# 2D Crystallization by Dialysis

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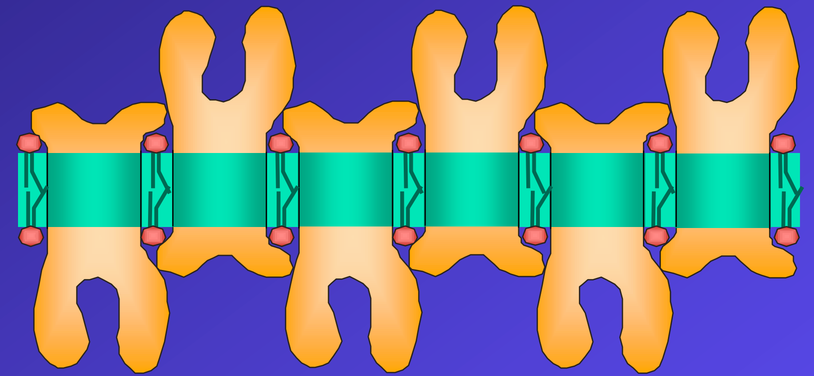
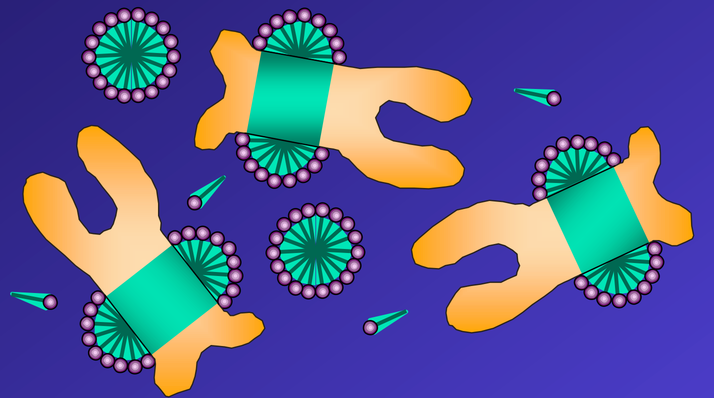
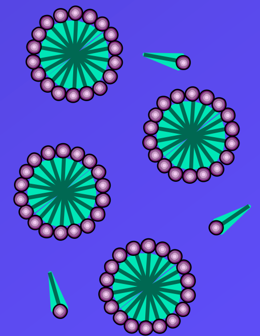
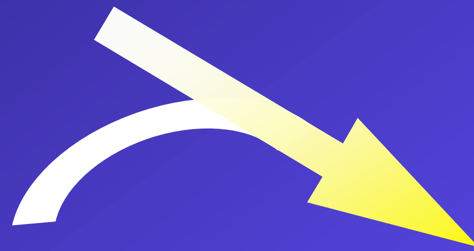
# Two-dimensional crystallization of a membrane protein



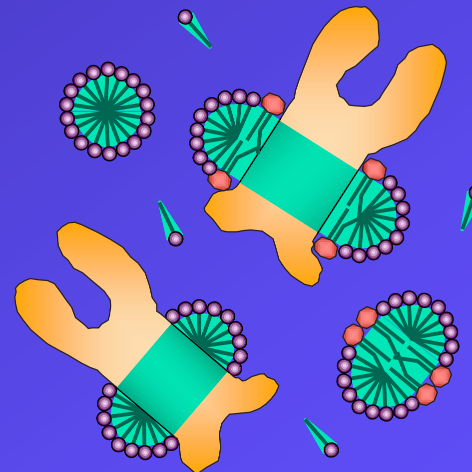


Removal of detergent

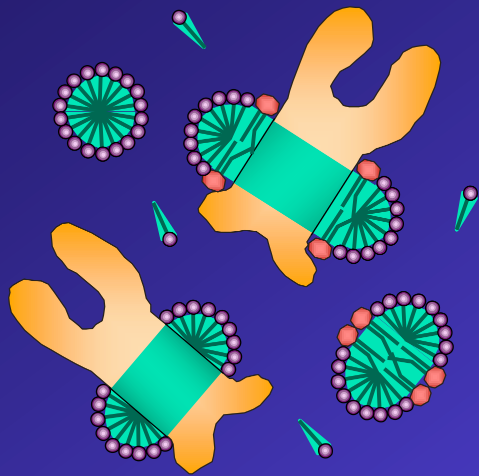




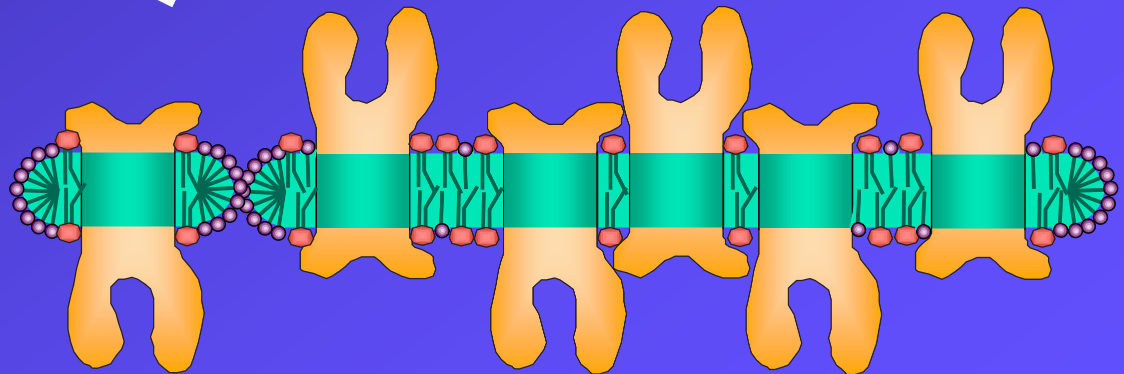
Addition of lipids



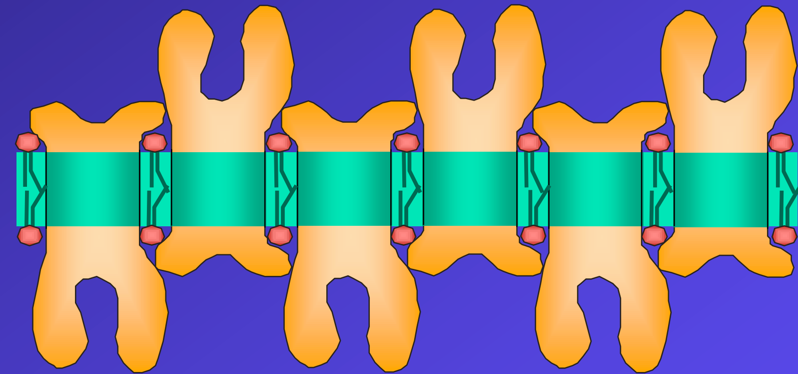
# Two-dimensional crystallization as a two-step process



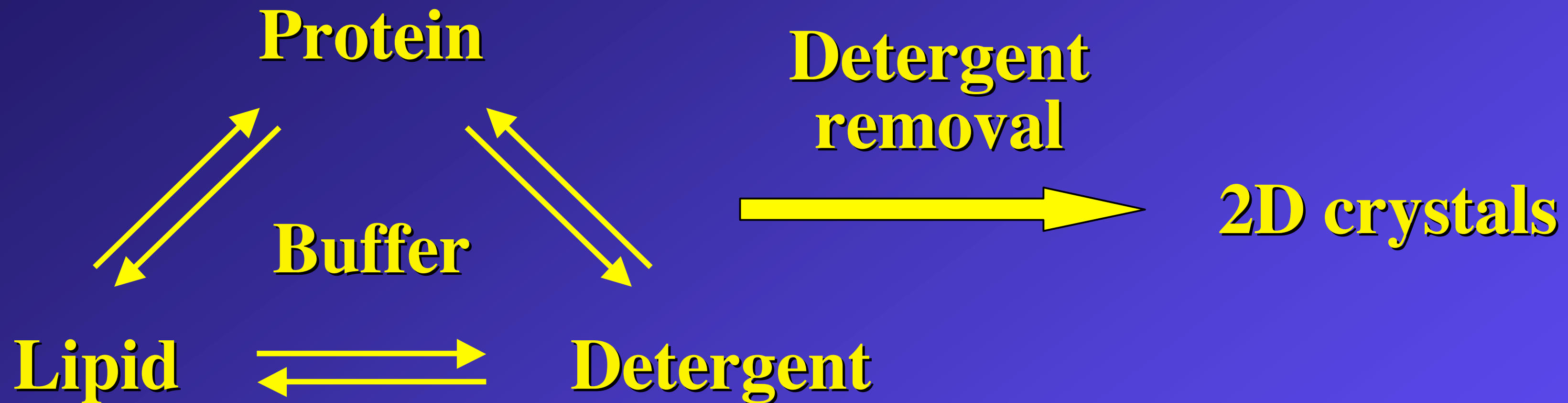
Reaching the cmc



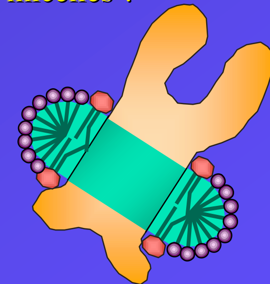
Removing the residual detergent



# The "system"




What about the mixed micelles ?



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

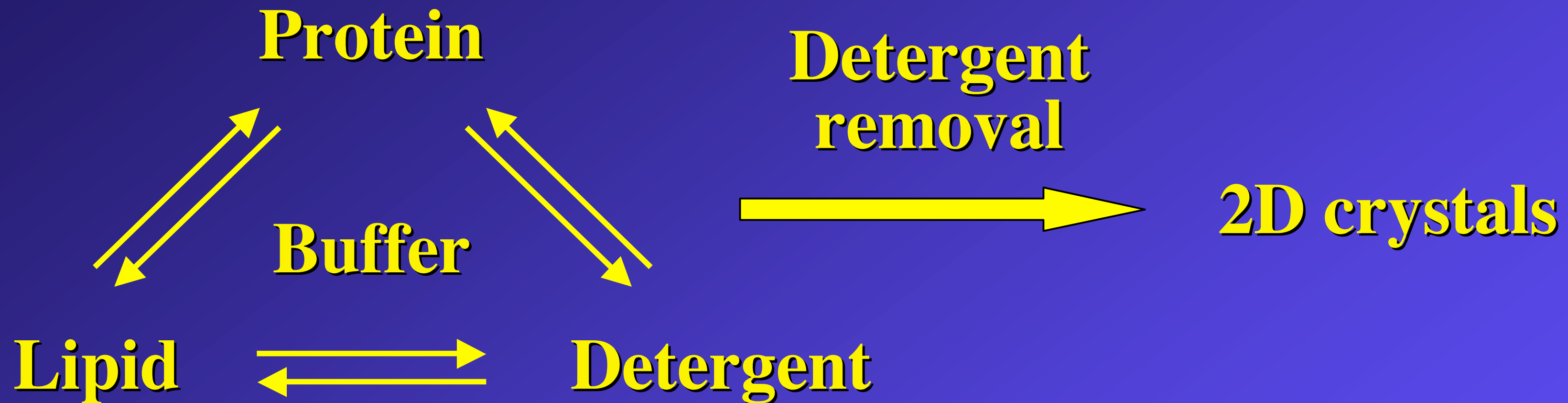
Protein ⇔ Detergent << Lipid ⇔ Detergent
–> protein aggregates later empty vesicles form

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

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

Is this a fruitful way to look at the problem ?

# The "system"




<u>Protein</u>

That's the given, but:

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

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

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

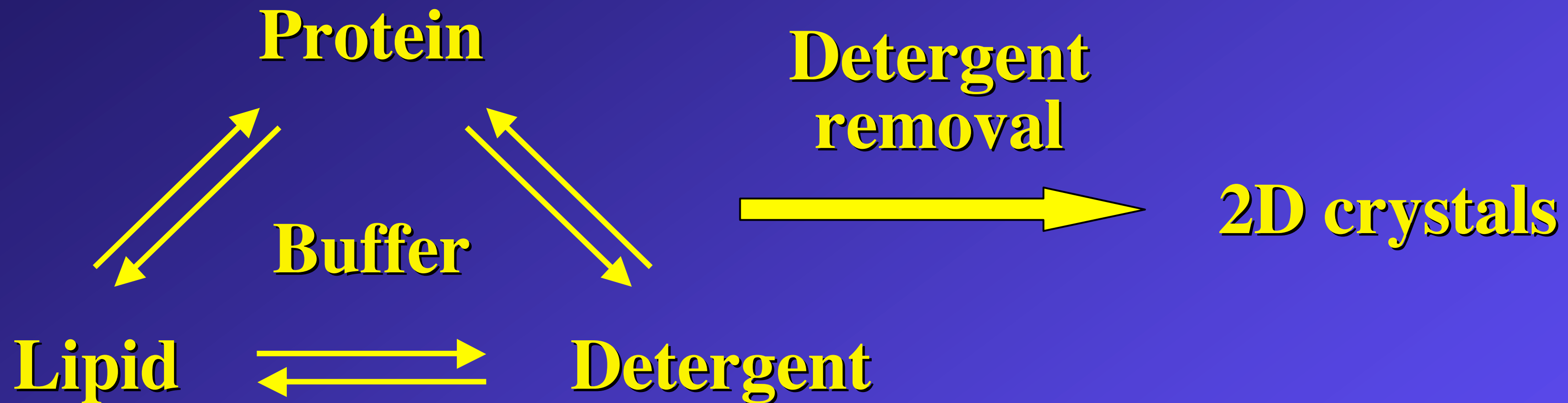
if there is an equilibrium, try to push towards higher oligomer

– purification tags - good or bad ?

solubility *versus* interference

– homologs from different species often behave differently !

# The "system"

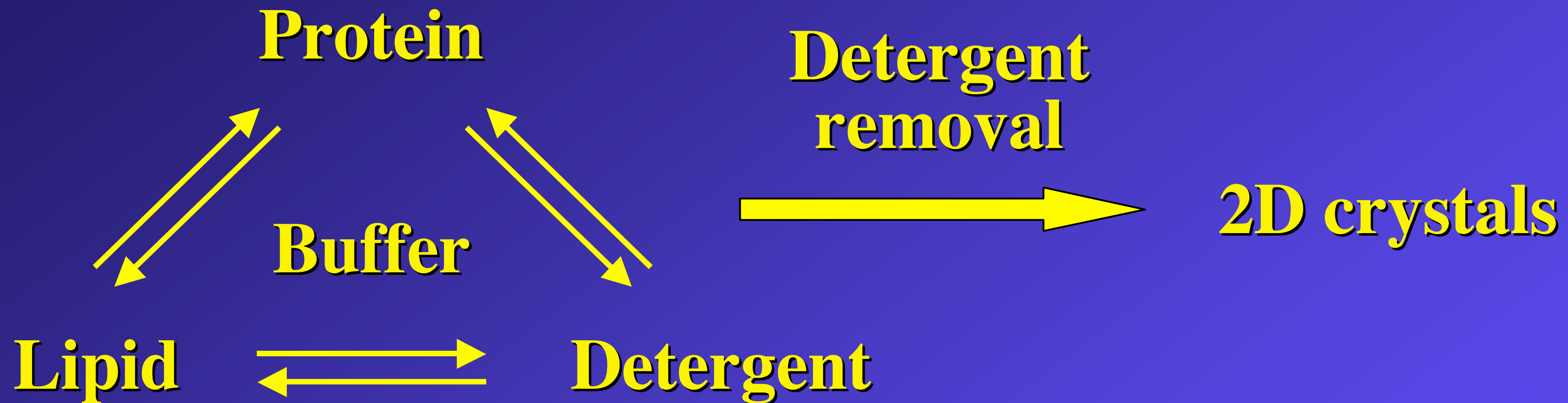



<u>Detergent</u> Essentially determined by the protein

- – 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

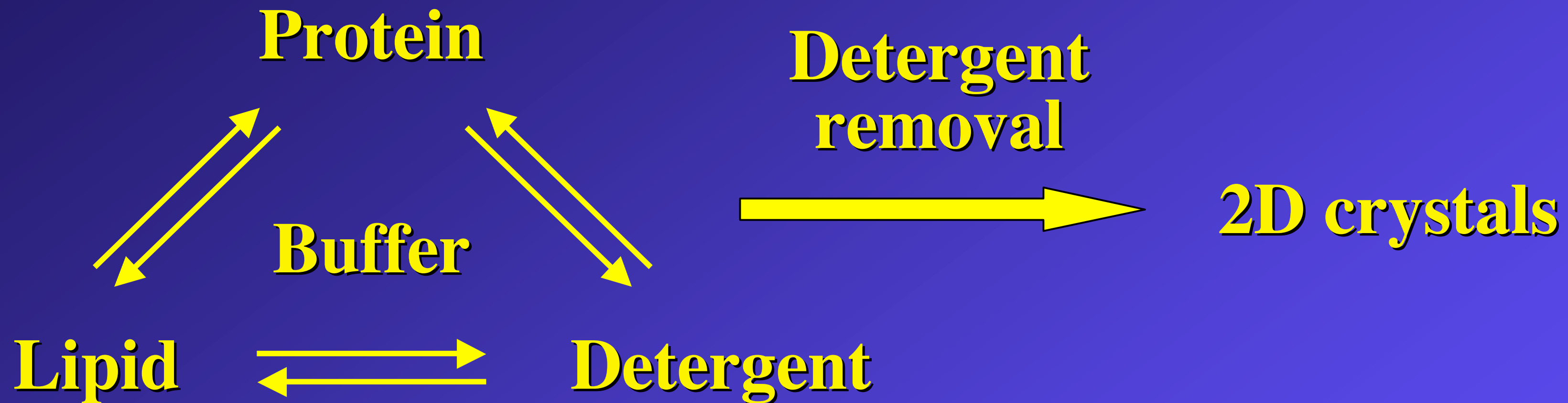
# The "system"




<u>Lipid</u> 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 <u>Removal</u>

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

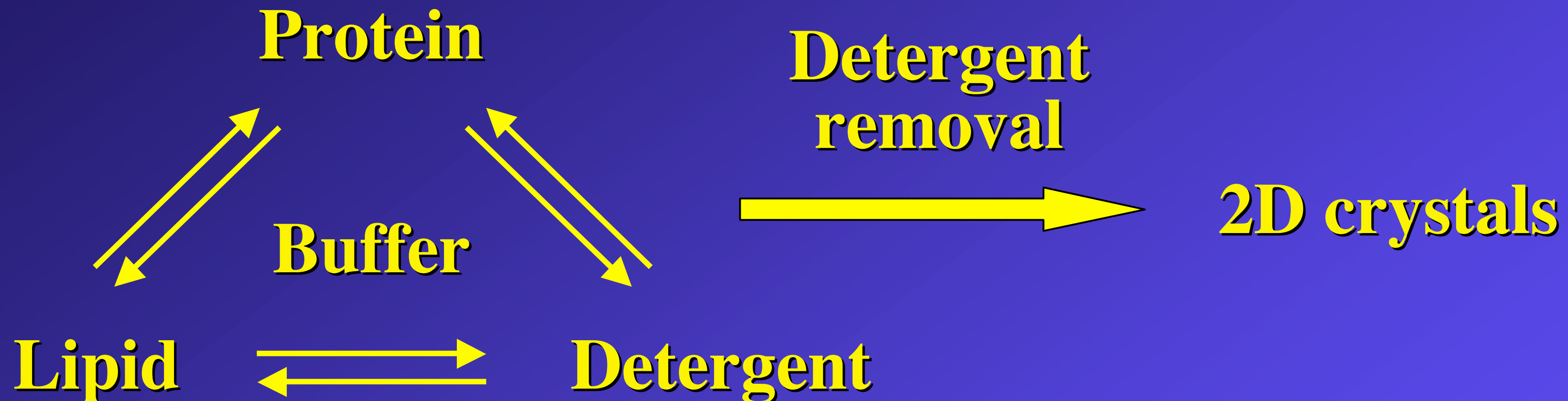
  What about adsorption of lipids and even proteins ?

- 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 10 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 provide limit the number of conditions that can be screened, but 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)

## Buffer exchanges

– prepare a stock solution

– exchange once or twice a day

# 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 sample for detergent using Teflon film

# 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 only membranes, 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

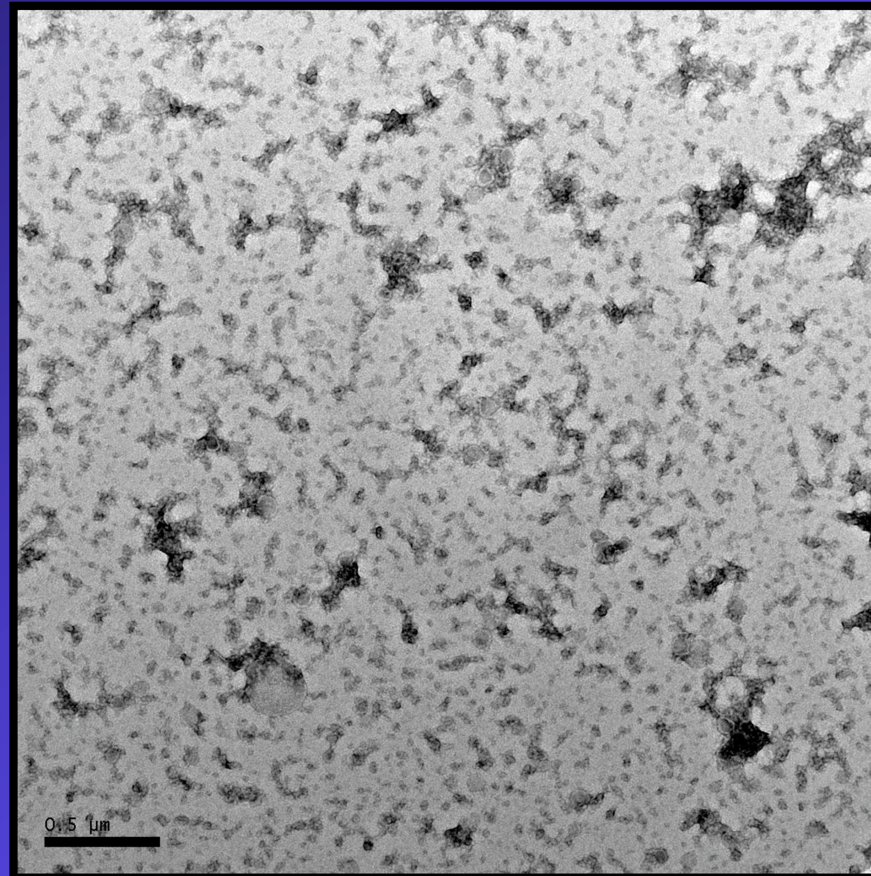
you are happy if:
– membranes are large (no guarantee that it is crystalline)
– membranes have a very defined shape
– membranes have sharp edges
– membranes have a rim of protein

## 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" - screening the samples

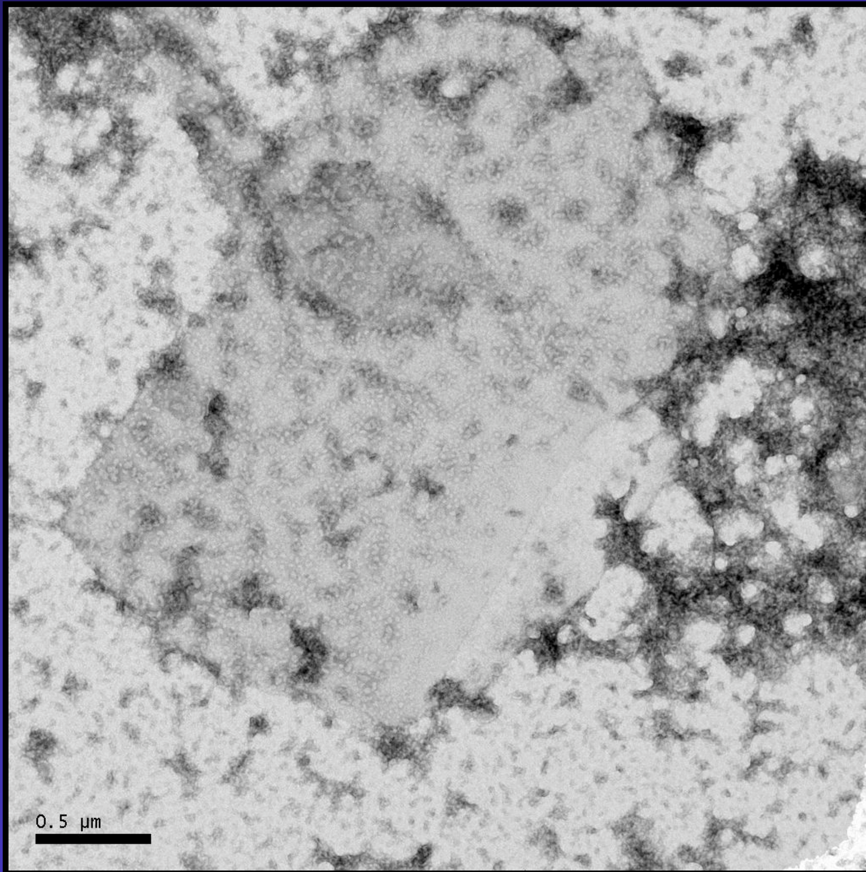
## Low magnification screening

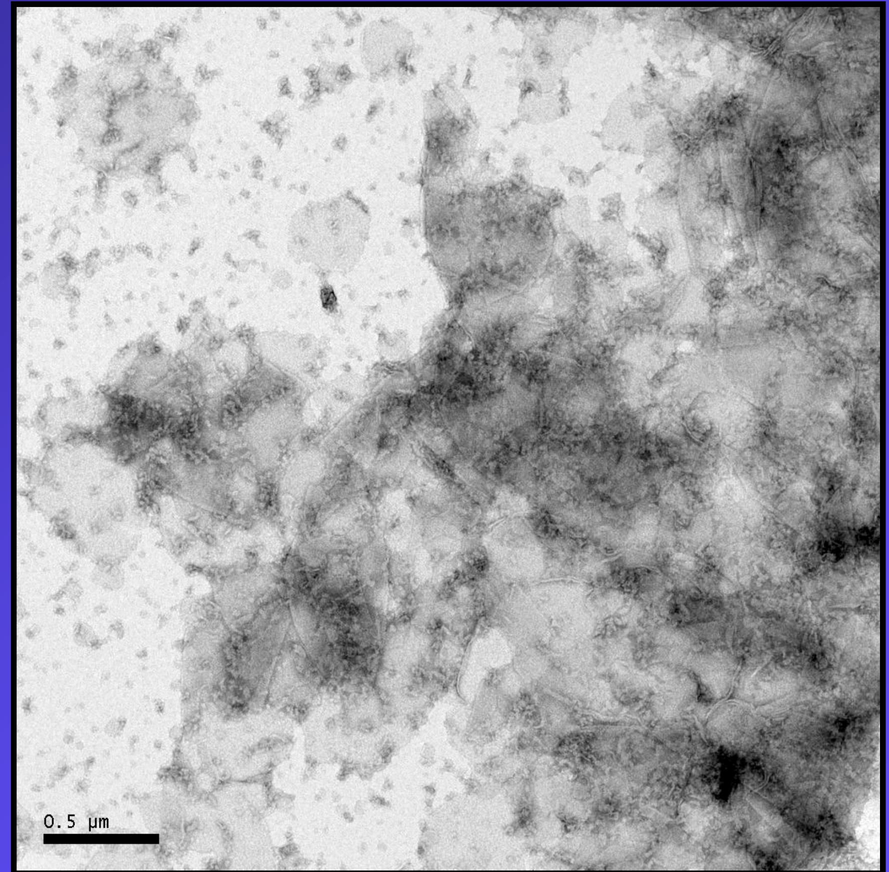



Protein aggregation

# The "experiment" - screening the samples

## Low magnification screening

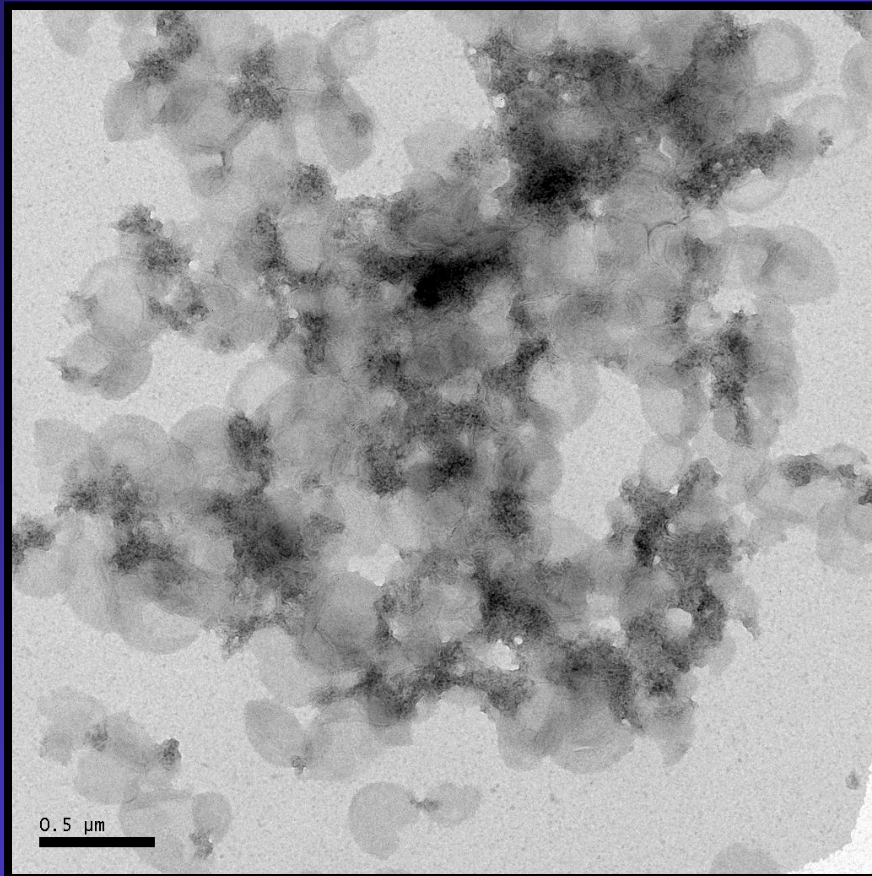


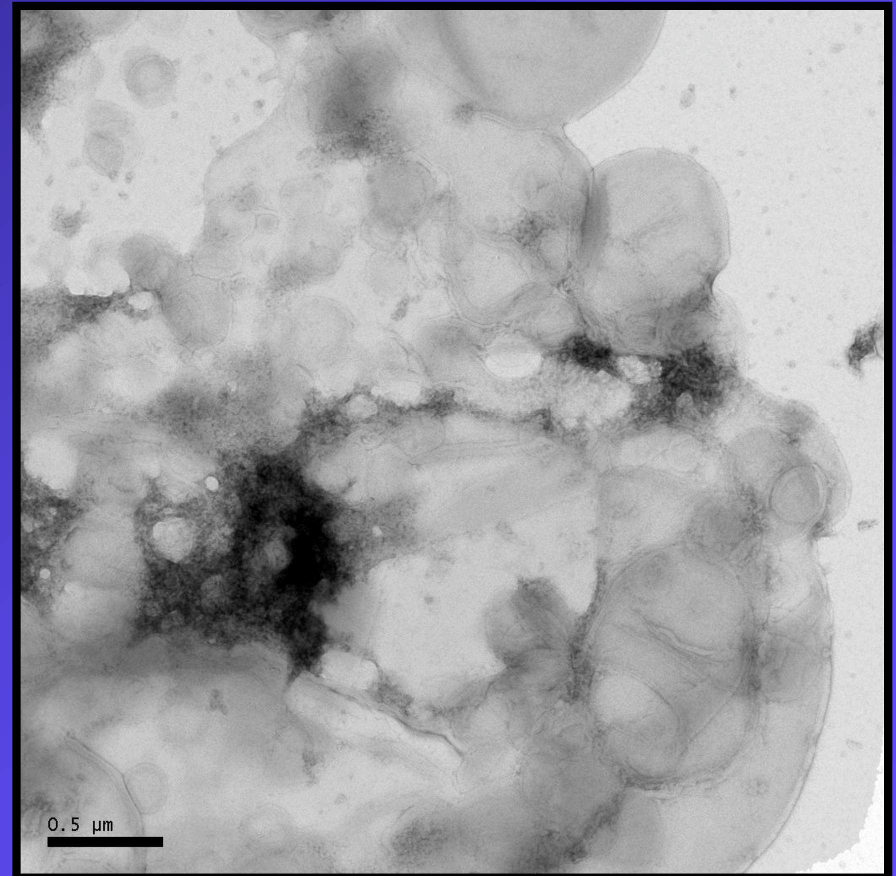




**Poor quality membranes with protein aggregation**

# The "experiment" - screening the samples

## Low magnification screening

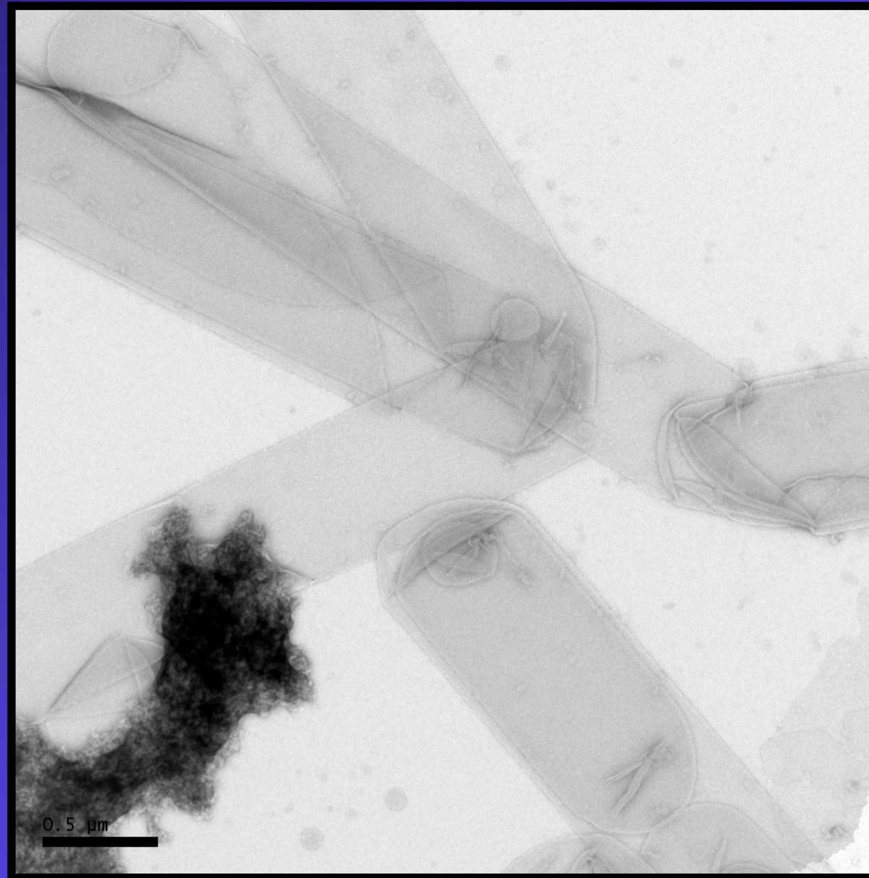



**Better quality membranes with protein aggregation**

# The “experiment” - screening the samples

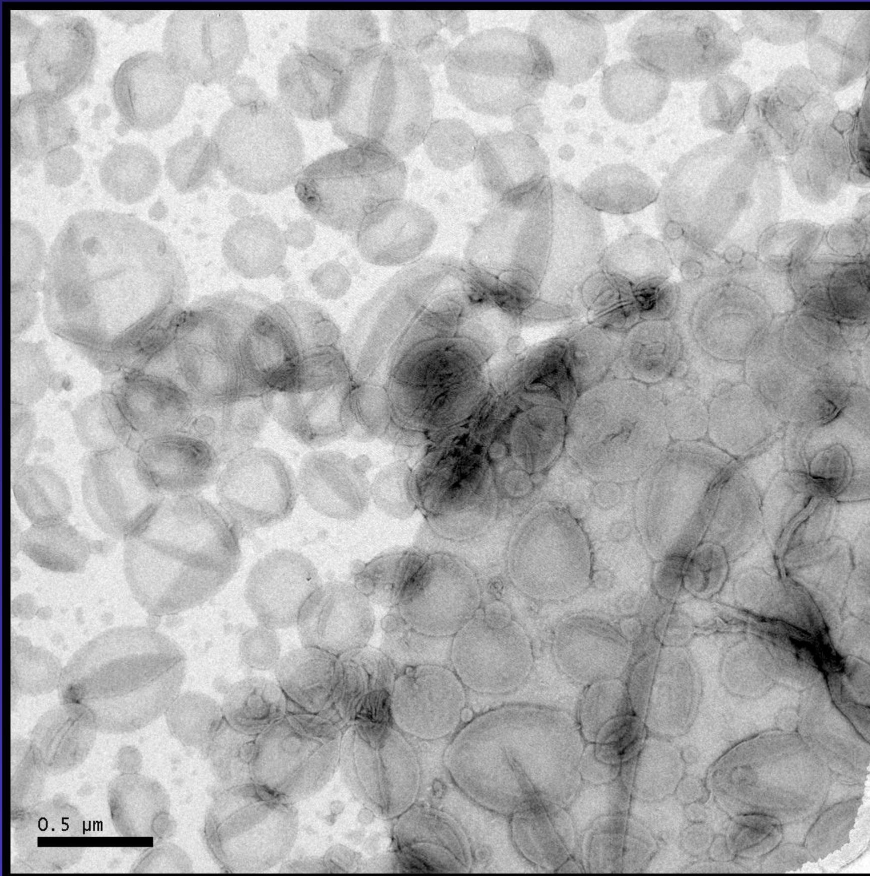
**Low magnification screening**

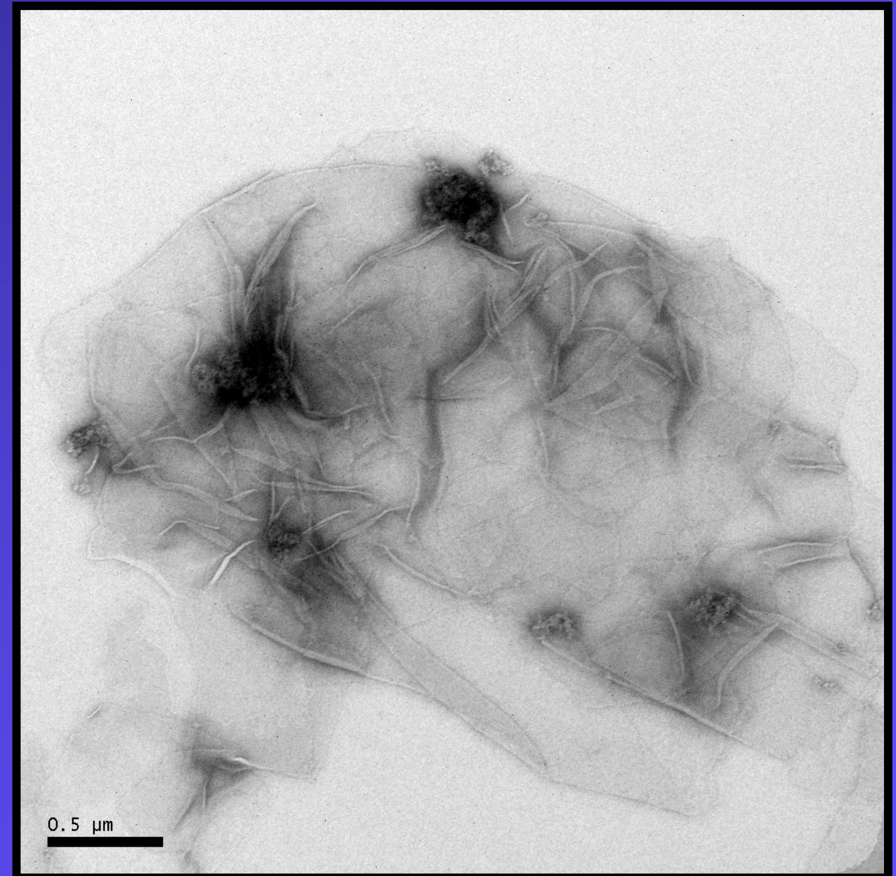



**High quality membranes with protein aggregation**

# The “experiment” - screening the samples

Low magnification screening

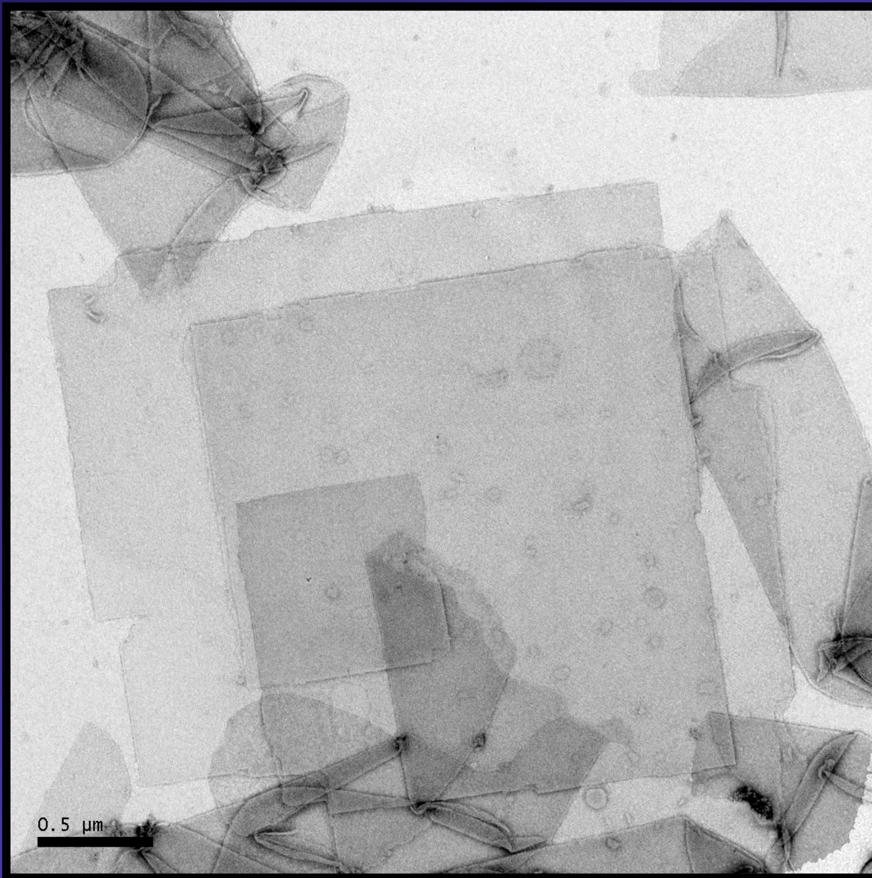


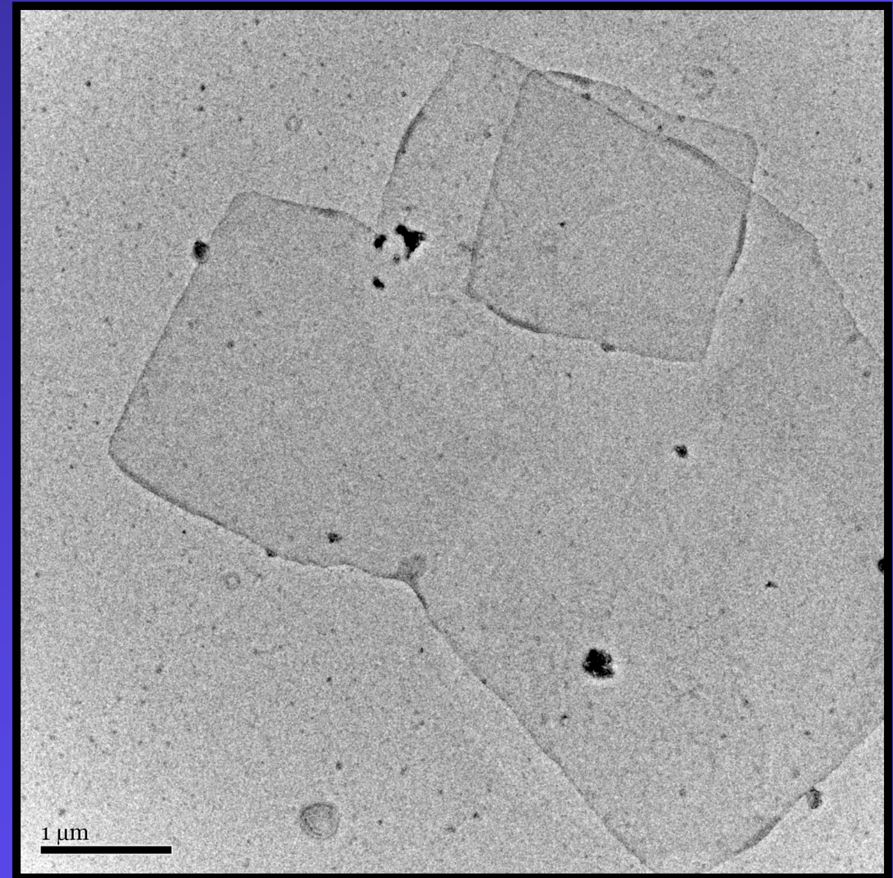

Vesicle size not crucial, but useful area matters

# The "experiment" - screening the samples

## Low magnification screening



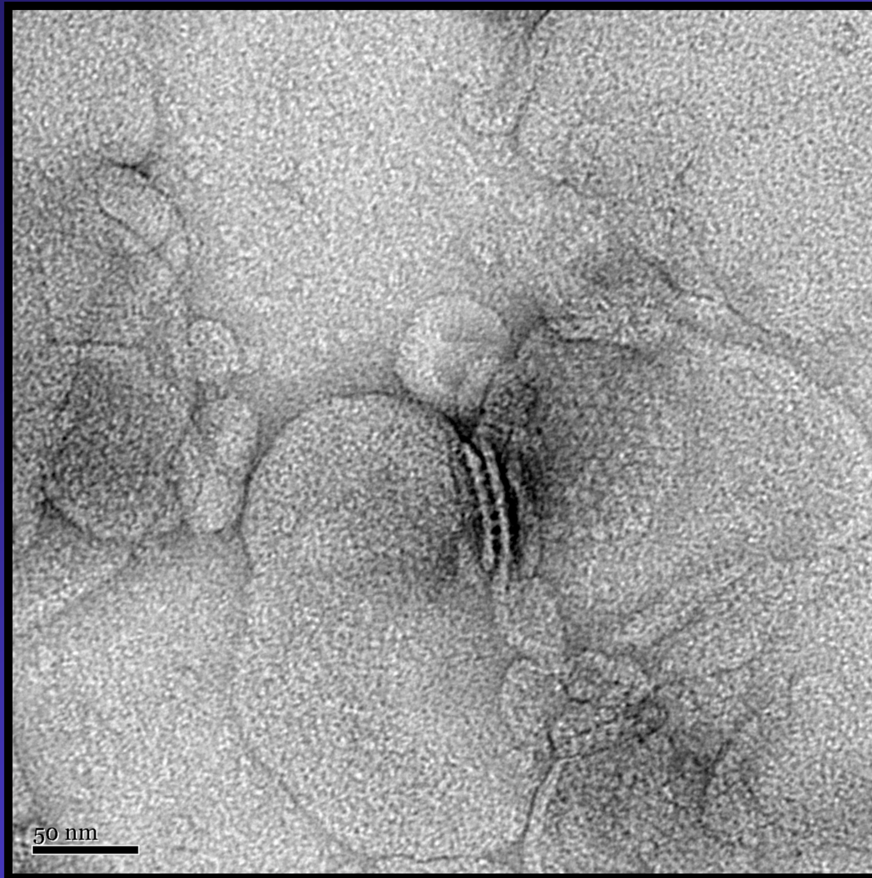

**Great sheets**
(square shape, sharp edges)




**Less great sheets**
(squarish shape, round edges)

# The "experiment" - screening the samples

## High magnification screening

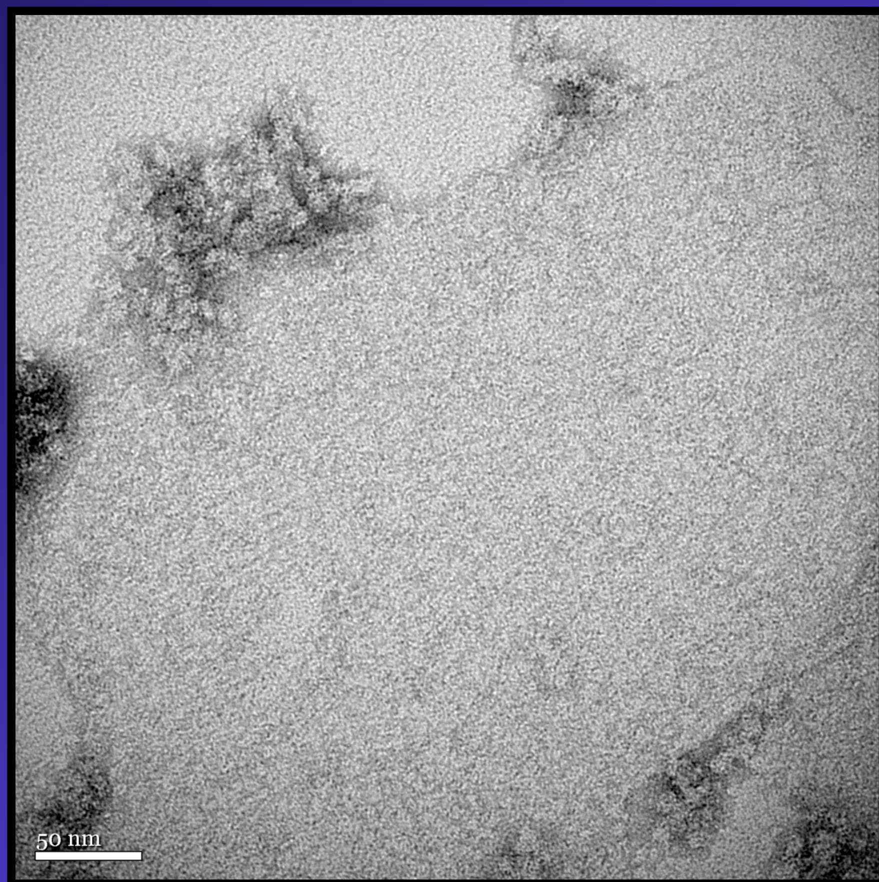


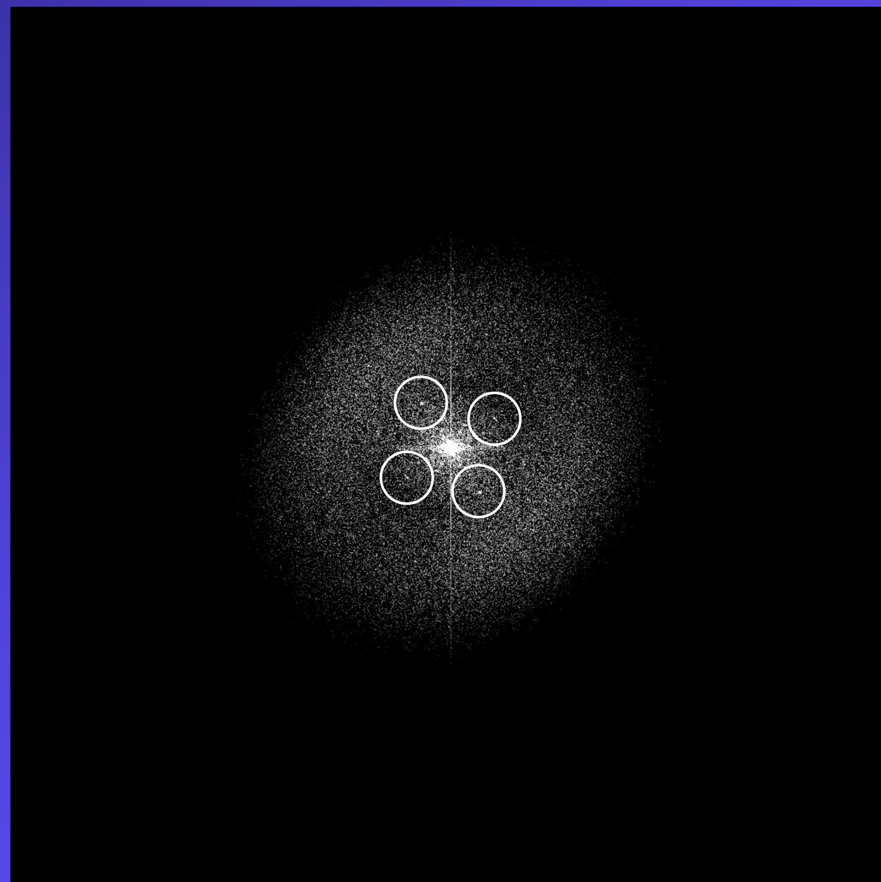

Small crystalline patch

Lipid structures

# The "experiment" - screening the samples

## High magnification screening

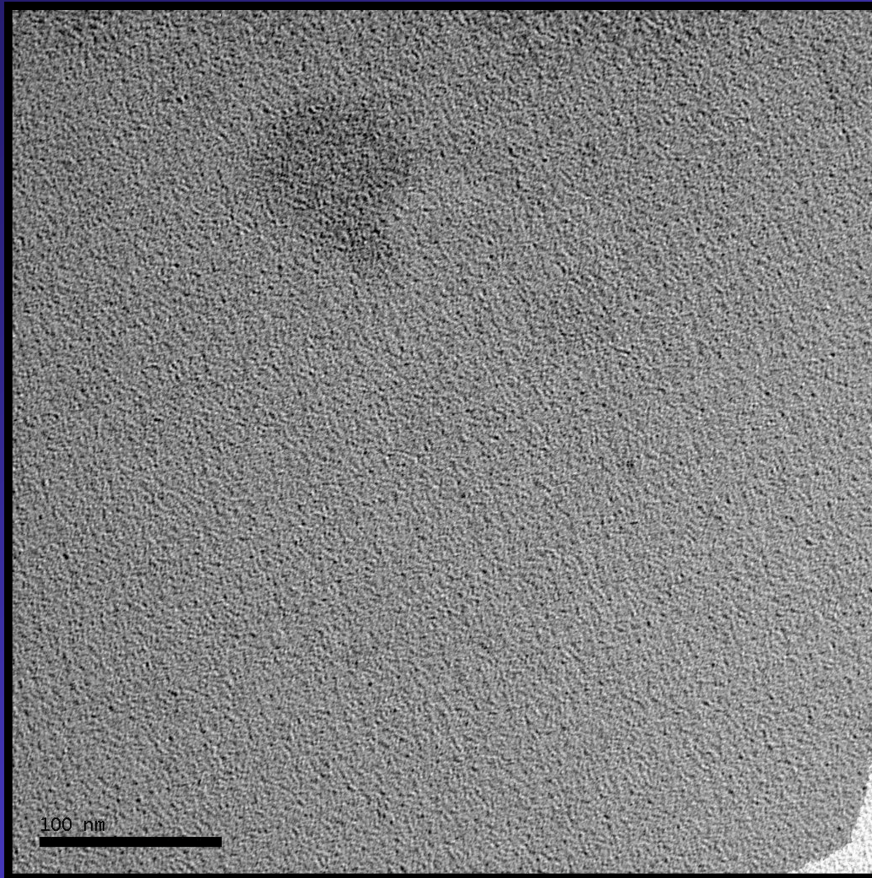


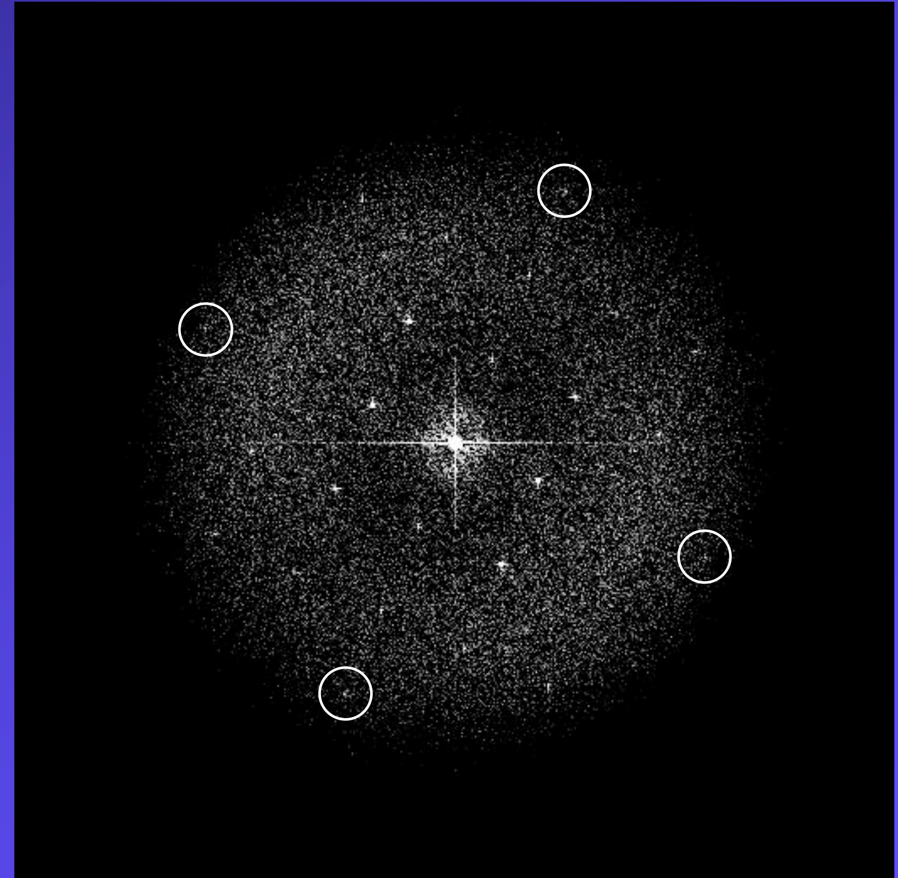



Poorly ordered 2D crystal

# The "experiment" - screening the samples

## High magnification screening



Well ordered 2D crystal

# The “experiment” - evaluating the results

**Always reproduce every reconstitution experiment at least one time, 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