Exploring lipid-based phases for membrane protein crystallization

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Part I:
Lipidic cubic phase

Part II:
Lipidic sponge phase
Bicelle crystallization
Part II:

Lipidic Sponge Phase and Bicelle crystallization methods

History, practical advice and successful examples
The Lipidic Cubic Phase - Recap

- Is a protein/lipid mixture used mainly for crystallization of membrane proteins
- Suitable for most types of membrane proteins
- Those containing cofactors (esp. hydrophobic ones) might not be suitable
- Standard techniques such as robots, additive screens, hanging and sitting drop are applicable.
- If the lipid MO is used, the crystallization must occur at room temperature
The Lipidic Cubic Phase (LCP)

- First membrane protein crystallized was bacteriorhodopsin (bR) by Landau & Rosenbusch in 1996
- Several GPCRs
- Photosynthetic proteins (RC, LHII)
- Rhodopsins

bR crystals in:
- a) MO (MAG 9.9)
- b) MP

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• Different MAGs are used
• Most common is Monoolein (MO), MAG 9.9
• Monopalmitolein (MAG 9.7)
• Monovaccenin (11.7 MAG)
• MAG 9.7
• MAG 7.7
• Semi-solid, “toothpaste”.
• a 60:40 mixture of lipid:protein, forms spontaneously
• Several phases are possible.
• Stable at room temperature, MO will revert to lamellar if colder.

A phase diagram of water and MO

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Clarifications

• The 60:40 ratio of lipid:protein refers to the w/w ratio
• The density of protein solution is assumed to be the same as that of water
• It is the amount of protein solution and not amount of protein which should be 40%
• Soaking? Perhaps better to try co-crystallization
Lipidic sponge phase

- A “liquid analogue” to LCP
- Is a swollen LCP (a precipitant is added)
- Liquid
- Can be used in hanging drop experiments
- Can be made in advance (the protein is added later)
- Is compatible with robots
• Just like the LCP it is compatible with most common crystallization agents (additives etc)
• Its liquid nature facilitates crystal visualization and harvesting
• It is possible to flow the sponge phase in a flow cell
• Is often referred to as L3 phase
• It is a ternary system consisting of lipid, water (or buffer) and a precipitant
• The protein is "replaced" by water-buffer
• Precipitants include PEGs (different MW), MPD and Jeffamine M600
• Salt is often included
The sponge phase area is very narrow
- Easy to end up outside when preparing a phase
- Often you end up in a cubic or lamellar phase
- For a PEG400 diagram, the water content is constant
If the phase diagram is not known for a particular sponge phase, start with a cubic phase.

Mix the lipid and the buffer in syringes.

Dispense the mixture in a glass vial and add precipitant on top in large excess.

The sponge phase will separate from the water phase within 24 hours.

A blue dye is contained within the lipid phase. After 23h, the sponge phase has formed!

If the phase diagram is known for a particular sponge phase it is possible to go directly to the sponge phase without creating the cubic phase first.

- Weigh in the required amounts into a glass vial, seal and incubate (up to 37°C).
- A transparent phase should form within 24 hrs.
- No phase separation should occur!
Requirements

- Buffer (including salts of choice)
- Lipid of choice
- Precipitant
- (Two gastight syringes with coupler)
- Glass vials with septa (or other airtight container)
- Crossed polarizers for phase inspection
Requirements

- Protein
- Crystallization plates (glass or plastic)
- Cover slides
- MiTeGen or nylon loops for harvesting
- No cryo protection necessary in most cases

In situ crystallization plates from Molecular Dimensions inc.
• Include MPD, Jeffamine, PEGs, PPO, ethanol
• All of these affect the phase through either swelling or shrinking (ethanol)
• The amount of precipitant necessary depends on the lipid and on the precipitant itself
• As an example, PEG400-based phases contain 20-40% PEG, while MPD-phases can go from 5% to 60% MPD
Various hydrophobic agents can be added to the phase for stabilization

- Cholesterol
- Cardiolipin
- Glycerol
- Co-factors (such as quinols)
- Drugs, inhibitors, pesticides
Destabilizing agents and actions

- Low (below 5) and high pH (above 9): the ester bond in the lipid will hydrolyse
- High temperature for a long will also hydrolyse the lipid
- Since the sponge phase is prepared without protein, it is usually not as sensitive in that respect as the LCP

Sponge phase in vial

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• The reaction centers (RC) from *R. sphaeroides* and *Bls. viridis* have been crystallized using swollen LCPs

• MO was used as the lipid and Jeffamine M600 as the the main precipitant (along with (NH$_4$)$_2$SO$_4$)

The LHII crystals were grown using 20 % (v/v) PPO as a swelling agent.

Cherezov et al. (2006). *JMB* **357**: 1605-1618
Successful examples: BtuB

200 nL protein:lipid mixture
3uL precipitant including:
10-12% (v/v) MPD

The crystals took 10-14 days to form.

Cherezov et al. (2006). *JMB* 357: 1605-1618
The addition of (at least 30%) PEG400 to a cubic phase will result in swelling of the phase to a sponge phase.

The GPCR conditions are: 50 nL of protein/lipid mix and 0.8µL precipitant (approx. 25% PEG)

Thus, most (or all) GPCR structures today, can be viewed as have proceeded via the sponge phase during crystallization!
Practical advice

- Requirements
- Experimental setup
- Optimization
- Finding and harvesting crystals
- Troubleshooting
Practical advice: choice of lipid

➢ The size of your protein, protruding membrane parts?
➢ MO easily available, but others need to be synthesized
➢ Temperature? If your protein is not stable at RT, try shorter MAGs
One a hit is found, optimization will be the next step.
The phase itself can be screened (varying the precipitant mainly)
Try optimizing: pH, salt (concentration), protein concentration, additives, bolus size, temperature, time
Try seeding
Change to other lipid is a big step!
Practical advice: phase interpretation

- Inspect the phase visually first
- It should look transparent without any granules or phase separation
- Inspect it between crossed polarizers
- Should appear dark (no shiny bits or layers should be present)
- SAXS measurements on the phase for characterization

While inspecting the phases you might think that they look good. Well, use crossed polarizers to be certain since looks can be deceiving...
To properly characterize the phase, SAXS (Small-Angle X-ray Scattering) must be performed.

- The rings are radially integrated.
- Shown to the left are:
  - PEG400, 1500 and 4000
  - Jeffamine M600
After the correct phase is confirmed, the next step is setting up crystals.

The phase is harvested from the vial (either the top layer or the whole layer dependent on preparation).

It is then used as in a vapour diffusion experiment.
Practical advice: finding crystals

- The LSP suffer from the same problems as LCP
- Often difficult to spot small colorless crystals
- Often phase separation in the drop
- Check edges for crystals
- Use polarizers
- Set up control plates without protein
Practical advice: harvesting

- Use MiTeGen or nylon loops
- Phase separation often occur in the drop due to dehydration
- Upon removal of cover slide, add a drop of the precipitant solution to avoid drying out.
- Often the lipid and precipitants act as cryo protectants

Litholoops from Molecular Dimensions Inc.

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Troubleshooting: cloudy phase

- If a phase turns cloudy after preparation it needs to be shifted towards the sponge phase by addition of buffer to the system.
- Often only a microliter will suffice.
- Reincubate.
- Repeat the steps if it is still cloudy.

Using crossed polarizers can give an indication of a cloudy phase.
Trouble shooting: phase separation

- Unwanted phase separation occur (e.g. three layers in a swollen cubic phase)
- Difficult to reverse the phase separation
- Better to prepare a new one
- If unsure, add less buffer to start with
- Happens often since the sponge phase area is very narrow

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Other problems

• Colorless proteins difficult to spot
• Chromophores might be lost
• Prepare enough phase in advance since it takes approx. 24 hours to form
• Novel data collection methods and micro-focus beamlines have greatly improved the success rate.
• Possible to merge data from several small crystals
Comparing LCP and LSP

**LCP**
- Stiff, “toothpaste”
- Can not be pipetted
- Harvesting can be difficult
- Easier to vary
- Monitor temperature mainly
- Room temp. Crystallization
- Does not flow in a flow system

**LSP**
- Liquid, though viscous
- Pipetting possible
- Harvesting easier
- Many phases to prepare
- Needs characterization before crystallization
- Room temp, crystallization
- Can be used in a flow system in an XFEL experiment
The lipidic sponge phase screen

Left to right, row by row:
(1) *plasmodium* aquaporin
(2) spinach aquaporin
(3) complex II from *Bacillus subtilis*
(4) RC from *Rhodobacter sphaeroides*
(5) RC from *Blastochloris viridis*,
(6) LH2 from *Rhodopseudomonas acidophila*
(7) LH2 from *R. sphaeroides*
(8) RC-LH1 from *Bl. viridis*

Time-resolved Laue crystallography using LSP

532 nm laser beam
Polychromatic X-ray beam

Jeffamine-based LSP was used to grow large crystals used for time-resolved laue crystallography

LSP for XFEL studies of membrane proteins

- XFEL sources can be used to determine the structure of various unknown proteins
- Requires large amounts of nanosized crystals
- Crystals in LSP can be flown in a jet-delivery system
- Both PEG and Jeffamine-based LSPs have been successfully injected
- RC crystals grown in batch have a different space group than those grown in hanging drop.

Johansson et al. *Under review*
LSP for XFEL studies of membrane proteins

RCvir Data to 2.7 Å

Collection in Feb. 2011 at the CXI beamline (LCLS)

- Repetition rate: 120 Hz and λ=1.5 Å
- The crystallization conditions were optimized (higher hitrates)
- Approx. 12 hrs of data collection

Data processing is in progress!
Molecular Dimensions inc has a screen with 48 different LSP conditions including Jeffamine M600, PEG400, 1500 and 4000 at various pH.

Tools such as syringe couplers can be obtained from Emerald Biosciences among others.

Glass vials can be purchased from Sigma.

MAGs from Nu-Chek prep inc, Sigma, Avanti Polar Lipids among others.


Bicelle crystallization

- Fairly new method
- Suitable for room temperature crystallization
- Is a detergent-lipid type of crystallization method
- Relies on using lipids such as DMPC and DMPG along with detergents such as CHAPS and CHAPSO to form a suitable crystallization environment
Bicelles

- Bicelles are small bilayer disks that form in lipid/amphiphile mixtures
- Lower viscosity at low temp (solid at 0°C)
- Crystallization takes place at room temperature or higher
- Gel-like at room temperature and thought to form a perforated lamellar structure
The protein solution is mixed with a lipid/amphiphile mixture on ice

A precipitant is added

The resulting mixture is dispensed onto a cover slip

Incubate at room temp (or above)
The lipid/detergent mix needs to be vortexed or sonicated to form (done on ice)

Sometimes the mixture is incubated over night

Freeze-thawing is also possible

Once the phase is formed it is mixed with protein (on ice) and transferred to a crystal plate, incubated with precipitant at higher temp to allow formation of a gel
Most additive screens are compatible with bicelles, however salt or detergent crystals may form.

It is possible to add other lipids into the mix (use chloroform to dissolve them into the lipid first, remove chloroform) and proceed as usual.

Possible to use the robot if dispensing occurs quickly or at 4°C.
• Ratios of bicelle:protein can be varied extensively
• Choice of lipid and detergent
• Vary precipitant, salt and pH
• Additive screens (useful for robot screens too)
• Hanging vs. Sitting drop
• Batch crystallization
Successful examples: mVDAC1

4:1 protein: bicelle ratio
DMPC:CHAPSO (2.8:1 ratio)

Other additives include
10% PEG400 and 18-20% MPD

Ujwal et al. (2008), PNAS 18:17742-7.
Successful examples: bR

- a) DTPC and CHAPSO
- b) DMPC and CHAPSO

These crystals look very similar to those grown in LCP!

Faham et al. (2005). *Protein science* **14**:836-840
Successful examples: Xantorhodopsin

Bicelle: 16.7% Dimyristoyl- PC and 20 % nonyl maltoside

This type of bicelle composition is fairly unusual and is not commercially available.

Luecke et al. *PNAS* **105**: 16561-65

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Successful examples: $\beta_2$-adrenergic GPCR

Bicelle: DMPC and CHAPSO
Crystals grew within 10-14 days in hanging drops.

• Kits are available from Anatrace containing DMPC, DMPG, CHAPS and CHAPSO. Requires mixing of the lipid and detergent!
• MemX Biosciences’ screen contains: DMPC and CHAPSO. No mixing needed!

MemX Biosciences Bicelle kit

Faham et al. (2005) Crystallization of bacteriorhodopsin from bicelle formulations at room temperature. Protein science 14:836-840

Acknowledgements

LCLS:
Christoph Bostedt
John Bozek
Mike Bogan
Sebastien Boutet
Ryan Coffee

DESY:
Henry Chapman
Thomas A. White
Anton Barty
Karol Nass
Daniel DePonte
Andrew Aquila
Mengning Liang

Heidelberg- MPI:
Ilme Schlichting
Robert L. Shoeman
The CAMP detector group

Arizona State University:
Petra Fromme
John H. Spence
Uwe Weierstall
R. Bruce Doak
Richard Kirian

Uppsala:
Jan Davidsson

University of Gothenburg:
Richard Neutze
David Arnlund
Gergely Katona
Erik Malmerberg
Annemarie Wöhri
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