents, temperature, and additives plays a significant role in crystals formation.

Table-1: Different concentrations of salts and additives used for intact monoclonal antibodies.

Antibody	Salt	Conc. of Salt (M)	Additives	mAb Conc (mg/mL)	Reference
IDEC-151	Lithium Sulphate	1.5	Jeamine 2000/ PEG 4000	9	36
IDEC-152	Ammonium Sulphate	0.7	-	25	22
	Lithium Sulphate	1.0	-	5	
	Ammonium Sulphate	0.8	PEG3350	10-15	
mAb01	Sodium Chloride	0.06	-	25	39
Anti-CD20	Sodium sulphate	1.1	PEG400	30	38
	Sodium sulphate	0.7	PEG400	60	
HuCC49CH2	Sodium Formate	4.0	PEG 3350 Anapoe X-405/ Triton X-100	16	40
IgG (mAb1)	sodium acetate buffer	0.1	PEG 4000	10	37
IgG4	HEPES, Caffeine	0.05	PEG 3350	20–40	41

Methods of intact Monoclonal Antibody Crystallization

Prior to crystallization, intact monoclonal antibodies are in the dissolved form within the surrounding environment and need to attain a supersaturated state to confine in a concentrated and organized form. Therefore, several methods have been recommended to crystallize monoclonal antibodies which are classified based on the volume and assembly of apparatus (Vapor diffusion, batch, micro-batch, etc.). After the crystal formation of purified intact monoclonal antibodies, the crystals are then investigated for proper shape, size, purity, and productivity under several analytical methods, including X-ray crystallography and microscopy, AFM.[42]. The following sections represent preparative and analytical techniques applicable to mAb crystallization.

Production methods for crystallization: The protein solution is deployed physically and chemically to propel super-saturation for the crystallization of protein[43]. There is a number of methods, such as vapor diffusion, hanging-drop vapor diffusion, sitting-drop vapor diffusion, batch, micro-batch, and micro-dialysis found to be financially feasible methods of crystallization[44]. All these techniques applied for intact monoclonal antibody crystal growth, follow distinct time intervals to achieve the nucleation and metastable zones, at which the molecules ultimately released from the solution in crystalline nature[45]. In experimental set-ups, numerous methods have been performed in series to obtain high-efficiency crystal formation. In which, various parameters, such as sample concentration, time, temperature, and different additives have also been considered[46]. Among all these methods vapor diffusion is used widely for macromolecules crystallization[47]. In the development process of crystal formation, sitting-drop or hanging-drop vapor diffusion are applied extensively, a small droplet entailing of a mixture protein and precipitating agent in aqueous solution is suspended on the micro-titre plate, which is kept at top of the closed container having a precipitating agent as a reservoir solution (Figure-5a). The diffusion of water from the drop causes the continuous aggregation of protein molecules leading to crystallization in the hanging or sitting drop method[2]. However, vapor diffusion refuses to overcome several difficulties associated with crystal formation which can be diminished by using the batch method, and the super-saturation is achieved immediately rather than diffusion[48], where the aqueous mixture of protein and the precipitating agent is covered with an oil layer (Figure-5-c) to prevent diffusion in a tapering container. Another conventional and effectual approach of Micro-batch crystallization to form a crystallized antibody has been commonly projected. It is easy to set up, based on the same experimental condition based on vapor diffusion and batch technique[45]. Another technique in macromolecular crystallization is the floating-drop method, which resolves the problems regarding automation, and adherence to mixture vessels, emerged from hanging-drop and sitting-drop. It also produces protein crystals possessing high-quality, which can easily detectible under X-ray diffraction and microscopy[49]. Crystallization methods can be effective, due to their equipment's compactness, and the mechanical firmness of the system for protein crystals[50]. Since, various kinds of proficient and reproducible techniques operated for macromolecular crystallization, which carried out on an industrial scale to provide a sufficient amount of crystals. The conditions required for antibody crystallization can be inducted by empirical methods to boost productivity[15]. All these methods were modified intentionally to reduce the protein impurities, to obtain acceptably purified crystal yield.

Several methods have been used in the formation of appropriate crystal yield and to obtain adequate purity. The spiking of impurities was also had investigated to evaluate the effectiveness of crystallization methods. Zang, et. al[51] crystallized the antibody IgG4 (mAb04C) by using the vapor diffusion and micro-batch method. The initial conditions were screened by a sparse matrix, and it was then transferred to the micro-batch method. After having attainment in the HCP spiking test, the efforts were diverted to make antibody crystals from cell culture supernatant, by one step of crystallization. The yield obtained was 31.3% and having a purity of 90% with coffin-shaped crystals obtained within the time of 5 days. The inspection on problems regarding the removal of major impurities of HCP and HCDNA made a process capable to attain great purity in a single crystallization run of the micro-batch method. The same technique of crystallization was used by Hekmat, et al[52] to achieve the maximum purity of crystals of mAb from Harvested cell culture fluid (HCCF) directly. The experiment was successful to confer 3030 fold HCP reduction and almost complete removal of host cell DNA i.e. 97% purity, which is nearly equal to chromatography.

The anti-CD 20 antibody crystallized by using the hanging drop and batch method with several pre-treatments. Crystals resulted from both the technique were morphologically the same but showed a huge time difference from several days (2-3 days) to hours (12 hr.) with reduced crystal size[38]. One more approach to check the effect of sedimentation on crystal formation is the microgravity crystallization method performed by Reichert, et. al. The crystallized form of mAb IgG1 (Pembrolizumab) was obtained with the help of PEG 3350 in a batch crystallization experiment[41]. The flight experiment gave the monomodal population of crystalline particles compared to the ground-based experiment, which produced bimodal particles. The concentrated flight samples were sedimented more uniformly than the ground-based experiment. This study reveals the identification of sedimentation and temperature gradient as a key variable to control crystal nucleation and growth that gives uniform size crystals. In the case of intact monoclonal antibody crystal formation, the conventional method of crystallization as a purification step may offer significant economic advantages along with various concentrations of additives, including PEG and salt. In accordance with the yield, the micro-batch technique was noticed to be preferable giving maximum production of crystals compared to other techniques at a small scale level at any surrounding.

Analyses of crystallization: The purpose of using an analytical technique is to examine the properties, such as crystal size distribution, crystal habit, crystal shape, interactions, breakage, and agglomeration[53]. This aid to optimize the crystallization process by improving process parameters, including the rate of stirring, cooling, and seeding time for improving the rate and quality of the crystal[50]. Numerous analytical techniques have been established and applied to identify crystal yield and to reduce the errors raised throughout the process. In crystallization, temperature dependent parameter B22 can be used to examine the favorable salts and effective temperature in the phase behavior analysis prior to crystallization[54]. Several types of microscopic techniques play a pivotal role in crystallographic research in many aspects in the examination of crystal growth. The growth of crystals is analyzed using a digital camera attached to a polarizing microscope. The microphotographs are processed under image processing software to determine the presence and length of the crystal. Along with microscopy, fluorescence dye detection is also a preferred technique by researchers to recognize the progress of nucleation. Christian Hildebrandt, et. al[55], investigated the exchange of mAb1 between the solution and the solid-state to analyses the origin of crystal formation using red fluorescence-labelled IgG1 antibodies with help of Confocal Laser Scanning Microscopy (CLSM). A0fter attaining maximum crystallization using PEG4000 within 2 weeks, the solution of unlabeled supernatant was substituted with a fluorescence-labelled antibody solution. CLSM has displayed the migration of the labelled antibodies to the crystal phase from the supernatant and also showed a steady increase in crystal growth[56]. Figure-6, shows photographs of several crystallized intact monoclonal antibodies at different concentrations.

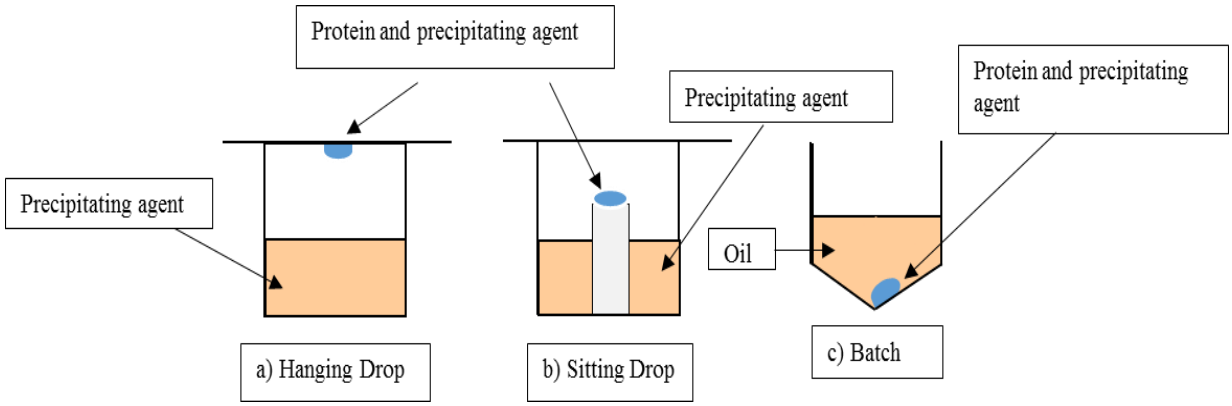

Figure-5: Fundamental methods for small-scale monoclonal antibody crystallization.

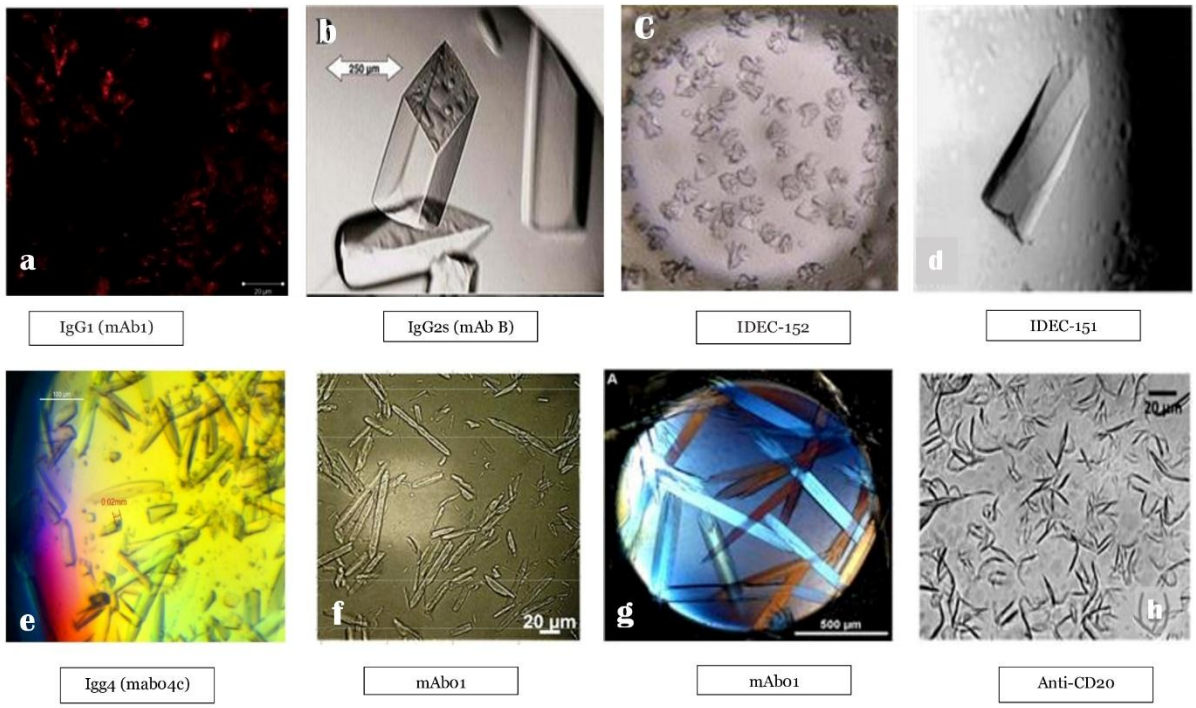

Figure-6: The microscopic view of crystallized intact monoclonal antibodies. (a) IgG 1(mAb1): 10mg/ml, 0.1 M sodium acetate, 24% w/v PEG 4000[55]; (b) IgG2s (mAb B): 140 g/L IgG2s, 0.3 M lithium sulfate, 20% PEG 1000 [63]; (c) IDEC-152: 27.1mg/mL IDEC-152, 0.9 M lithium sulphate[22]; (d) IDEC-151 : 9mg/ml IDEC-151, 1.5M $LiSO_4$, 2.5% PEG[36]; (e) Igg4 (mab04c): 20mg/ml mAb04c,12% (w/v) PEG 8000, 0.4M calcium acetate[57]; (f) mAb01: 16 mg/ml mAb01, 10 mM histidine[58]; (g) IgG 1(mAb1): 4.0mg/ml mAb01, 10mM NaCl[39]; (h) Anti-CD20: 30mg/mL anti-CD 20, 1.1 M Na_2SO_4 , 12% (w/v) PEG400[38].

X-ray crystallography and Atomic force microscopy are the favored techniques for three-dimensional structure determination of monoclonal antibodies and macromolecules. X-ray diffraction analyses the size and physical properties, where structural determination occurs[59]. The data obtained from anti-CD20 crystals by batch mode is exploited in Figure-7, to confirm the formation of pure antibody crystals. The augmented experimental set-up for low-resolution data was used to detect diffraction rings produced by arbitrarily oriented microcrystals at 66 Å, 57 Å, and 45 Å. The packing of antibody anti-CD20 in crystals approved by the diffraction rings gained in the analysis. The peculiar characteristic of a macromolecular crystal sample is the occurrence of rings at a low resolution[38].

Table-2 illustrates the number of experiments was carried out for intact monoclonal antibody crystallization using a different technique. The antibodies harvested from the supernatant showed a significant amount of yield. Among the mentioned antibodies some of the antibodies were found to be giving more than 90% crystallized yield. Batch and micro-batch techniques prove the suitability for application in large-scale crystallization of intact monoclonal antibodies. The time required for each antibody to crystallize depends on the provided conditions. As time increases, the crystal continues to grow further, which affects productivity. Generally, several analytical techniques are used for crystal analysis of which microscopy is the commonly preferred technique for the structural determination of crystals. Though there are variations in the span of the experiment the data obtained retained needle and rod-shaped crystals under microscopic observation.

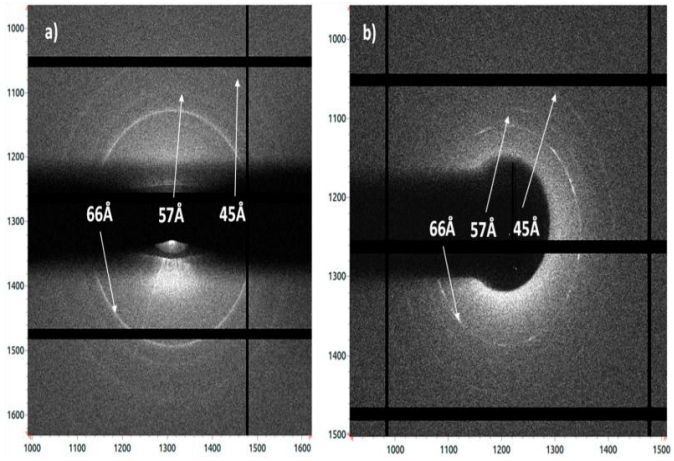

Figure-7: Photographic view of an X-ray diffraction pattern obtains for monoclonal antibody Anti-CD20 (Modified from[38]).

Scale-up of crystallization: The escalating demands for biotechnology to produce proteins in the crystallized form are accountable for the industrial-scale crystallization process. The

accomplishment to reach large-scale processing requires an understanding of all features of proteins that can affect crystal formation. The acceptable optimization of methods involves the adjustment and control over super-saturation and residence time to enhance the crystal growth, thereby the significant improvement in yield can be subjected while scaling up the process[64]. As well, the large-scale production empowered by various ameliorating strategies, which involves the addition of several additives to amplify the molecular aggregation and the implementation of techniques, such as Ultracentrifugation, Dialysis, etc. to enrich the purity and yield[65]. The stirred tank reactors are used widely in batch and continuous operations to crystallize the therapeutic proteins. Though the crystals of the intact monoclonal antibody obtained from baffled stirred tank reactor are considerably large, the chance of attachment of nucleated crystals to the baffles increases leading to amorphous precipitate or crystals breakage. Hence, it is preferred to perform the experiments in un-baffed stirred tanks to generate a high yield of crystallized mAb, though the small-sized crystals are produced. While dealing with large-scale systems, it is important to look after the agitation rate, time of mixing, impeller speed, turbulence rate, and mean power input[66]. Many bioprocesses apply this method to acquire efficient results through the steadily increasing volume from laboratory scale (5mL) to industrial scale (1L). Figure-8 depicts the un-baffled stirred tanks with a volume of 10mL, 100mL, and 1L used for monoclonal antibody crystallization.

The study on mAb01 for the crystallization of full-length IgG was conducted by Smejkal, et. al which had not been crystallized before. The experiment was performed on a non-agitated micro-batch reactor and scaled up in the tank from the mL to the L scale. Crystallization of mAb01 from partially purified solutions gives up to 97 % yield and from clarified cell, culture harvest gives a yield below 90% and purity above 97.3%. And the purified mAb01 antibody gives 92% yield within 2hr. at a 6mL scale. While in scale-up to 1 L, the yield reached 95.8%. The large crystals obtained at pH 6.3 using 8 mM Tris after increasing the concentration of mAb to 25g/L. Even if, the mAb01 is crystallized at a large scale directly from cell culture supernatant, it gives much more purity as compared to purified one. The execution of AEC and CEC to confirm the reduction of host cell protein impurities, prove the purity obtained from continuous crystallization. Besides, the efforts were taken by Hekmat et. al[58] for process intensification of mAb01 crystallization in order to get high productivity. The experiment was performed by using a continuously operated stirred tank to which the inlet of antibody solution and crystallizing agents were provided continuously and mixed with a stirrer to maintain ionic strength and the tubular reactor was used in bypass mode to reduce the temperature of the solution (Figure-9). The result showed that the mAb gets crystallized best in low ionic strength solution and at low temperature. The crystal yield of mAb was 90% and nearly 96% of antibody purified with a significant reduction of impurities. The tubular crystallizer/ reactor gives the orthorhombic crystals and yield of 12 g/L. The productivity of recrystallization was two times higher than that of crystallization from pre-treated harvest. A significant reduction in impurities was obtained and the nucleation rate was enhanced when the monoclonal antibody solution passed through the cooled tubular reactor.

Table 2: The methods of crystallization and analytical techniques for several monoclonal antibodies crystallized with the help of appropriate additives.

Monoclonal Antibody	Method of Crystallization	Analytical Technique	Yield	Shape of crystals	Duration	Reference
Adalimumab	Batch method	Light microscopy	95 %	Needle	5 Days	60
Rituximab	Micro batch	Light microscopy, LS Particle Size Analyser.	93%	Needle-like crystals	24 hrs	61
Trastuzumab	Micro batch	Light microscopy	85 %	Needle	12 hrs	
Infliximab	Batch method	Light microscopy	-	Rod-shaped	12 hrs.	
Anti-human IL-23 P19	Batch and vapour diffusion	Light microscopy, X-ray differaction	33-85 %	-	10 Days	62
ANTI-CD20	Batch method	Light Microscopy	100	Needle	24 Hrs.	
Anti-human IL-12	Batch method	Light microscopy	60-70	Sword like needle	13 days	63
Igg4 (mab04c)	Sitting drops, Hanging drop, Micro batch	Birefringence	31.3%	Coffin-shaped	48hr	57

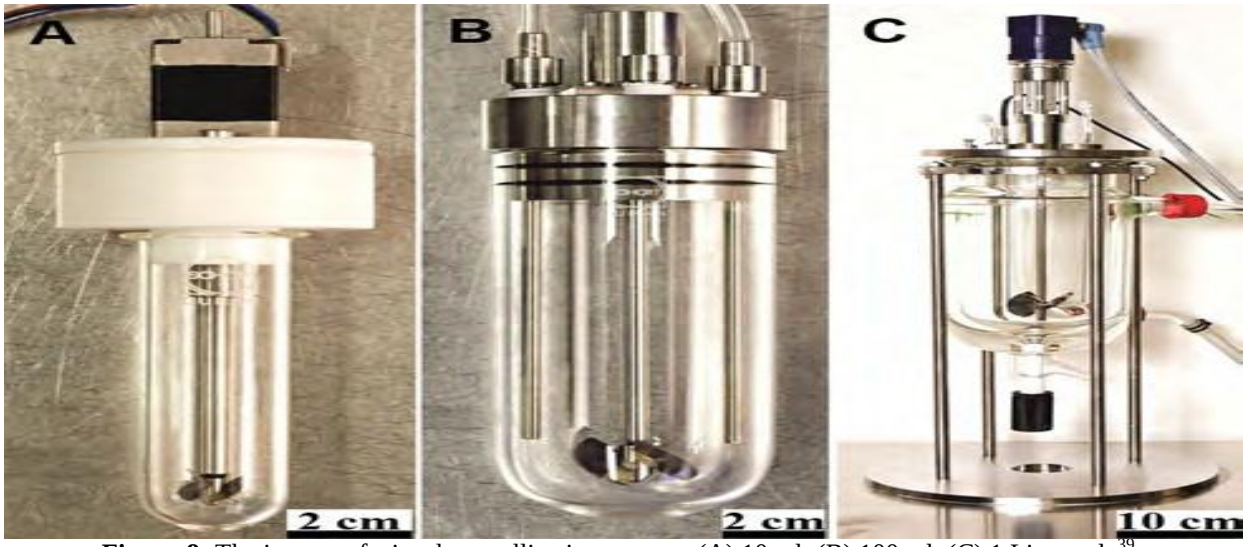

Figure-8: The images of stirred crystallization reactors (A) 10 ml; (B) 100 ml; (C) 1 Lit.- scale[39].

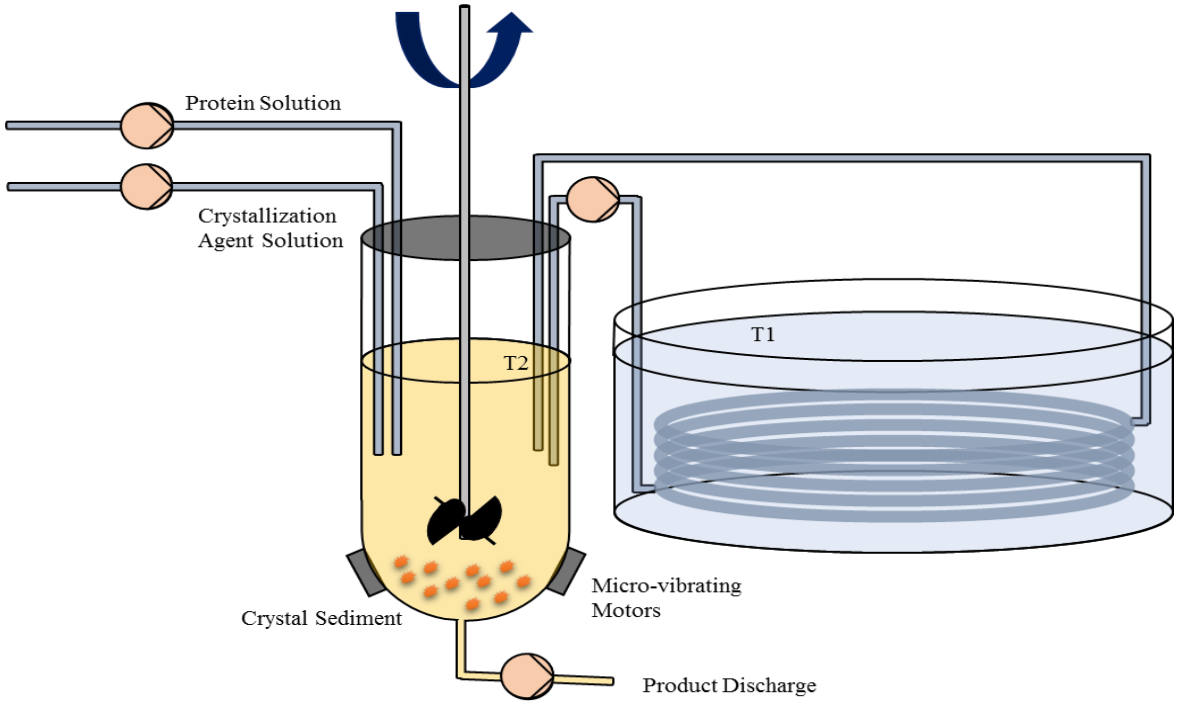

Figure-9: Schematic diagram of the continuous crystallizer attached to a tubular reactor for maintaining the temperature T1<T2, immersed in the chilled batch thermostat (Modified from[58]).

The production processes of monoclonal antibodies in crystallized form have been tried to optimize for large-scale formulation keeping the view of commercialization of product[67]. Though the purified crystals obtained from the solution are in relatively moderate quantity, it has been seen that crystallization at a large scale can be an efficient, fast, and cost-effective alternative to traditional chromatography processes.

Future aspects: The operating conditions of crystallization can be made feasible to crystallize the majority of intact monoclonal antibodies. The process of continuous crystallization is one of the rapid techniques to obtain a high yield compared to other purification techniques and can easily purify the product with the elimination of impurities, including HCP, HCDNA, and other contaminants. Hence, continuous crystallization can be applied widely for therapeutic drug purification. However, due to the unsatisfied results of multiple attempts, various studies are still going on to improve the process conditions and reactors for continuous crystallization. If the efforts can be taken into account, the process of continuous crystallization of monoclonal

antibodies can be industrialized with the help of distinct types of stirred tank reactors to satisfy the high throughput market ultimatum[67].

The endeavor on ameliorating pragmatic conditions to carry out laboratory methods might result in a reduction of tediousness and time consumption in the process. The mathematical predictions in the tactics used to optimize the concentrations and types of additives and determination of the rate of aggregation through osmotic second virial coefficient (B22), spinodal measurement and birefringence can be exploited to enhance the crystal formation and yield. While increasing the use of crystal screens and electronic gadgets while performing the process of crystallization resort to preciseness aiding to obtain pure and well-ordered crystals.

Recently, the formulation of fragments of monoclonal antibodies by means of crystallization is trending, and it is quitea simplified process than intact mAbs, which gives highly explicit purity that would be effective in site-specific defense[67]. However, the purification of Fabs from supernatant requires multistep processes, which makes the procedure complex and costlier as compared to the intact monoclonal antibody purification by crystallization. Hence, the process requires modifications to make it feasible.

Conclusion

The process of purification is one of the unavoidable stages in the formulation of therapeutic drugs. Among the techniques of purification, crystallization has its prime importance in the field of applicative immunotherapy, as it offers numerous predictable advantages over the conventional formulation processes. In this, the crystallized form of intact monoclonal antibodies imparts prolonged durability because of its solidified organization. Due to the structural firmness of monoclonal antibodies, other protein impurities can be easily avoided in crystal formation. The efficient crystallization prerequisites the understanding of phase behaviors of intact monoclonal antibody concerning the other substitutes, including salts and polymers. The presence of such additives in adequate proportion aids to formulate exceedingly concentrated dosages of the molecules by removing hydrophobic patches. The sulfate salts have been used maximally, owing to remarkable results in eluting out the antibody molecules from the solution. A very little amount of salts of sodium, lithium, and ammonium sulfate affects the process of crystallization. Whereas the broad array of polymers having a molecular weight ranging from 400 to 4000 (PEG/ Jaffamine) show effective activity in crystallization. The higher the molecular weight of the polymer, the lower is the volume required to be entailed to take the water molecules away from solubilized monoclonal antibodies. Hence, the purity with higher activity can be achieved prominently.

The methods executed for crystallization of monoclonal antibodies can predict the conditions required to produce crystals with high productivity aiding the newly applied techniques, such as continuous crystallization through a stirred tank batch reactor. Continuous crystallization can be a rapid and economically feasible technique to purify monoclonal antibodies in large-scale production. The obtained product concentrated in the form of crystal yield can reduce the storage cost. However, lots of studies are required to acclimatize the process. Monitoring the process of crystal formation with the help of XRD and modernizing microscopes can give insight into the shape and structure of crystalized monoclonal antibodies. The study elucidates the development in intact monoclonal antibody crystallization emphasizing mainly features of crystal formation from phase separation to analysis, which depicts that, crystallization can be attractive and promising processing, purification, and formulation platform from an industrial perspective compared to chromatography.

Acknowledgments

We would like to thank the Department of Biotechnology from Kolhapur Institute of Technology's College of Engineering, Kolhapur for the technical support.

Abbreviations: mAb- monoclonal antibody; PEG- Poly-Ethylene Glycol; HCP- Host Cell Protein; HCDNA- Host Cell-DNA; UF- Ultrafiltration; DF- Diafiltration;B22- Osmotic second virial coefficient; CLSM- Confocal Laser Scanning Microscopy; XRD- X-ray diffraction.

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