Porous nucleating agents for protein crystallization

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ABSTRACT

Solving the structure of proteins is pivotal to achieving success in rational drug design and in other biotechnology endeavors. The most powerful method for determining the structure of proteins is X-ray crystallography, which relies on the availability of high-quality crystals. Obtaining such crystals is, however, a major hurdle. Nucleation is the crucial prerequisite step, which requires overcoming an energy barrier. The presence in a protein solution of a nucleant, a solid or semiliquid substance that facilitates overcoming that barrier allows crystals to grow under ideal conditions, paving the way for the formation of high-quality crystals. The use of nucleants provides a unique means for optimizing the diffraction quality of crystals, as well as for discovering new crystallization conditions. We present a protocol

for controlling nucleation of protein crystals that is applicable to a wide variety of nucleation-inducing substances. Setting up crystallization trials using these nucleating agents takes an additional few seconds compared to conventional set up and can accelerate crystallization, which typically takes several days to months.

INTRODUCTION

Overview

Knowing the three-dimensional structure of proteins is a primary tool of modern medical and biotechnology research. X-ray crystallography is the most powerful technique for structure determination at high resolution and is of key importance in the rational design of therapeutic agents. The production of the high-quality crystals, which crystallography relies upon, is a major challenge (Fig.1) and is crucial to progress. The problem associated with the experimental bottleneck of obtaining diffraction-quality protein crystals is becoming more acute as the number of proteins identified as potential drug targets increases.

Nucleation is the prerequisite step for crystal growth; hence means of controlling nucleation have a profound impact on the success of crystallization trials. Nucleants are agents that are not themselves protein crystals or nuclei and that induce heterogeneous nucleation of protein crystals in a controlled manner by providing a substrate on which a crystal nucleus can form and grow. Nucleant research for protein crystallization was kick started by a study published in 1988[1] and has progressed since with varying degrees of success. A variety of materials have been demonstrated to induce nucleation, including non-protein agents such as functionalized mica[2] and charged surfaces[2,3], as well as protein-based ones, such as Langmuir-Blodgett (LB) protein homologous thin films (i.e. thin films made of the protein to be crystallized)[4-6], and horse[7] and human[8] hair amongst others. In the case of hair, it was shown that it is not only the surface chemistry of keratin but also the cuticle microstructure on the hair surface that are relevant[8]. Research in this area has experienced a revival in recent years following the discovery of effective non-protein nucleants which are porous in nature[9-13].

Application

This protocol describes the use of porous nucleants to produce diffraction-quality protein crystals for X-ray crystallographic structure determination. Furthermore, it may be adapted to other fields where crystallization is required such as synthetic organic chemistry and the production of pharmaceuticals.

Development of porous nucleants

In 2001, a new approach to protein crystallization introduced the idea of utilizing as nucleating agents porous materials (porous silicon being the first), in which the pores are of a similar size to the proteins present in solution[9]. The hypothesis was that the pores would entrap protein molecules, thereby encouraging them to aggregate in crystalline order. Over the years that followed, a host of porous materials were designed and proven to be effective for protein crystallization, Bioglass[10] in 2006, coated carbon nanotubes[11] (Fig.2) and, very recently, nanoporous gold[12]. The determining factor in the success of such nucleants is the size distribution of their pores. Further development of these nucleants has involved improving their nucleating ability, modifying their porosity and/or texture (both in terms of handling, cleanliness and visibility within trials), harnessing their surface chemistry, and testing their suitability for high throughput trials.

Bioglass, a disordered porous medium with a distribution of pore sizes and shapes (Fig. 3), was found to promote nucleation of a wide range of proteins (including difficult-to-crystallize proteins and other biomacromolecules) of various molecular weights, at various pHs, precipitants and crystallization setups[10]. Bioglass is a mesoporous gel-glass, of chemical composition $CaO\text{-}P_2O_5\text{-}SiO_2$[14]. It is available in the form of grains measuring 30–100 μm in diameter and containing pores of diameters in the 2–10-nm range (Gaussian distribution). This material has been commercialized as 'Naomi's Nucleant' (by Molecular Dimensions Ltd.).

A different type of nucleant with the added benefit of specifically attracting proteins are Molecularly Imprinted Polymers (MIPs). MIPs are polymers formed in the presence of a molecule that is subsequently extracted, leaving cavities that retain a memory of the molecule and exhibit a tendency for the highly selective rebinding of it. MIPs can be imprinted with the protein to be crystallized (cognate) or with a more widely available protein of similar molecular weight (non-cognate). MIPs have been successfully used for promoting nucleation of various proteins in both screening and optimization trials[13,15] (Fig.4).

The discovery of porous materials as nucleants for protein crystallization has set a trend for utilizing such materials to induce nucleation e.g[16-20]. The use of Bioglass as a nucleant is described in detail in this protocol because it is already commercialized and therefore easy to obtain. Most other solid nucleants would also be utilized to obtain protein crystals according to the procedure described below. MIPs have been discovered more recently and are in the process of being commercialized. A patent for their use in protein crystallization has been granted to protect commercial exploitation but does not preclude scientific research using this method. Their fabrication is detailed in Saridakis *et al*.[13]. To date MIPs

are the only semi-liquid nucleants and are applied by a slightly different procedure, which is also described in this protocol. Although several theories have been proposed for the mechanism of protein nucleation within pores[21,22,23], this mechanism has yet to be determined experimentally. In the case of Bioglass, the distribution of pore sizes on the surface is such that proteins can 'select' the optimal pore size for their nucleation. This size distribution explains the effectiveness of Bioglass over a wide range of proteins. When considering MIPs, it is left to the researcher to utilize a cognate MIP or to select a 'reference' MIP imprinted with a protein of similar molecular weight as the protein to crystallize.

Bioglass and MIPs have been demonstrated to work successfully for globular proteins, membrane proteins and complexes[10,13,24,25]. Their application to trials containing membrane proteins is no different to that of globular proteins. These materials have yet to be tested in conjunction with the cubic phase and other alternative methods for crystallizing membrane proteins[26,27].

Comparison with other methods

Another method to assist protein crystallization that is most directly comparable to that described in this protocol is seeding, a well-established technique based on adding ready nuclei to metastable solutions. Seeding can involve the transfer of whole crystals, fragments of crystals or even ordered precipitate[28-30]. Recently developed automated means for performing seeding trials[30] are powerful but focus mainly on screening. Optimization of crystallization using seeding is still mostly performed manually, it involves very careful handling and usually requires crystalline material to use as seeds. The use of heterogeneous nucleants overcomes these limitations. Furthermore nucleants are stable over long periods of time and their dispensing is less delicate an operation than crystal transfer. The use of nucleants for protein crystallization was initially treated as a type of seeding[28], but it has since evolved into a field of its own where the 'state of the art' has been extended beyond the similarity to seeding with the requirement for a distinct approach and procedure.

Other methods exist for controlling nucleation, but none is directly comparable to applying nucleants. These alternative methods include incubating trials at high supersaturation, where nucleation takes place spontaneously, and after a given time lowering the supersaturation to conditions that are metastable with respect to crystallization, i.e. where nucleation does not occur spontaneously but where supersaturation is sufficient for already nucleated crystals to grow. This goal can be achieved by diluting a crystallization drop after a given time[31], by transferring vapor diffusion trials over to a more dilute reservoir[32], or even by changing the temperature[33]. The main drawback of this method is that unless transfer is timed very

accurately, the method fails. The induction time for nucleation varies for each protein and condition and detecting aggregation for accurately predicting the time of transfer can be done using *in situ* Dynamic Light Scattering, which requires, however, specialized equipment[34].

A notable advantage of this method over the above alternatives of seeding and dilution is that much less special planning is required. All that is needed is to set up a few trials at lower precipitant concentration than those leading to spontaneous nucleation and insert the nucleant from the outset, irrespective of the nature of the protein, precipitating agent and buffer, temperatures and the crystallization method. Bioglass and MIP nucleants are stable and easy to handle, and they can be dispensed in very small quantities into numerous crystallization trials. Also, it is not necessary to have the same size of grain in all drops (for solid nucleants), or precisely the same volume (for semi-liquid nucleant). Due to the nature of the materials, they are easy to observe under a light microscope and to distinguish from the growing crystals. Many of the other nucleants referred to in the "Overview" (non-porous nucleants such as hair[7,8]) and in the "Development of porous nucleants" (porous nucleants such as porous silicon[9], carbon nanotube films[11] and nanoporous gold[12]) sections, are made of thin wafers or materials that are dark, thereby significantly limiting the visibility of the crystals.

Limitations

We have noticed that, besides assisting the crystallization of globular proteins, protein complexes and membrane proteins[10,13,24], porous nucleants can also facilitate salt crystallization, if the crystallization cocktail or protein buffer contains significant levels of salt. Approaches for dealing with this occurrence during screening and optimization are detailed in the Troubleshooting section.

EXPERIMENTAL DESIGN

Sample checking for crystallizability. The protocol begins with a description of routine approaches for assessing protein quality. Crystallographic studies have stringent requirements with respect to protein purity; it is essential that protein preparations be examined to ensure that they contain the single protein of interest rather than a mixture of proteins. SDS-PAGE electrophoresis is the most standard technique for assessing sample purity and separates proteins, in the form of bands, on an acrylamide gel. This separation is based on the ability of proteins to move within an electrical current (which is a function of the length of their polypeptide chains or of their molecular weight). Several protocols are

available, the most popular being the Laemmli[35] system which uses Tris-glycine gels comprised of a stacking gel component and the resolving gel with varying acrylamide gel percentages. A purity of 90–95% is recommended[28]. Generally, a major band on a Coomassie blue stained gel is considered indication of sufficient protein purity for crystallization (Fig. 5).

Some laboratories are also equipped with Dynamic Light Scattering (DLS) apparati. DLS is a non-invasive technique that can be used to assess the monodispersity of a sample, an indicator of homogeneity. A monodisperse sample (i.e. a sample composed of either a monomeric or a single-size oligomeric particle population) is often, but not always, an indicator of good crystallization propensity[36,37] (Fig. 6).DLS requires protein solutions at concentrations (<1 mg/ml) that are not nearly high enough for crystallization or precipitation. Size-exclusion chromatography (SEC) is a powerful technique that can complement or even supplant DLS. An assessment of a chromatogram's peaks can provide sufficient information as to the monodispersity and aggregation state of the protein sample. A single, narrow peak, devoid of 'shoulders' and corresponding to the expected molecular weight of the monomer (or of a specific oligomer) is often satisfactory indication of a good quality sample[38].

Protein Concentration. The protein concentration typically required for crystallization experiments is 5–10 mg/ml. It is not always possible to have a solution that is that concentrated, however, in which case one should endeavor to concentrate the protein sample to the highest possible concentration. As an amount of protein can be retained in the filter during filtration (see relevant paragraph below) or it can precipitate while the solution is being concentrated, the final sample concentration should be measured by spectrophotometry.

Protein concentration can be determined with good accuracy from absorbance at 280 nm using the formula $C = A/b \cdot \varepsilon$, where C is the protein concentration, b the path length in cm (generally normalized to 1 cm) and ε the extinction coefficient, given by: $\varepsilon = (nW \times 5500) + (nY \times 1490) + (nC \times 125)$, where nW, nY and nC are the numbers of tryptophan, tyrosine and cysteine residues, respectively, in the protein.

If the buffer present in the original protein solution is not suitable for crystallization, buffer exchange can be performed during the concentration step. The ultimate goal is to obtain a protein solution with a low (10–20 mM) concentration of a buffer in which the protein is stable (e.g. Tris or HEPES for slightly basic/neutral pH, sodium cacodylate or acetate for acidic pH). A low (20–50 mM) salt concentration may also help to keep the protein soluble[39].

Filtration. Filtration is performed prior to commencing crystallization trials to remove contaminating particles within the protein sample (dust, protein aggregates, fungi, bacteria, etc.). Performing this procedure reduces the amount of nucleation centers, which leads to the growth of fewer, larger crystals. When screening for crystallization conditions, it is preferable to perform filtration through a 0.22-µm cut-off filter. This is the conventional approach to filtering for protein crystallization[40], and it is beneficial to the reproducibility of 'leads' (see definition in the next paragraph) obtained during screening. When performing optimization trials (such as inserting nucleants into metastable protein solutions) greater stringency is necessary, so as to substantially increase the probability that nucleation events are facilitated by the added nucleants. In this instance filtering through a 0.1-µm cut-off filter is advised[41].

Initial screening. The first step in any crystallization experiment is to expose the protein to be crystallized to a wide range of chemical mixtures, in order to find conditions that yield microcrystalline suspensions, clusters or even ordered precipitate, and which are referred to as 'hits', or 'leads'. Such 'leads' point to conditions that may be conducive to crystallization. This step is called screening and is usually performed using commercially available screens (see below for their characteristics) and automated robotic systems. A wealth of commercial screens exist and the question of how many to use in order to maximize output is still debated[42,43]. Laboratories and large consortia that have access to robotic equipment tend to use as many screens as possible. However, an efficient way for individual laboratories is to start with two 96-condition commercial screens that are as diverse as possible in terms of the chemical cocktails they contain[44]. Most commercially available screens are sparse-matrix screens, designed to sample conditions that have been successfully used to crystallize other proteins. Possible alternatives are incomplete factorial screens, where a pre-determined parameter space is sampled in a non-exhaustive but more systematic fashion than sparse-matrix screens. If pure protein is only available in very small amounts and some prior crystallization information (e.g. of highly homologous proteins) exists, customized screens around conditions similar to the known crystallization conditions are applied.

The use of stabilizing ligands that specifically interact with protein molecules should be considered when screening. Interaction with these ligands reduces the flexibility of the protein, producing a tighter folding and greater conformational homogeneity. Three general approaches exist to ligand-based protein stabilization: (i) co-crystallizing the protein of interest with an inhibitor, substrate analog or other compound or ion known to interact with the protein; (ii) using a commercially available or custom-made additive screen[45] in the hope that one (or more) of the additives will stabilize the protein; (iii) using an initial screen, such as the Silver Bullets[46] or the Morpheus[47], which is based on a Protein Data Bank search of

ligands that have been observed to bind specifically to proteins and which, therefore, may have helped to stabilize them.

Screening trials can be set up using commercially available crystallization robots[48] or manually, using vapor diffusion[28], microbatch[49], counter-diffusion[50] or microfluidic platforms[51]. In this protocol we focus on vapor diffusion and microbatch, as they are the most widely employed approaches in the field. For vapor diffusion trials, the robots will typically dispense 100–400 nl of crystallization mixture, which is air tightly sealed inside an experimental chamber in the presence of a reservoir containing 60–100 µl of screen solution. The crystallization mixtures consist of protein stock solution mixed with screen solution at different protein: screen solution ratios, Manual screening typically uses 0.4–1 µl of protein solution and 300 µl–1ml of screen solution per trial. For microbatch trials, protein stock solution is mixed directly with screen solution. The screen solution volume is typically the same as the protein solution volume, in the 50-200 nl range. The standard hanging-drop vapor diffusion technique is most suitable for manual trials, whereas sitting-drop vapor diffusion and microbatch are optimal for small-volume automated liquid handling.

Once a lead is identified, optimization can be performed by fine-tuning of the crystallization conditions (varying the concentration of the protein, type and concentration of precipitant, pH, temperature, and introducing additives). This fine-tuning may improve the quality of crystals to some extent, but, more often than not, additional work is needed to reach the desired results as described below.

Determination of the metastable zone of the crystallization phase diagram. In order to produce crystals the protein solution needs to be in a supersaturated state. Crystal nucleation, however, requires higher levels of supersaturation than those ideal for growth. This phenomenon stems from the free energy barrier that must be overcome for the formation of the nucleus–solution interface. Solutions that, despite being supersaturated, will not yield crystals within a practicable time because of a lack of nucleation events are said to be metastable. Such metastable conditions are more suitable for crystal growth, but growth can of course only take place if nuclei are introduced into the solution or if nucleation is somehow induced. Growth at metastable conditions can lead to better diffracting crystals, for two reasons: (a) the lower the supersaturation the slower the crystal growth, thereby leading to better ordered crystals[52,53] (note that conditions below metastability are undersaturated and therefore cannot support crystal growth) and (b) uncontrolled spontaneous nucleation cannot take place in a metastable solution, thus avoiding formation of an excessive number of crystallization nuclei, which would compete for a limited amount

of protein. These principles lie at the basis of every attempt at nucleation control via heterogeneous nucleation-inducing substances or other methods.

As crystallization is a phase transition process, it can be represented by a phase diagram (Fig. 7). In a crystallization phase diagram, metastable conditions are separated from those where nucleation takes place spontaneously by the so-called supersolubility curve, which is generally much easier to determine and in practice more useful for designing crystallization and nucleation trials than the solubility curve. The most practical way to draw the supersolubility curve is by setting up a series of crystallization drops to cover a grid of conditions around some known crystallization condition (lead). In doing so, a 'working' phase diagram is created (Fig. 8), where the protein concentration is mapped against that of the primary precipitant or against some other variable, such as the pH. The resulting grid should be wide enough to cover conditions of spontaneous nucleation as well as conditions at which the solution remains clear. It should also be narrow enough for the boundary between the two conditions to be sufficiently well defined. Knowledge of the position and behavior of the supersolubility curve means that the conditions that cause a protein solution to be metastable with respect to nucleation can be estimated when the supersolubility curve has been determined, therefore, metastability can be exploited by the use of nucleants.

Although a proper phase diagram has protein concentration as one of its variables (since protein solubility and supersolubility are defined as protein concentrations at equilibrium and at spontaneous phase transition, respectively), if the amount of available protein is scarce, it can be more practical to construct a 'working phase diagram' using an alternative parameter to protein concentration. This parameter should be the concentration of a precipitating agent, since it is an easily varied parameter to which crystallization is extremely sensitive. This parameter can be mapped against, for example, the pH or the concentration of a secondary ingredient (additive), both of which are parameters that crystallization systems are also very sensitive to and which can also be easily varied. The resulting curve on such an alternative diagram would not strictly speaking be a supersolubility curve, but would play the same role in determining the boundary between metastable and spontaneous nucleation conditions.

Introduction of nucleants. The selected nucleant should be introduced in a clear protein solution, but one whose conditions are sufficiently near in terms of supersaturation to the situation where crystals are known to spontaneously nucleate. As described above, such conditions are to be found just below the supersolubility curve of the crystallization phase diagram (marked with arrows in Fig. 8). New drops are set up at the selected metastable conditions (also see an alternative application under "Further application of the method: use of nucleants in screening" below) and the nucleant is introduced as described in the

Procedure. Solid nucleants such as Bioglass are introduced using forceps or an acupuncture needle, ensuring that a single grain is inserted[10]. With a little practice, this procedure can be performed efficiently within a matter of seconds (Supplementary Video1). Semi-liquid nucleants such as MIPs are dispensed to trials like an additive using a pipette[13].

Harvesting crystals. A common cause of concern is how to harvest nucleant-grown crystals and whether nucleant presence interferes with subsequent diffraction. This concern is only relevant when dealing with solid nucleants. In most cases, crystals disengage from the nucleant surface during growth or harvest with a cryo-loop. In the rare occurrence of a crystal being attached firmly to a nucleant, an acupuncture needle can be used to gently pull the nucleant away. Occasionally a small fragment of nucleant can remain adherent to the crystal surface. In such instances, the nucleants utilized in this protocol do not adversely affect diffraction.

Further application of the method: use of nucleants in screening. Nucleants are used mostly during optimization, but they can also be used during screening[13,54]. When nucleants are placed in supersaturated protein solutions, crystals will usually appear faster than in their absence, even at conditions of spontaneous nucleation. Furthermore, several screen conditions happen to be metastable at the protein concentration present in the sample. Several screen samples that would, therefore, remain clear (and be discarded) under conventional screening, would produce crystals if nucleants were added to them. Nucleants can therefore be added indiscriminately to all conditions of a screen[54].

The automated introduction of solid nucleants to screening trials poses a technical challenge. The creation of a nucleant suspension is the best solution, but it requires the determination of an optimal serial dilution. MIPs, on the other hand, have a viscous consistency and can be dispensed by robots similarly to PEG solutions.

MATERIALS

This Protocol assumes the availability of a dilute solution of purified protein to be crystallized. Methods to obtain such pure protein solutions, which are not the object of this Protocol, are detailed in numerous general and protein-specific protocols[55-57] and are in themselves an active field of scientific research.

Reagents

A dilute solution of purified protein to be crystallized. The solution composition is protein-specific, but should in general contain a low concentration (10-20 mM) of buffer in which the

protein is stable (often Tris, HEPES, or sodium acetate) and a low concentration (20-50 mM) of salt (often sodium chloride) in order to maximize its solubility, as discussed in the "Protein concentration" section of Experimental Design.

SDS-PAGE analysis

Acrylamide/bisacrylamide 30% (wt/vol) solution 29:1 (Sigma-Aldrich, cat. no. A3574)

SDS 10% and 20% (wt/vol) solutions (Sigma-Aldrich, cat. nos. 71736 and 05030)

Ammonium persulfate (Sigma-Aldrich, cat. no. A3678)

Tetramethylethylenediamine (TEMED; Sigma-Aldrich, cat. no. T9281)

Tris-HCl (Sigma-Aldrich, cat. no. T3253)

Dithiothreitol (DTT) or 2-mercaptoethanol (BME) (Sigma-Aldrich, cat. nos. 43817 and M6250)

Glycerol (Sigma-Aldrich, cat. no. G5516)

Coomassie brilliant blue R (Sigma-Aldrich, cat. no. B8647)

Glycine (Sigma-Aldrich, cat. no. G8898)

Molecular Weight Markers (BIO RAD, 161-0317)

EQUIPMENT

Crystallization Screens

A selection of the following screens should be acquired, depending on the availability of protein and on the time and funds that can be devoted to your project. This list is by no means comprehensive and there are a wealth of alternative commercial screens available from a variety of companies. Two screens are a usual minimum. See also discussion under the Initial screening section of Experimental Design**.**

Molecular Dimensions Ltd: Structure Screen 1 MD1-01, Structure Screen 2 MD1-02, Morpheus® MD1-46, Clear Strategy™ Screen I MD1-14, Clear Strategy Screen II MD1-15, PGA Screen™ Premixed MD1-50, MemGold™ MD1-39, MemStart™ MD1-21, ProPlex MD1-38.

Hampton Research: Crystal Screen HR2-110, Crystal Screen 2 HR2-112, Index HR2-144, PEG/Ion Screen HR2-126, PEG/Ion 2 Screen HR2-098

Qiagen Screens: NeXtal Tubes JCSG Core Suite I 130724, NeXtal Tubes JCSG Core Suite II 130725, NeXtal Tubes JCSG Core Suite III 130726, NeXtal Tubes JCSG Core Suite IV 130727

Crystallization plates

Linbro: Molecular Dimensions Limited 24-well XRL Plate MD3-11.

Easy –Xtal with screw caps (Qiagen 15 well tool X-seal 132008)

96-well sitting drop (The MRC Crystallization Plate ™ MD11-00-100)

HLA plates Nunc ™ 72 well 470378

Oils for microbatch

Paraffin oil (BDH GPR ™ 294365H)

Silicone oil (Dow Corning 200/1 cs BDH GPR 630024N)

Nucleants

Naomi's nucleant (Molecular Dimensions, MD2-07)

Hydrogel based Molecularly Imprinted Polymers (MIPs): to be imprinted with your protein of choice (as described in Saridakis *et al*.[13])

http://www.pnas.org/content/suppl/2011/05/31/1016539108.DCSupplemental/pnas.1016539108_SI.pdf.

Filters

0.1-µm filter (Millipore UFC30VV00)

0.22-µm filter (Millipore UFC30GV00)

Amicon® Ultra Centrifugal Filters: 3,000 MWCO UFC500396, 10,000 MWCO UFC501096, 30,000 MWCO UFC503096, 50,000 MWCO UFC505096, 100,000 MWCO UFC510096

Crystallization Robots

Any crystallization robot can be used for this protocol. In our laboratory we utilise;

TTP Labtech's Mosquito® LCP

Douglas Instruments Oryx 8

X-ray Diffraction

In house X-ray generator, detector and cryocooling equipment (in our laboratory Rigaku 007HF-M X-ray generator operating at 40 kV and 30 mA with VHF optics, Saturn 944+ CCD detector and Oxford Cryosystems 700 liquid nitrogen cryostream).

Cryoloop ™ Hampton Research, Magnetic CryoVials with Plain CryoCaps MD7-404.

Access to synchrotron radiation is desirable.

Instruments

Liquid handling robot (Tecan group liquid handling and Robotics Freedom EVO® 75)

Dynamic Light Scattering (DLS, Zetasizer Nano S, Malvern)

Microscope (Leica M165C)

Incubator (Qualicool LTE Scientific)

Spectrophotometer (Nanodrop ND-1000)

Temperature-controlled benchtop centrifuge (Eppendorf, 5417 R)

Other

CleneGlass Siliconized Cover slides Si 22mm round 1000 No 2 Glass MD4-04

Forceps. Any stainless steel fine tip forceps for insertion of solid nucleants

Acupuncture needle, any type available

Air duster (Office Depot 1513823)

Crystal Clear Sealing Tape (Duck**,** HP260)

PROCEDURE

Please find a flow diagram of the Procedure in Fig. 9.

Protein characterization **TIMING (24 h)**

1 *Sample Preparation:* to characterize the protein solution sample via SDS gel electrophoresis[35], prepare the sample to a final concentration of 2 mg/ml protein with 1% SDS, 10% glycerol, 10 mM Tris-HCl, pH 6.8, 1 mM ethylene diamine tetraacetic acid (EDTA), a reducing agent such as dithiothreitol (DTT) or β-mercaptoethanol, and a pinch of bromophenol blue to serve as a tracking dye (~0.05 mg/ml). Heat the samples to at least 60°C for 10 minutes to allow SDS to bind in the hydrophobic regions and complete the denaturation. .

Critical Step: Avoid heating the protein sample to the boiling point, as it can cause protein aggregation. Insufficient heating may result in incomplete denaturation, as such a trial and error series of experiments may be required to achieve the best results. Although heating is not necessary when preparing some protein samples, this procedure is essential when preparing samples of membrane proteins.

PAUSE POINT: Once denatured, the samples can sit on a bench top for a few hours. They need to be kept frozen at –20 °C if they are to be used at a later date. Denatured samples can be stored safely at -20 ºC for several years.

2 *Gel Preparation and Assembly:* The separating and stacking gels can be purchased commercially or prepared by mixing 40% Acrylamide, gel buffer (comprising 3M TRIS, 10% w/v SDS and 60mM DTT), TEMED and 10% Ammonium persulfate as described in reference 35. The assembly of the gel running stand itself varies with the type of apparatus. Ensure the top of the cassette is continuous with the upper buffer chamber and the bottom with the lower chamber so that current will run through the gel itself. The cassette must be sealed in place using gaskets or a sealant such as agarose. The upper and lower buffer compartments are filled with a running buffer typically consisting of 25 mM Tris pH 6.8, 192 mM glycine and 0.1% w/v SDS.

3 *Gel Loading and Gel running:* Load 5-10 µl of this sample into the well of the gel using a Hamilton syringe. The glycerol in the sample causes it to sink neatly to the bottom of the well. The correct amount of protein to load depends on the distribution of individual proteins in the sample. Load 5 µl of the reference molecular weight marker sample into another well. Connect the anode to the bottom chamber and the cathode to the top chamber. Gels are usually run at a voltage of about 150 volts.

Critical Step: Please note that sample overloading results in precipitation and aggregation of proteins, producing streaks and smears. Under loading may lead to the detection of only the most abundant of polypeptides. A rule of thumb for mini-slab gels is to load about 0.5 micrograms of protein per expected band. Since complex mixtures contain proteins of widely varying concentrations, there is no ideal single amount to load.

4 *Disassembly and Staining:* When the dye front is nearly at the bottom of the gel it is time to stop the run. For low percent gels with a tight dye front, the dye should be on the verge of running off the gel. Stain the gel with 0.1% Coomassie Blue dye in 50% methanol and 10% glacial acetic acid. Leave the gel overnight whilst agitating to ensure uniform staining.

5 *Destaining and Storage:* Agitate the gel in 50% methanol and 10% acetic acid overnight, followed by agitation in 7% methanol and 10% acetic methanol for 4 to 24 hours to complete destaining. Dry the gel and scan it for later analysis.

Critical Step: Please note that protein purity should typically be above 95% for optimum results.

Dynamic Light Scattering **TIMING (5 min)**

6 (OPTIONAL) If DLS equipment is available, check monodispersity of the sample (see Fig. 6) using instructions provided by the equipment supplier.

Protein concentration **TIMING varies depending on the final concentration required, typically 20–40 min**

7 Once the centrifuge rotor is in place and fixed, close the lid and cool the centrifuge to 4^{o} C.

8 To remove any excessive cellulose or other residual material adhering to the filter membranes before concentrating the protein, pipette 500 µl of whichever buffer the given original dilute protein sample is dissolved in, so as to cover the entire length of the membrane .

9 Place the filter in the rotor along with a counter balance and spin for 5 min at 2000 g and at 4^{o} C. Increase the run time if buffer still remains on the membrane.

Load the dilute protein sample. Set the run time and speed of the centrifuge and spin the protein solution to the desired concentration. As mentioned under Experimental Design, the protein concentration typically required for crystallization experiments is 5–10 mg/ml. If low solubility of the protein does not allow such concentrations to be achieved, one should endeavor to concentrate the protein sample to the highest possible concentration. Experience acquired during the protein isolation/purification stage should help deciding on a realistic goal concentration, but chances have to be taken at this stage and if prior information is totally lacking and the protein proves rather insoluble, it can be expected that some protein may precipitate and be lost.

Critical Step: Gauge the amount of protein filtering through at low speed. This is a trial and error process with a 1 min spin at 2000 g providing a good starting point to judge the optimal speed.

10 Measure the final protein concentration using a spectrophotometer (absorbance at 280 nm, see relevant discussion in the Experimental Design).

Troubleshooting

PAUSE POINT: Most proteins are stable enough so as not to require immediate continuation to the next step. Stability can be very variable though. Overnight storage at 4°C is almost always safe. Most proteins can withstand storage for a couple of weeks at -20°C. Other possibilities, such as storing at 4°C for a few days or "flash freezing" in liquid nitrogen and storing at -80°C for several weeks are frequently possible, but, if the behavior of the given protein is not known beforehand from other work, there is always a risk involved. Repeated freezing/thawing of the sample is not advisable.

Filtration TIMING 2–3 min

11 Filter the protein solution obtained in step 11 through a 0.22 µm–cutoff filter. This filtration is performed in a benchtop centrifuge at 2–9 g for 2 min at room temperature (20˚C in our laboratory).

Critical Step: It is essential to filter at least 40 µl of protein solution so that hardly any protein is lost during filtration. It is advisable to skip this step if your protein is scarce or 'sticky'.

Screening TIMING ~1 h to manually set up a 48-condition screen, ~2–10 min to automatically dispense a 96-condition screen, ~3–4 weeks for observation of the trials

12 Select commercially available 48 or 96-condition sparse-matrix, incomplete factorial or customized screens and set up trials using the crystallization method of your choice (vapor diffusion[28] or microbatch[49], as described in the Initial screening section of Experimental Design. Each well in the screen will consist of a different solution, to which a drop of protein solution from step 11 will be added. The trials should be incubated, usually at either room temperature or 4°C. Depending on protein availability, trials can be set up more than once, so that they can be incubated at different (usually two) temperatures.

Critical Step: Some proteins, membrane proteins in particular, require incubation at more than one temperature. If possible, incubate the trials in a temperature-controlled environment, as fluctuations can hinder reproducibility.

13 Observe the drops under a light microscope or with an automated visualization system at regular intervals after setup. Perform one observation a day for the first 2–4 days, then once a week is sufficient.

Critical Step: It is important to observe the trials at time 'zero', to ascertain the protein's immediate response to the various crystallization cocktails.

Troubleshooting

14 Select 'hits', namely conditions that give some indication of crystallinity: typically clusters of microcrystals, ordered precipitate or even phase separation[42].

PAUSE POINT: It is at this stage that optimization trials are commenced if high quality crystals are not obtained directly from screening. There are a variety of well documented optimization methods available for the different scenarios encountered (including excessive nucleation, a lack of nucleation, crystal twinning, poor diffraction etc.[58-60]). The optimization approaches specific to this protocol include filtration, the determination of metastability and the exploitation of metastability for crystal optimization through the use of nucleants..

Filtration **TIMING 2–3 min**

15 (OPTIONAL) Filter the protein through a 0.1 µm–cutoff filter following the directions in Step 11.

Determination of the Metastable Zone **TIMING (protein-specific, typically 30 min to manually prepare a 24-condition matrix grid, ~1 week to draw a working phase diagram).**

16 Note the conditions in one or more hits of a screen. If several hits are obtained, select the visually most promising ones. Please note that diffraction analysis is the preferred means of determining the quality of a lead.

17 Dispense (manually or by a robot) 10–24 trials using a crystallization method of your choice, varying the protein and precipitant concentrations (or another useful pair,

such as the precipitant and additive concentrations or the precipitant concentration and the pH or temperature) in steps on a matrix grid, centering on the selected 'hit' conditions, see **Box 1**.

Critical Step: The concentrations of protein and precipitating agents (or alternative pair of parameters to which solubility is sensitive) should be above and below the concentrations that gave the conditions of the hit.

Critical Step: When performing heterogeneous nucleation trials it is of utmost Importance to ensure that the cover slides (onto which the crystallization trials are dispensed) and plates are free from any debris (air borne, residual from fabrication etc.). An air duster is invaluable in this regard.

18 Plot the results to obtain a crystallization phase diagram, focusing on the supersolubilty curve (Fig. 8), or curve that separates the clear drops from those where spontaneous nucleation took place. Please note that the area just below the supersolubility curve is the metastable zone. Pink squares represent conditions in the labile zone, yielding crystals (at moderate supersaturation), crystalline precipitate or amorphous precipitate (with increasing supersaturation), whereas blue diamonds represent clear drops. The arrows indicate optimal conditions for nucleant insertion. Those clear drops that are sufficiently close to the supersolubility curve correspond to the metastable.

BOX 1. DETERMINING METASTABILITY

If, for example, a screen produced 'hits' at a protein concentration of 10 mg/ml in 18% (wt/vol) PEG of average Molecular Weight 4000, the experiments are set at concentrations ranging from 7 mg/ml to 15 mg/ml in protein versus 12–20% PEG 4000, thus covering a range of conditions above and below the 'hits'. In theory, in true metastable conditions the solutions remain clear for an indefinite period of time if left undisturbed. In a real experiment however, time is limited, and therefore, if crystals have not appeared within ten days to two weeks, these conditions are considered metastable.

Insertion of nucleants **TIMING (~20 s to manually introduce to each trial, 3–10 min to automatically incorporate liquid nucleant to a 96-well plate, depending on the robot used).**

19 Insert the nucleant at selected metastable conditions (see step 18). For each condition, set up a number of crystallization drops to which the nucleant will be introduced. The number of crystallization drops set up for each metastable condition depends on how scarce the protein is (triplicate would be ideal). The method of insertion depends on the type of nucleant used.

Critical Step: Dispensing metastable crystallization drops in triplicate on a single cover slide (equilibrated against a single reservoir) saves resources. This is not advisable for trials containing membrane proteins however as the presence of the detergent reduces surface tension, causing the drops to spread.

20 Add the nucleant to the crystallization drops according to option A, if the nucleant is a solid, or option B, if it is a MIP. Please refer to Box 2 for additional tips on nucleant handling.

BOX 2. MORE NUCLEANT HANDLING TIPS

- *For vapor diffusion drops it is important to insert the nucleant quickly, to minimize mechanical disturbance and exposure of these trials to air.*
- *Use only ONE grain/flake per drop. Failing to do this will result in excessive nucleation!*
- *Remember to wipe the forceps clean between trials.*
- *The positioning of the nucleant within the drop has little impact on the outcome of the trial.*
- *Do not worry if bubbles are observed in the crystallization drop following the insertion of Bioglass. They disappear within a few minutes.*
- *In Microbatch trials it is preferable to insert the nucleant prior to the addition of the oil to ensure the presence of the nucleant within the drop.*
- *Observe at less frequent intervals (e.g. weekly) since control of nucleation requires minimal mechanical disturbance of the trial.*

a. Adding a solid nucleant to the crystallization drops:

Pick up a single grain (as in the case of Bioglass) or a small flake (as in the case of nanoporous gold) using a fine tip forceps (refer to Fig. 10 and Supplementary Video 1) and insert it into the crystallization drop. Please note that it can be helpful to tip a few grains onto a clean glass coverslip to aid picking up a single grain. Return any unused grains back into the vial. If the grains prove difficult to grasp, it helps to wet the forceps in the reservoir solution. All the grains are much smaller than even a

nanolitre-scale crystallization drop, but their individual sizes vary appreciably. The size of grains does not influence the effectiveness of the nucleant.

Troubleshooting

b. Adding MIPs to the crystallization drops:

When working with a semi-liquid nucleant (MIPs), add to each crystallization drop a volume of nucleant that is ~1/10th of the overall drop volume to prevent excessive dilution.

Incubation and Further Monitoring **TIMING (protein specific, up to 2 months, albeit faster than equivalent trials without nucleants).**

21 Once the nucleant has been inserted, incubate the trial as described in Step 12 and observe at regular intervals for evidence of crystallization as described in Step 13.

Troubleshooting

TIMING

Protein Characterization (steps 1-6): 24 h

Protein Concentration (steps 7-10): 20–40 min

Filtration (step 11 and 15): 2–3 min

Screening (steps 12-14) manual: 2 h, automated: 2–10 min/screen

Determination of the Metastable Zone (steps 16-18): 30 min

Nucleant Insertion (steps 19 and 20) manual: 20 s/trial, automated: 3–12 min/screen

Incubation and Monitoring (step 21): up to 2 months

TROUBLESHOOTING

Troubleshooting advice is detailed in Table 1.

ANTICIPATED RESULTS

The purity of the starting protein sample is one of the most crucial factors that will determine the success of macromolecular crystallization in general and accordingly this Protocol. The presence of various kinds of impurities (such as proteolytic fragments of the protein, misfolded protein molecules, random oligomers, or even unrelated proteins that co-elute with the protein of interest) are frequently observed to prevent nucleation during screening, or to provoke inherent, severe limitations to crystal quality that cannot be overcome by any optimization procedure. The presence of impurities that may be deleterious to crystallization can be assessed by gel electrophoresis and Dynamic Light Scattering, as described in Steps 1-5 and Fig. 5, and Step 6 and Fig. 6, respectively. These are also discussed in the "Sample checking for crystallizability" section of Experimental Design.

The unpredictability and irreproducibility associated with crystallization precludes guarantees of success when crystallizing a target protein. There is no *a priori* way of knowing if a protein will crystallize, what conditions it will crystallize in and how long the process will take. What we can however confirm is that we have had considerable success utilizing this protocol to crystallize target proteins, both in terms of optimizing crystal quality and obtaining new leads from screening experiments[9-13,54]. The targets mentioned include globular proteins, peptides, protein complexes and membrane proteins. The porous nucleants utilized in the present protocol have reproducibly enabled crystal growth at lower supersaturation than previously possible (leading to improved crystal order[10,13]). We stress the importance of ensuring a single nucleant grain being added in this case. Nucleants have also been beneficial when introduced to screening trials, enabling the discovery of new 'hits' that would have been missed in their absence. The troubleshooting section details the alternative approaches required should one experience difficulty. The protocol described is not limited to the application of Bioglass and MIPs and can be utilized for most other heterogeneous nucleating templates.

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FIGURES

Figure 1: Illustration of the ~18% success rate in obtaining diffraction quality crystals from purified protein. Results compiled from leading Structural Genomics centers and Protein Science Initiatives worldwide on 8/09/13 (Target Track PSI).

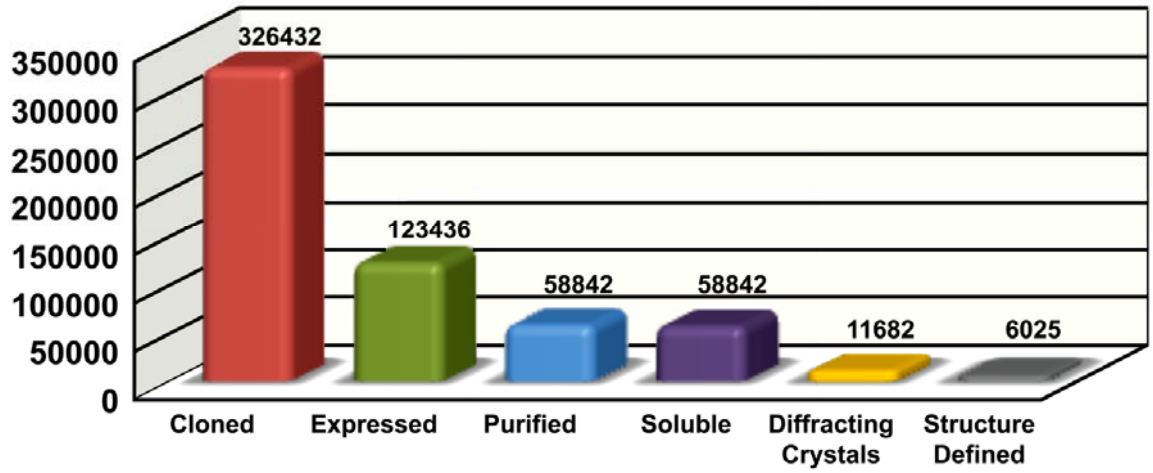

Figure 2: A Light microscope image of *Human Cardiac Myosin Binding Protein C crystals growing from a carbon nanotube nucleant at metastable conditions. Scale bar corresponds to 150 µm (reprinted with permission from reference 11, Asanithi et al. Carbon nanotube-based materials for protein crystallization. ACS Applied Materials & Interfaces 1:1203-1210, Copyright 2009 American Chemical Society).*

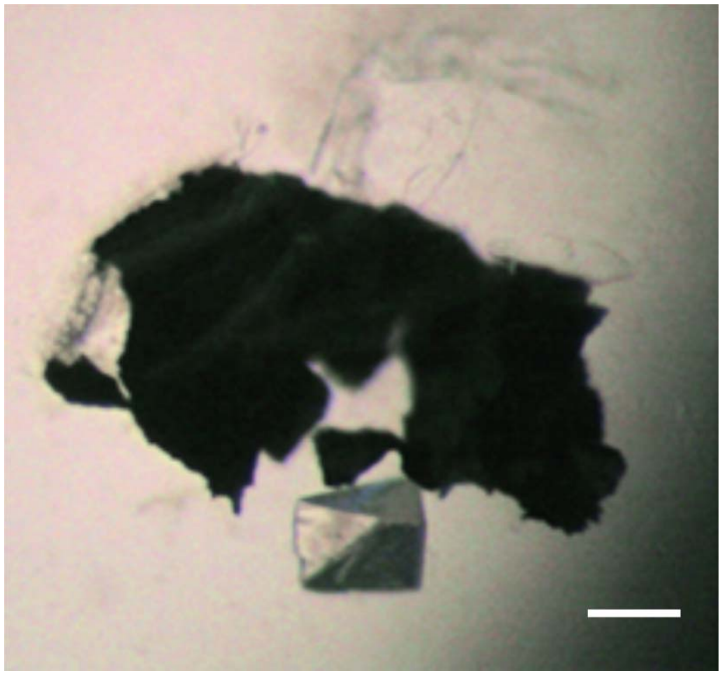

Figure 3: The porous nature of Bioglass and its suitability for protein crystallization. (a) Scanning electron micrograph of Bioglass showing its porosity (reprinted from reference 14 with permission of John Wiley and Sons, Inc. copyright 2001). Scale bar corresponds to 5µm. (b) A light microscope image of *a gguanCD crystal growing from the Bioglass surface at metastable conditions. Scale bar corresponds to 100 µm. The Bioglass grain is indicated by the arrow.*

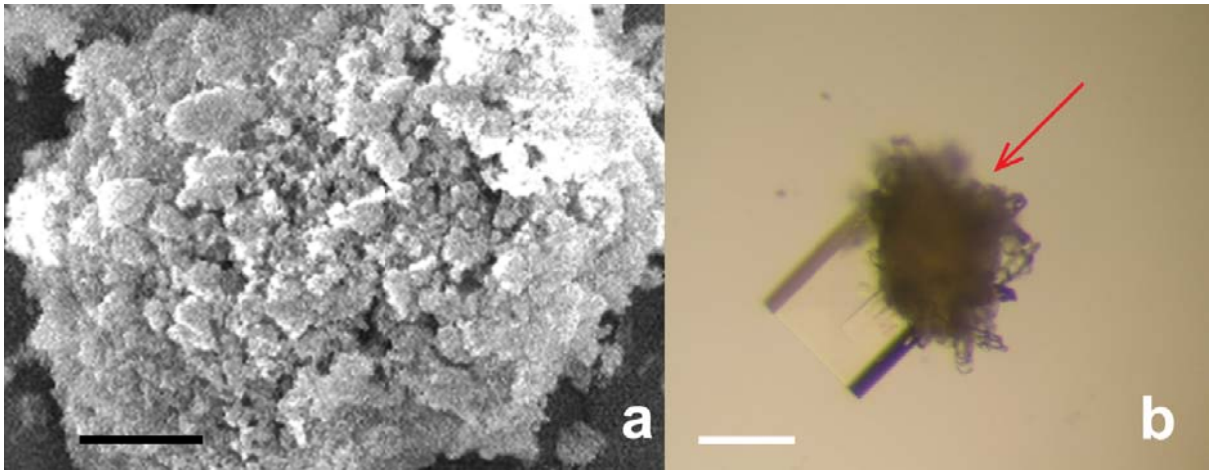

Figure 4: A light microscope image of spear shaped Human Migration Inhibitory Factor (MIF) crystals growing from a MIPs nucleant surface. MIPs have a translucent gel-like appearance. When added to crystallization drops they can spread out and fragment. The MIP is indicated by the arrow. Scale bar corresponds to 100 µm (reproduced with permission from reference 13).

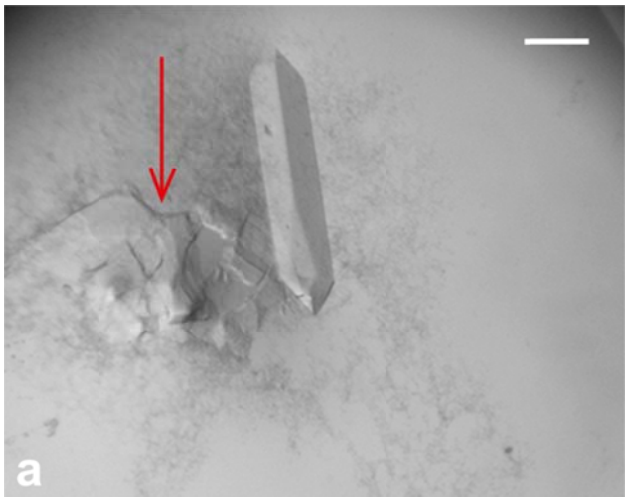

Figure 5: Illustrating protein sample quality using SDS-PAGE. (a) A photograph of a gel where the protein was not of suitable purity for crystallization. Note the multiple bands within the target protein lane. (b) A photograph of a gel following further purification of the same protein, the arrow indicates a clean single band at the appropriate molecular weight.

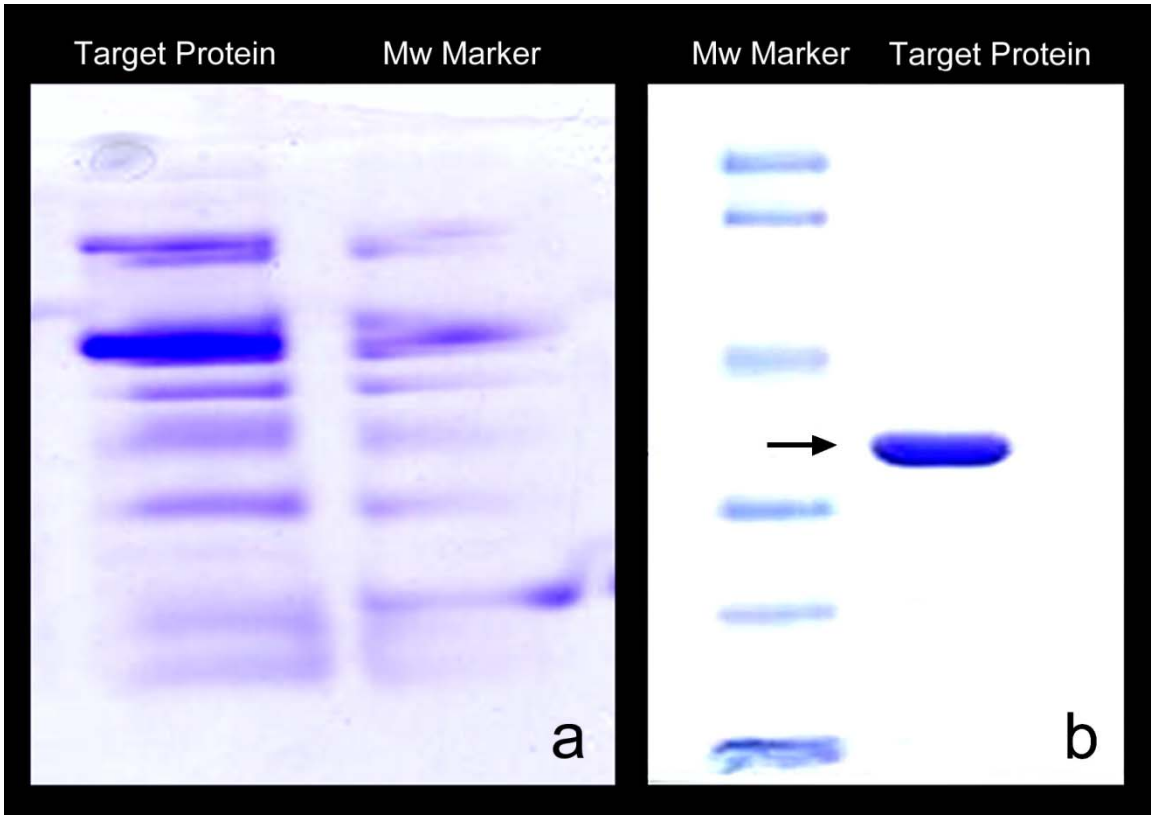

Figure 6: Monodisperse and polydisperse size distribution plots obtained using DLS when characterizing protein samples. Polydispersity (Pd) is representative of the protein particle size distribution width. If the DLS data analysis assumes a Gaussian distribution of particles, given as a single peak, polydispersity of the sample is the peak width at half its height. %Pd can be calculated by dividing Pd by mean hydrodynamic radius (R_H) and expressing as a percentage. A protein sample with less than 20% polydispersity is regarded as monodisperse. As detailed in Experimental Design, having a monodisperse protein sample is often a good indicator of crystallization propensity. The x axis is indicative of the protein hydrodynamic radius in nanometers. The y axis represents the percentage intensity fluctuation of scattered light. Adapted from Malvern.com.

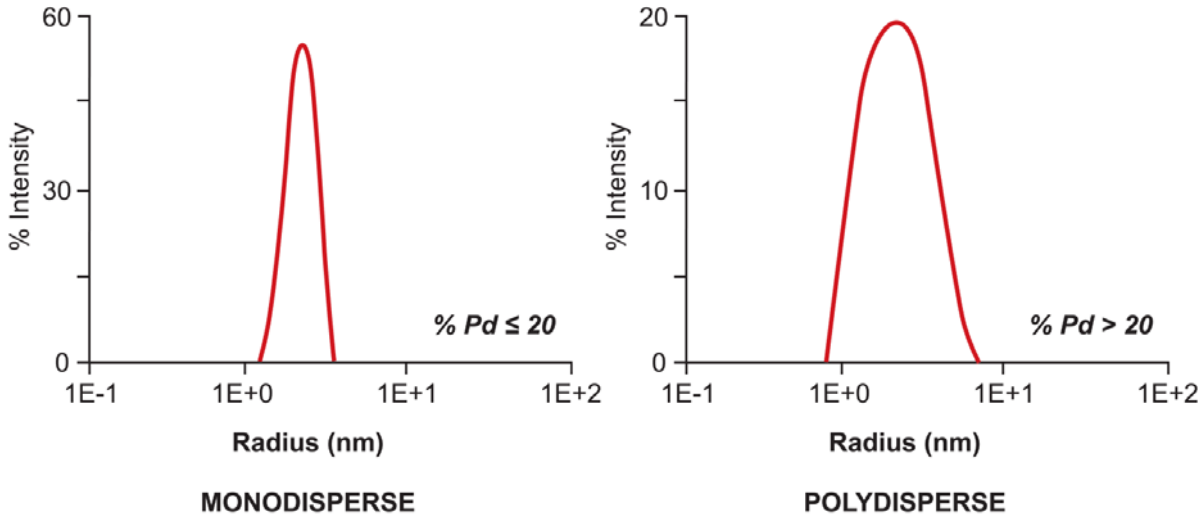

Figure 7: Schematic illustration of a protein crystallization phase diagram. The adjustable parameter can be precipitant or additive concentration, pH, temperature and so on. The solubility is defined as the concentration of protein in the solute that is in equilibrium with crystals. The supersolubility curve is defined as the line separating conditions where spontaneous nucleation (or phase separation, precipitation) occurs from conditions where the crystallization solution remains clear if left undisturbed (this figure was adapted from Fig.1 of reference 53).

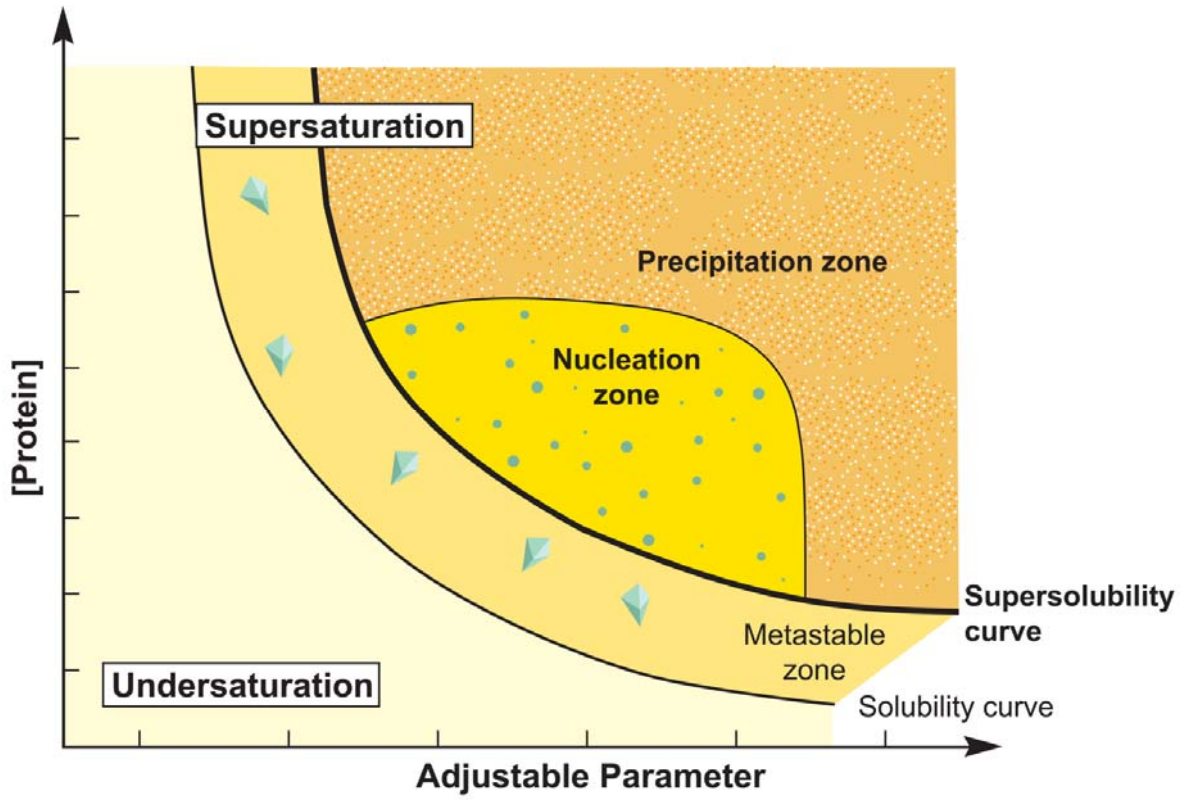

Figure 8: A Working Phase Diagram. Squares represent conditions in the labile zone, yielding crystals (at moderate supersaturation) or crystalline precipitate/amorphous precipitate (at increasingly greater supersaturation); diamonds represent clear drops; Arrows indicate suitable conditions for nucleant insertion. (adapted with permission from reference 10).

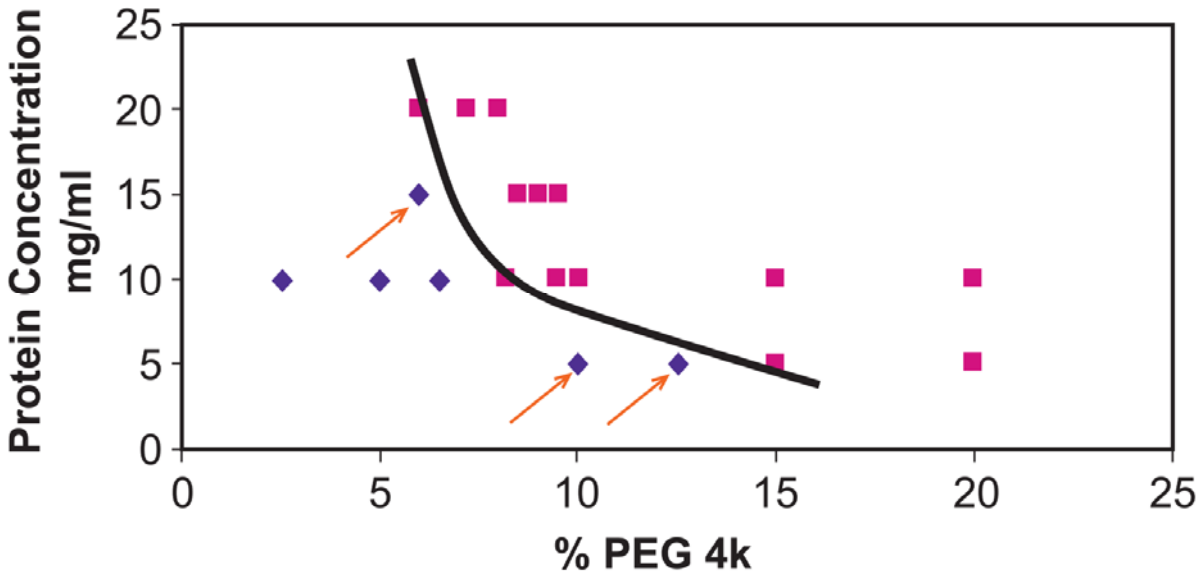

Figure 9: Flow Chart illustrating the downstream procedure involved when commencing crystallization trials.

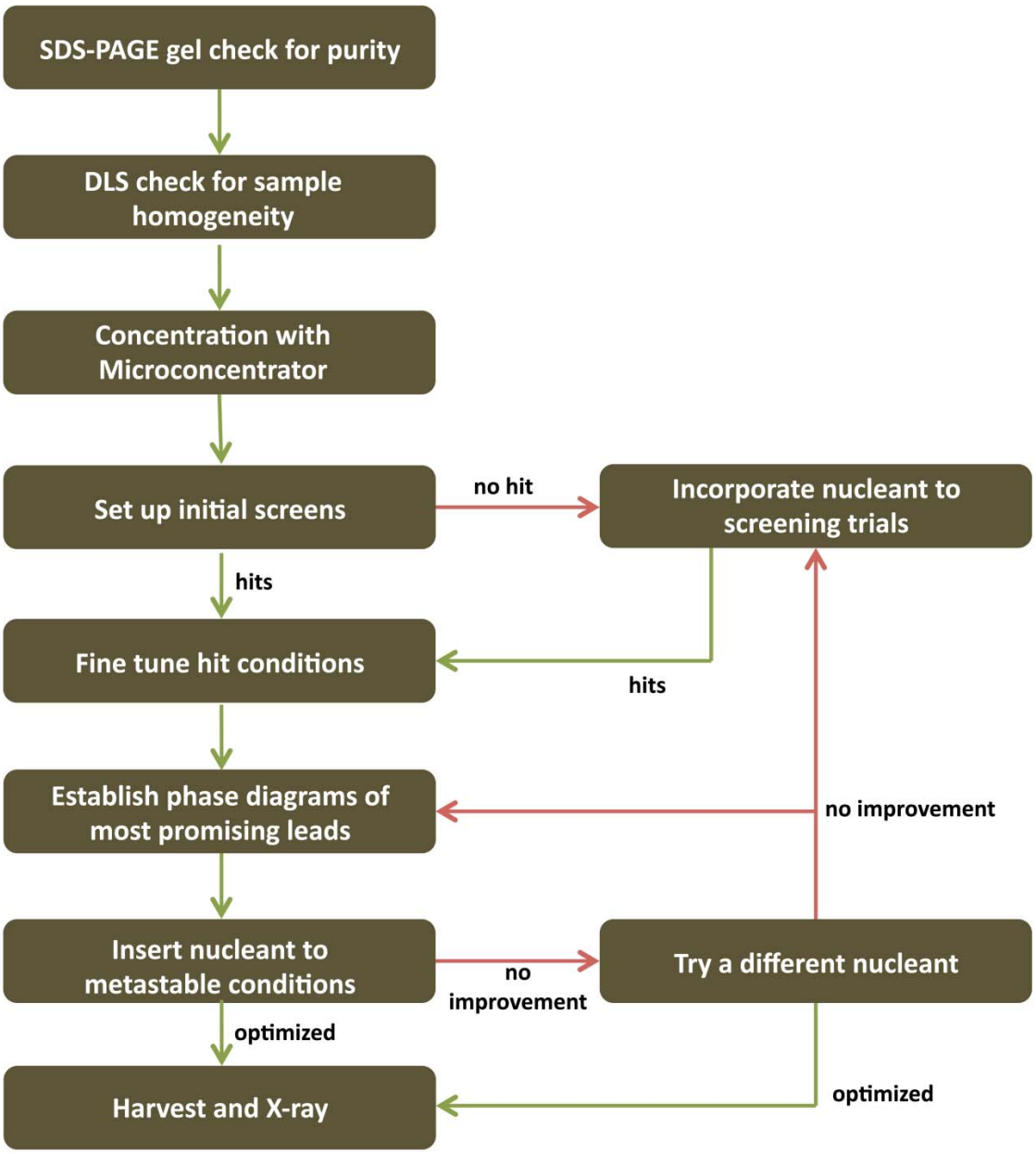

Figure 10: Technique utilized to add nucleant grains to crystallization drops. (a) crystallization droplet is dispensed, (b) vial containing nucleant is tipped slightly to dislodge a few grains and a single grain is picked up using fine tip forceps (c) addition of this single grain to a trial.

TABLES

Table 1: Troubleshooting advice for potential scenarios encountered.

Step	Problem	Possible reason	Solution
10	After concentrating the protein, very low absorbance reading at 280 nm	Protein retention by the membrane or attempted to concentrate beyond the limits of solubility of the protein, which caused a substantial amount of protein to precipitate	Choose a filter with different membrane material or molecular weight cutoff. Plan for lower final concentration. Adjust the time and speed of centrifugation on a trial-and-error basis
13	All drops remain clear during screening trials	Protein concentration too low	If possible, concentrate the protein further. If not, allow further evaporation of the trials by unsealing the trials (vapor diffusion) or reduce the amount of oil covering the trials (microbatch)
13	A precipitate is visible in all, or in the large majority of drops	Protein concentration or precipitating agent concentrations too high for the protein at hand	Dilute the protein sample or use a screen with less concentrated crystallization cocktails (e.g. Light Screens)
20a	When inserting the nucleant, several grains appear in the trial	Nucleant is very powdery and the smallest grains tend to stick together and disperse when entering the liquid	Disperse the grains first on a coverslip and pick one up with an acupuncture needle from the coverslip. Return the rest to the vial
21	Crystals appear spontaneously (i.e. in the absence of nucleant) in what was thought to be the metastable zone	Different batches of protein can behave differently. Nucleation is a stochastic phenomenon dominated by kinetics, which can cause metastable zone boundaries to shift	Conduct experiments using the same batch, aliquoting and freezing upon protein production. Set up nucleation experiments at lower precipitant concentration. Try as much as possible to avoid mechanical disturbance of trials

21	Crystals not visible	Nucleant material made of a dark wafer, e.g. porous silicon	Using the same microscope, illuminating from above or at an angle rather than using the standard setting
21	Salt crystals obtained in crystallization trials. Salt crystals are distinguishable from protein crystals by a higher than usual birefringence under polarized light, from greater resilience to mechanical pressure when probed with an acupuncture needle or, ultimately, from their X-ray diffraction pattern.	Substantial salt content in crystallization reagents/buffer. Some nucleants are particularly potent and will initiate precipitation indiscriminately	When optimizing, insert nucleant deeper into the metastable region (at lower precipitant concentration). When screening, try using a 'Light' screen'

Author contributions

SK: Designed research, performed research and wrote the paper

ES: Designed research, performed research and wrote the paper

LG: Designed and performed research

NEC: Designed research, coordinated research and wrote the paper

Competing Financial Interests

The authors declare that separate patents for the use of Bioglass and MIPs for protein crystallization have been granted. Bioglass is available as a commercial product called 'Naomi's Nucleant'. In the case of MIPs, the patent protects commercial use but does not preclude its use for scientific research.