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Review Article

Diamonds in the Rough: Protein Crystals from a Formulation Perspective

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The focus of the present review is to address the use of protein crystals in formulation design. Although this idea has been present for some time, i.e., insulin crystals were first reported back in 1920s, macromolecular crystallization has not received as much attention as the other methods for stabilizing protein drug candidates. The prospective potential of crystalline protein formulations in light of new advances in the field of macromolecular crystallization was reviewed, and the basic concepts and the tools now available for developing protein crystals into drug formulations are introduced. In addition, formulation challenges and regulatory demands, along with examples of current applications of protein crystals, are presented.

KEY WORDS: protein formulation; crystalline formulation; protein crystals; macromolecular crystallization.

GENERAL FORMULATION STRATEGIES FOR THERAPEUTIC PROTEINS

Advances in recombinant technology and tailored drug design have provided the pharmaceutical industry with numerous new therapeutic substances in the form of proteins. This class of macromolecules performs the function of their natural blueprints in soliciting desired responses from the body. The identity or resemblance of therapeutic proteins to host-generated proteins, however, frequently creates difficulties for drug delivery and formulation. Many such molecules are quickly broken down and processed *in vivo* within presystemic and systemic regulatory mechanisms. In environments other than their physiologic ones, therapeutic proteins may also rapidly denature or easily lose their biologic activity. Formulation design for such macromolecules, therefore, must be customized to protect their biologic, chemical, and physiologic stability during processing and for the desired effective period for storage and upon delivery.

The focus of the present review is to address the use of protein crystals in formulation design. Although this idea has been present for some time, i.e., insulin crystals were first reported back in 1920s, macromolecular crystallization has not received as much attention as the other methods for stabilizing protein drug candidates, e.g., by embodiment in polymers, by use of stabilizing additives, or by lyophilization. Previous understanding of the art of macromolecular crystallization may have daunted pharmaceutical scientists because protein crystallization has been identified as the "bottleneck" in X-ray structural determination (1). Recent advances, however, have alleviated many of the obstacles to crystallizing proteins. In this article, we review the prospective potential of crystalline protein formulations in light of the new developments. The basic concepts of macromolecular crystallization are introduced followed by the tools now available for developing protein crystals into drug formulations. In addition, formulation challenges and regulatory demands, along with examples of current applications of protein crystals, are presented.

ADVANTAGES OF CRYSTALLINE DRUG FORMS

Crystals of small therapeutic substances have been used as drug formulations for decades. The main advantages include better handling, stability, and varied dissolution characteristics, which allow better control over bioavailability. In addition, chemical degradation may be significantly reduced in crystalline vs. amorphous or soluble forms. Although current examples cannot confirm all the small molecular crystal traits, some of the same advantages could be envisioned for selected protein crystals. Protein crystals could protect the integrity (biologic, physical, and/or chemical) of the therapeutic agent in the lattice structure during processing, upon storage, and after delivery. Protein crystals may allow sustained release of the therapeutic agent for an effective duration, thus avoiding the labor- and cost-intensive need of repetitive dosing. Dose reduction could also be achieved by protecting the depot for more economic use of therapeutic proteins (2).

PROTEIN CRYSTAL BASICS

Protein crystals, like small molecule crystals, are solids with ordered packing of the molecular units into defined lattice space groups. The crystalline form is generally accepted to be an energetically favorable state, an estimated 3–6 kcal/mol more stable than in solution (3). Of the 230 potential

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crystal unit symmetries, only 65 space groups are possible for protein crystals. The limitation is due to the enantiomer building blocks of proteins (L-amino acids), which will not crystallize with inversion symmetries (4). The birefringence of protein crystals under polarized light may also be weaker than of small molecule crystals due to the isotropic globular character of the protein units (4).

The overall dimensions of protein crystals usually are smaller than nonbiologic crystals and seemingly restricted to a "terminal" size (5). Protein crystals generally are held together by the hydrophobic interactions, H-bonds. and salt bridges between/within the proteins. Because of fewer and weaker intermolecular interactions, protein crystals are more fragile than nonbiologic crystals. Often, a "crunch" instead of a "crack" under compression can distinguish a protein from a mineral crystal, respectively (5).

A large percentage of solvent is almost always associated with protein crystals. Water molecules are found both within the crystal structure, as well as loosely associated in channels within the crystal lattice around the protein molecules. This moisture may be required to maintain a hydrated configuration of the proteins (6). For structural analysis, the hydrated proteins within crystals are generally assumed folded in their native forms. Crystallized protein-ligand structures (see listed examples in 5), as well as active enzyme crystals (6), support this assumption.

Despite the aforementioned differences, most general theories on nucleation dynamics and crystal growth kinetics can be applied to macromolecular crystals. (7,8). As with small molecules, the processes of crystal nucleation and growth depend on the solubility and supersaturation of the protein in its environment. The driving force for crystallization is the difference between the supersaturated chemical potential of the molecule and its chemical potential in the saturated solution, $\Delta\mu$. Mathematically, it is expressed as

$$\Delta\mu = \kappa_B \, T \, ln\frac{C}{C_S} \qquad (1)$$

where κ_B is the Boltzmann constant, T the absolute temperature, C the actual concentration before crystallization, and C_s the concentration of the protein at equilibrium.

In crystallization literature, supersaturation usually is divided into the following two regions: the higher labile region, which promotes spontaneous nucleation, and the lower metastable region, which allows crystal growth. Figure 1 shows a typical solubility (or phase) diagram used for crystallization. The parameter in the x-axis can also be temperature, pH, or another factor that influences the solubility behavior. In general, the nucleation of protein crystals requires a high degree of supersaturation, about 2–10 times that of saturation, i.e., several hundred percent (9). However, too high of a saturated state should be avoided to prevent random precipitation.

Supersaturation of a protein in solution can be propagated by adding a precipitant to the protein in solution. During nucleation and growth, protein crystals are extremely dependent on the electrostatic and electrodynamic effects of the electrolyte solutions in which they bathe (7; An extensive list of common precipitants used in macromolecular crystallization can be found in Reference 1.) For small-scale protein crystal production, slow vapor diffusion of the solvent over a miniaturized reservoir (hanging and sitting drop method), direct mixing of protein solution with a strong precipitant (batch method), and dialyzing the protein-electrolyte solution are the most common methods (10). Changing the pH or temperature of a protein solution can also push the system into supersaturation (11,12). Possible conditions are limited, however, by the risk of denaturation and degradation.

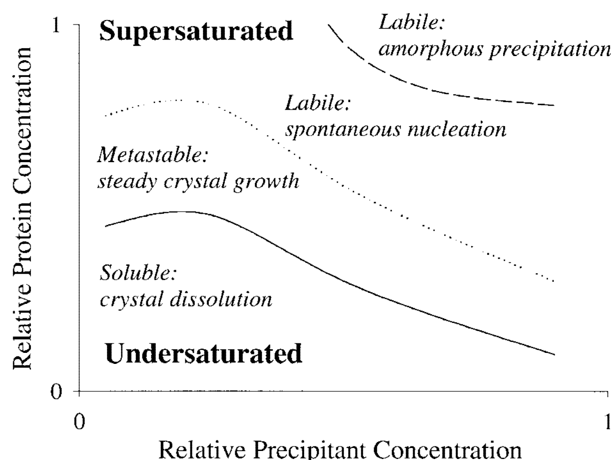

Fig. 1. Typical solubility (phase) diagram used for crystallation of proteins, shown here for a theoretical molecule as a function of precipitant concentration (adapted from 5).

High supersaturation as needed for spontaneous nucleation often leads to rampant growth with defects in the crystals. To provide better growing conditions, one can seed a metastable supersaturated solution with existing crystal nuclei onto which further growth can propagate. Two classes of seeding are defined: homogenous, which uses crystal seeds of the same protein and technically includes spontaneous nucleation in the bulk fluid, and heterogeneous, which uses seeds of a different nature, usually crystals of another protein having structural similarities. Microseeding is done with small crushed particles of crystals whereas macroseeding transfers entire crystals as the nuclei. Usually the seeds are first washed to achieve a defect-free growth surface before being introduced into the new environment (13).

The mechanisms of protein crystal growth are similar to those for small molecule crystals. (A detailed discussion of traditional growth mechanisms can be found in Reference 14.) Briefly, the process can be separated into following two stages: 1) mass transfer of building blocks to the nucleus and 2) incorporation of building blocks into the crystal lattice after sticking to the growth surface. At the surface, crystals can grow through spiral dislocations, normal growth from random intensive nucleation, two- and three-dimensional nucleation on surfaces, and addition of aggregates from solution (5). It has been shown that for most proteins, the growth kinetics in small volumes is surface controlled (7).

In contrast to miniaturized crystallization typical in biophysical analysis, preparative crystallization in formulation design, which is of a larger scale, could be severely diffusion limited without mechanical agitation. The diffusivities of proteins in solutions generally are low, and a concentration gradient can form around growing crystals (15). It has been shown that mechanical agitation and microgravity, which alter transport phenomena, can change the crystallization in diffusion-controlled systems (16). Whereas microgravity re-

duces the solutal convection flow to allow very slow growth, agitation creates convection and a more constant bulk concentration profile. For large crystallization batches as purposed for pharmaceutical applications, mechanical agitation would be indispensable as diffusion of the building blocks to the nuclei would be insignificant over large distances. Convection must be present to promote uniform crystallization throughout the reactor. However, high shear should be avoided because it can lead to premature growth cessation (17).

The unique characteristics of protein crystallization, as discussed in this section, are summarized in Table I. The knowledge is based on many crystallography studies in the past few years, which have been geared towards understanding the crystallization phenomena, to convert the present art of macromolecular crystallization into a more exact science (5). Although conditions must be customized for each macromolecular candidate, knowledge of general systemic behavior has enhanced control over the crystallization process (1). Recent developments have convinced most crystallographers that macromolecular crystallization is a subset of general crystallization (8). Macromolecular crystallization has also been compared with that of colloids and polymer systems because both contain large molecules interacting over small intermolecular distances (18).

RECENT ADVANCES IN MACROMOLECULAR CRYSTALLIZATION

The hazards of macromolecular crystallization have been significantly alleviated through the sheer volume of experience published in the X-ray crystallography literature over the past few years. An extensive collection of known protein structures can be accessed through the internet from the Protein Data Bank (http://www.rcsb.org/pdb/). Also available through the internet is the databank of successful crystallization conditions for these proteins, which can be consulted as starting points for other macromolecules (19) (http://www.cstl.nist.gov/biotech/carb/gilliland_group/database/database.html). In addition, seminars and short courses are offered regularly by various societies and companies to provide both old hands and neophytes alike with the opportunity to learn the newest theories and techniques. Some crystallization journals now publish separate "biologic" editions or conference proceedings on macromolecular crystallization. These publications can be referenced for details of techniques as well as new general developments in the field (e.g., *Acta Crystallographica Section D* and *Journal of Crystal Growth*).

Several systematic strategies have been proposed for crystallizing macromolecules in a less-random trial-and-error process. These methods include using statistical design to identify influential factors (20), examining the solubility behavior of the protein with a specific precipitant (21), finding favorable conditions based on the databank of successful crystallization trials with similar molecules (19), and sequentially adding different precipitants to propagate supersaturation (22). The sparse matrix approaches of (23) and (24) to screen precipitants are now available commercially (Hampton Research, Laguna Niguel, CA). In addition, a "windows" screening approach, which simultaneously alters three crystallization parameters, has been introduced for easy adaptation to large-scale process (25).

Certain technologic advances have also eased the actual task of protein crystallization. Besides crystallization screening kits, standard crystallization tools and reagents can be purchased commercially (see http://www.hamptonresearch.com and http://www.emeraldbiostructures.com). Crystal analysis software can now almost automatically categorize the size and shape of screened crystals (e.g., CrystalScore™ from CyberLabs of Gilson, Inc., Middleton WI, or Sysmex FPIA-2100 from General Microtechnology & Photonics SA, Renens, Switzerland). These tools can be used to analyze mass crystallization trials using small quantities of protein, made possible by the computer modulated robotic systems now available on the market (see http://www.douglas.co.uk and http://www.gilson.com/crystal.htm). A computerized method for better control during vapor diffusion crystallization is also under development (26). The incorporation of computers and robotics into the process of macromolecular crystallization may make high-throughput crystallization soon possible. (For a more complete review on high-throughput acrocrystallization, refer to Reference 27.)

CURRENT APPLICATIONS USING PROTEIN CRYSTALLIZATION

As expected, most modern experts in macromolecular crystallization are found in structural biology labs, where they nurture the growth of perfect single crystals, i.e., crystals of constant space group packing with no growth defects. Large crystals of such quality (>100 μm in all dimensions) can generate high-resolution X-ray diffraction patterns that may reveal the molecular structure of biomolecules through analysis with sophisticated computer modeling (28). Since being introduced in 1934 (29), this method has remained the most accurate one available for structural analysis of large biomol-

Table I. Unique Characteristics of Protein Crystallization

Properties	Special protein crystal characteristics
Intermolecular interactions	Hydrophobic forces, H-bonds, and salt bridges
Space group	Due to symmetry limitation only 65 of 230 configurations possible
Solvent content	Many water molecules in structure, as well as loosely associated in channels; to keep proteins in native conformation
Optical properties	Weak birefringence and often lower resolution X-ray diffraction patterns
Size	Usually limited to small dimensions (μm)
Crystallization conditions	Limited due to risk of denaturation and degradation
Supersaturation	Showing colloidal behavior, such as the liquid-liquid immicibility phases and gel phase
Nucleation	High saturation needed; generally slow due to larger size of the building blocks with respect to the small intermolecular distances
Growth kinetics	Under nonconvection conditions in small volumes, dominated by surface kinetics

ecules. Using improved high-intensity X-rays with better beam-line optics and equipment set-up, one can now achieve atomic resolution diffraction patterns with protein crystals of only 10–50 μm on all faces (27,30). Such technological advances remove the necessity of large perfect crystals.

Whereas protein crystallization for structural analysis usually is conducted in microliter volumes, examples of large-scale efforts can be found in the biotechnology industry. Crystallization usually serves as a means for product recovery and purification (31). Even before the 1930s, crystallization has been used for isolation of biologic products from complex media (see references in 5). One well-studied example is the isolation of extracted or recombinant insulin to produce a "pure" therapeutic product (32). Recent studies have been performed to extend the use of large-scale macromolecular crystallization for recovery of other proteins directly from biotechnology fermentation broths (33). Crystallization is still viewed as an alternative for some more expensive separations methods, although as with crystallization of small molecules, impurities and imperfections can be incorporated into the crystal structure of proteins up to observable percentages (34 and references within).

Crystals of enzymes are industrially in use today as biologic catalysts. Enzymes are much more efficient for catalyzing organic reactions than synthetic methods (31). Crystals can be immobilized to retain longer activity with better stability than enzymes in solution (35). The high solvent content of protein crystals easily allows the diffusion of reactants into the crystal lattice to react with the bound catalysts (6). Cross-linking the crystals can further increase the longevity of the biologic catalysts without significant loss of activity, even under harsh conditions (36). In addition, the success of this cross-link stabilization technique has prompted the investigation into cross-linking protein crystals for other applications, e.g., vaccine and oral protein delivery (37,38). An extensive review of current and potential applications for cross-linked protein crystals is found in (39).

One well-studied specific example in the field of protein crystal drug delivery is of therapeutic insulin for treating diabetes mellitus. Although amorphous insulin formulations are fast acting, medium- and slow-acting insulin crystals can be formulated with finely tuned individual dissolution and release features. In general, the slow-acting insulin formulations are crystallized with Zn^{2+} (32). Variations in coprecipitation strategies and conditions, e.g., amount of coprecipitated zinc, temperature, and pH during the reaction, addition of protamine, etc., have led to many formulations of varying action profiles (32). Use of bovine or porcine insulin forms can also alter the action rate from that of human insulin (40,41).

In our group, protein crystals have been investigated as a potential sustained release formulation for transforming growth factor TGF–β3 (42,43). TGF-β3 is envisioned for local applications in wound healing, bone regeneration, or as a cotreatment against side effects of chemotherapy (see the review on TGF-β3 in Reference 44). The growth factor can be crystallized in three precipitant solutions (42,45). The most physically stable form is bound with one dioxane per protein in an internal hydrophobic pocket (45). The dioxane stabilizes the crystals by promoting three instead of one crystal contact interfaces for a closely packed formation (45). Stable TGF-β3 crystals are physically robust and exhibit slow release properties under physiologic conditions (43). A faster or slower release can be triggered by altering the pH or the fluid exchange profile in the microenvironment around the crystal depot. For example, an acidic pH, as created during bone remodeling or by degradation of polymer carriers, can enhance the crystal/protein solubility for faster release. Also, mechanical stimuli at the delivery site, e.g., through physical movement, can be used to enhance release by increasing fluid flow around the crystals (43).

FORMULATION CONSIDERATIONS, STRATEGIES, AND CHALLENGES

Collaboration with X-ray crystallographers can facilitate the screening efforts for promising protein crystal drug candidates. Often, the X-ray crystallographers already have found favorable crystallization conditions for their structural studies and may know the handling characteristics of discovered crystal forms. The downstream drug development potential of the crystallographers' work renders careful documentation of all crystallization trials of protein candidates very important. It is possible that disappointments for X-ray diffraction studies may turn out to be "jewels" for formulation development. Although crystallographers aim for large perfect crystals, the formulation scientist can work with smaller crystals, preferably of regular size and shape.

Good crystal drug candidates should have sufficient physical robustness that allows handling as a solid dosage form. Unfortunately, this is generally not the case with fragile protein crystals. Crystals may rapidly "melt" or dissolve when brought into an environment different from their supersaturated crystallization medium. Moreover, crystals may loose their integrity upon mechanical processing, e.g., by stirring, shaking, or simply during transfer, e.g., by pipetting. Several strategies based on the examples provided in the previous section are at hand to overcome the physical deficits: 1) cross-linking fragile forms of protein crystals with a reversible cross-linker, e.g., done with enzyme crystals (35); 2) coprecipitation of the protein with biocompatible multivalent metal ions like Zn^{2+}, e.g., for insulin crystals (32); and 3) coprecipitation with another kind of stabilizing agent, such as a natural or artificial binding ligand, e.g., dioxane for TGF-β3 (45). The discovery of suitable ligands is an absolute prerequisite to obtain physically stable crystals. Once a physically stable form of the protein crystal is made, its dissolution could be tested for controlled/sustained release properties. Physical stability, such as aggregation and conformation changes under temperature and humidity stress, should also be examined (46).

As with all pharmaceutical entities, chemical and biologic stability of crystal drug candidates must also be evaluated. The examples of insulin and TGF-β3 crystals suggest that crystallization alone may not be sufficient to protect proteins from chemical degradation. Insulin formulations, both crystalline and amorphous, have been extensively studied (47,48 and references within). *In vivo,* the crystalline formulations are efficient in protecting the insulin to allow delayed action upon delivery. However, *in vitro* it was found that in the form of suspensions, crystalline insulin is only two times more stable than amorphous insulin, instead of an order of magnitude greater as is typical for small molecules (47). Dried insulin crystals have higher denaturation temperatures but are extremely sensitive to moisture. Below the denaturation temperatures, vacuum-dried crystal preparations degraded

faster at the B3 deamidation sites than freeze-dried amorphous insulin, especially under <15% water content (48).

Like insulin, TGF-β3 crystals dried under vacuum have comparable chemical stability under storage and temperature/moisture stress as freeze-dried amorphous samples. The TGF-β3 crystals have higher denaturation temperature than amorphous preparations but are very sensitive to moisture (43). We propose the use of excipients with the crystals to improve chemical stability to benefit from the other advantages for drug delivery possible with protein crystals. Likely, an optimal excipient combination can be used to enhance the protein's stability in both amorphous and crystalline forms.

Once a crystal candidate has shown promising properties for pharmaceutical development, the crystallization effort must be up-scaled. The batch and dialysis methods are likely the easiest options for adaptation to large-scale crystallization because similar constructions already exist for chemical, pharmaceutical, and biotechnological processes. The conversion of microliter-size crystallization trials into industrial dimensions, however, may be a challenging task. Successful upscaling requires careful documentation and understanding of favorable conditions found during the screening process, optimally defined in terms of particle shape, size, and solubility (degree of saturation). The size and shape of the products from production trials would indicate which operating factors, such as protein and precipitant concentration, could be adjusted to achieve the desired product. Once the operating parameters have been optimized, crystal form and size can be used to monitor product quality.

At this point, protein crystallization under aspects of mechanical agitation has not yet been much scrutinized, although convection would be very important for large-scale crystallization. Large-scale batch production of enzyme crystals currently employs shaking, which is also readily available for lab-scale trials (25). Production in stirred tank reactors, as performed for insulin preparations, is another standard production method with much empirical data and theoretical models available to help with process design. Ultrasound reactors as available for small molecule crystallization may also soon be adaptable for crystallization of proteins. The high-energy input of sonication may even be able to overcome the nucleation barrier to germinate spontaneous nuclei in metastable supersaturated solutions for stable growth without seeding (49).

With TGF-β3, we have explored the effects of shaking, stirring, and ultrasound during lab-scale crystallization. Operation parameters, such as shaking or stirring speed, frequency, and exposure time to ultrasound, were all found to be very influential on the resulting crystal products (43). No chemical or biologic changes were observed due to the agitation methods tested. These parameters that can be controlled during production must be optimized during upscaling process design. Validation of the processes would be also necessary to gain approval from regulatory agencies for use of the protein crystals as pharmaceutical products.

REGULATORY CONCERNS

Protein crystals are subjected to the regulatory specifications for biotechnologic/biologic products. Most of the analytical methods and tools developed for proteins can be used directly for physical, chemical, and biologic characterization of the protein within the crystals. The key is to understand the relevance of the crystal properties with respect to the *in vivo* performance of the protein. Analysis of the protein within the crystals requires that the crystals be dissolved under conditions that minimize interference with the analytical protocol. Use of additives or adjustment of temperature and pH may be necessary both to dissolve the crystals and solubilize the protein. Extreme conditions should be avoided to prevent artifacts from denaturation and/or degradation.

Electrophoresis, chromatography, spectroscopy, light or electron microscopy, calorimetry, immunobiochemic, and biologic assays are some major categories of useful techniques for protein analysis. These analytical methods can characterize the proteins, as well as monitor product quality and stability later in the development process. The specific features of these protein methods will not be discussed here. The reader is referred to the extensive literature available on each topic as well as general reviews on protein analytical methods (50–52).

Protein crystals are also subjected to the typical testing requirements for solid dosage forms. The pharmaceutical industry already has experience with solid crystalline formulations, with many analytical techniques available. Fourier transforms infrared (FTIR), atomic force microscopy (AFM), scanning electron microscopy, and light scattering are some techniques gaining recognition for protein crystal analysis. (An instructive summary of standard testing requirements for solid dosage forms can be found in Reference 53.)

SUMMARY

Protein crystals represent a valuable formulation option for the stabilization and the delivery of macromolecular therapeutics. Examples presented in this article show that the protein crystals can provide some of the advantages found with small molecule crystal formulations, such as better physical handling stability and controlled sustained release. Stabilization may synergistically benefit from combining crystallization with another strategy, such as the use of additives and drying. Formulation design can then take advantage of the crystal properties to achieve a well-controlled and economical delivery system for protein drugs. The approach, however, may not be possible for all protein candidates because physically stable and robust crystals can be elusive. The significant advances in the area of macromolecular crystallography have made the candidate-screening task much easier than before, with possibilities to automate the process. Although each individual protein must be separately examined, the list of crystals "discovered" by X-ray crystallographers provide a group of promising development candidates waiting to be explored. Follow-up formulation studies would then reveal whether the crystals of pharmaceutically interesting proteins have desirable release characteristics and acceptable stability for development as therapeutic agents.

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REFERENCES

1. A. McPherson. Current approaches to macromolecular crystallization. *Eur. J. Biochem.* **189**:1–23 (1990).
2. S. D. Putney and P. A. Burke. Improving protein therapeutics with sustained-release formulations. *Nat. Biotechnol.* **16**:153–157 (1998).

3. J. Drenth and C. Hass. Protein crystals and their stability. *J. Crystal Growth* **122**:107–109 (1992).
4. R. Giegé and A. Ducruix. An introduction to the crystallogenesis of biological macromolecules. In A. Ducruix and R. Giegé, (eds.), *Crystallization of Nucleic Acids and Proteins: A Practical Approach*, Oxford University Press, New York, 1992, pp.1–15.
5. A. McPherson. *Crystallization of Biological Macromolecules*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1999.
6. M. Frey. Water structure associated with proteins and its role in crystallization. *Acta Crystallographica D* **50**:663–666 (1994).
7. R. S. Feigelson. The relevance of small molecule crystal growth theories and techniques to the growth of biological macromolecules. *J. Crystal Growth* **90**:1–13 (1988).
8. J. M. Wiencek. New strategies for protein crystal growth. *Ann. Rev. Biomedical. Engineer.* **1**:505–534 (1999).
9. V. Mikol and R. Gieg è. The physical chemistry of protein crystallization In A. Ducruix and R. Giegé, (eds.), *Crystallization of Nucleic Acids and Proteins: A Practical Approach*, Oxford University Press, New York, 1992, pp. 219–238.
10. A. Ducruix and R. Gieg è, R. Methods of crystallization. In A. Ducruix and R. Giegé, (eds.), *Crystallization of Nucleic Acids and Proteins: A Practical Approach*, Oxford University Press, New York, 1992, pp. 73–98.
11. J. R. Luft, D. M. Rak, and D. G. DeTitta. Microbatch macromolecular crystallization on a thermal gradient. *J. Crystal Growth* **196**:447–449 (1999).
12. C. A. Schall, J. S. Riley, E. Li, E. Arnold, and J. M. Wiencek. Application of temperature control strategies to the growth of hen egg-white lysozyme crystals. *J. Crystal Growth* **165**:299–307 (1996).
13. E. A. Stura and I. A.Wilson. Seeding techniques. In A. Ducruix and R. Giegé, (eds.), *Crystallization of Nucleic Acids and Proteins: A Practical Approach*, Oxford University Press, New York, 1992, pp. 99–125.
14. R. Boistelle and J. P. Astier. Crystallization mechanisms in solution. *J. Crystal Growth* **90**:14–30 (1988).
15. F. Rosenberger and E. J. Meehan. Control of nucleation and growth in protein crystal growth. *J. Crystal Growth* **90**:74–78 (1988).
16. A. Rosenberger. Specific phenomena and general insights on crystallization kinetics. *10th International Summer School on Crystal Growth, ISSCG-10*, Rimini, Italy, pp. 241–256.
17. M. L. Pusey. Continuing adventures in lysozyme crystal growth. *J. Crystal Growth* **122**:1–7 (1992).
18. C. C. Young, R. C. DeMattei, R. S. Feigelson, and W. A. Tiller. Some implications of colloid stability theory for protein crystallization. *J. Crystal Growth* **90**:79–85 (1988).
19. G. Gilliland. A biological macromolecule crystallization database—a basis for a crystallization strategy. *J. Crystal Growth* **90**: 51–59 (1988).
20. C. W. Carter Jr., E. T. Baldwin, and L. Frick. Statistical design of experiments for protein crystal-growth and the use of a precrystallization assay. *J. Crystal Growth* **90**:60–73 (1988).
21. E. A. Stura, A. C. Satterthwait, J. C. Calvo, D. C. Kaslow, and I. A. Wilson. Reverse screening. *Acta Crystallographica D* **50**:448–455 (1994).
22. C. Quain, L. Lagacé, M. J. Massariol, C. Chabot, C. Yoakin, R. Déziel, and L. Tong. A rational approach towards successful crystallization and crystal treatment of human cytomegalovirus protease and its inhibitor complex. *Acta Crystallographica D* **56:**175–180 (2000).
23. J. Jancarik and S. H. Kim. Sparse matrix sampling: a screening method for crystallization of proteins. *J. Applied Crystallography* **24**:409–411 (1991).
24. B. Cudney, S. Patel, K. Y. N. Weisgraber, and A. McPherson. Screening and optimization strategies for macromolecular crystal growth. *Acta Crystallographica D* **50**:414–423 (1994).
25. T. Lee, J. Vaghjiani, G. Lye, and M. Turner. A systematic approach to the large-scale production of protein crystals. *Enzyme Microbial. Technol.* **26**:582–592 (2000).
26. P. D. Collingsworth, T. L. Bray, and G. K. Christopher. Crystal growth via computer controlled vapor diffusion. *J. Crystal Growth* **219**:283–289 (2000).
27. R. C. Stevens. High-throughput protein crystallization. *Curr. Opin. Struct. Biol.* **10**:558–563 (2000).
28. D. E. McRee. *Practical Protein Crystallography*, Academic Press, San Diego, 1999.
29. J. D. Bernal and D. Crowfoot (Hodgkin). *Nature* **133**:794–955 (1934) (as referenced in Giegè and Ducruix (4), original source not available).
30. A. Perrakis, F. Cipriani, J.C. Castagna, L. Claustre, M. Burghammer, C. Riekel, and S. Cusack. Protein microcrystals and the design of a microdiffractometer: current experience and plans at EMBL and ESRF/ID13. *Acta Crystallographica D* **55**:1765–1770 (1999).
31. J. H. Northrop, M. Kunitz, and R. M. Herriot. *Crystalline Enzymes*, Columbia University Press, New York, 1948.
32. J. Brange and L. Langkjaer. Insulin formulation and delivery. *Pharm. Biotechnol.* **10**:343–409 (1997).
33. C. Jacobson, J. Garside, and M. Horare. Nucleation and growth of microbial lipase crystals from clarified concentrated fermentation broths. *Biotechnol. Bioeng.* **7**:666–675 (1998).
34. C. L. Caylor, I. Dobrianov, S. G. Lemay, C. Kimmer, S. Kriminski, K. D. Finkelstein, W. Zipfel, W. W. Webb, B. R. Thomas, A. A. Chernov, and R. E. Thorne. Macromolecular impurities and disorder in protein crystals. *Proteins* **36**:270–281 (1999).
35. C. P. Govardhan. Cross-linking of enzymes for improved stability and performance. *Curr. Opin. Biotechnol.* **10**:331–335 (1999).
36. M. A. Navia and N. L. St. Clair. Cross-linked enzyme crystals. US Pat. 5618710, Vertex Pharmaceuticals, Inc. 1997.
37. N. St.Clair, B. Shenoy, L. D. Jacon, and A. L. Margolin. Cross-linked protein crystals for vaccine delivery. *Proc. Natl. Acad. Sci. USA* **96**:9469–9474 (1999).
38. A. L. Margolin, R. A. Persichetti, N. L. St.Clair, and N. K. Khalf. Controlled dissolution cross-linked protein. US Pat. 6140475, Altus Biologics Inc. 2000.
39. A. L. Margolin and M. A. Navia. Protein crystals as novel catalytic materials. *Angew Chem. Int. Ed*. **40**:2204–2222 (2001).
40. P. Hildebrandt, A. Berger, A. Volund, and C. Kuhl. The subcutaneous absorption of human and bovine ultralente insulin formulations. *Diabet. Med.* **2**:355–359 (1985).
41. J. A. Hoffmann. Insulin formulation. EP 646379A1, Eli Lilly and Co., USA 1995.
42. U. Gietz. Therapeutic protein formulation for sustained delivery: Formulation aspects and stability. Ph.D. Thesis, ETH Zürich, Zurich (1997). Document available from corresponding author on request.
43. A. Jen. Protein crystals for drug formulation: TGF-beta 3 case study. Ph.D. Thesis, ETH Zurich, Zurich (2001). Document available from corresponding author on request.
44. D. Cox. Transforming growth factor-beta 3. *Cell Biology International, Special Issue Polypeptide growth factors with special emphasis on wound healing* **19**:357–571 (1995).
45. P. R. E. Mittl, J. P. Priestle, D. A. Cox, G. McMaster, N. Cerletti, and M. G. Grütter. The crystal structure of TGF-β3 and comparison to TGF-β2: Implications for receptor binding. *Protein Sci.* **5**:1261–1271 (1996).
46. B. Shenoy, Y. Wang, W. Shan, and A. L. Margolin. Stability of crystalline proteins. *Biotechnol. Bioengineer.* **73**:358–369 (2001).
47. J. Brange and L. Langkjaer. Insulin structure and stability. *Pharm. Biotechnol.* **5**:315–350 (1993).
48. M. J. Pikal and D. R. Rigsbee. The stability of insulin in crystalline and amorphous solids: Observation of greater stability for the amorphous form. *Pharm. Res.* **14**:1379–1387 (1997).
49. C. J. Price. Take some solid steps to improve crystallization. *Chem. Engineer. Progress* **93**:34–43 (1997).
50. D. M. Bollag and S. J. Edelstein. *Protein Methods*, Wiley-Liss, New York, 1991.
51. R. L. Garnick, N. J. Solli, and P. A. Papa. The role of quality control in biotechnology: An analytical perspective. *Anal. Chem.* **60**:2546–2557 (1988).
52. T. Vogl and H. J. Hinz. Characterization of recombinant proteins: biochemical and biophysical criteria for the identity of native and renatured human tissue plasminogen activator and its mutants. *Biotechnol. Appl. Biochem.* **20**:1–22 (1994).
53. S. Byrn, R. Pfeiffer, M. Ganey, C. Hoibery, and G. Poochikian. Pharmaceutical solids: A strategic approach to regulatory considerations. *Pharm. Res.* **12**:945–954 (1995).