Nucleation and seeding in protein crystallization: an old story retold

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The Crystallization flowchart

Purification and pre-screening

Gels, SEC, DLS, MS, NMR?

Screening

Nucleation and seeding

Observation

Real hits and false positives

Optimisation

Nucleation and seeding
Growing protein crystals

We have 2 basic questions to answer:

1. Is the protein crystallizable?
2. How good are the crystals?
Good or bad protein?

- Impurities
- Aggregation
- Flexible domains
- Flexible tail
- Proteolysis
- Unfolding
- „Pure“ protein
- Crystals
Classes of proteins (an oversimplified view!)

Proteins that cannot crystallize

Aggregation/wrongly folded

Proteins that are easy to crystallize

Proteins that are perfect but difficult to crystallize
Why don’t they crystallize readily?

- ~ 30% of “well behaved proteins don’t crystallize”

Glycosilation  Flexible termini/domains  Too soluble
Protein very soluble, no nucleation without seeding
Nucleation and seeding in protein crystallization

- Nucleation in general
- Seeding methods
- Matrix seeding, method and results
Nucleation

The growth of protein crystals requires 2 sequential processes and optimum conditions for these processes differ:

- Nucleation (high concentrations of protein and/or precipitant)
- 3D growth (lower concentrations of protein and/or precipitant)

In screens there are often too many clear drops because spontaneous nucleation cannot occur.

Have we neglected the nucleation event?
What effects nucleation?

Sample concentration (1-100mg/ml)
Sample preparation (filtering, mixing etc.)
Method used, VD, MB, FID
Surfaces
Mixing drops

Drop set up (mix?)
Filtering sample to reduce nucleation

Gooey mess too many crystals

Reduced nucleation 2.0 Å diffraction
Nanocrystallization is not a simple miniaturization of a protein-crystallization experiment and that one cannot reduce the crystallization volume without paying a penalty. In nanovolumes, surface-tension forces become more prominent and might have effects on the nucleation event. Furthermore, Bodenstaff et al. (2002) showed that the mean number of nuclei formed per unit volume is linearly proportional to the volume of the mother liquor. Moreover, when working in a nanolitre regime, the time before the first nuclei are formed increases dramatically.
The effect of increasing drop size

100nl +100nl

300nl +300nl

2ul +2ul

100nl +100nl

300nl +300nl

2ul +2ul
Different seeding methods
Different seeding methods: **Streak**

Streak seeding with needle or hair
Different seeding methods: Seed bead

Seed bead no dilution

Seed bead 1:10 dilution

Seed bead 1:100 dilution

Seed bead 1:1000 dilution
Different seeding methods: Macro

Crystal displacement
Different seeding methods: Heterogeneous
Crystals of chicken triose phosphate isomerase, the first TIM barrel 1970

- Courtesy of Dave Banner (Roche)
The first artificial snow crystals

- 1954 – Ukichiro Nakaya
- Nakaya's real triumph came from growing artificial snow crystals in the laboratory under controlled conditions.
- This success was apparently due to rabbit hairs falling from his parka hood into the experiment!!!
Hair as a successful nucleant

Using natural seeding material to generate nucleation in protein crystallization experiments

Allan D’Arcy, Aengus Mac Sweeney and Alexander Haber


Heterogeneous nucleation of three-dimensional protein nanocrystals


Cause and effect-proof of concept

- Horse hair is responsible for nucleation

Control: no hair added  Concentrated hair added  Diluted hair added
Hair is visible within crystal
Other nucleation agents

- McPherson and Schlichta 1987: Crushed mineral materials
- Punzi et al. 1991: Polyvinylidene Difluoride
- Chayen et al. 2001: Porous silica
- Rong et al. 2004: Porous silica
- Fermani et al. 2001: Polymeric films.
- Haushalter and McPherson 2002: Nanoengineered Surfaces
- Molecularly imprinted polymers 2011 Saridakis
A major breakthrough

“Microseed Matrix Seeding”
A crystallization strategy termed `microseed matrix screening.

This method is an extension of conventional seeding techniques in which microseeds from the nucleation step are systematically seeded into new conditions where all components of the drop are allowed to vary to screen for subsequent growth of well ordered specimens.
Separating nucleation and growth

(~28% PEG 8000, 0.1 M sodium cacodylate pH 6.5)

25% PEG 8000, 0.1 M calcium acetate, 0.1 M sodium cacodylate pH 6.5

All attempts to grow the new crystal form under these conditions by de novo nucleation (without transfer of microseeds) were unsuccessful.
Microseed Matrix Seeding: preparation of seeds

- Seed stock preparation:
  - Select best crystals possible
  - Crush crystals in their reservoir solution using Hampton tools
  - Transfer to Hampton seed bead and vortex for at least 3 minutes or place on Thermoshaker 10“ on 2“ off for 10-30 mins
  - Store at -80°C
Matrix Seeding: Automated seeding

- **Screen Setup**
  - Use Oryx robot with seeding function
  - Dispensed drops 0.6µl drops containing 0.3µl protein
  - 0.2µl reservoir solution
  - 0.1µl seed stock
Screening for initial crystallization conditions

- ~ 300 “precipitating agents are tested in the first screens
- 300nl of protein are mixed with 300nl of precipitating agent
Things you see in drops (some could be used as seeds)

Bushels  Spherulites  Phase separation

Precipitate and xtals  Sea urchins  Skins

http://xray.bmc.uu.se/terese/crystallization/tutorials/tutorial2.html
Seed anything that might be crystalline”
(Terese Bergfors)
Standard seeding and MMS

Normal seeding, into same conditions at lower concentrations

Matrix seeding, into many new conditions at different concentrations

New screen
You may generate more false positives..

A lot of time and effort can be wasted, testing or optimizing conditions that turn out to be salt crystals.

Recognizing false positives?
Recognizing typical morphologies
Using UV to identify protein crystals

Filter 1: Transmission from 260 – 395 nm
Filter 2: Transmission from 270 - 320 nm
Salt crystals
Example of confirmed screen hit
Automated microseesd matrix seeding

Results and observations
CYS protease

- Starting crystals using published conditions
  - 3 hits without seeding
  - 53 hits with seeding

Result:
More hits better morphology
Viral protein

Original hit in Peg screen

1 hit without seeds
64 hits with seeds

Result:
More hits better morphology led to first inhibitor complex
Metallo Protease

Only one crystal grew spontaneously in published conditions

- No hits without seeding
- 39 hits with seeding

Result: Many more conditions made it possible to obtain apo crystals
From no hope to new conditions, new crystal form and new structure.

Result: New conditions made it possible to solve structure and establish crystallization system.
Eliminate twinning

Result:
Twinning solved
reproducible crystallization
Using spherulites

Result:
New conditions
New crystal form
Complex structure solved of natural compound
Fab complexes, another powerful crystallization aid

Fab complexes combined with matrix seeding, gives crystals of target protein for the first time.
Is it matrix seeding or just an additive effect?

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<th>Prot #1</th>
<th>Prot #2</th>
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<td>Adding original condition</td>
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<td>3</td>
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<tr>
<td>Adding Seeds 1:100:</td>
<td>7</td>
<td>52</td>
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<tr>
<td>Adding Conc. Seeds:</td>
<td>9</td>
<td>59 (*)</td>
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Different conditions from original:
- Succinic acid
- Malic acid
- Succinic acid +1% peg
- Tacsimate +2% peg
Conclusions and questions

- In protein crystallization the devil is in the details
- Nucleation is the single most important event in crystallization
- Seeding is the most powerful tool for optimization.
- Automated matrix seeding is a major breakthrough in increasing the number of hits and finding new conditions to optimize
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Backup