2D Crystallization by Dialysis

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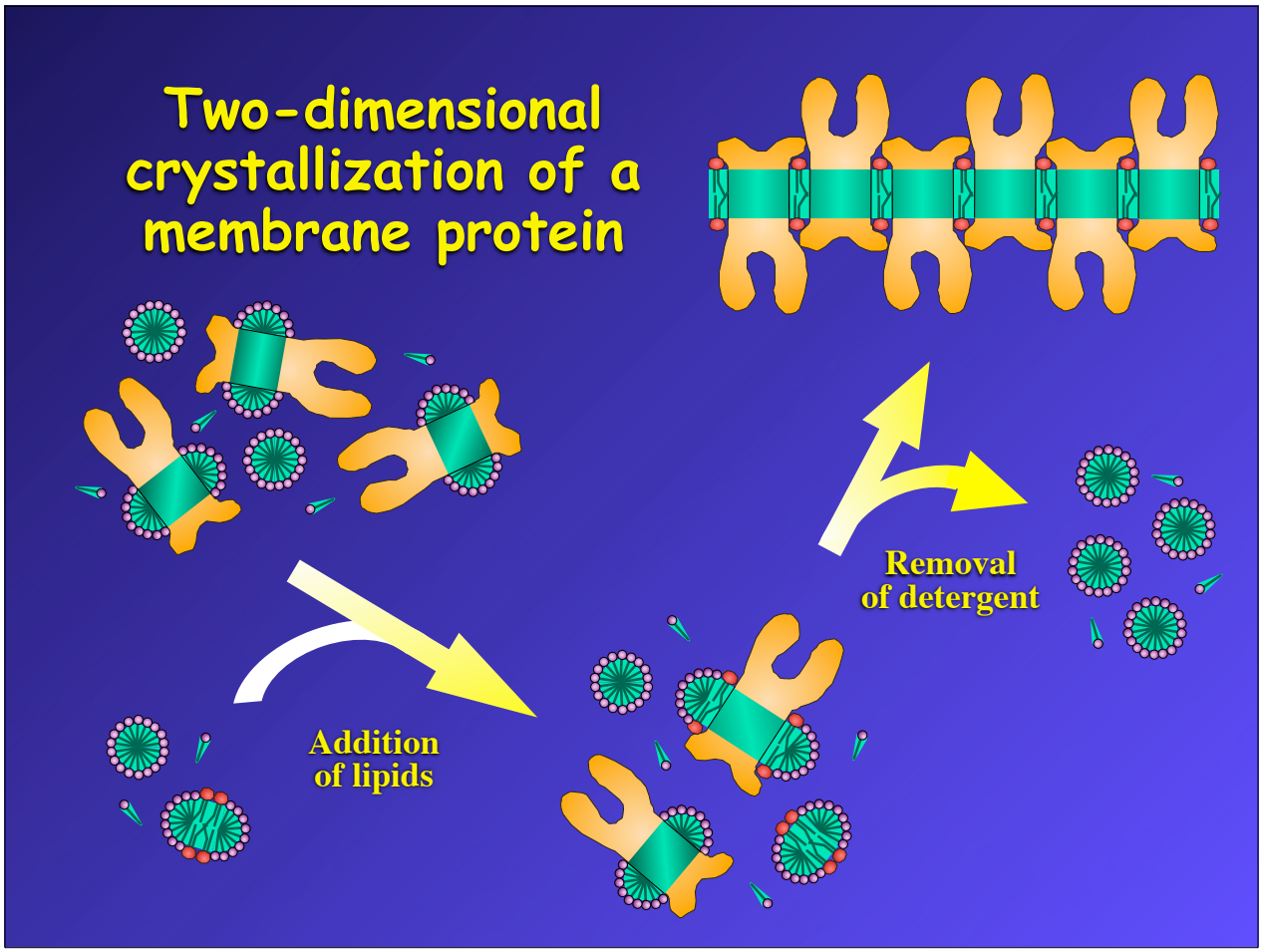
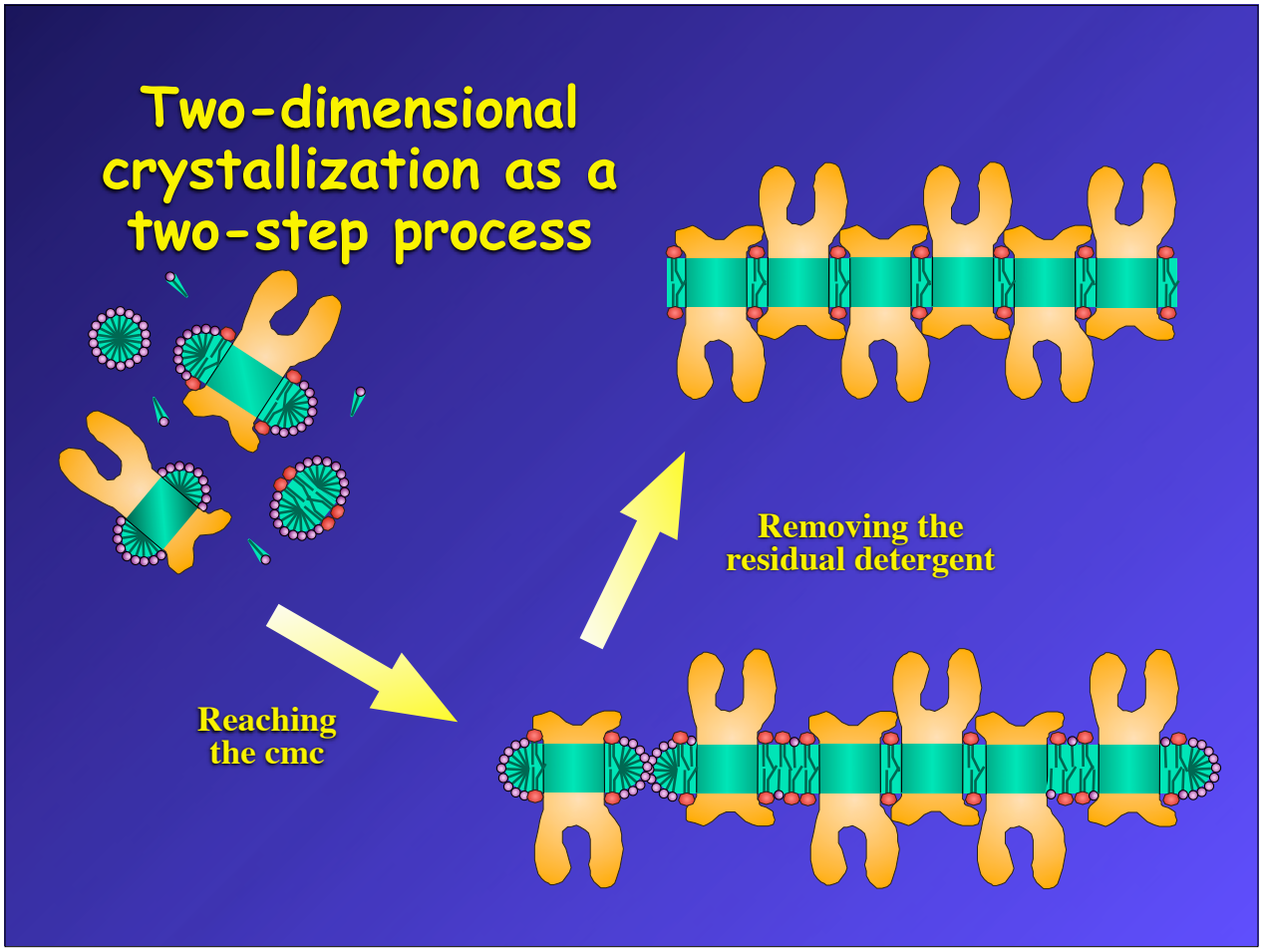
Black Magic with Superstitious Belief Systems Depending on the Lab and PI

A lot of information is distributed by hear-say while not solidly proven
(anecdotal science)

Only successful experiments are reported in the literature
Are we constantly repeating the same mistakes ?


Two-dimensional crystallization of a membrane protein
Removal of detergent
Addition of lipids


Two-dimensional crystallization as a two-step process
Removing the residual detergent
Reaching the cmc

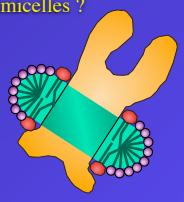
The "system"

Protein ⇔ Detergent >> Lipid ⇔ Detergent
–> empty vesicles form, later protein aggregates

Protein ⇔ Detergent << Lipid ⇔ Detergent
–> protein aggregates, later empty vesicles form

Protein ⇔ Detergent ≈ Lipid ⇔ Detergent
–> protein reconstitutes into lipid membranes

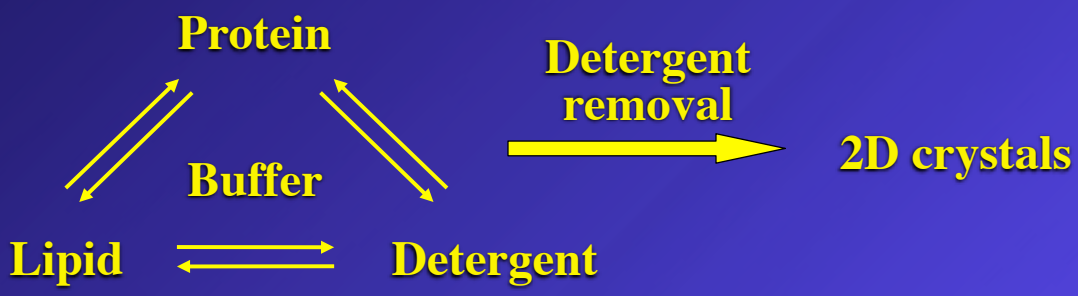
What about the mixed micelles ?



Is the detergent likely to interact differently with protein and lipids ?

Is this a fruitful way to look at the problem ?

The "system"



Protein

That's the given, but:

– better if pure and homogeneous (functional states, oligomeric states)

– higher oligomers more likely to form well-ordered 2D crystals

– purification tags - good or bad ?

– homologs from different species often behave differently !

– check in EM for aggregates
– pushed into one state in the crystal
– separation into different patches

if there is an equilibrium, try to push towards higher oligomer

solubility *versus* interference

The "system"

Detergent

Essentially determined by the protein

– solubilization efficiency *versus* stability of protein in solution

– high cmc detergents easier to dialyze, but usually harsher

– use of detergent mixtures

– initial detergent concentration

detergent does not have to stabilize the protein as much as for X-ray

The "system"

Lipid

Can be freely chosen ! How crucial for 2D crystals ?

– phase transition temperature and membrane fluidity

what is the influence of the protein ?

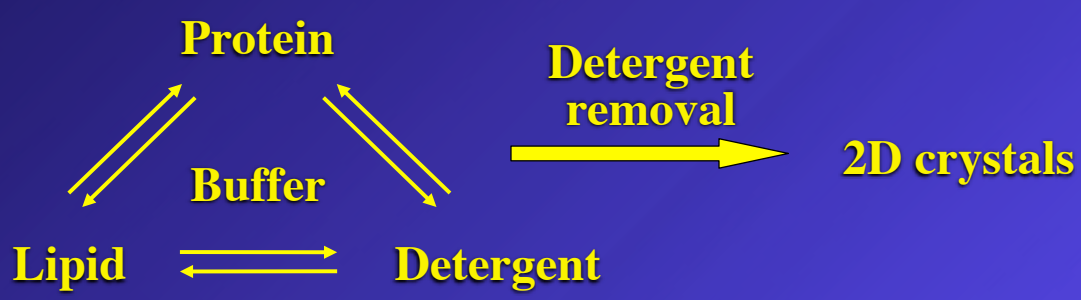
– make fresh every time or keep a stock solution ?

lipid oxidation *versus* reproducibility

– single lipids or mixtures ?

– prepare lipid in protein buffer or water ?
in what detergent should the lipid be prepared ?

The "system"



Detergent Removal

Dialysis is the classic way – all high-resolution structures were obtained with 2D crystals prepared by dialysis.

– BioBeads: difficult to control, but well suited for low cmc detergents

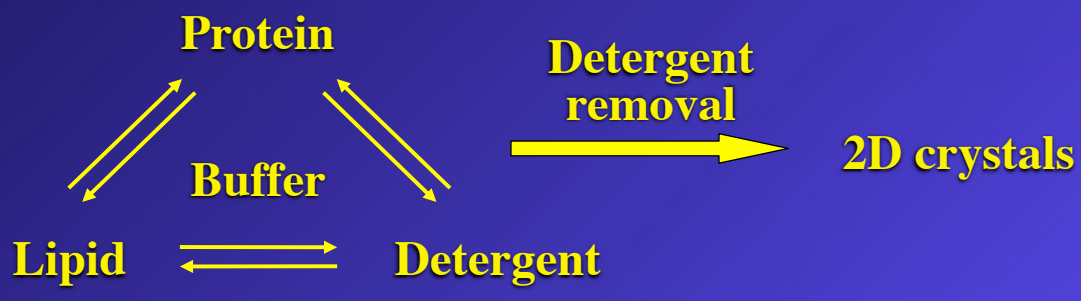
What about adsorption of lipids and even proteins ?

– Dilution: can be well controlled, but the protein concentration also drops (especially for low cmc detergents)

Does it need the special dilution apparatus ?

– Chelation: looks very promising, but has not been widely used yet

The "system"



2D crystals

The size but mostly the order of the 2D crystals determine the resolution that can be obtained.

– a size of 1 µm or larger is needed for electron diffraction (not overall size of membrane, but coherent crystalline area)

– sheets: ideal specimen for structure determination
vesicles: problems for el. diff., but good for functional assays
tubes: very difficult to get to high resolution, but no tilting

– crystal stacking is an unresolved problem

The "experiment" - making the sample

Protein solubilized and purified in detergent

– minimize the time between solubilization and adding the lipid (ideally within one day)

– determine the protein concentration always as accurately as possible and always in the same way (important for LPR)

Prepare the lipid solution

– make lipid solutions at high concentrations (e.g. 10 mg/ml) to minimize dilution of protein when mixing

– make sure the lipid is completely solubilized – begin with high detergent concentration and then dilute; high temperature and sonication; OG if needed

Mixing the sample

– aim at protein concentrations of 0.5 to 1 mg/ml for screening (can be increased at a later stage); always use same protein concentration

– choose lipids and lipid-to-protein ratios

– keep the protein concentration constant (fill with buffer to final volume)

– pre-incubation before start of dialysis - needed to form mixed micelles ?

The "experiment" - physical parameters

Dialysis devices

– continuous flow system: most sophisticated, efficient dialysis, much control

– dialysis buttons: simple set-up, easy to prepare many samples

– slidelyzers: expensive, fast dialysis because of large area

– hockey sticks: still being used ?

Dialysis membrane

– choose cut-off size (usually 12-14 kDa)

– prepare by boiling in EDTA (remove heavy metals) and ethanol (kill bacteria)

– store in NaN_3

Sample size

– small samples allow more conditions to be screened, but crystals have to be reproduced for data collection

– large samples limit the number of conditions that can be screened, but provide enough material for data collection

The "experiment" - the dialysis

Composition of the dialysis buffer

– type & concentration of salt (ionic strength, special salts)
– pH (slightly acidic pH values usually better - good for lipid)
– divalent cations (Ca^{2+} stronger effect than Mg^{2+})
– DTT (add fresh at every buffer exchange)
– special molecules (e.g. ligands, inhibitors, solutes)
(can be expensive for dialysis – BioBeads)
– glycerol or sugars (for embedding)

Start simple - you can always get more complicated …

Buffer exchanges

– prepare a stock solution (dilute, adjust pH) – reproducibility
– exchange once or twice a day (depends on detergent)
– stirring or not (depends on detergent)
– disturbance of the crystallization samples ?

The "experiment" - the dialysis

Dialysis Speed

Slow dialysis: low-cmc detergents, slow growth = few crystals with good order
Fast dialysis: high-cmc detergents, fast growth = many crystals with poor order

– depends on initial detergent concentration
– depends on MW cut-off and area of dialysis membrane
– detergent can initially be added to dialysis buffer to slow down dialysis
– temperature can be dropped/increased to slow down/speed up dialysis
(temperature cycles)

When is the dialysis complete ?

– sample turns opaque (continue another day or two to remove all detergent)
– if in doubt, check a single sample for detergent using Teflon film or make grid

The "experiment" - screening the samples

Make negatively stained samples
(we usually make 2 grids per sample, in case one grid is bad
when to prepare grids – immediately, after a day, after a few weeks ?)

Look at grid at low magnification (~5,000x)

check for membranes and/or protein aggregates
– if only aggregates and no membranes, go to the next sample
– if no aggregates, protein must be reconstituted
– if a mixture of aggregates and membranes, there may still be crystals
– if too little material on the grid, there may be an adsorption issue

you are happy if:
– membranes are large (no guarantee that it is crystalline)
– membranes have a very defined shape
– membranes have sharp edges
– membranes have a rim of protein

Look at grid at high magnification (~50,000x)

– try to see lattice lines
– if possible, always take CCD image and calculate FFT
  • crystal order: number and sharpness of spots
  • otherwise check for powder diffraction

The "experiment" - evaluating the results

Always reproduce every reconstitution experiment at least once, before drawing final conclusions
(there is a severe issue with reproducibility)

Comparisons within a set of reconstitution experiments are most informative, because all the parameters are identical except the ones that were varied on purpose
(it is much less reliable to compare results between different sets of reconstitution experiments)

Trends within a set of reconstitutions are most informative
what happens to vesicle size and/or crystal order with changing LPR, salt concentration, pH etc.

Refine parameters in the next set of reconstitutions according to observed trends

Problems - reproducibility

Variations in concentrations and thus in the LPR

– inaccurate protein assays, partial protein aggregation

– lipid solutions (better when using stock solutions)

– pipetting inaccuracies (use Hamilton syringes)

Other causes for variations

– variations during dialysis
  - air bubbles
  - temperature

– other factors we do not consider ?

Makes it difficult to screen reconstitution conditions and to identify important parameters

Problems - crystal morphology

Usually not obvious what determines the crystal morphology or how to change it

– different morphologies are often present in the same sample, e.g. tubes & vesicles, vesicles & sheets

– parameters that may influence crystal morphology: lipids, Mg^{2+}, LPR

Multi-layered crystals

– prevents merging of data

– no known solutions (can try salt, pH)

– try different protein construct

– ideally computational solution

Our approach

Primary Screens – Reconstitution of Protein

– protein concentration: 1 mg/ml
– standard buffer (10 mM MES, pH 6, 150 mM NaCl)
– LPRs: 0.5, 1, 2
– vary lipids: first screen: DMPC, DOPC, POPC, *E. coli* lipids
second screen: PE and PS lipids, other lipid mixtures

Identify the lipid
Identify the approximate LPR

Our approach

Secondary Screens – Produce 2D Crystals

– protein concentration: 1 mg/ml
– vary buffer (divalent cations, pH, salt)
– vary LPRs

Identify the buffer conditions
Identify the LPR

Ternary Screens – Produce the "Golden Batch"

– increase protein concentration
– vary LPR around the identified LPR
– vary divalent cation concentration

Get the perfect 2D crystals

Some Statements

Make the protein happy !
(quality is more important than quantity)

Minimize the time between solubilization and reconstitution !
(aim for one-day purifications if possible)

Don't believe the results of a single reconstitution trial !
(repeat conditions with different protein batches 2 or 3 times)

Don't assume that different membrane proteins behave the same !
(try everything with every new membrane protein)

Never assume that you will be able to reproduce your 2D crystals !
(take very good care of the crystals you already have)

Don't give up !
Great crystals are just
one reconstitution away !

Some papers to read

Engel *et al*. (1992) Assembly of 2-D membrane protein crystals: dynamics, crystal order, and fidelity of structure analysis by electron microscopy. *J. Struct. Biol*. 109: 219-234

Jap *et al*. (1992) 2D crystallization: from art to science. *Ultramicroscopy* 46: 45-84

Kühlbrandt (1992) Two-dimensional crystallization of membrane proteins. *Q. Rev. Biophys*. 25: 1-49

Dolder *et al*. (1996) The micelle to vesicle transition of lipids and detergents in the presence of a membrane protein: towards a rationale for 2D crystallization. *FEBS Lett*. 382: 203-208

Hasler *et al*. (1998) 2D crystallization of membrane proteins: rationales and examples. *J. Struct. Biol*. 121: 162-171

Mosser (2001) Two-dimensional crystallogenesis of transmembrane proteins. *Micron* 32: 517-540

Schmidt-Krey (2006) Electron crystallography of membrane proteins: two-dimensional crystallization and screening by electron microscopy. *Methods* 41: 417-426