Preventing Protein Aggregation

Protein aggregation cause artifacts in most biophysical techniques, leads to uncertainty in the active concentration of protein in solution and loss of biological activity. Unfortunately, it is a common problem for many protein constructs when removed from their biological context (e.g. solution conditions, domain boundaries, post-translational modifications, chaperones, interaction partners), and present at high concentrations for measurements.

Detection of Aggregates

Aggregation can be detected or inferred in various ways e.g.:

- Visual observation of particulate matter in suspension.
- Detection of very large species eluting in the void-volume during size exclusion chromatography.
- Detection of abnormally high light-scattering in absorbance measurements.
- Detection of very large particles by Dynamic Light Scattering.
- Inference from loss of activity
- Inference from experimental artifacts, the nature of which depends on the type of measurement and the technique.

Screening for Thermal Stability *vs* Screening against Aggregation

A popular general approach to increase protein stability is to employ dye-based thermal shift assays to screen for buffer conditions or additives that increase the thermal denaturation midpoint of the protein. This is a good approach if one is primarily concerned to ensure thermal stability, i.e. that the protein essentially exclusively populates the native (folded) state at a given temperature.

Screening to prevent aggregation isn't *necessarily* so straightforward. If the protein aggregates only from the denatured (unfolded) state, then thermally stabilising the protein should prevent aggregation. This is because thermal stabilisation will decrease the concentration of the denatured state – the aggregating species – at a given temperature. However, if aggregation occurs from the native state, an intermediate state, or an *apo-* state, thermal stabilisation may not help at all. It may in fact *increase* the concentration of the aggregating state(s).

One can screen more specifically for conditions that prevent aggregation by Dynamic Light Scattering, or by a simple light-scattering assay in an absorbance plate reader. Scattering from particulate aggregates in suspension can give an increase in apparent absorbance in the range 300-600 nm over time.

Tips for Reducing Protein Aggregation

1. **Tip: change the pH of the solution.**
   Compare the theoretical pI of the protein (from Protparam or EMBOSS) with the current buffer pH.
   If pI > buffer pH, lower the pH by 1 unit.
   If pI < buffer pH, raise the pH by 1 unit.
   If pI ≈ buffer pH, flip a coin and try one or the other.
   Rationale: Proteins are expected to be least soluble when pH = pI, since the net charge on the protein is zero. Moving the pH away from the pI should increase solubility. N.B. theoretical pI values are not very reliable. In any case, changing pH changes the net charge on the protein and the surface charge distribution. This can modulate both specific and non-specific interactions that lead to aggregation. To avoid erroneous conclusions when making significant changes in pH, try to keep the ionic strength constant (accounting for the contribution from buffering species). You can obtain recipes for buffers at varying pH but constant ionic strength using the tool at the URL below:
   `http://www.liv.ac.uk/buffers/buffercalc.html`

2. **Tip: change the salt concentration or change the salt.**
   Try both higher and lower ionic strength (account for buffer ionic strength)
   Try different salts (e.g. KCl for NaCl, sodium sulphate, sodium iodide, low concentrations of GdmCl)
   Rationale: changing ionic strength changes the energetics of electrostatic interactions within and between proteins. These can be stabilising or destabilising, and prevent or cause aggregation. Changing the type of salt can affect stability, oligomerisation and aggregation through direct binding of ions, or through Hofmeister effects (complex changes in the physical properties of the the water-solute system, the nature of which are still much debated). Low concentrations of chaotropes can disrupt hydrophobic interactions in aggregates.

3. **Tip: add a stabilising osmolyte.**
   Usually included at molar concentration, e.g. glycerol, sucrose, proline,TMAO.
   Rationale: Stabilising osmolyte co-solvents are thought to interact unfavourably with the exposed amide backbone of proteins (Bolen *et al.* JMB, 310, 955–963.). Thus the presence of stabilising osmolytes favours the population of states with less-exposed backbone (e.g. the native state) over states with more exposed backbone (e.g. denatured state). Such osmolytes are therefore generally thermodynamically stabilising. This is protective against aggregation from the denatured state. They may also al-

ter the relative population of different conformational states in the native state ensemble, thus altering aggregation propensity from the native state.

4. **Tip: add a mixture of arginine and glutamate.**
   Add 50 mM of an equimolar mix of arginine and glutamate to your buffer. Either adjust the pH of the Arg / Glu solution before you add it, or make the buffer up with Arg / Glu in it before you adjust the pH. When making a stock solution, add the Arg first, as it helps to solubilize the Glu.
   Rationale: Arg / Glu can dramatically increase protein solubility. The mixture is thought to act by direct binding to charged and hydrophobic regions, and by charge screening, for details see: Golovanov *et al.* JACS 126, 8933–8939, 2004.

5. **Tip: use a reducing agent, or change your reducing agent.**
   Try DTT instead of B-mercaptoethanol. Try TCEP (tris 2-carboxyethyl phosphine) instead of DTT.
   Rationale: B-mercaptoethanol can form covalent adducts with surface cysteines. If you get aggregation over time in crystallization drops, try using TCEP instead as it has a longer half-life (n.b. check pH when adding TCEP, which is very acidic). For details see: Getz *et al.* Analytical Biochemistry, 273, 73–80, 1999.

6. **Tip: try a slightly different construct.**
   If the protein construct has a cleavable His-tag, try it with and without the tag. Try a Thrombin cleavage site instead of a TEV-protease cleavage site. Try small variations in domain boundaries if possible.
   Rationale: small changes in domain boundaries can make dramatic changes in expression levels, solubility and stability, without compromising the biological validity of the construct.

7. **Tip: add a ligand before concentration.**
   Add a ligand to the protein solution at high-enough concentration to promote binding. Do this before concentration, as the ligand may prevent aggregation during concentration.
   Rationale: adding a ligand will result in preferential population of the native state of a protein, and sometimes of a particular ligand-bound state. The former will thermodynamically stabilise the protein, protecting against aggregation from the denatured state. The latter can reduce the exposure of e.g. hydrophobic or highly-charged patches in the ligand binding site, which can promote aggregation. It can also restrict the conformational heterogeneity of the native state ensemble, promoting crystallisation. This

is commonly observed for protein kinases, which are significantly stabilised by addition of a ligand and rarely crystallize without one.

8. **Tip: phosphate buffer for nucleic acid or NTP binding proteins.** For a valid comparison, use a phosphate buffer with identical pH and ionic strength to the buffer that you have previously been using.
   Rationale: phosphate ions can stabilise these proteins by binding to phosphate binding sites. This can be a disadvantage in binding studies, if phosphate competes with the nucleic acid or NTP.

9. **Tip: Try adding metal ions.**
   Add metal ions Mg^{2+}, Ca^{2+}, Fe^{x+}, etc... Look in the literature to see whether they are a common cofactor for your protein class of interest. Or try dialysing against EDTA or EGTA to get rid of bound metal ions. Beware of buffers (e.g. phosphate) that can form insoluble metal salts.
   Rationale: The metal ions may stabilise the native state (c.f. adding a ligand), or may promote the binding of a stabilising ligand. Sometimes, a metal-bound form, or the wrong metal ion, will be less stability or have a higher tendency to aggregate.

10. **Tip: Try adding non-denaturing detergent, (or non-detergent!)**
    Add a low concentration of non-denaturing detergent e.g. 0.1 % CHAPS, 0.05 % Tween20, or add non-detergent sulphobetaines.
    Rationale: The detergent molecules can help to solubilise aggregates that are self-associated through hydrophobic patches. The amphiphilic nature of detergents means that the protein-detergent complex can present a more hydrophilic surface than the protein alone, resulting in a preference for interaction with solvent rather than self-association. Non-ionic (e.g. Tween20) or zwitterionic (e.g. CHAPS) detergents are milder detergents, being less likely to denature proteins. Non-detergent sulphobetaines act in a similar way to zwitterionic detergents but don't form micelles.

11. **Tip: try stabilising polymers:**
    For example the NVoy polymers from Expedeon:
    `http://shop.expedeon.com/products/13-NVoy-Polymer-Technology`
    Rationale: The polymers bind to exposed hydrophobic sites on the surface of proteins, they are typically used at lower concentrations than detergent, where they are non-micellar. For an unusually well-characterised application of these polymers to the purification of a very difficult viral polymerase see: Voros *et al.* Journal of Virology, 88, 2584–2599, 2014. Or for GPCR solubilisation see: Klammt *et al.* Protein Science, 20, 1030–1041, 2011.

Reaching High Concentration for Crystallization

Professor Timm Maier suggested the following two points specifically relevant to avoiding aggregation and precipitation when seeking very high concentrations for crystallisation:

1. Test solubility at different temperatures 4,12,20 and 30 °C. This has often shown an effect in our hands.

2. If the protein slowly aggregates over time, the points above are all you can do. However, sometimes proteins precipitate directly during concentration in a centrifugal concentrator, when using the manufacturers protocol (e.g. fill concentrator, spin at 4000g until desired volume is reached). In such cases, every aspect of concentration should be examined. The concentrator type, centrifugal speed, concentration time, frequency of sample mixing, and centrifuge temperature often have a huge effect on the success of concentration. This is due to the formation of local maxima of protein concentration inside the device (e.g. at the bottom of v-shaped concentration devices). One protein could be concentrated only to 6 mg/ml with a Vivaspin device and standard protocol, while it could be concentrated to 20 mg/ml using an Amicon, 500g only and mixing every 5 min.