Effects of Detergents on the Crystallization of Bacteriorhodopsin

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Summer Undergraduate Research Fellowship

NIST National Institute of Standards and Technology U.S. Department of Commerce

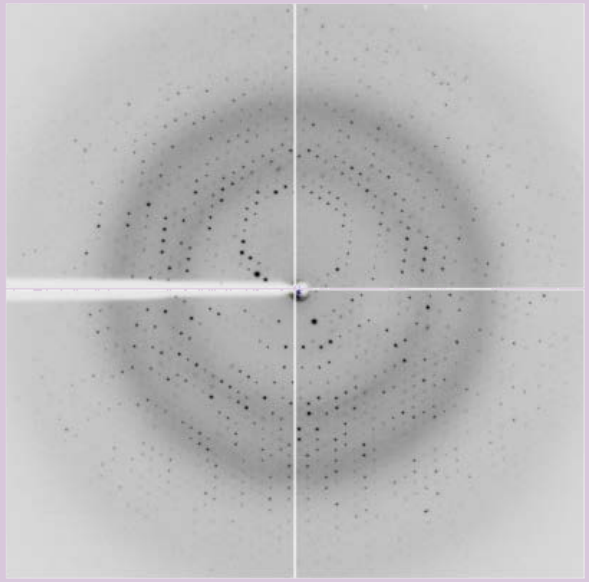
NIST Center for Neutron Research

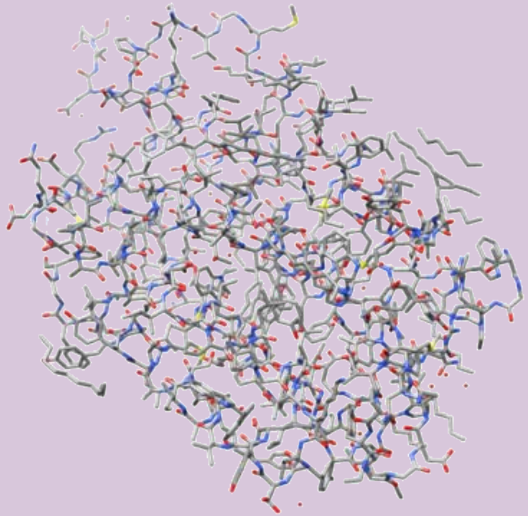
NSF CHRNS Center for High Resolution Neutron Scattering NIST

OUTLINE

- Protein crystallization
  - Why?
  - How?
- Membrane proteins
  - How is approach to crystallization different?
  - Lipidic cubic phase: what is it, and why use it?
- Project Aims
- Approach
- Results

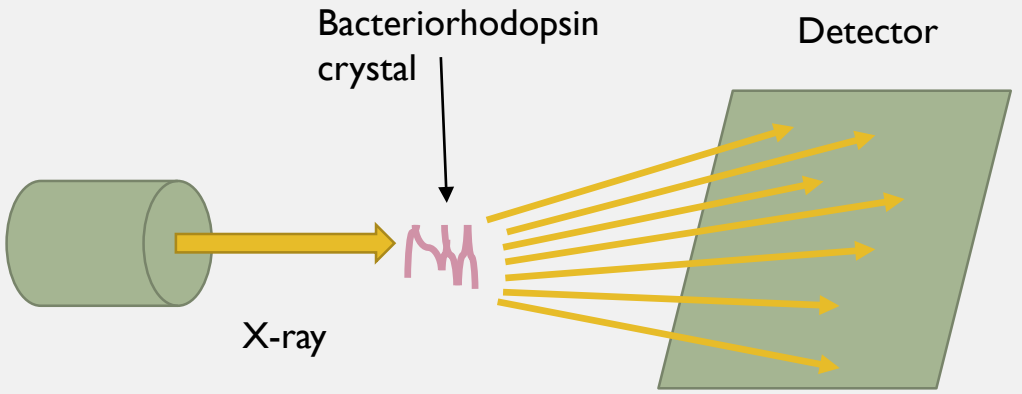
PROTEIN CRYSTALLIZATION





Bacteriorhodopsin diffraction patterns and resulting structural determination.

- Protein crystals are necessary for structure determination by x-ray crystallography
  - Protein crystal is exposed to x-ray beam
  - Results in diffraction patterns from electron clouds
  - Determines three dimensional structure of proteins and macromolecules
  - Structures necessary for understanding protein function
    - binding to other proteins
    - binding to drugs
    - enzyme mechanisms

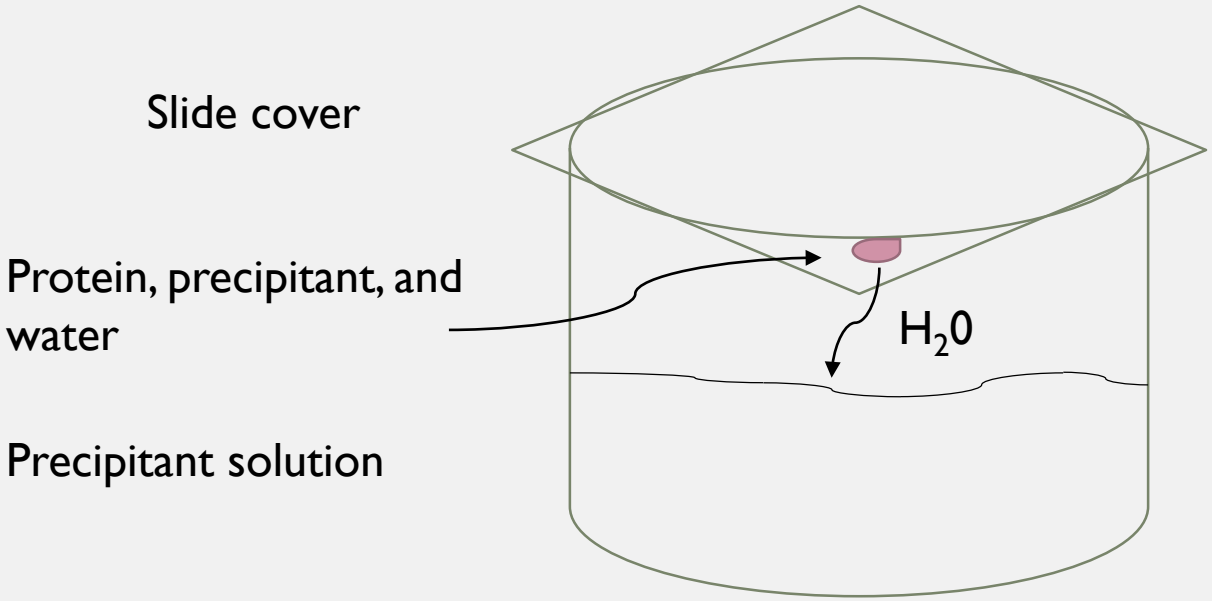


X-ray diffraction

HOW IS IT USUALLY DONE?

SOLUBLE PROTEINS

- Highly concentrate target protein
- Introduce precipitant to encourage crystal growth
- Hanging drop vapor diffusion



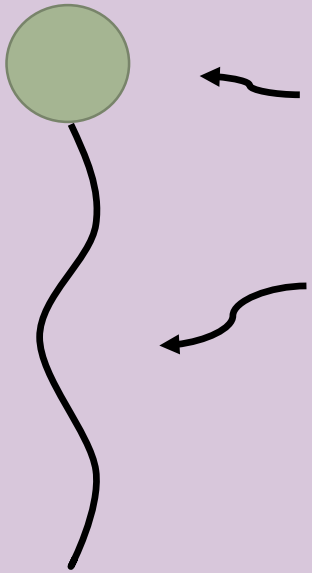
MEMBRANE PROTEINS

- Proteins are not soluble
  - Need detergents!
- Highly concentrate target protein
- Introduce precipitant to encourage crystal growth
- Protein and detergent complexes may also be incorporated into the lipidic cubic phase

OVERVIEW

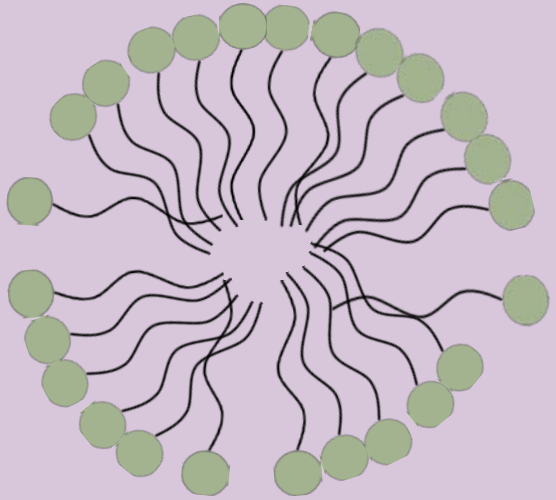
- *In meso* crystallization is important method for some classes of membrane proteins
  - Small proteins with few crystal contacts in hydrophilic domains
  - Better crystal packing can lead to higher-resolution crystal structures
- Detergents are used to solubilize proteins
  - Importance of detergent identity unknown in the cubic phase
  - Currently expensive and highly purified detergents in use
- Protein and detergent complexes are incorporated into lipidic cubic phase
  - Compare success of wide span of detergents

THE ROLE OF DETERGENT

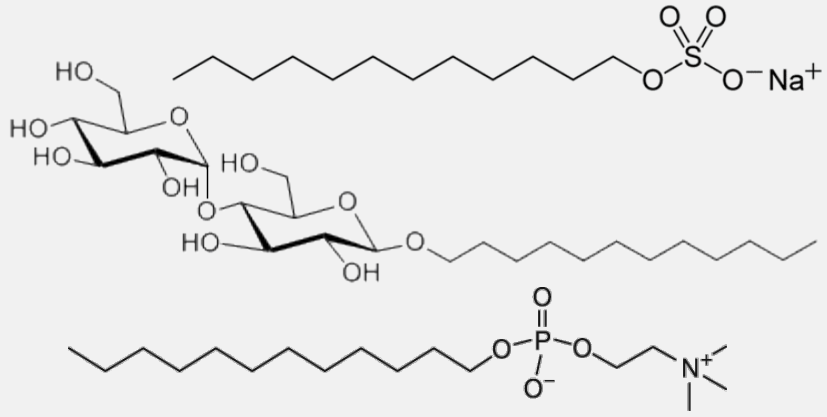


Hydrophilic head group

Hydrophobic tail

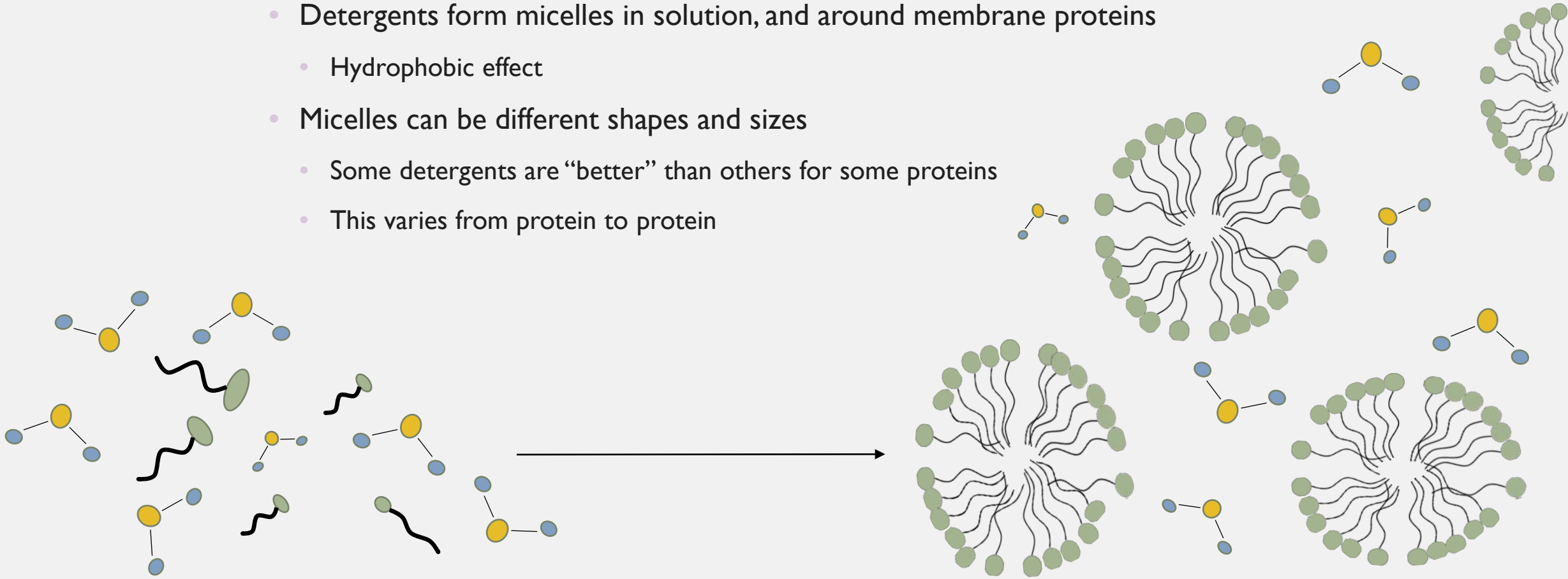


- Amphiphilic nature allows hydrophobic parts of membrane proteins to be solubilized
  - Forms lipid bilayer
- Large span of detergent properties
- Detergent characteristics affect chemical properties
  - Detergent monomers
  - Micelles have different shapes and sizes
- Prepares for incorporation into lipidic cubic phase

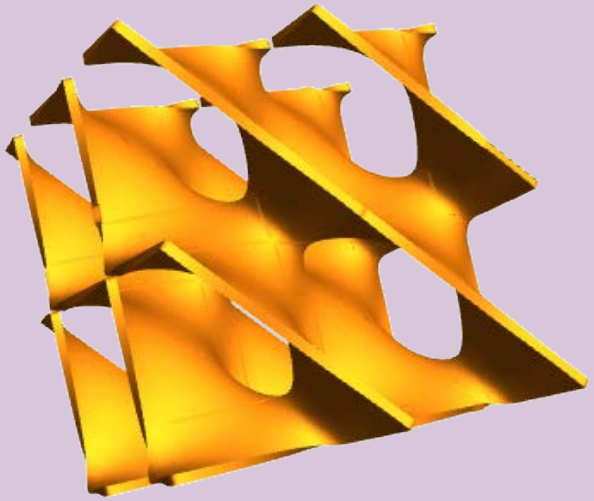


DETERGENTS IN SOLUTION

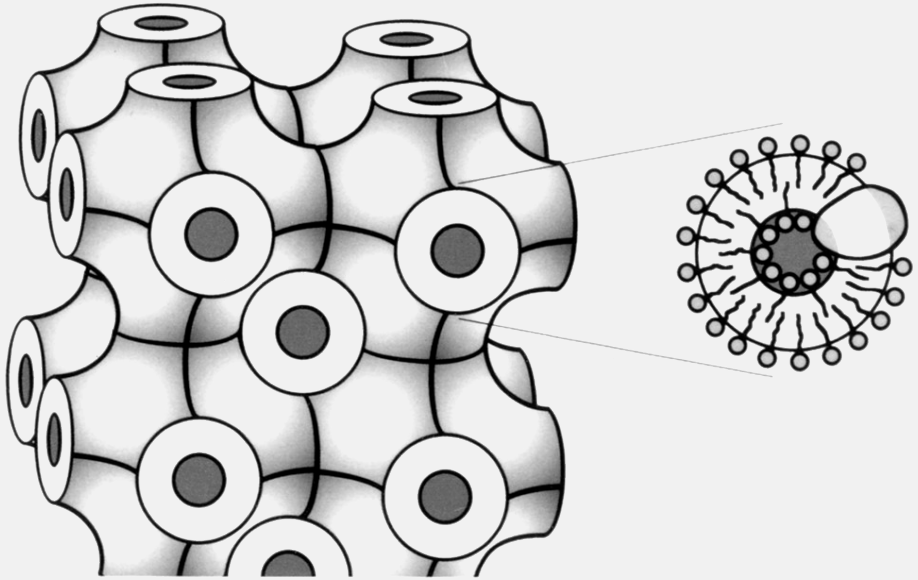
- Detergents form micelles in solution, and around membrane proteins
  - Hydrophobic effect
- Micelles can be different shapes and sizes
  - Some detergents are "better" than others for some proteins
  - This varies from protein to protein



THE CUBIC PHASE

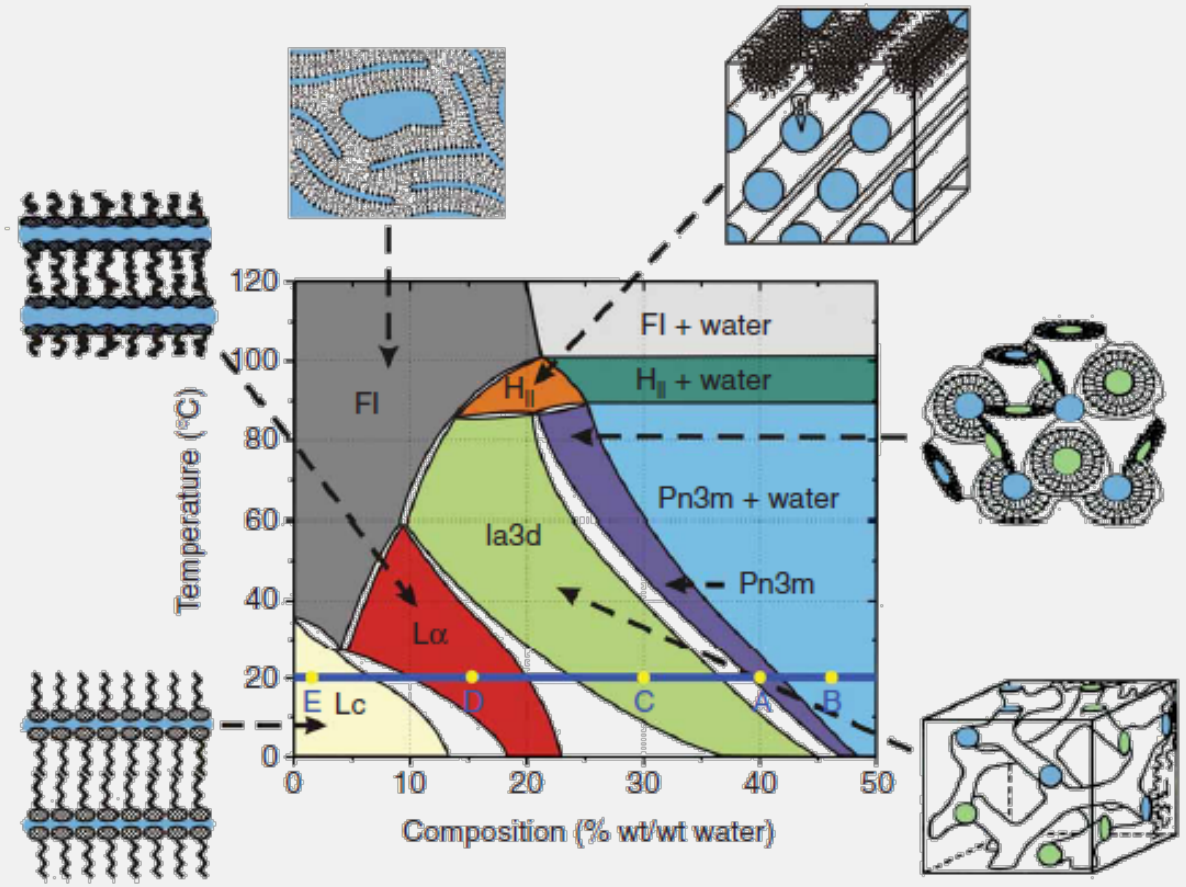


- Three dimensional bilayer with water, lipid, and protein
  - Forms a bicontinuous phase
- Protein reconstituted into the lipid bilayer
  - Protein remains in native and active conformation
  - Protein mobility
- Precipitant added to induce phase separation
  - Phase that has high levels of protein can encourage crystal growth
- Viscous and difficult handling

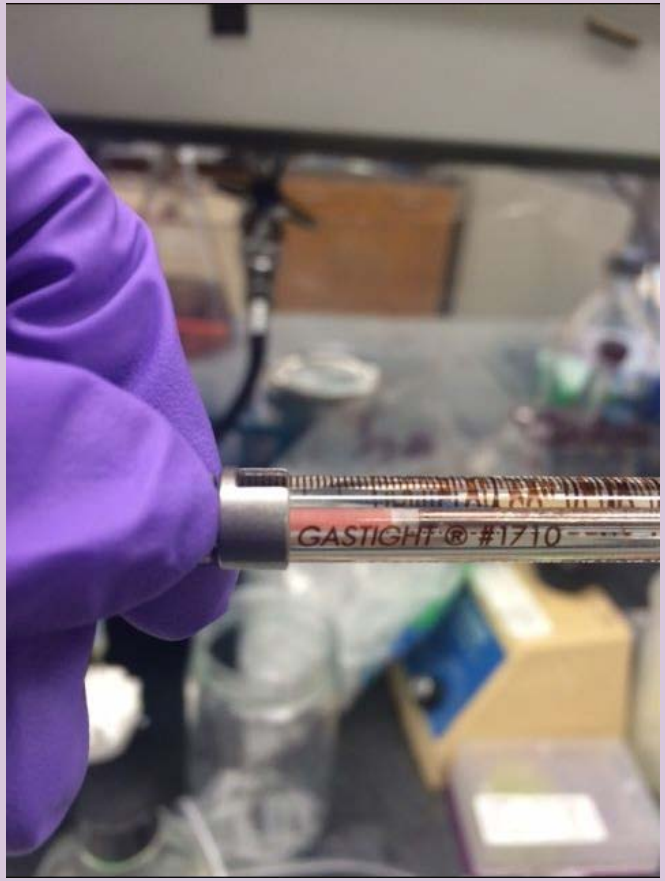


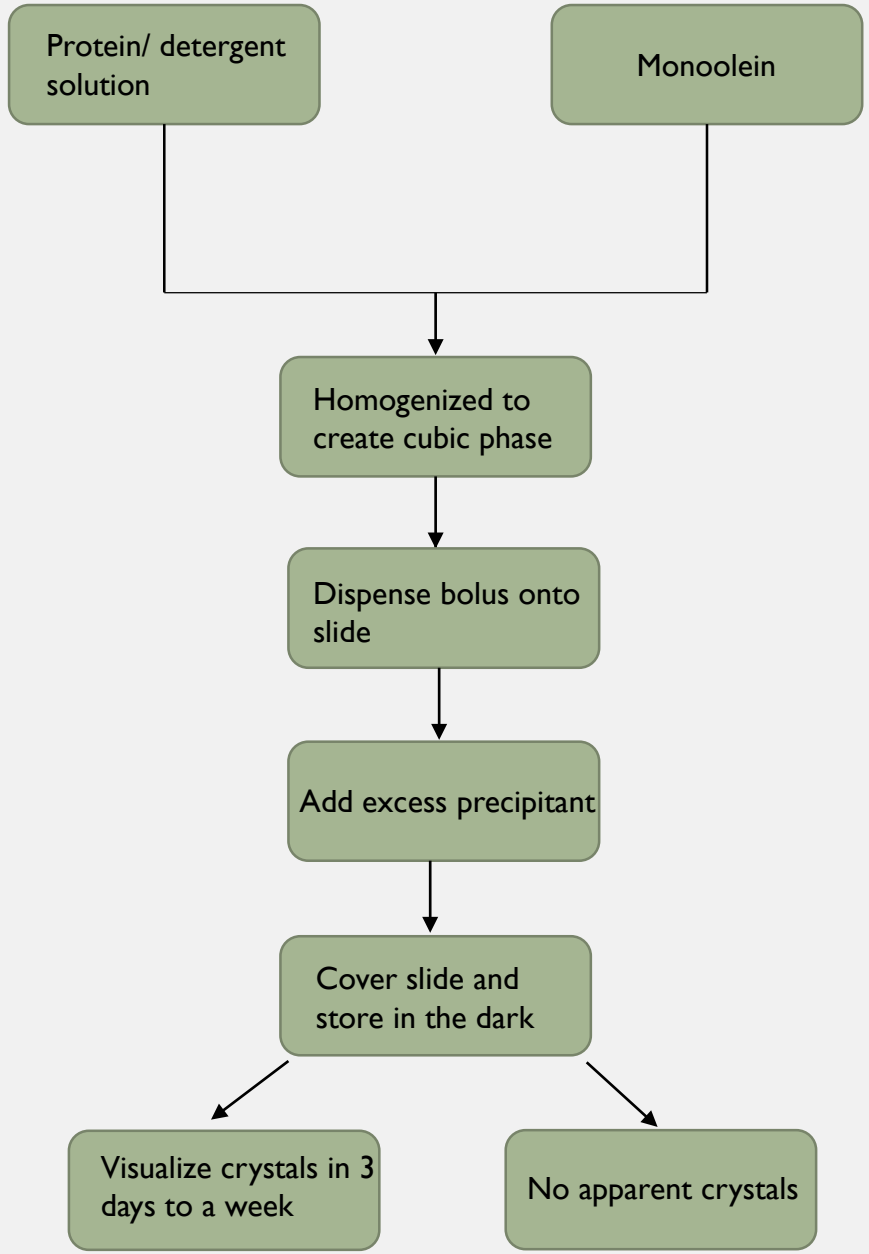
IN MESO CRYSTALLOGENESIS

- Lipidic cubic phase formed through lipid hydration
  - 40% hydration for protein incorporation
- Cubic mesophase as environment for crystallization
- Hydrated monoolein to Pn3m stage

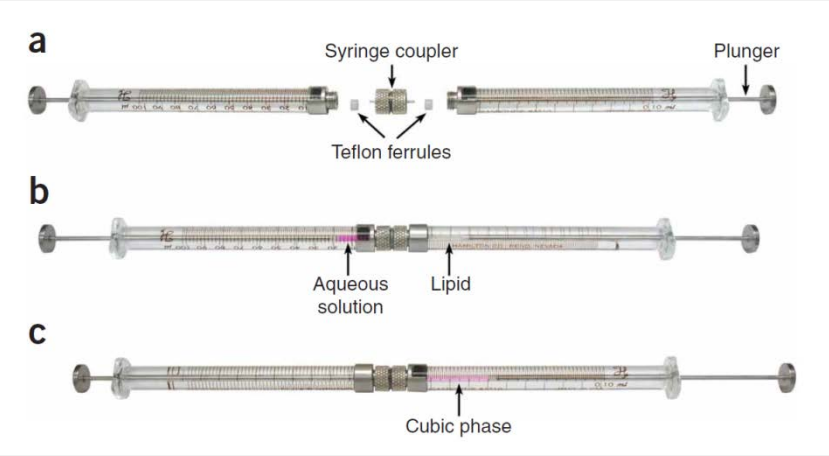


CRYSTALLIZATION OVERVIEW

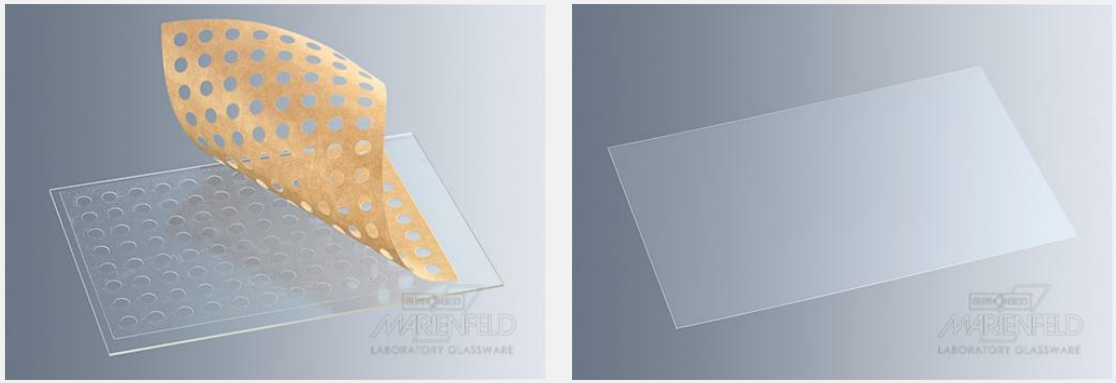




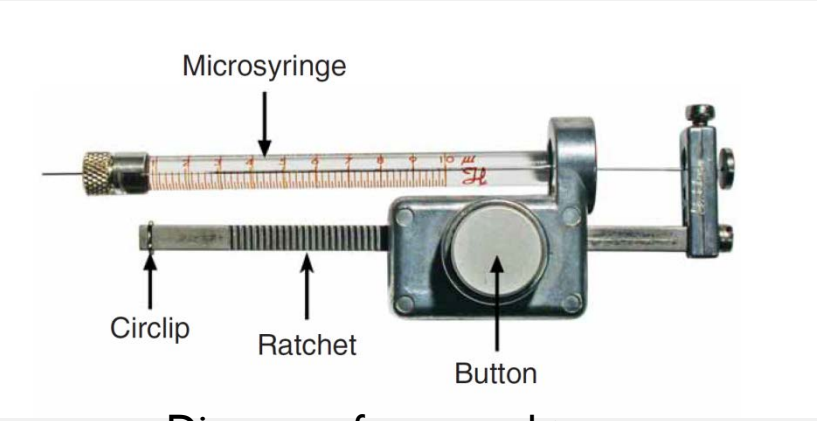
MAKING LCP AND PERFORMING CRYSTALLIZATION TRIALS



Mix lipid:protein in correct ratio (60:40 for monoolein)

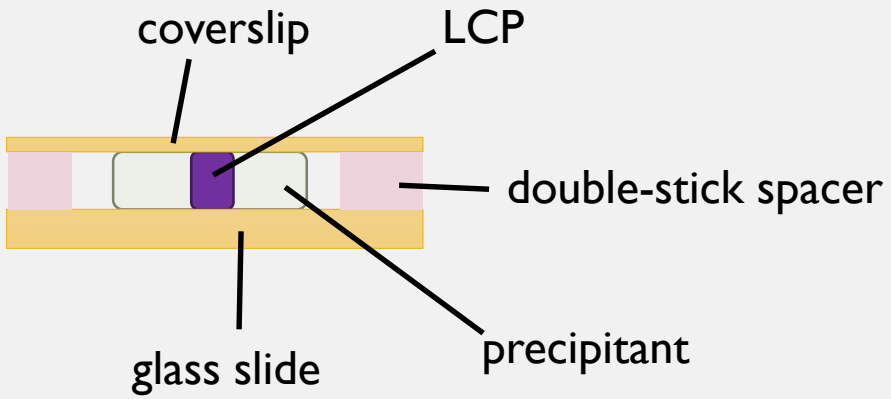


Sandwich plates



Dispenser for manual trays

Side View:



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- **Project Aims**
- Approach
- Results

PROJECT AIMS

1. **Determine whether a variety of detergents (other than the standard octyl glucoside) support crystallization of bR in LCP.**

   Other crystallization-related side issues:

   - SANS is done in D_2O. We need to verify that crystals can still be obtained.
   - Crystallization is usually done in bR after size exclusion chromatography. For rapid detergent screening, we will only use centrifugation.

2. **Use scattering to measure shape/size of micelles for common detergents**
3. **Determine what happens to these detergents upon incorporation into the LCP, and after addition of precipitant.**

PROJECT AIMS

"***Does*** the detergent matter?"

1. **Determine whether a variety of detergents (other than the standard octyl glucoside) support crystallization of bR in LCP.**

   Other crystallization-related side issues:

   - SANS is done in D_2O. We need to verify that crystals can still be obtained.
   - Crystallization is usually done in bR after size exclusion chromatography. For rapid detergent screening, we will only use centrifugation.

Why does it (not) matter?

2. **Use scattering to measure shape/size of micelles for common detergents**
3. **Determine what happens to these detergents upon incorporation into the LCP, and after addition of precipitant.**

MOTIVATION

- In solution, detergents that are good for protein stability can be bad for crystallization:
  - Large micelle
  - Heterogeneous
- Detergents that are good for crystallization can be bad for stability, or can present other practical difficulties:
  - Can be extremely costly
  - Other poor properties (e.g., low solubility, complex pH/temperature behavior, etc.)
- **Hypothesis: micelles dissociate upon incorporation into the lipidic cubic phase, so the detergent identity becomes less important than in solution.**
- **It would be nice to be able to work with any detergent that your protein is stable in *without* having to separately consider whether that detergent will allow crystallization.**

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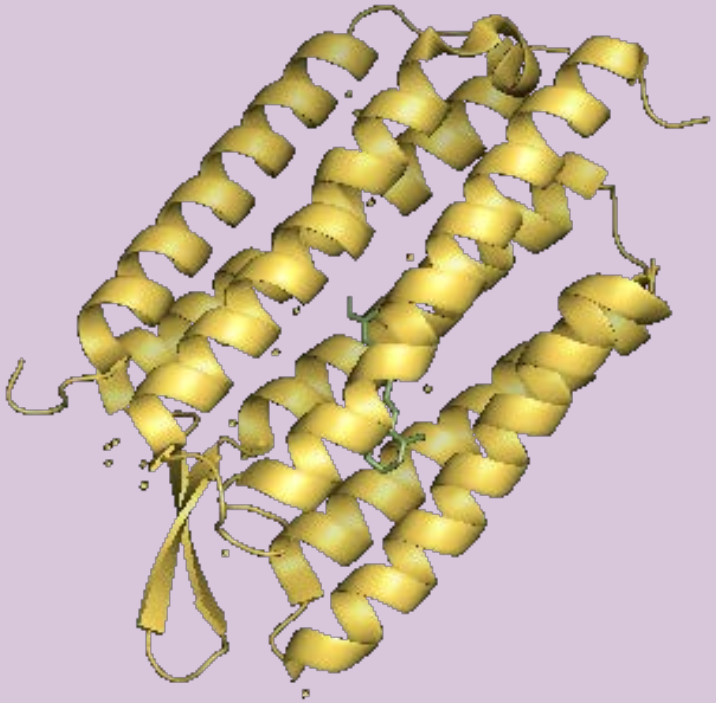
MODEL SYSTEM: BACTERIORHODOPSIN (BR)



- Photosynthetic transmembrane protein in *Halobacterium salinarum*
  - Converts light energy into proton gradient
  - Naturally present in "purple membrane:" two-dimensional crystals embedded in cell membrane
- Structure and function studied in detail
- Stable
- Crystallization propensity

BR CAN BE EXPRESSED AND PURIFIED IN LARGE AMOUNTS

Cell Pellet

Low-Salt Lysis

Washed Membranes

Sucrose Gradient

bacterioruberin

bR

debris

"Purple Membrane"

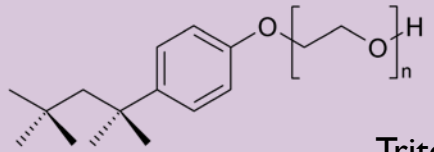
FURTHER PURIFICATION BY SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Addition of OG to purple membrane:

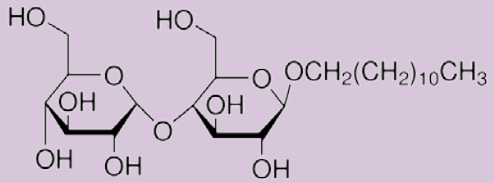
t = 0 1 day

Size exclusion chromatography column

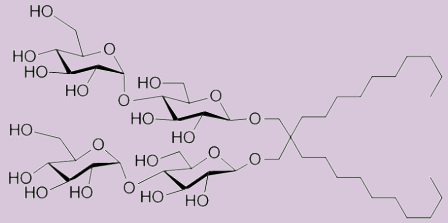
DETERGENT CLASSES SELECTED FOR STUDY



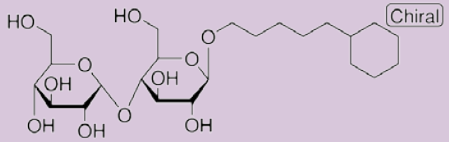
Triton Detergent



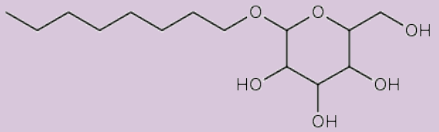
Maltoside Detergents



Neopentyl Glycol Detergents

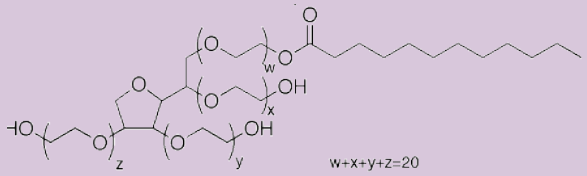


5-Cyclohexylpentyl β-D-maltoside

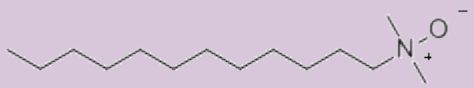


n-Octyl-β-D-Glucopyranoside **$20.12**

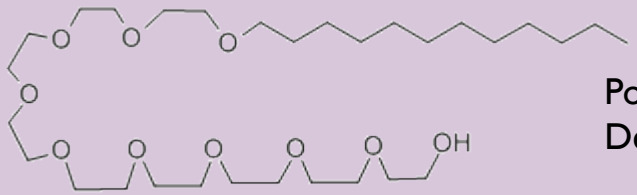
Elugent **$1.48**



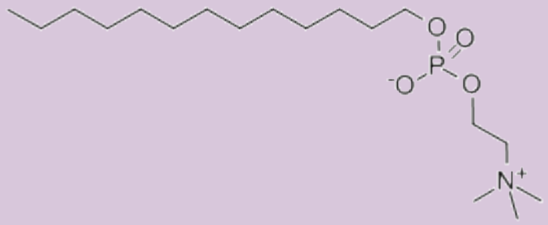
Tween Detergents



n-Dodecyl-N,N-Dimethylamine-N-Oxide

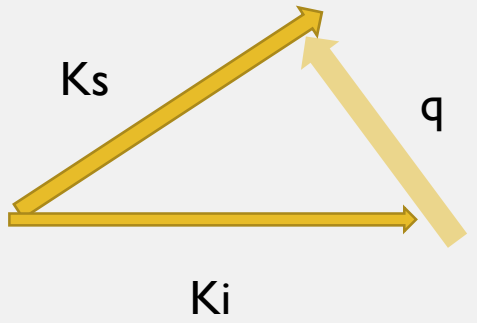


Polyoxyethylene Ether Detergents



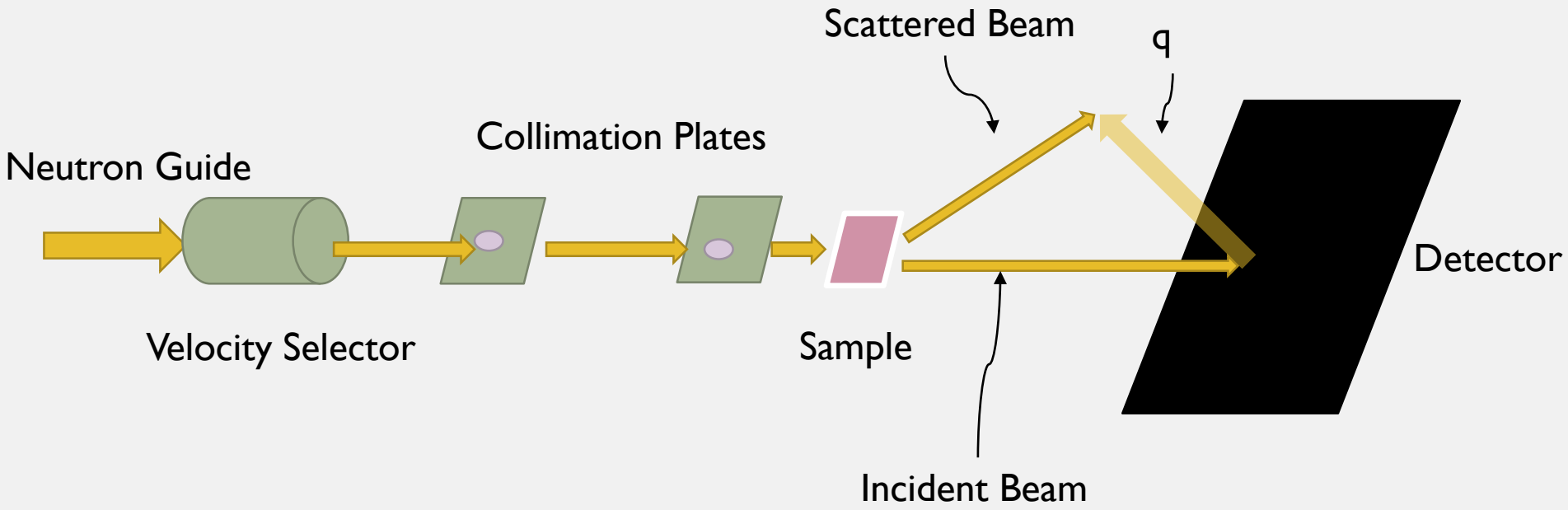
Fos-Choline

SMALL-ANGLE NEUTRON SCATTERING

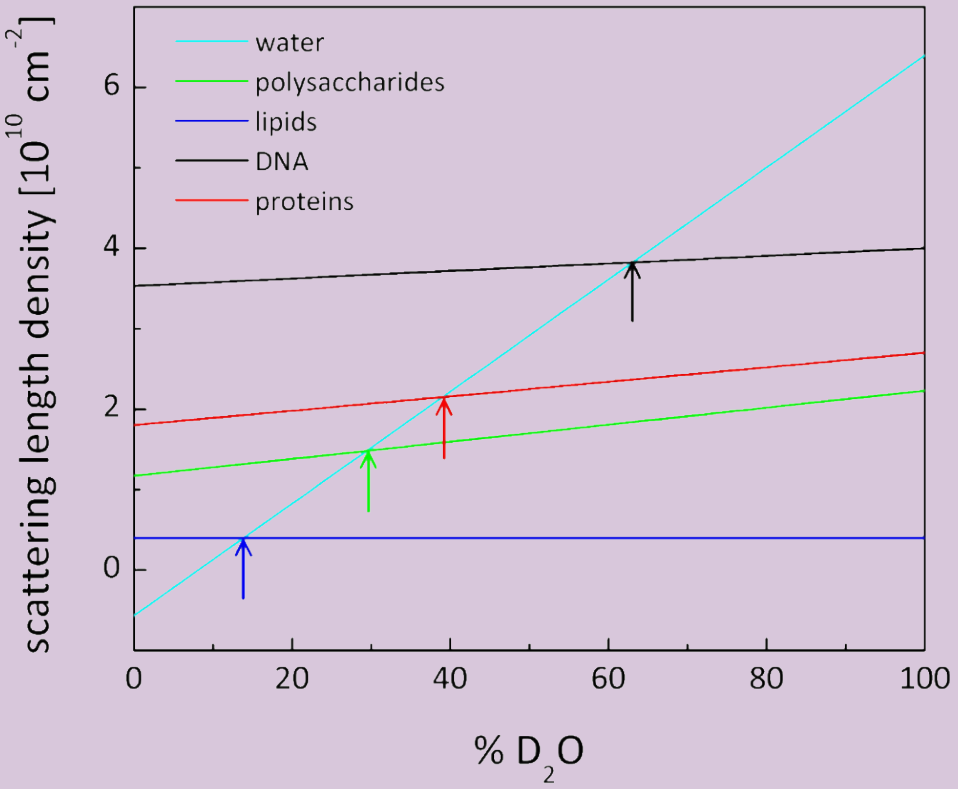


- Scattering of neutrons through interaction with nuclei
  - Scattering is result of inhomogeneities in sample (scattering length density)
- Detector can move to reach a range of scattering angles

$$Q = 2Ki\sin\theta = \frac{4\pi}{\lambda}\sin\theta$$

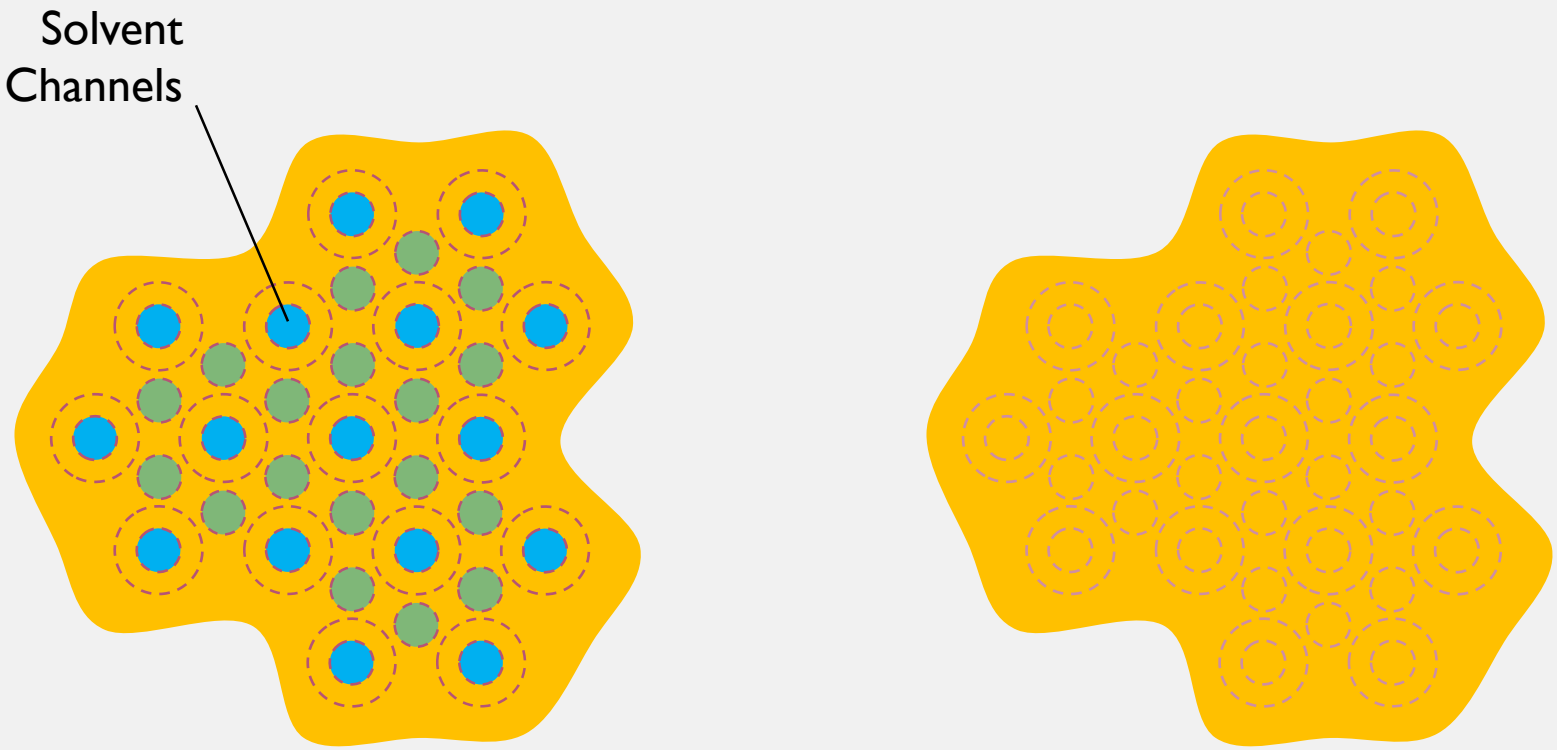


WHY NEUTRONS?



- Contrast matching
  - Exchanging hydrogen for deuterium in order to match your solvent to a component of sample
    - D_2O has scattering length density of 6.3×10^{-6} Å^{-2}
    - H_2O has scattering length density of -0.56×10^{-6} Å^{-2}
    - H_2O/D_2O mixture can match any sample component with SLD between those values
  - Can be used to silence some features and examine others

SANS WITH CONTRAST-MATCHED LIPIDS



Without contrast match:
Scattering will be mostly from the lipid (which makes up most of the sample)

Adjust $[H_2O]/[D_2O]$ of solvent so that its scattering length density matches the lipid

With contrast matching:
Scattering will be mostly from structures embedded in the (now-invisible) lipid

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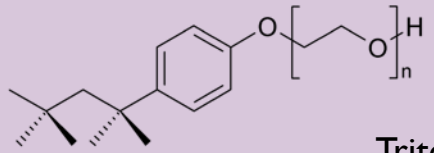
RESULTS

1. **Crystallization trials**
   - **Different detergents?**
   - **H2O vs D2O?**
   - **Size-exclusion chromatography necessary or not?**
2. Scattering Measurements
   - Detergent micelles
   - Detergents in the lipidic cubic phase
     - ✓ After initial mixing
     - ✓ Immediately after precipitant addition

PROTEIN SOLUBILIZATION



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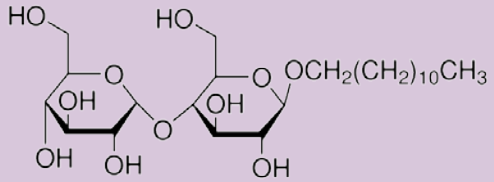

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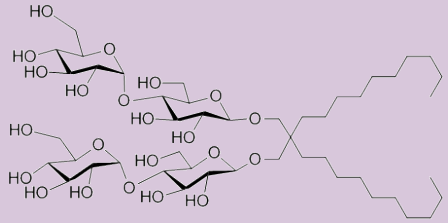

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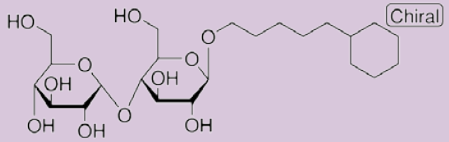

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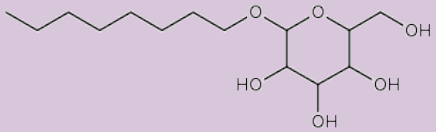

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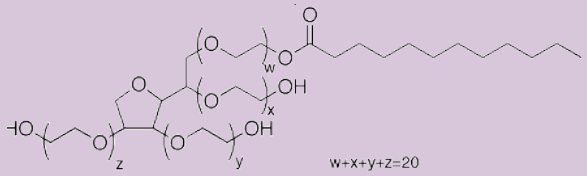

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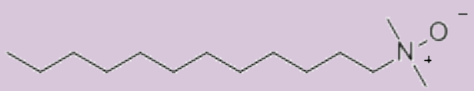

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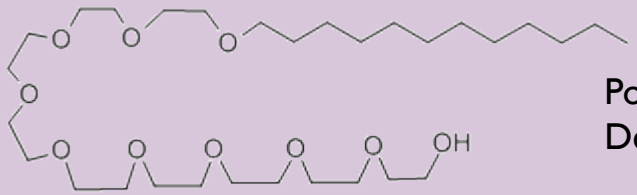

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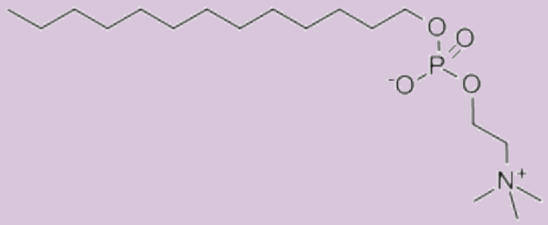

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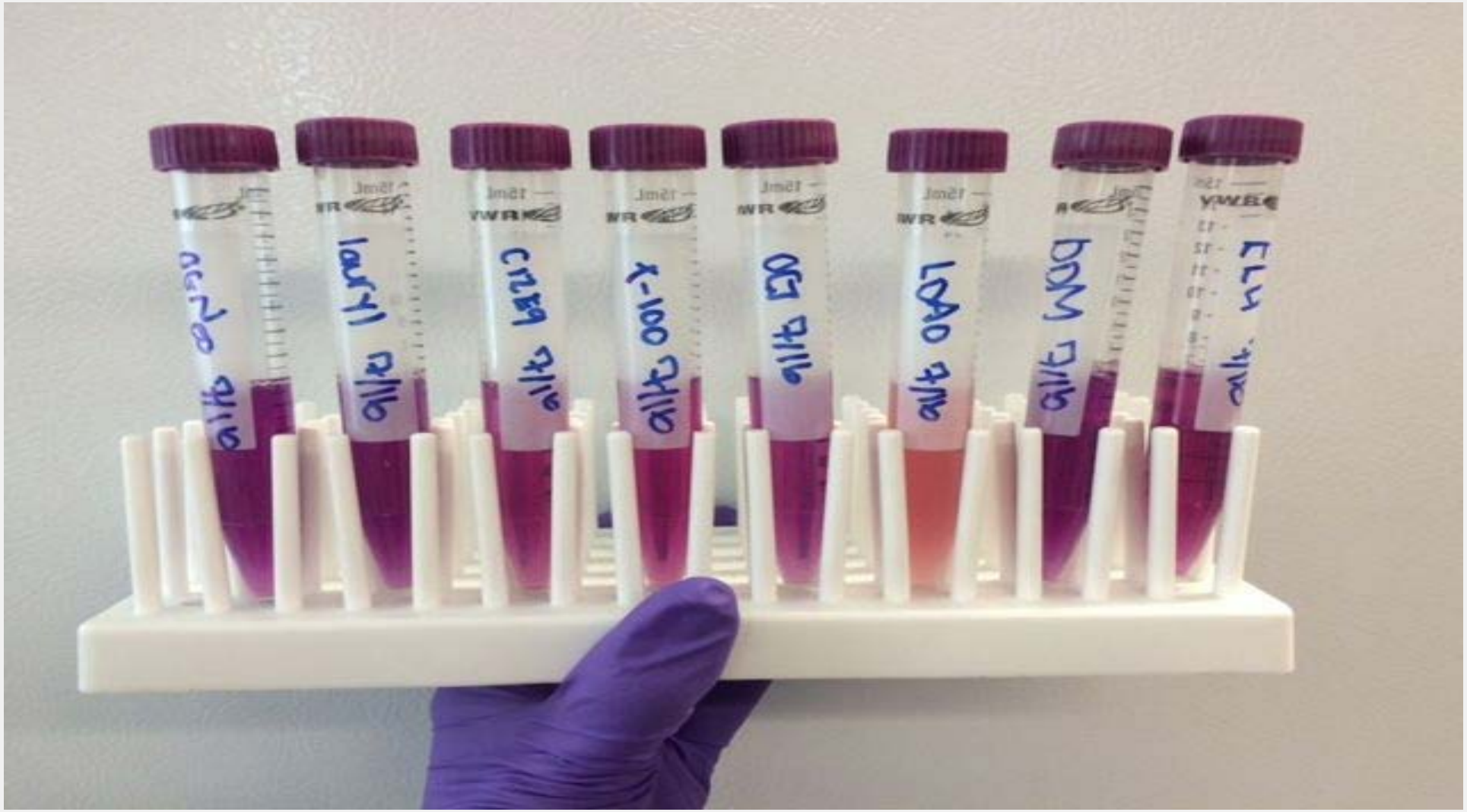
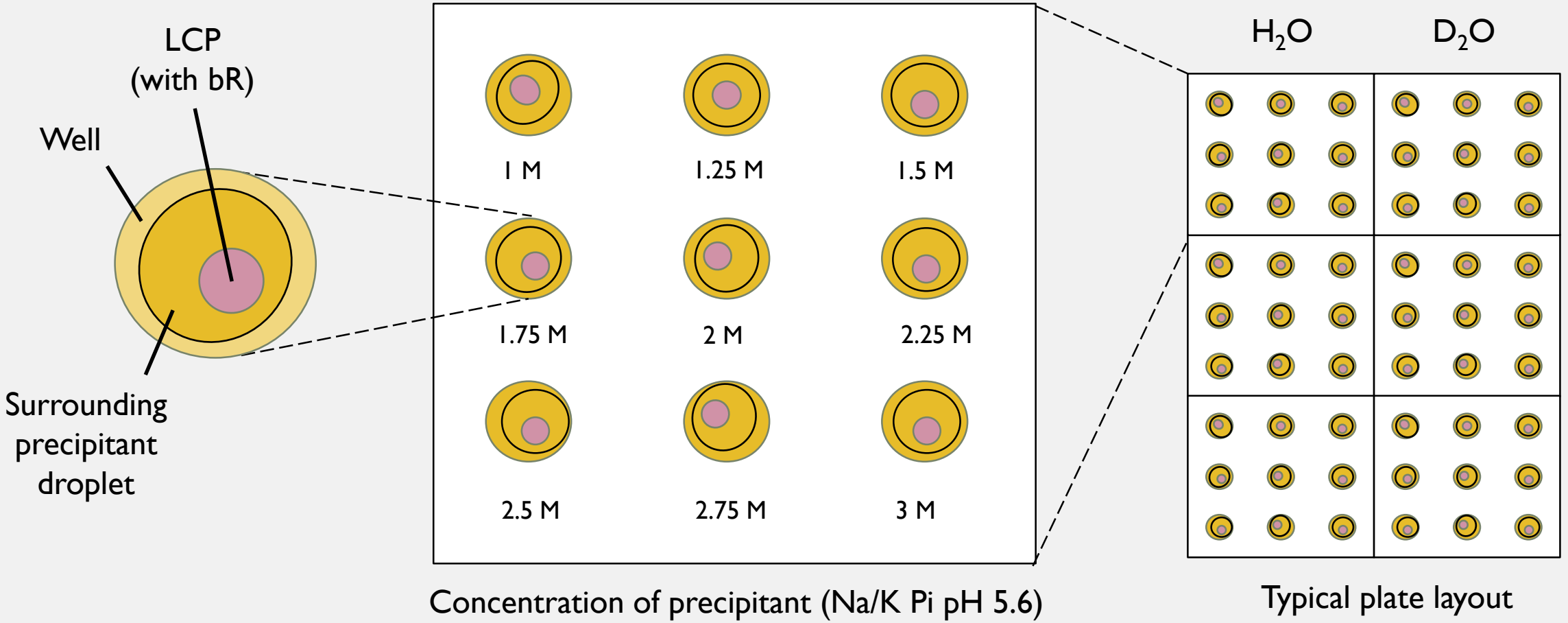


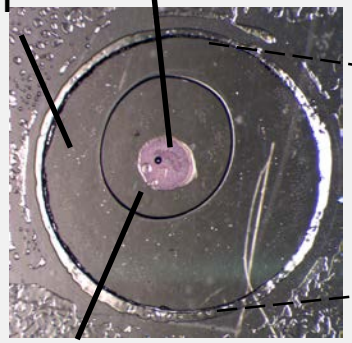
CRYSTALLIZATION TRIALS

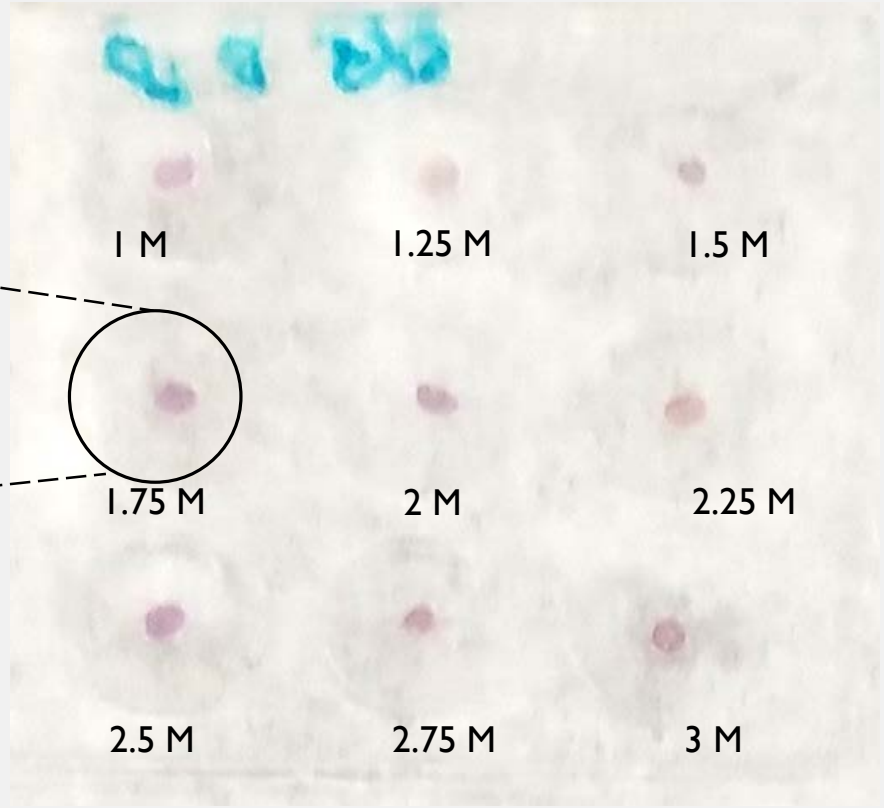


Concentration of precipitant (Na/K Pi pH 5.6)

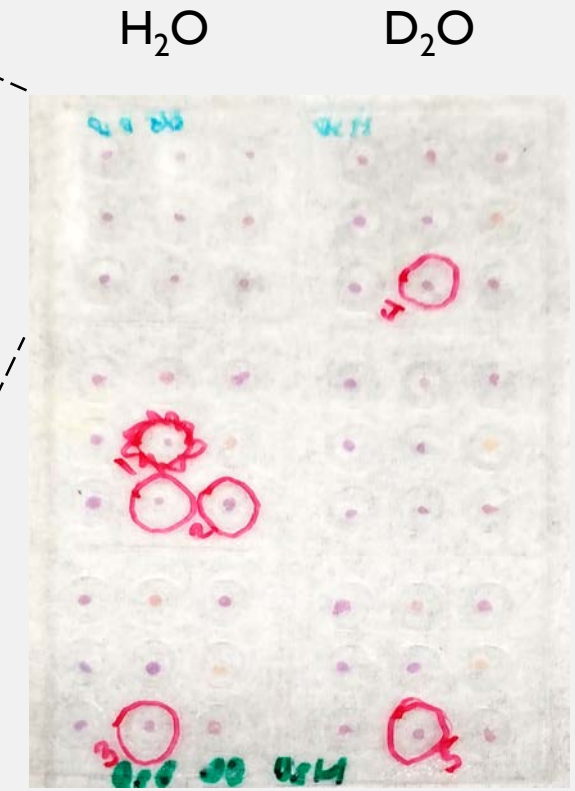
Typical plate layout

CRYSTALLIZATION TRIALS





Concentration of precipitant (Na/K Pi pH 5.6)

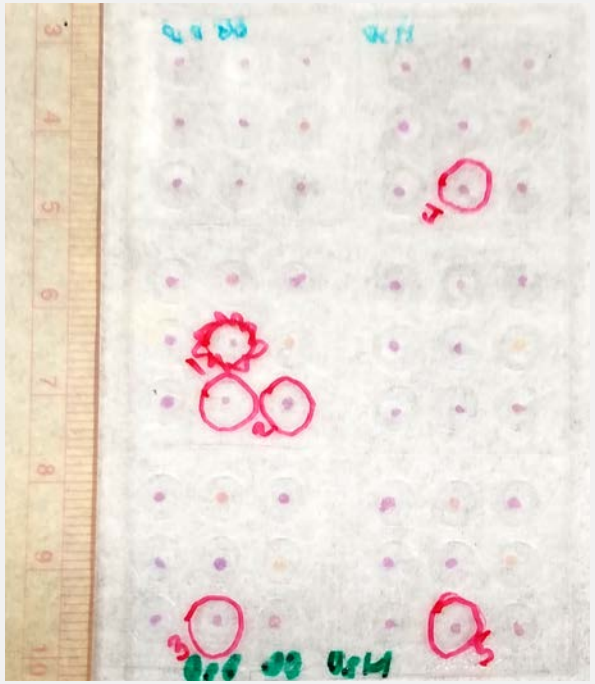


Typical plate layout

CRYSTALLIZATION TRIALS

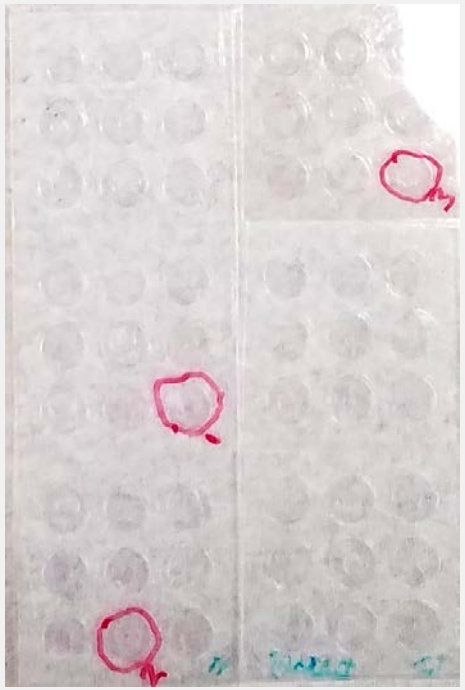
Crystals after ~5 days:

Octyl Glucoside



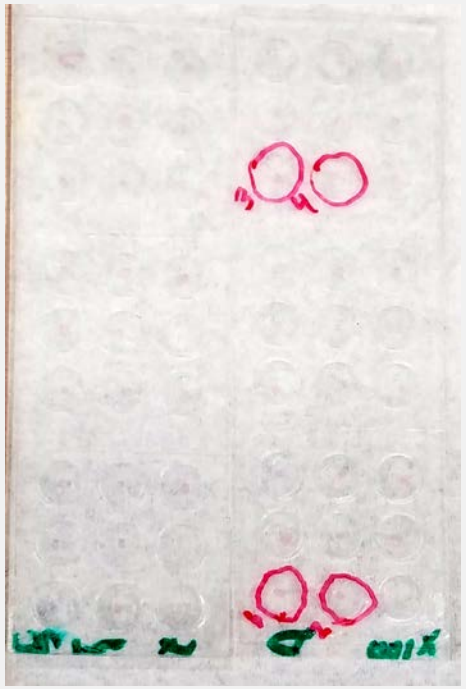
D_2O H_2O

Elugent



H_2O D_2O

Triton X-100



H_2O D_2O

No hits yet:

OGNG
LMNG
C12E9
DDM
LDAO

Key

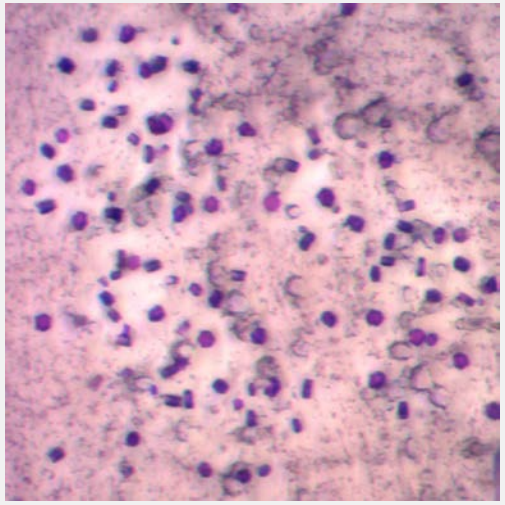
1.0	1.25	1.5
1.75	2.0	2.25
2.5	2.75	3.0

(Na/K Pi precipitant concentration in M)

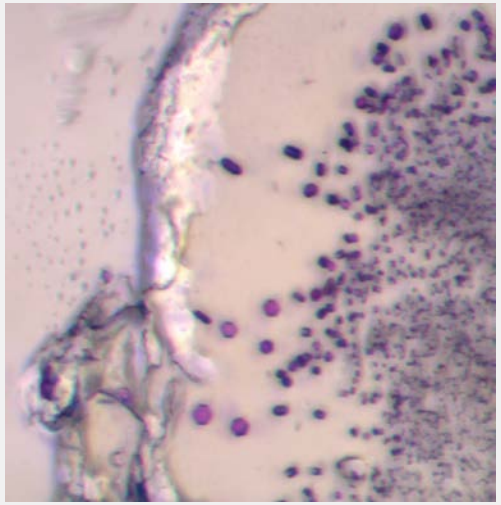
CRYSTALLIZATION TRIALS

Crystals after ~5 days:

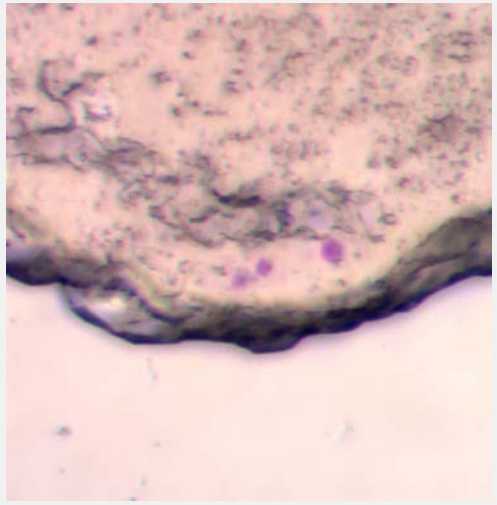
Octyl Glucoside



Elugent*



Anapoe X-100**



No hits yet:
OGNG
LMNG
C12E9
DDM
LDAO

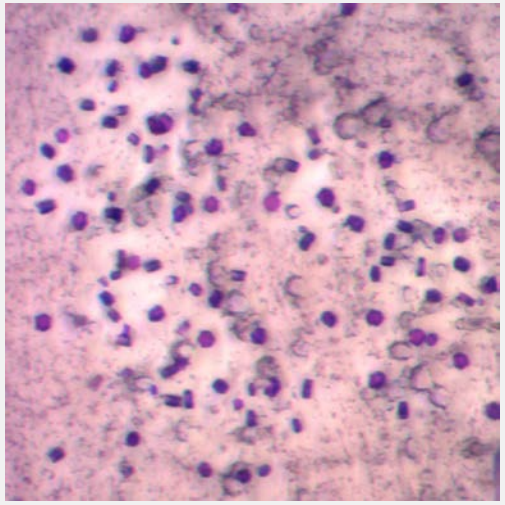
*Essentially a less-pure (far less expensive) form of octyl glucoside that has a distribution of different carbon chain-lengths.

**The same compound as Triton X-100, but this specific product is supplied with low peroxide content and packaged under inert gas.

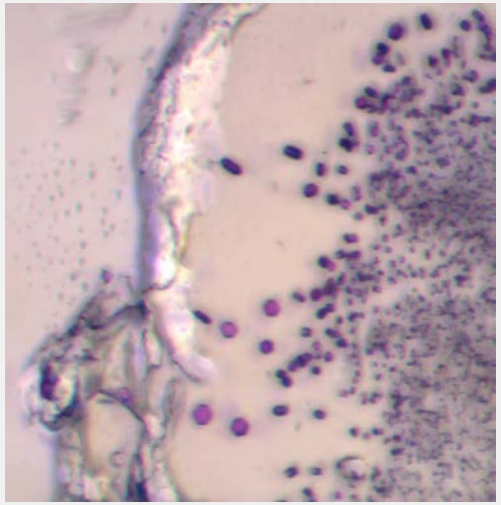
CRYSTALLIZATION TRIALS

Crystals after ~5 days:

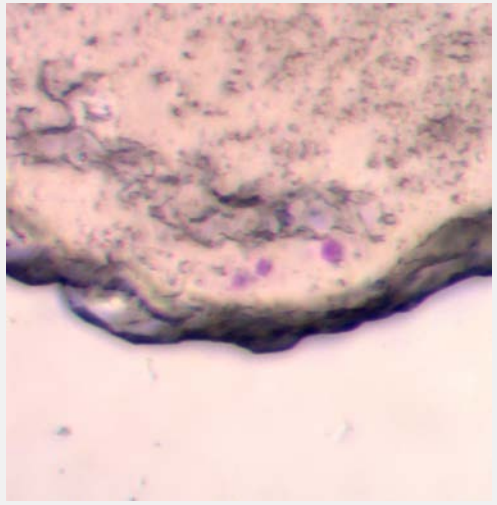
Octyl Glucoside



Elugent*



Anapoe X-100**



No hits yet:
OGNG
LMNG
C12E9
DDM
LDAO

Neither Elugent nor Triton X-100 are typically used for solution crystallization of membrane proteins!

- Both of them are heterogeneous, low-purity detergent mixtures.
- Both are extremely inexpensive

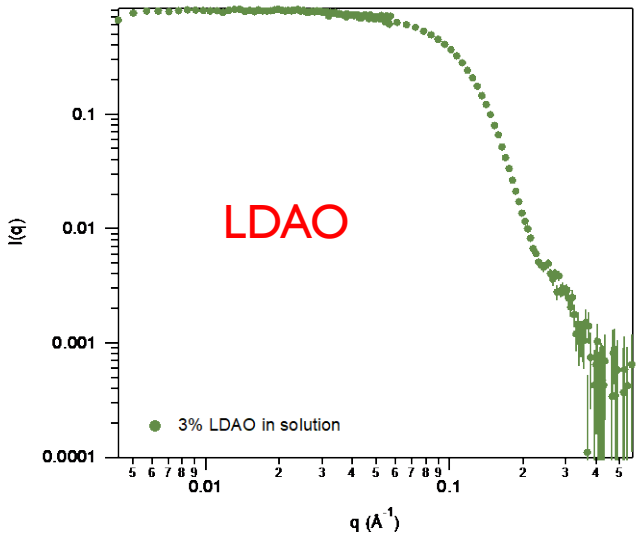
RESULTS

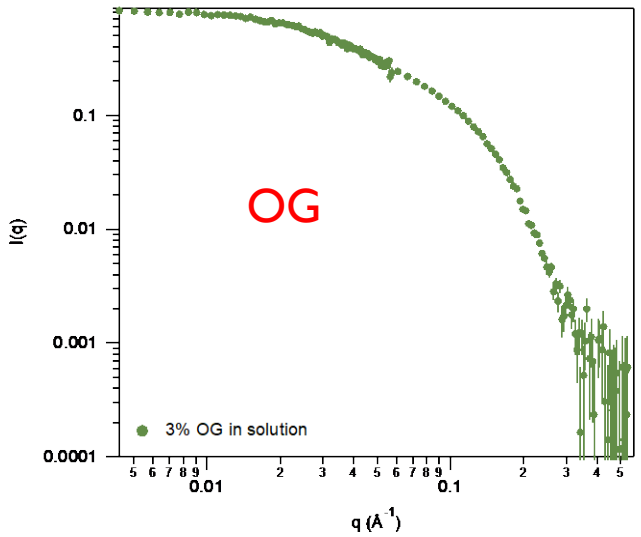
1. Crystallization trials
   - Different detergents?
   - H2O vs D2O?
   - Size-exclusion chromatography necessary or not?
2. **Scattering Measurements**
   - **Detergent micelles**
   - **Detergents in the lipidic cubic phase**
     - ✓ **After initial mixing**
     - ✓ **Immediately after precipitant addition**

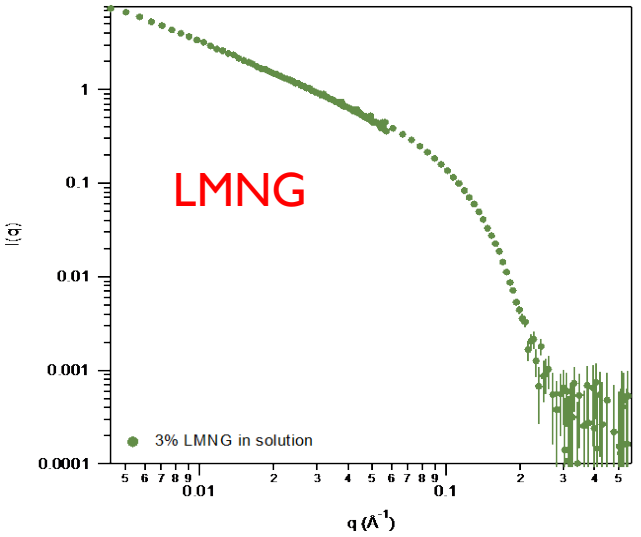
DETERGENTS IN SOLUTION

Detergent micelles can have a variety of shapes/sizes.

Example scattering curves:







Shapes:
(cartoon only)

MICELLE SHAPES AND SIZES

ELLIPTICAL CYLINDER

Detergent	Length (A)	Radius (A)
n-Tridecyl	17.998	17.998
n-Tetradecyl	18.089	18.089
LMNG	27.256	18.507
Fos-Choline	31.147	14.006
Triton	35.261	22.887
Anapoe-40	41.354	18.898
Anapoe-100	42.076	18.773
Anapoe-58	43.043	33.713
C10E9	43.589	15.863
C12E10	50.307	18.717

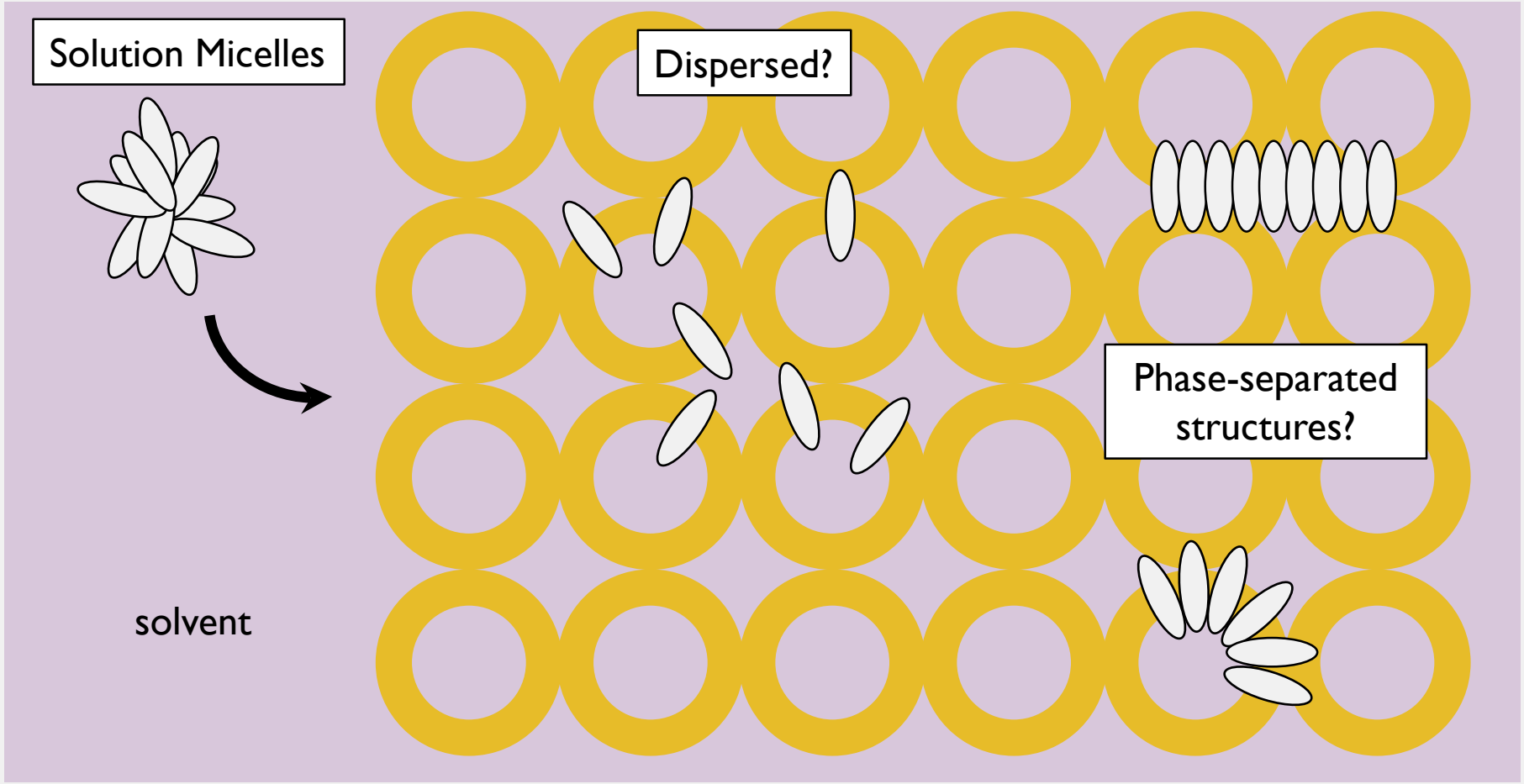
Detergent	Length (A)	Radius (A)
C12E9	54.262	22.839
C10E6	55.898	16.142
Anapoe-35	56.375	20.153
Anapoe-305	58.193	18.127
Anapoe-20	62.894	21.363
C13E8	64.197	19.776
Anapoe-80	73.597	27.083
OG	139.471	10.454
ELUGENT	187.012	13.901
OG Neo	224.900	9.3717

SPHERE

Detergent	Radius
LDAO	20.271
n-Decyl	21.648
CYMAL 5	21.885
n-Dodecyl	25.192
C12E8	29.102

WHAT HAPPENS TO THE DETERGENT IN THE LIPIDIC CUBIC PHASE?

WHAT HAPPENS TO THE DETERGENTS IN THE LIPIDIC CUBIC PHASE?



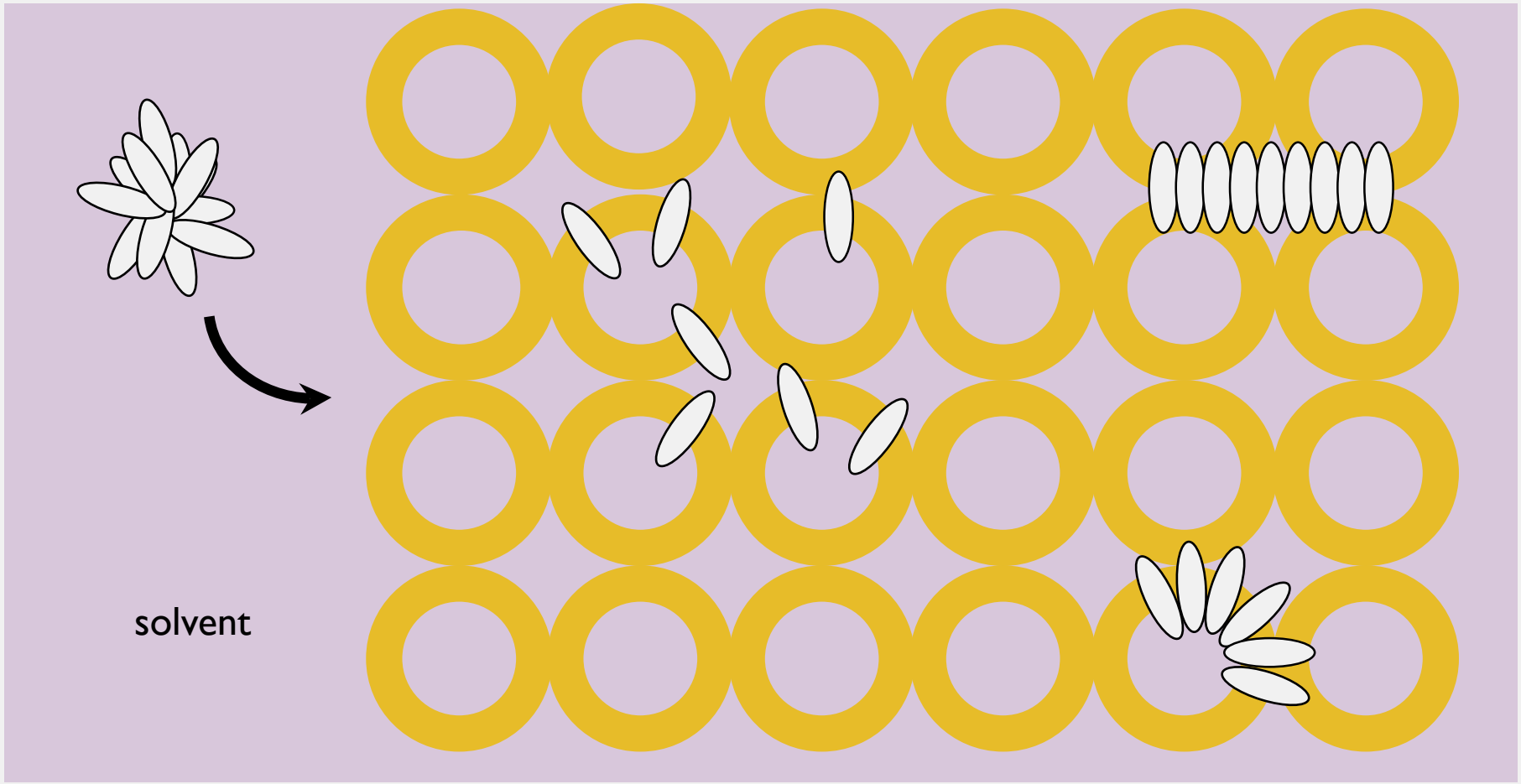
Scattering Length Density

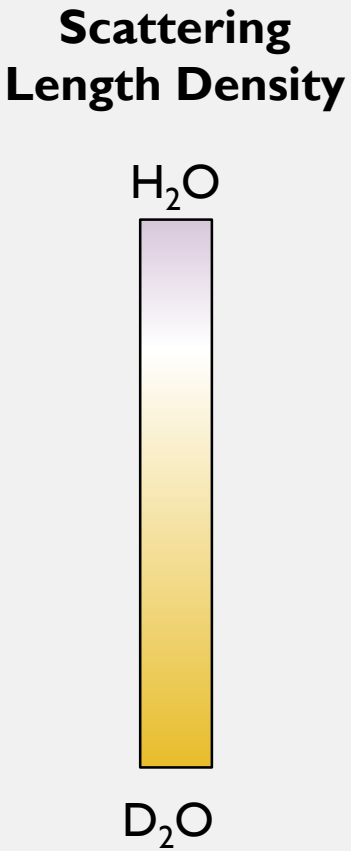
H_2O

D_2O

Without contrast matching: most observed scattering will just be from the LCP

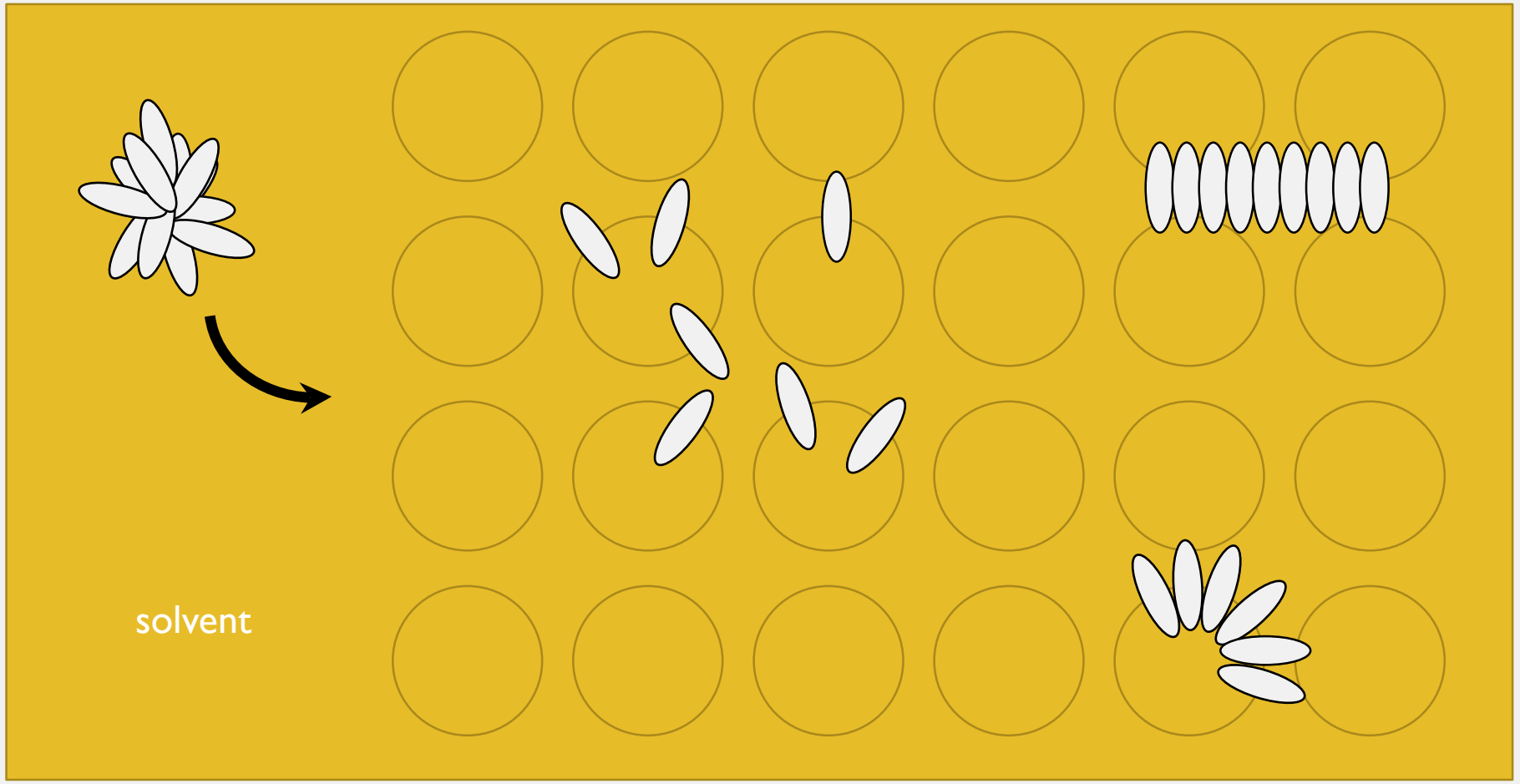
WHAT HAPPENS TO THE DETERGENTS IN THE LIPIDIC CUBIC PHASE?





Without contrast matching: most observed scattering will just be from the LCP

WHAT HAPPENS TO THE DETERGENTS IN THE LIPIDIC CUBIC PHASE?



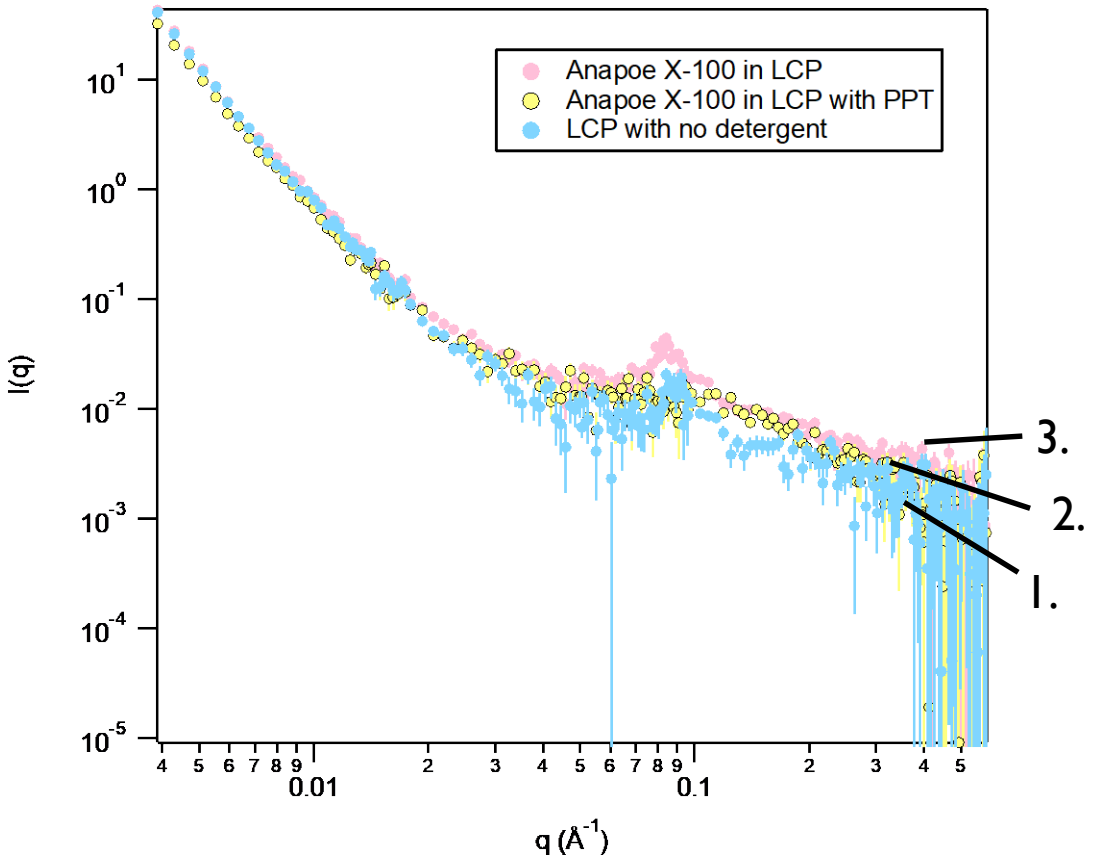
Scattering Length Density

H_2O

D_2O

With contrast-matched lipids: scattering from any structures formed by detergents will be observed

DETERGENTS IN THE LCP – SANS

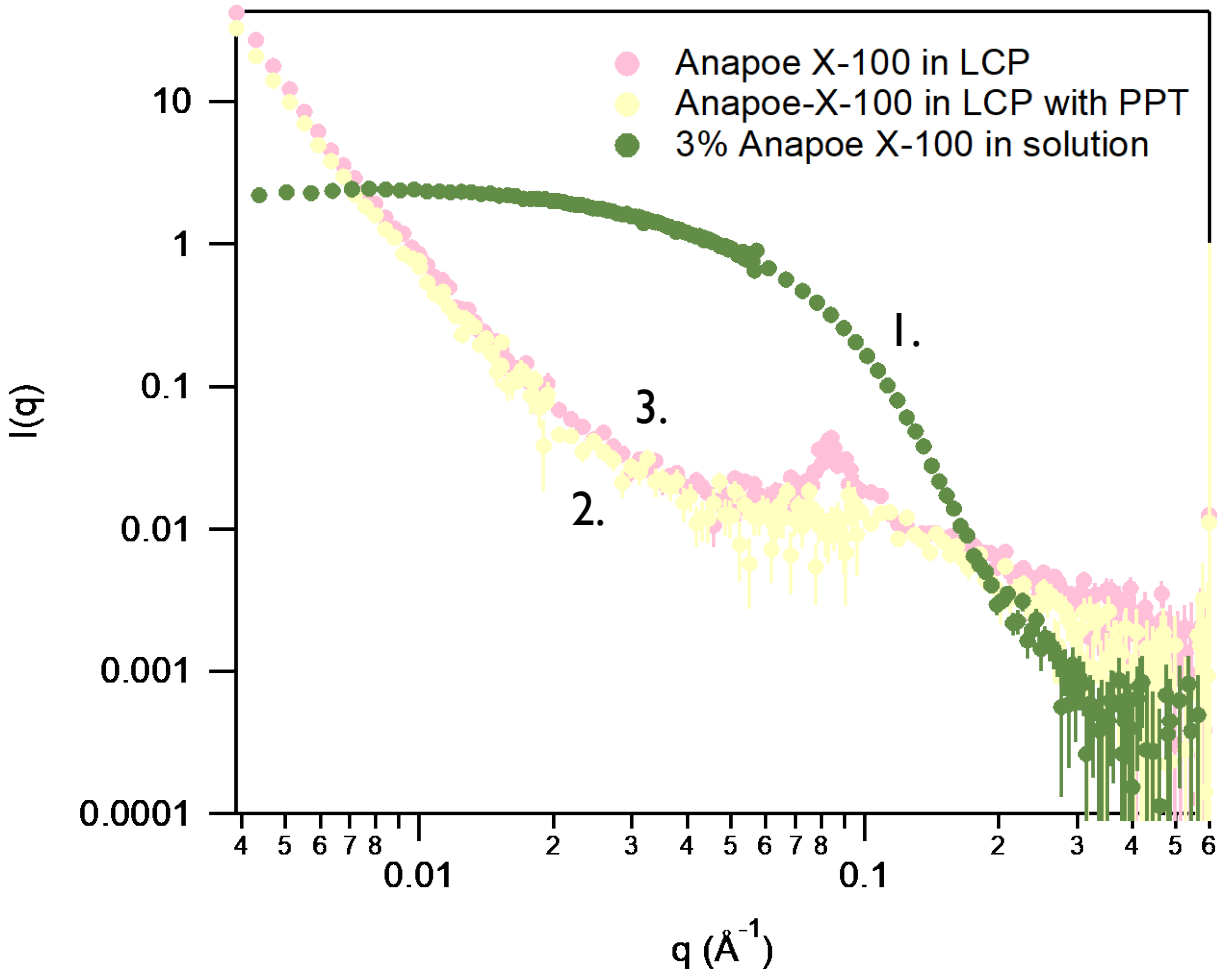


<u>Compare cubic phases with/without detergent:</u>

1. Without detergent
2. With detergent
3. With detergent + 2 M Na/K Pi Precipitant

Very little change

DETERGENTS IN THE LCP – SANS



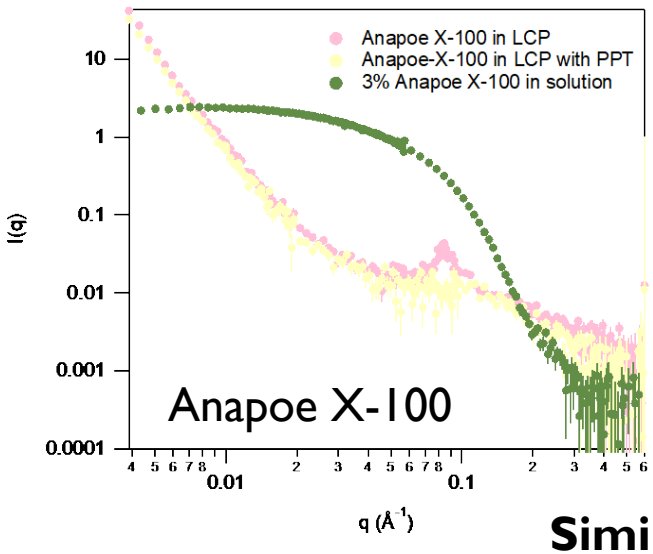
Compare cubic phase scattering to what we would see if there were aggregated structures such as micelles

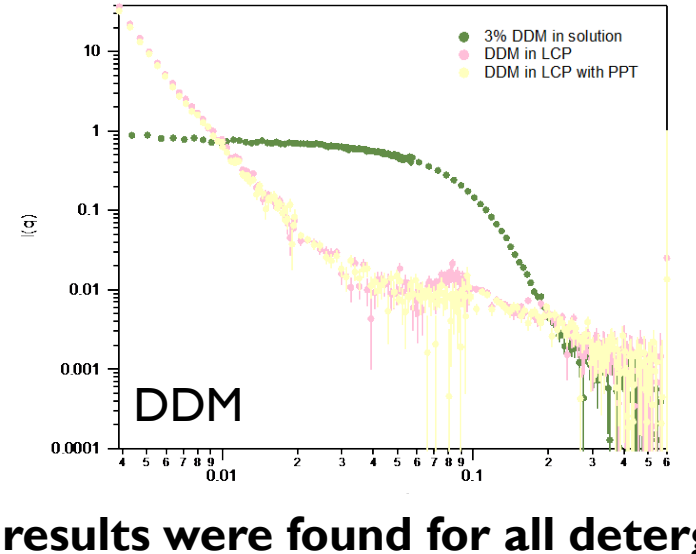
Curves

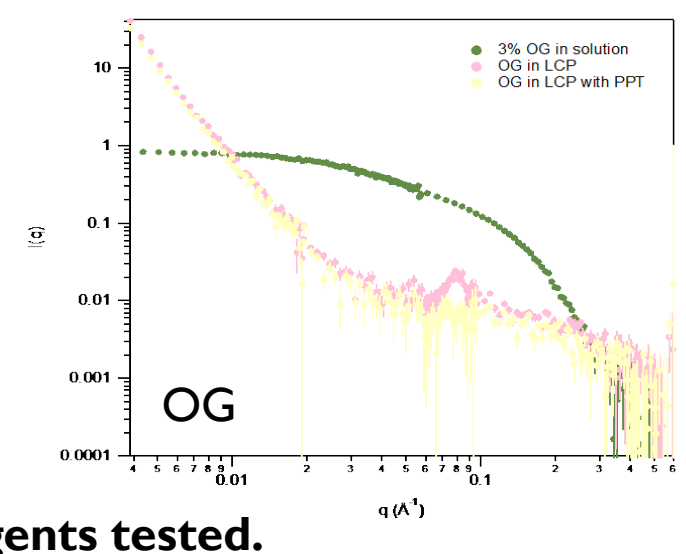
1. Detergent solution micelle
2. Similar amount of detergent in LCP
3. LCP/detergent after addition of precipitant

Detergent aggregates are not seen.
Detergent is dispersed in the cubic phase.

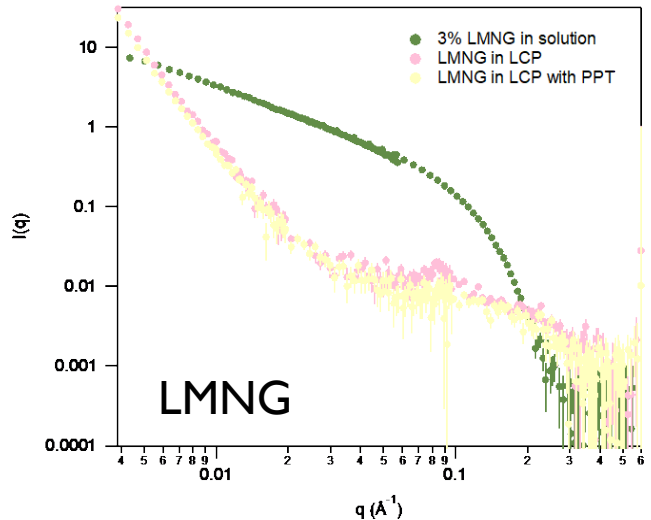
DETERGENTS IN THE LCP – SANS

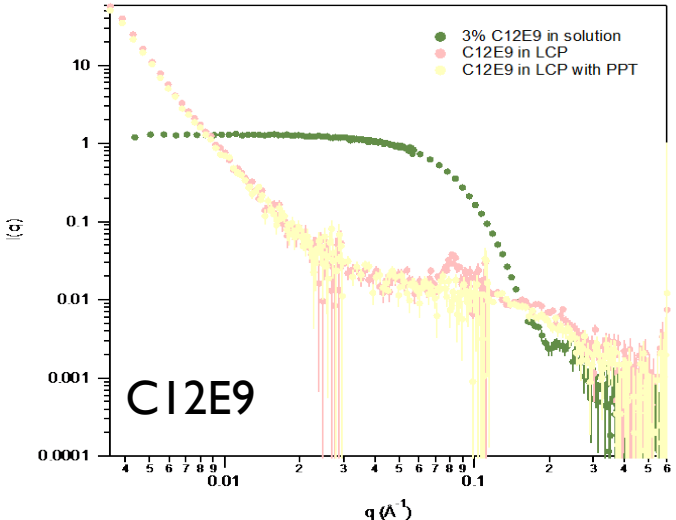


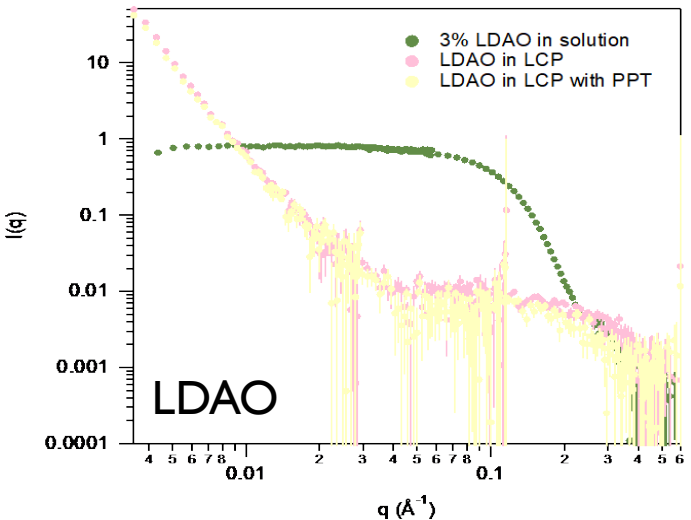


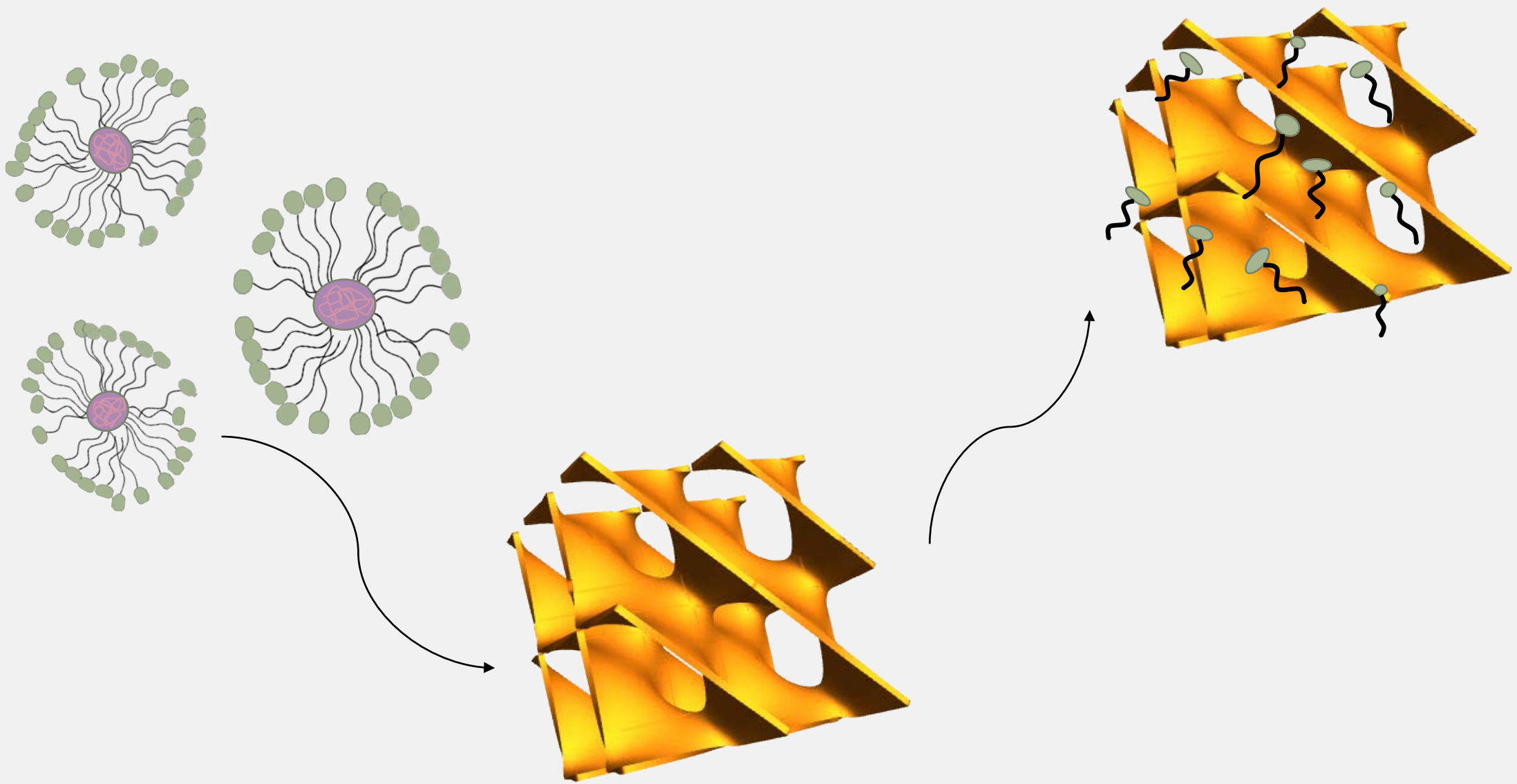


Similar results were found for all detergents tested.

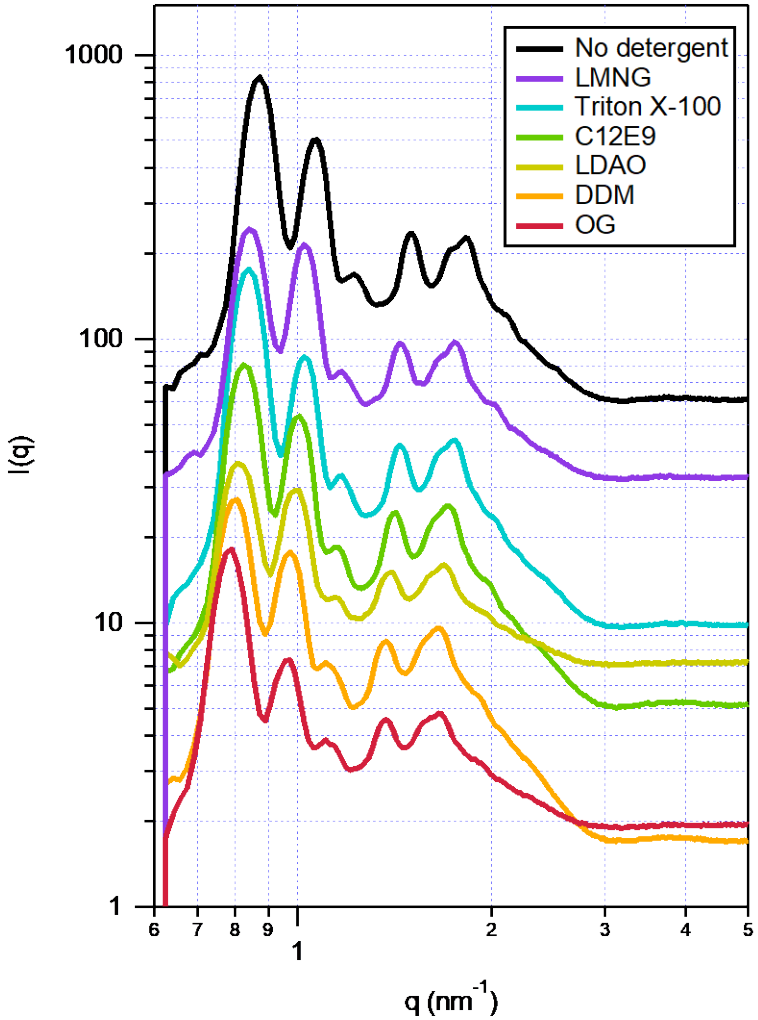








DETERGENTS IN THE LCP – X-RAY



Detergent aggregates were not seen; but what effects do they have on the cubic phase lattice? Used x-ray diffraction:

- Detergent solutions (3%) were mixed with the lipid to form cubic phases
- Bragg peak positions can be used to determine the lattice spacing of the cubic phase
- Detergents cause swelling in the cubic phase
- Crystals were obtained from Triton X-100 and Octyl Glucoside
- No direct correlation between lattice parameter vs. crystallization hits.

Detergent	Lattice Par. (Å)
OG	113.8
DDM	111.8
LDAO	108.1
C12E9	108.1
TX100	106.4
LMNG	106.4
None	101.4

CONCLUSIONS

- In LCP crystals of bR can be obtained in additional detergents aside from the traditionally-used octyl glucoside
  - include atypical (for crystallization studies) detergents such as Triton X-100 and Elugent
- Less-refined detergents in advantageous due to:
  - Low cost
  - May be most suitable for protein stability in certain proteins
- Absence of LCP crystals other detergents may not be due to "incompatibility" with crystallization but instead:
  - Poor solution stability before LCP incorporation (LDAO which showed color changes indicative of bR denaturation)
- Detergents matter less in the cubic phase because they disaggregate
- Properties depend more on the lipid then dispersed detergent

ACKNOWLEDGEMENTS

- My mentor: Thomas Cleveland
- SURF Coordinators: Julie Borchers, Joseph Dura, and Brandi Toliver
- Center for High Resolution Neutron Scattering
- NIST Center for Neutron Research
- My fellow NCNR SURFers.

NIST
National Institute of Standards and Technology
U.S. Department of Commerce

NIST Center for Neutron Research

CHRNS
CENTER FOR HIGH RESOLUTION NEUTRON SCATTERING
NSF
NIST

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- http://www.chem.uwec.edu/Chem455/expressbR.pdf