# In Search of Lipid Rafts: Evidence of Phospholipid/Cholesterol Complexes

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# Interactions at Lipid Interfaces

**Human Lung Surfactant**

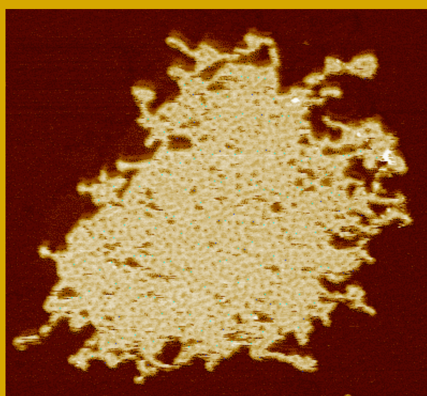
**Antimicrobial Peptides**

**Alzheimer's Aβ Peptides**

**Poloxamers as Membrane Sealants**

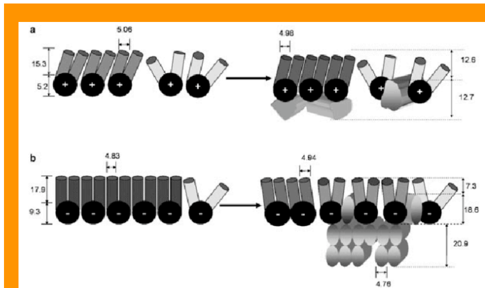
**Phospholipid/Cholesterol Interactions**

# Interactions at Lipid Interfaces

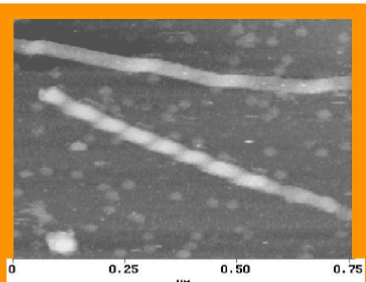


**Human Lung Surfactant**

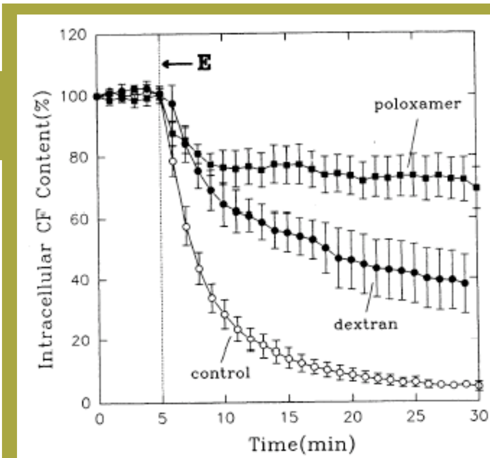

**Antimicrobial Peptides**



**Alzheimer's Aβ Peptides**



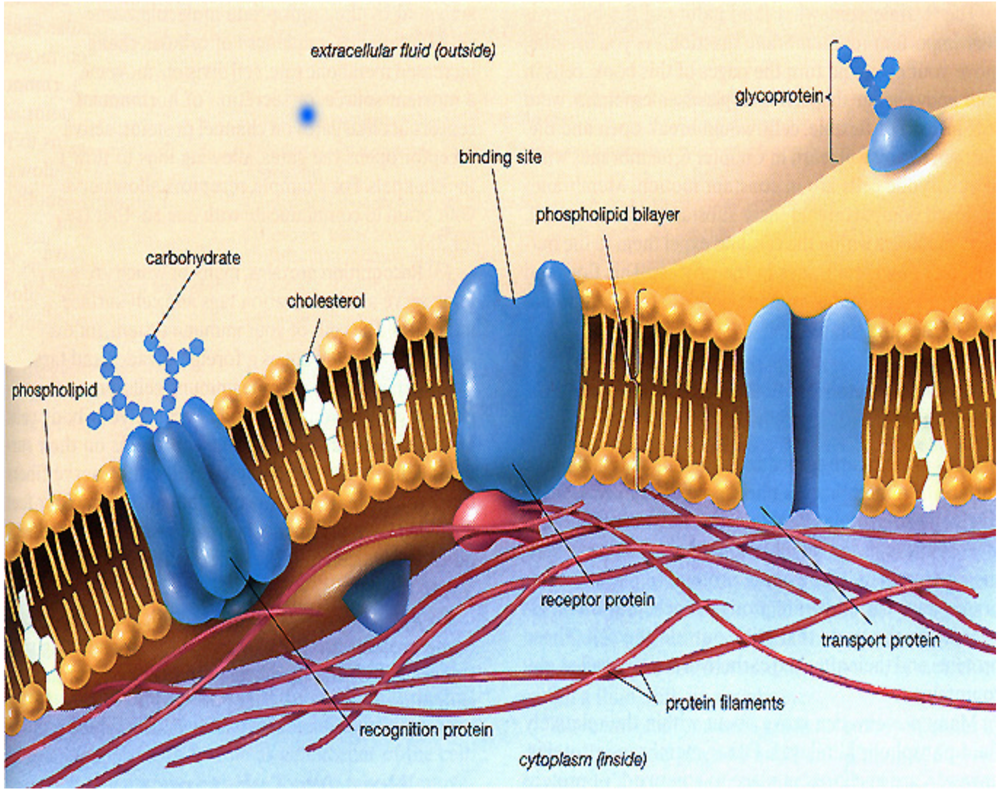

**Poloxamers as Membrane Sealants**




**Phospholipid/Cholesterol Interactions**

# Background

- 1972: Fluid Mosaic Model
- "Lipid raft": enriched in cholesterol and sphingolipids
  Important in signal transduction
- Lengthscale: predicted ~100 nm
- Model systems:
  GUVs
  Monolayers
- Thermodynamic phases?
- Dynamic structures?

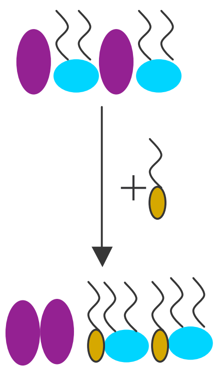


# Outline

- Background & Motivation
- Can phospholipid/cholesterol interaction be disrupted?

  Phase diagrams in the presence of displacing agent

  Cholesterol desorption by cyclodextrin in monolayers



- Ordering of phospholipid/cholesterol systems

  Grazing incidence X-ray diffraction and X-ray reflectivity results

- Evidence of displacement in live cell systems

  "Inhibiting" and "promoting" sterols on cholesterol activity in RBC

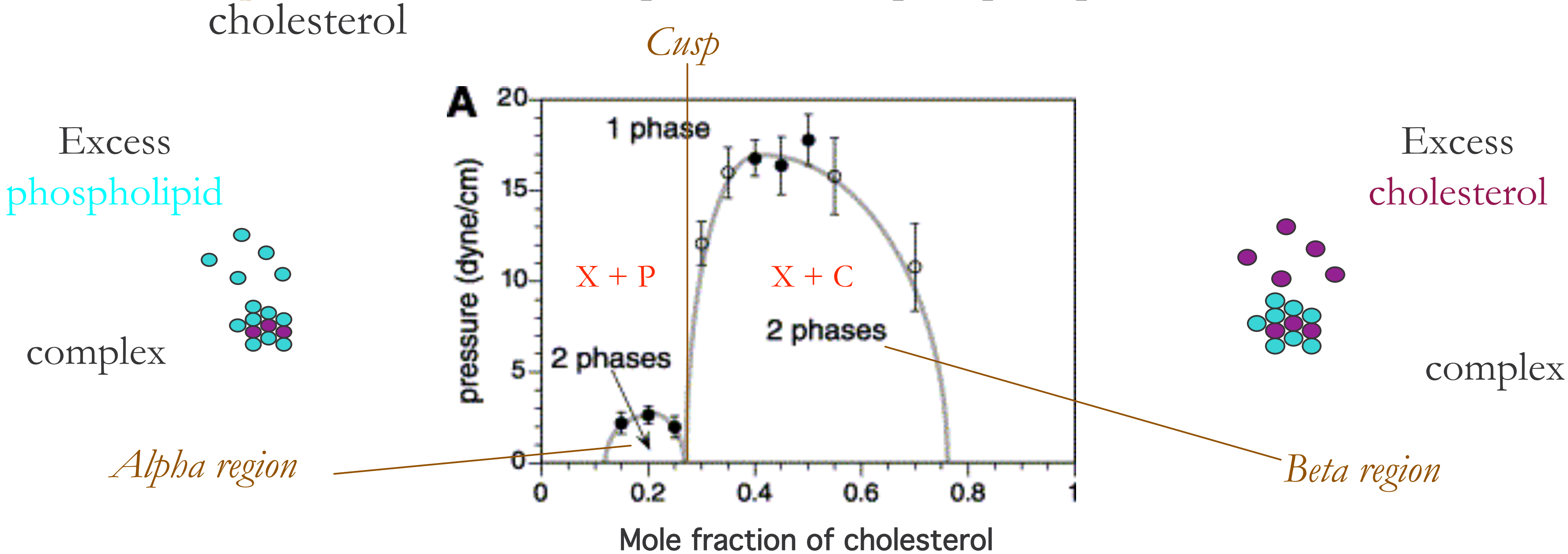
- Conclusions

# Postulation of Complex Formation

- McConnell postulates the existence of lipid-cholesterol complexes in model membranes
- *Alpha region:* region of low cholesterol concentration; complex + lipid (X + P)

  *Beta region:* region of high cholesterol concentration; complex + cholesterol (X + C)

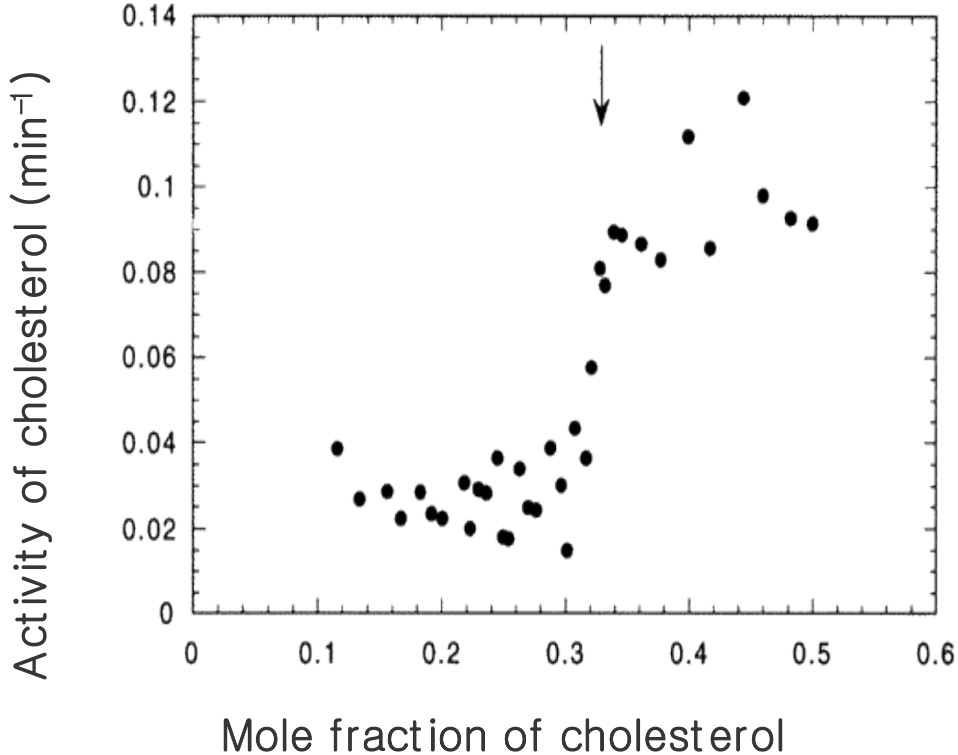
  *Cusp:* stoichiometric complex between phospholipid & cholesterol




A. Radhakrishnan and H.M. McConnell. *Biochemistry* **39** (2000), pp. 8119–8124

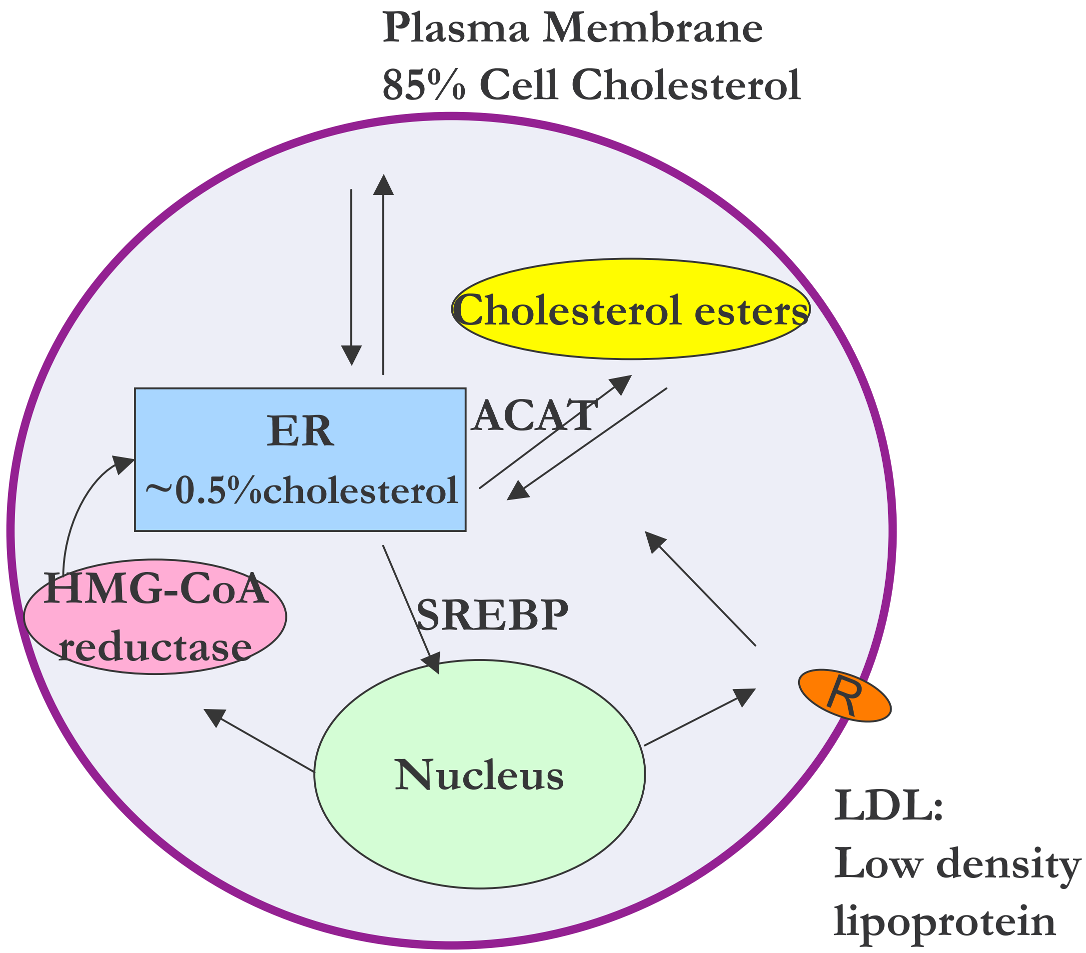
# Active (Free) Cholesterol Monomers

Uptake of cholesterol by beta-cyclodextrin (βCD)



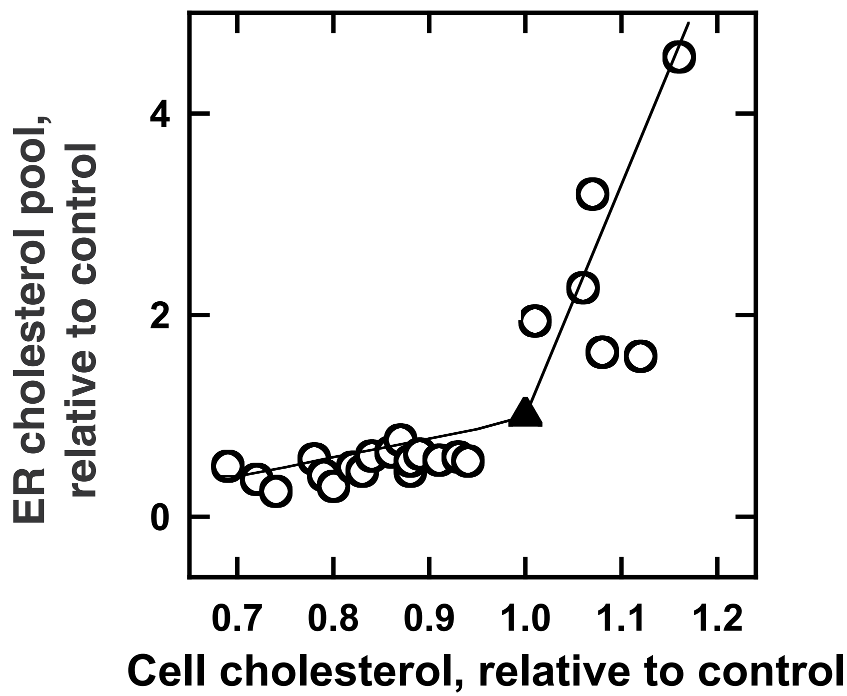

A. Radhakrishnan and H.M. McConnell. *Biochemistry* **39** (2000), pp. 8119–8124

# Cholesterol Traffic



# Biological Motivation: Cholesterol Homeostasis

- Lange *et al.*: n-octanol can increase cholesterol transfer and ER pool size
- Hypothesis: displacement cholesterol from plasma membrane by n-octanol from the complex, thus freeing up cholesterol monomers
- Understand the J-curve response observed in biological systems




Lange Y, Ye J, Rigney M, Steck TL. (1999) J Lipid Res, 40, 2264-70; also PNAS (2004)

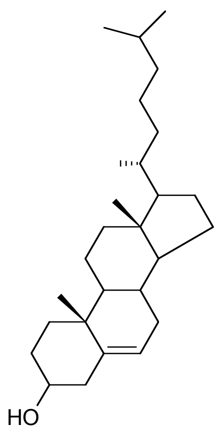
# Objectives

- Hypothesis:
  - cholesterol forms **complexes** with phospholipids

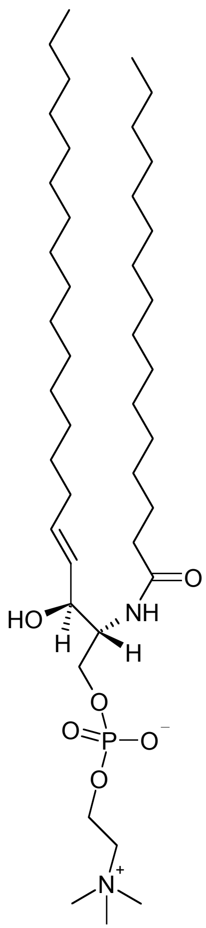
- Implications:
  - Cholesterol can exist in two different states:
    **bound cholesterol** *(low activity)*
    **free cholesterol** *(high activity)*
  - Other molecules that can form tighter complexes with phospholipids can be **displacing agents**, freeing cholesterol from its bound state

- Mechanistic Model
  - **cholesterol activity modulation by specific displacing agents.**

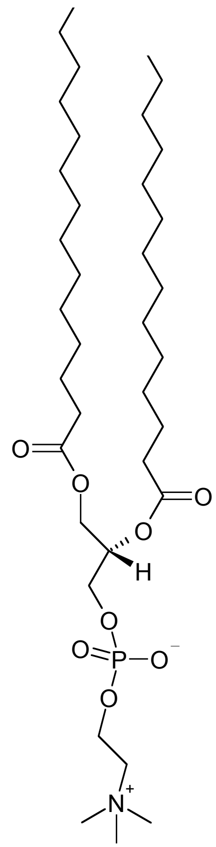
# Lipid Systems




Cholesterol




Typical SM




DMPC

# Four Different Approaches

- Phase diagrams
- Cholesterol desorption by beta-cyclodextrin (CD)
- X-ray diffraction and x-ray reflectivity
- Effect of "inhibiting" and "promoting" sterols on cholesterol activity in RBC

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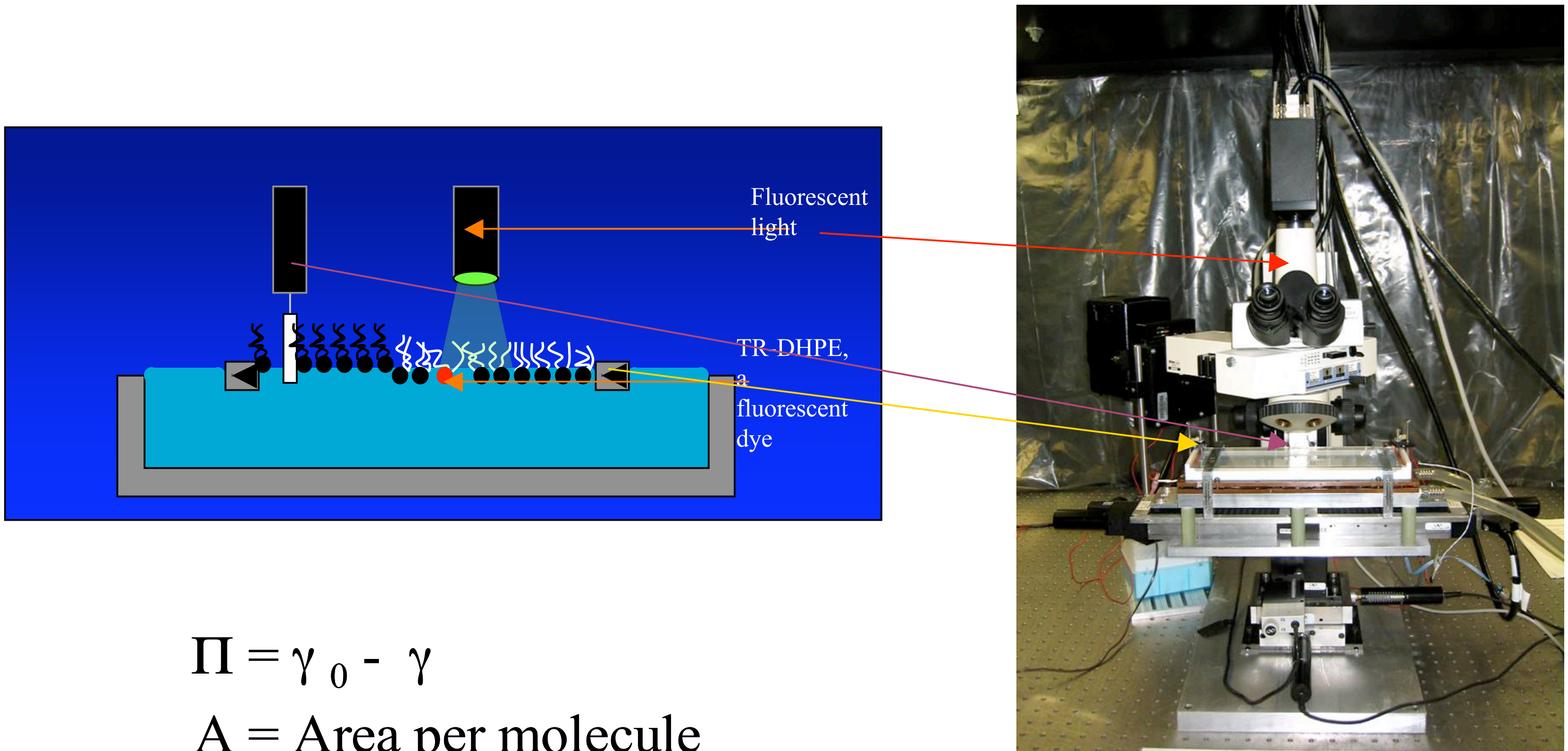
# Experimental Techniques



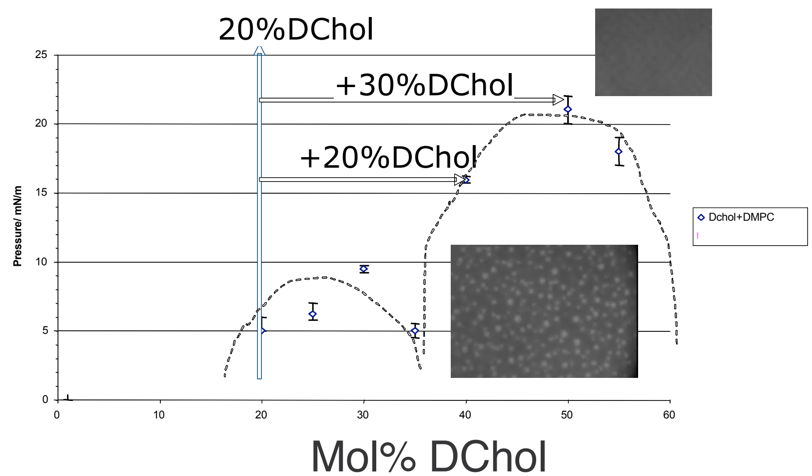

$$\Pi = \gamma_0 - \gamma$$

A = Area per molecule

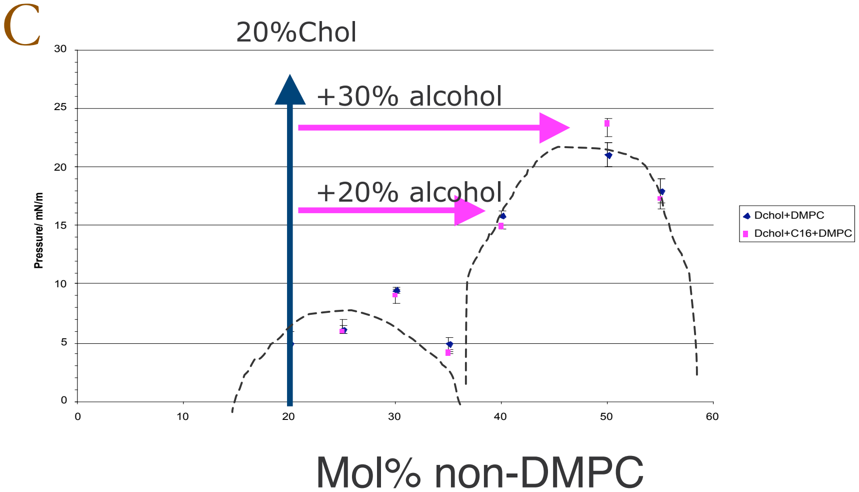
**Fluorescence Langmuir Surface Balance**

# Phase Diagram: Point Shifting

1. DChol:DMPC



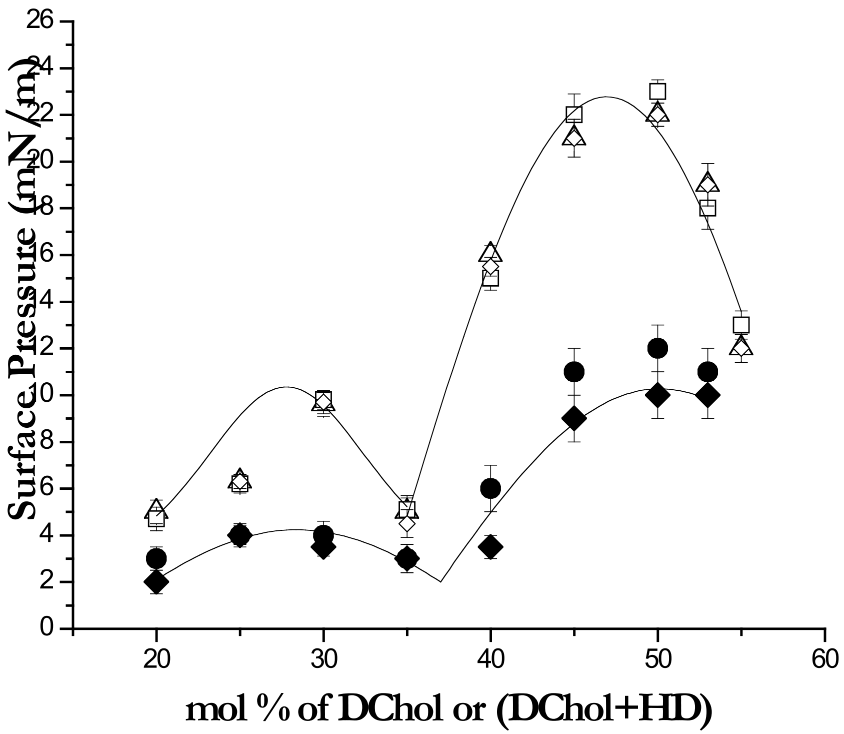

2. 20%DChol:Hexadecanol (C16):DMPC




3. C16:DMPC

No liquid-liquid immiscibility observed

# Cholesterol fixed at different mole fractions




| | |
|---|---|
| Open square | 0% DChol |
| Filled circle | 10% DChol |
| Filled diamond | 15% DChol |
| Open triangle | 20% DChol |
| Open circle | 25% DChol |

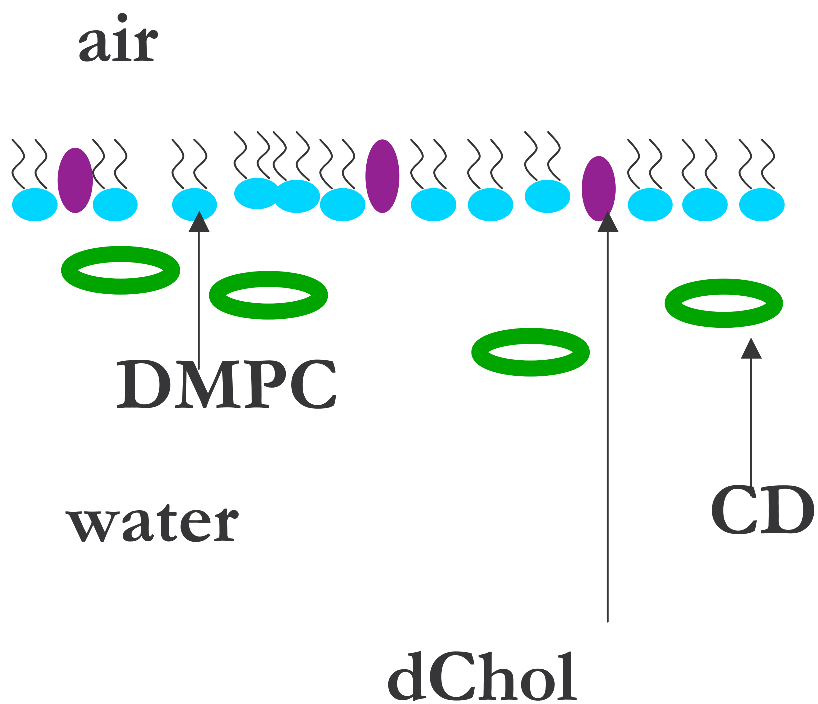
# Conclusions from Phase Diagram Study

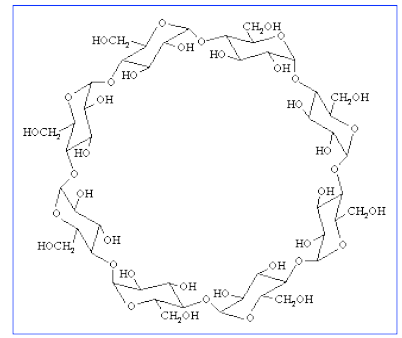
- Phase diagrams in the presence of displacing agent is equivalent to ones with just pure cholesterol, even at low cholesterol content
- Displacement of cholesterol from its association with phospholipid by alcohol
- The displacement seems to be 1 C16:1 DChol, since we observe identical phase diagrams
- **Does displacement results in the release of free cholesterol?**

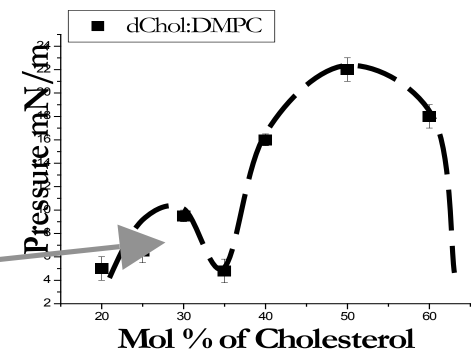
# Four Different Approaches

- Phase diagrams
- Cholesterol desorption by cyclodextrin (CD)
- X-ray diffraction and x-ray reflectivity
- Effect of "inhibiting" and "promoting" sterols on cholesterol activity in RBC

# Cholesterol Transfer to CD





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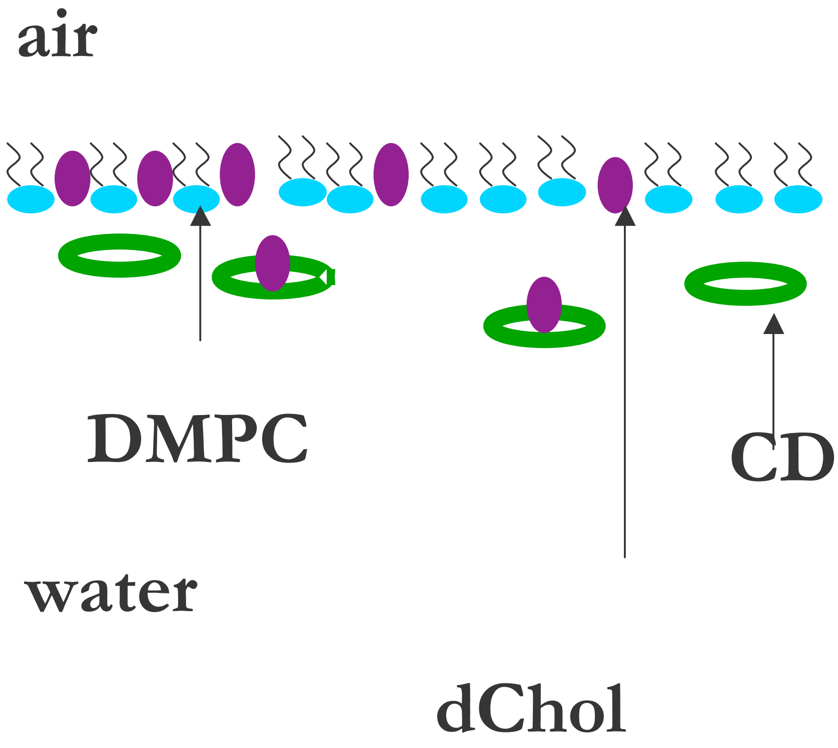
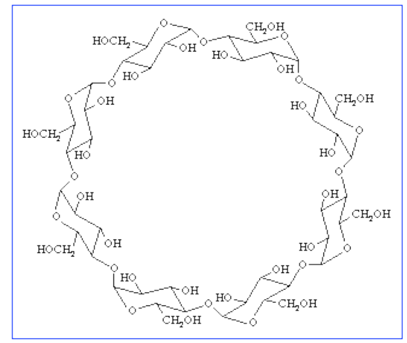
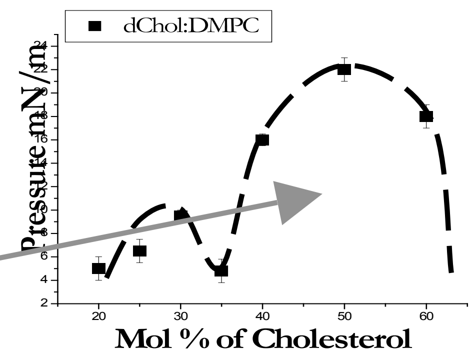
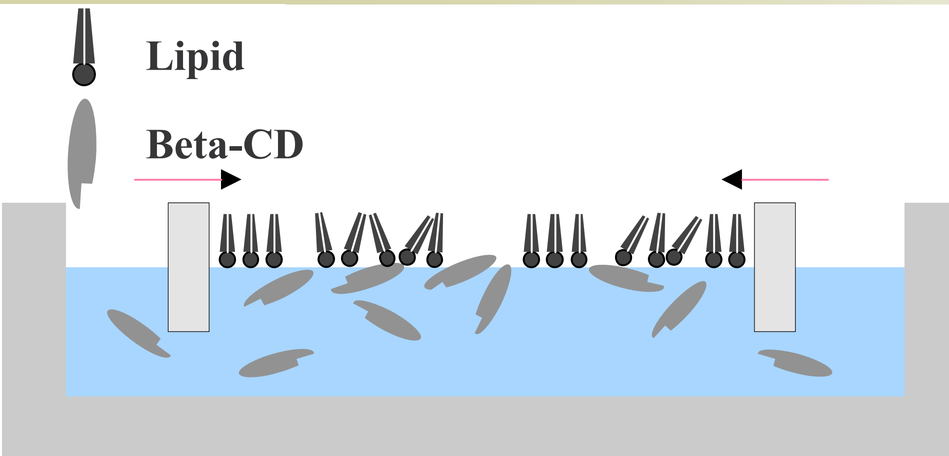


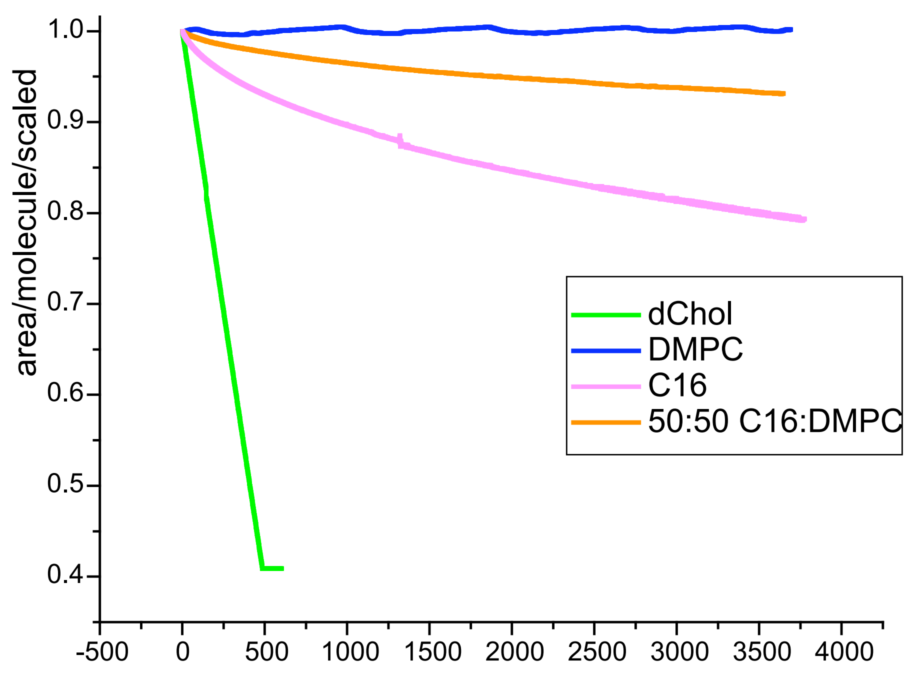


# Selectivity of Cyclodextrin



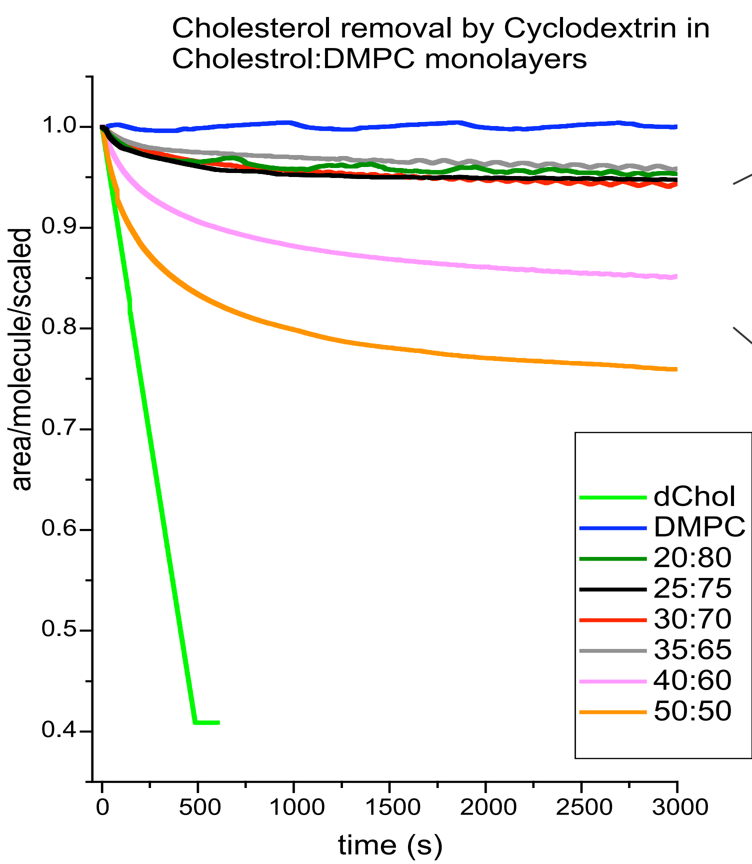

1. Monolayer Compression memb. equib. Π≈28 mN/m (20~35 mN/m)
2. Beta-CD in subphase
3. Area Contraction

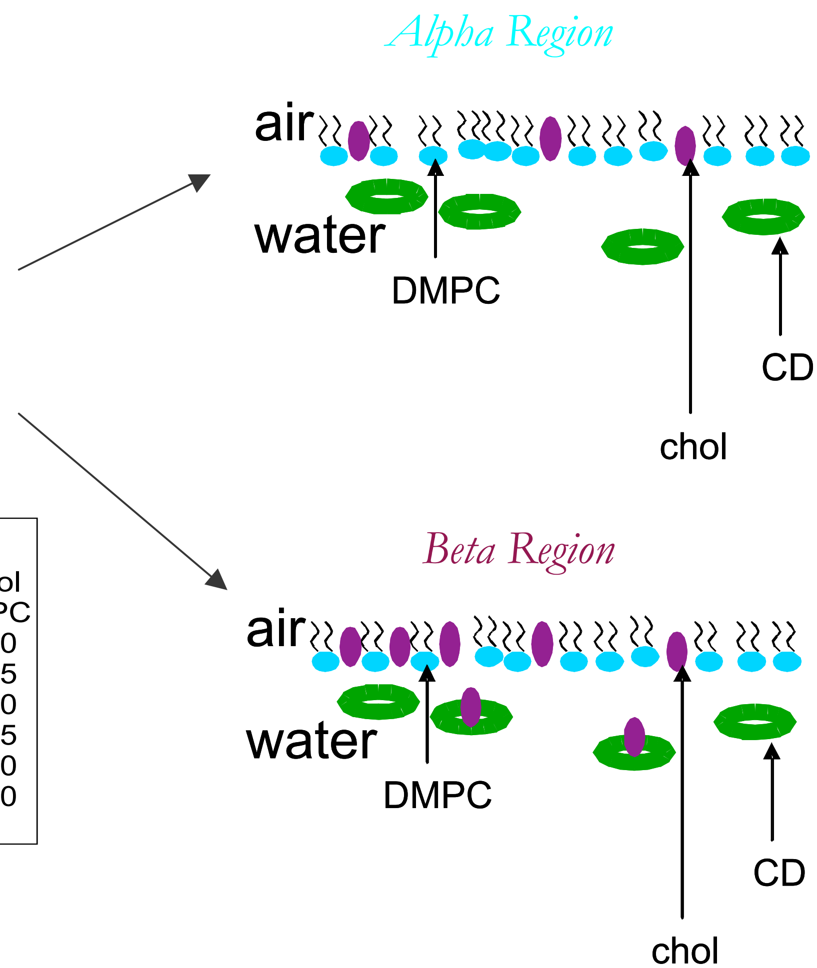
Cholesterol removal by Cyclodextrin in Cholestrol:DMPC monolayers



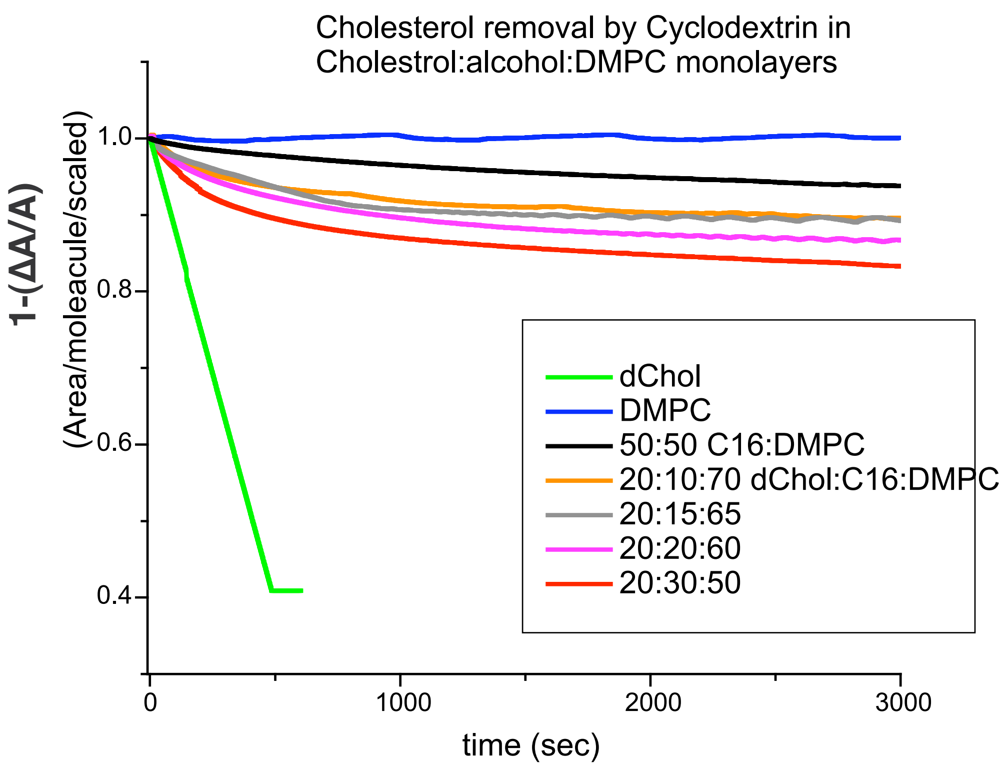

- β-CD removes sterols from monolayers
- Desorption rate of cholesterol to CD is much greater than the rates for DMPC or hexadecanol (C16)

# Cyclodextrin on System 1: DChol:DMPC



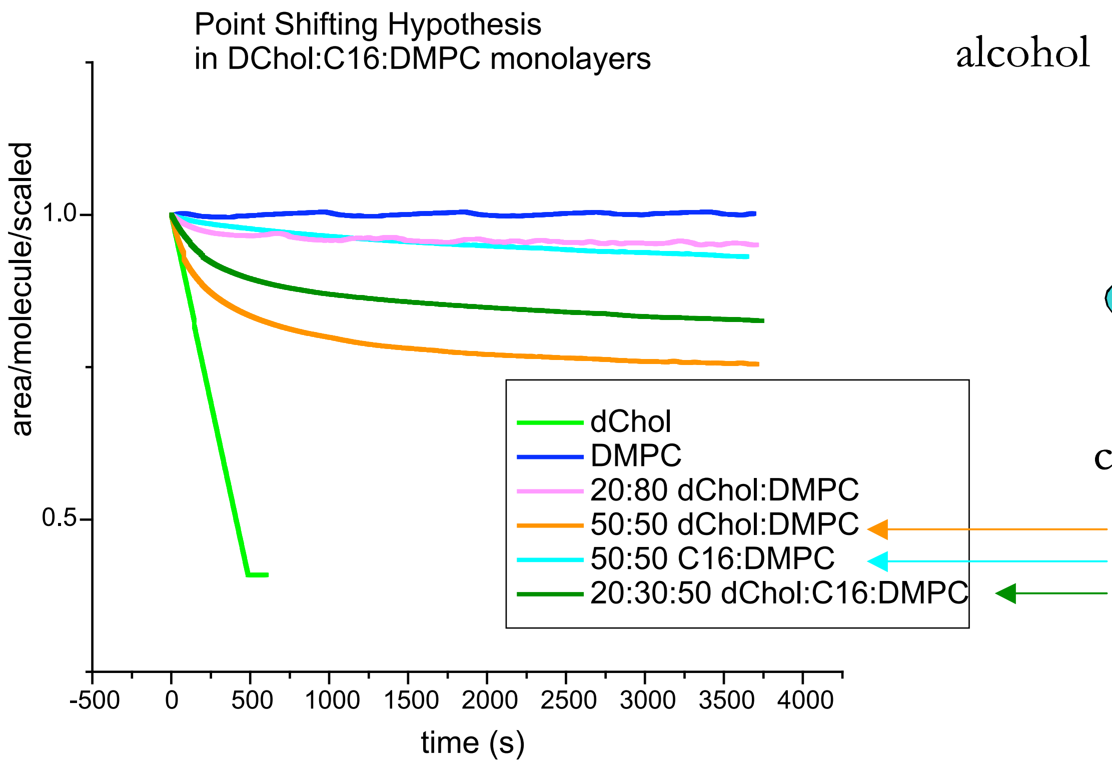
# Cyclodextrin on System 2
# 20% DChol: C16:DMPC

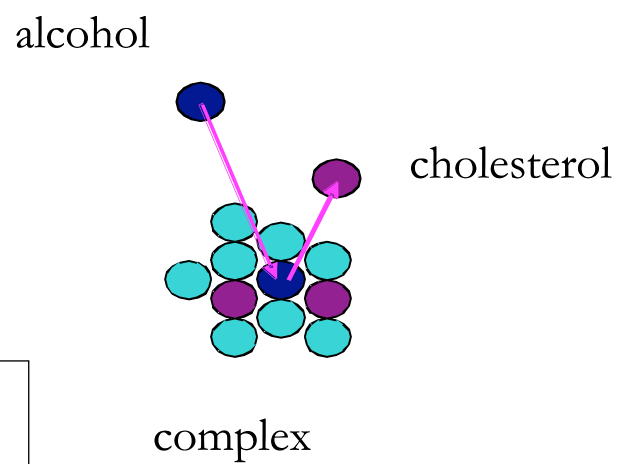



- Ternary mixtures displays similar behavior to βCD as binary mixtures of DChol:DMPC
- Even with equally low cholesterol content we see changes in desorption rates indicating that alcohol is displacing cholesterol
- Compare **black** and **red** curves

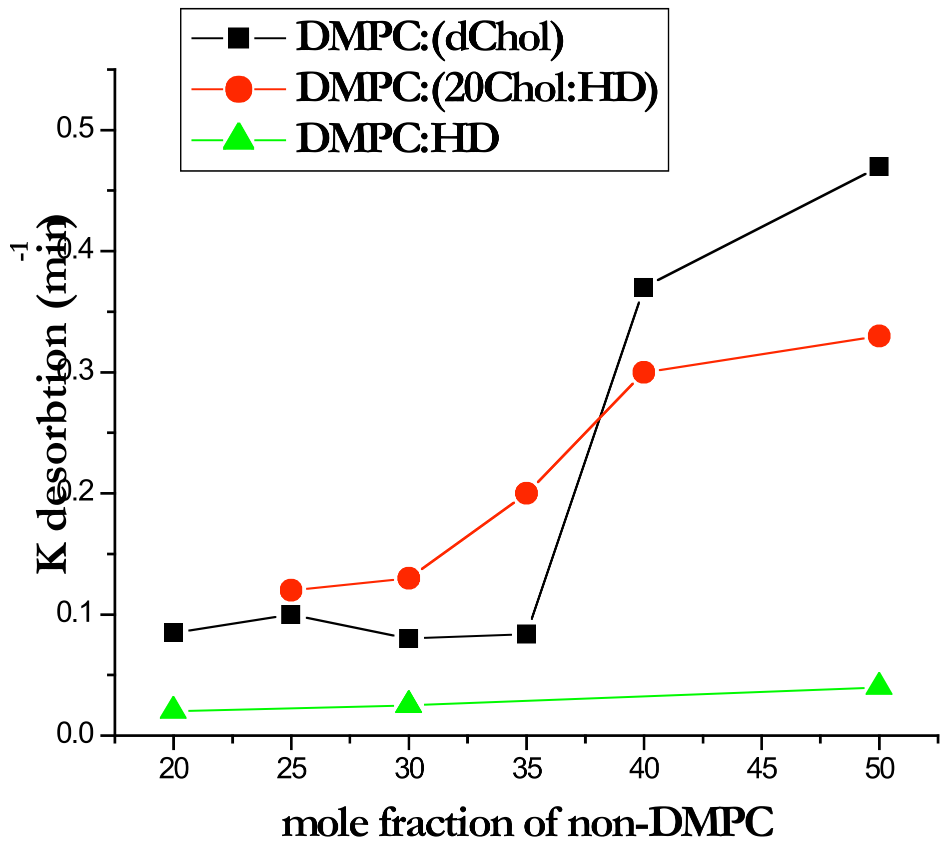
| | |
|---|---|
| 50:50 | C16:DMPC |
| 20:30:50 | DChol:C16:DMPC |

# Cyclodextrin II: point shifting

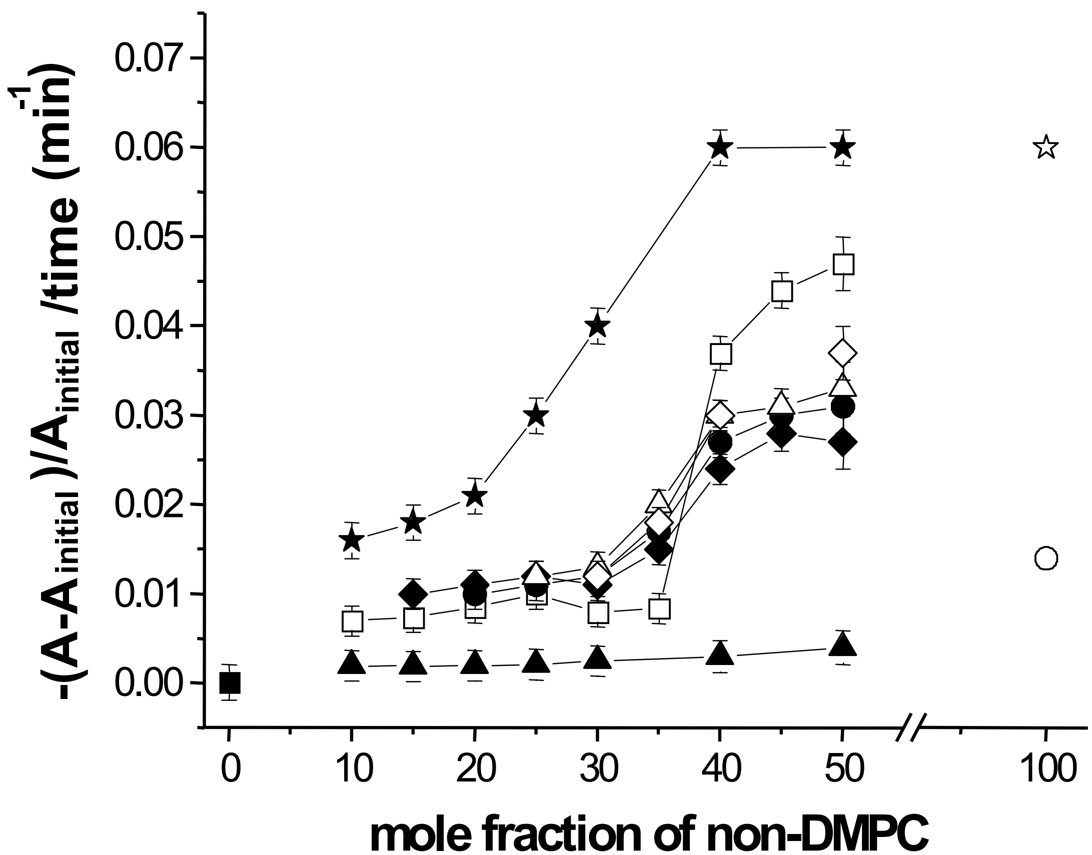


# Cholesterol Transfer to CD

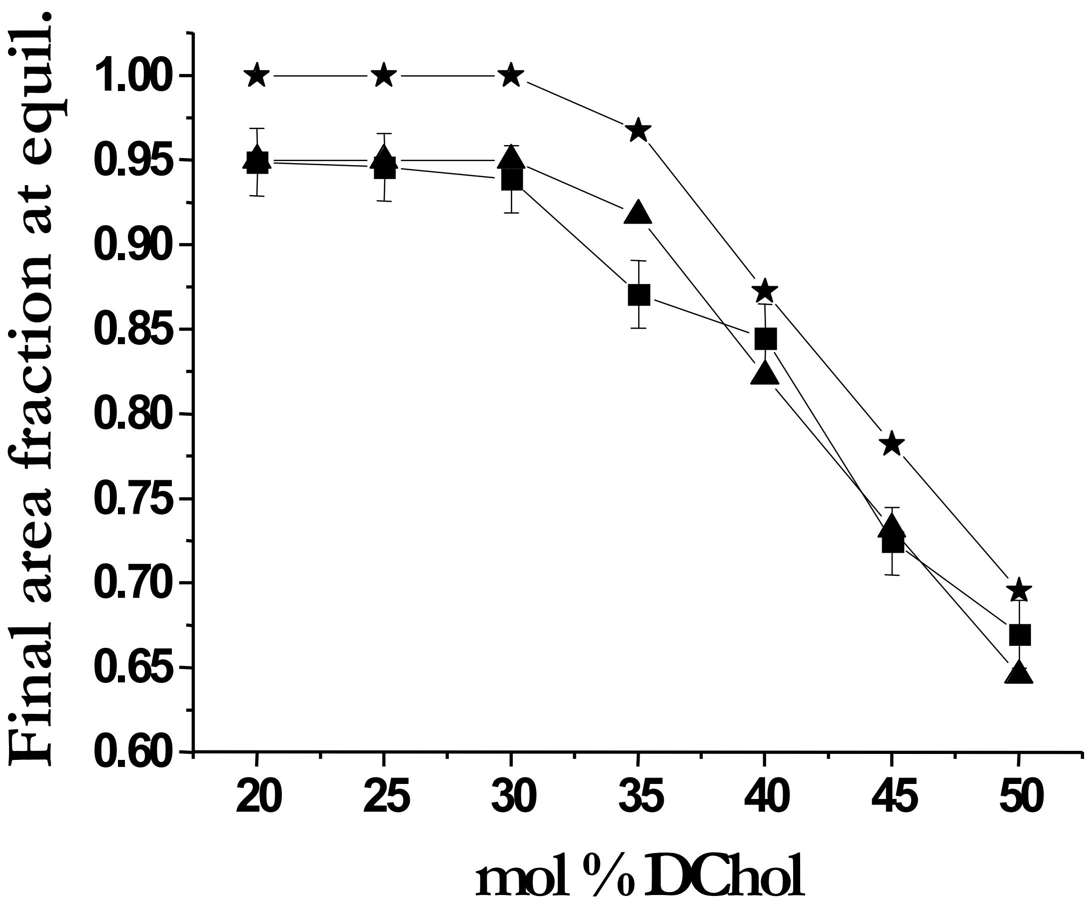


# Cholesterol Fixed at Different Mole Fractions




| | |
|---|---|
| Filled squares | DMPC |
| Open Star | DChol |
| Open circle | HD |
| Open square | DMPC/DChol |
| Filled triangle | DMPC/HD |
| Filled star | HD/DChol |
| Filled diamond | Ternary/10%DChol |
| Filled circle | Ternary/15%DChol |
| Open tirangle | Ternary/20%DChol |
| Open square | Ternary/25%DChol |

# Final Equilibrium Area for DMPC/DChol Mixtures




| | |
|---|---|
| Square | Expt. Results |
| Star | Model (assuming 2:1 DMPC:DChol) |
| Triangle | Model (5% area loss + 2:1 DMPC:DChol) |

*MR, YL, TS, KYCL, Biophys. J., in press*

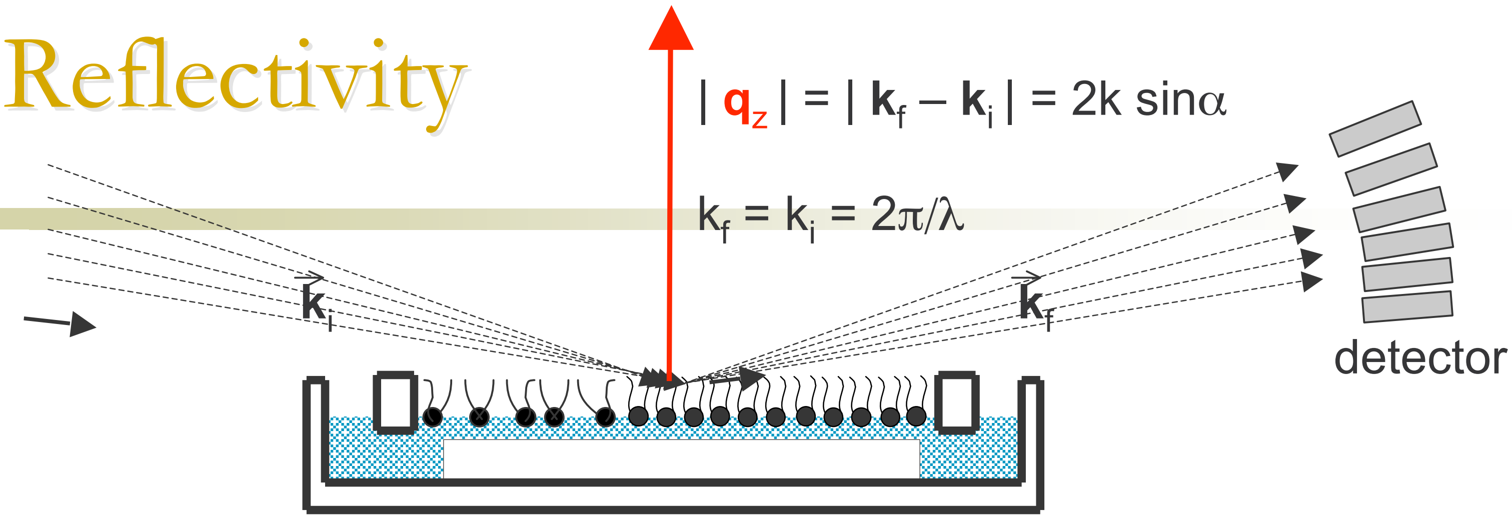
# Conclusions from CD Desorption Study

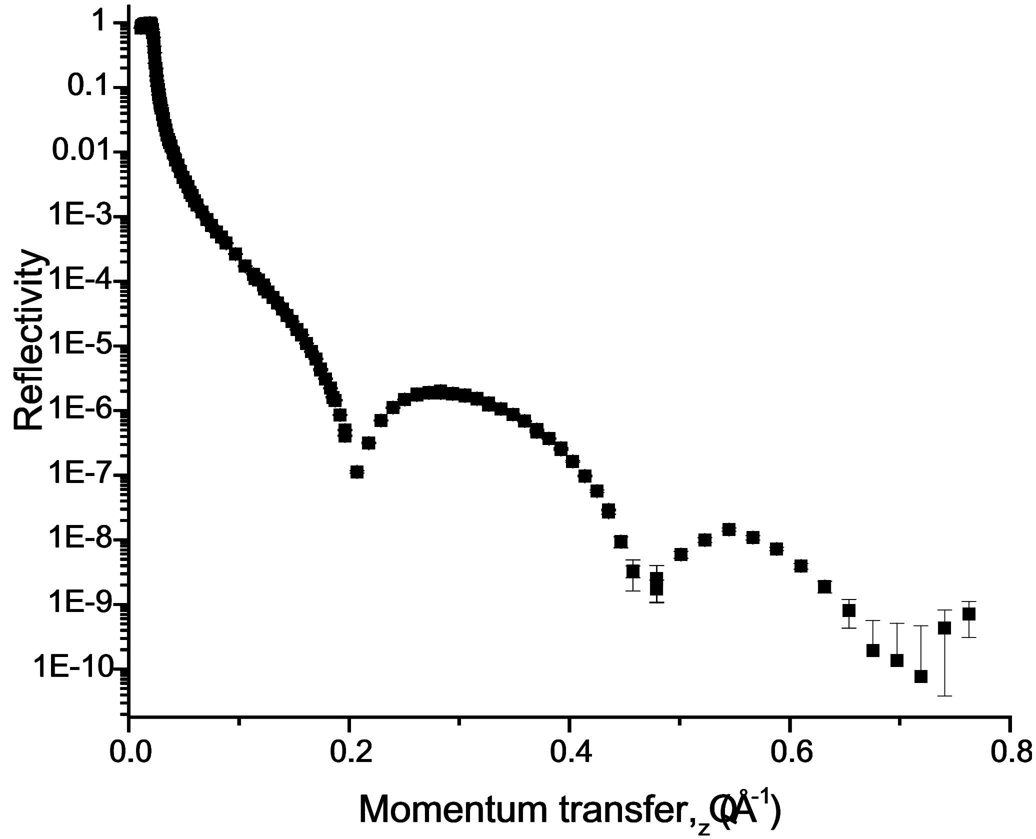
- Cholesterol can exist in two states of low and high chemical potential/activity/fugacity
- **In the presence of displacing agent cholesterol activity is increased**

# Four Different Approaches

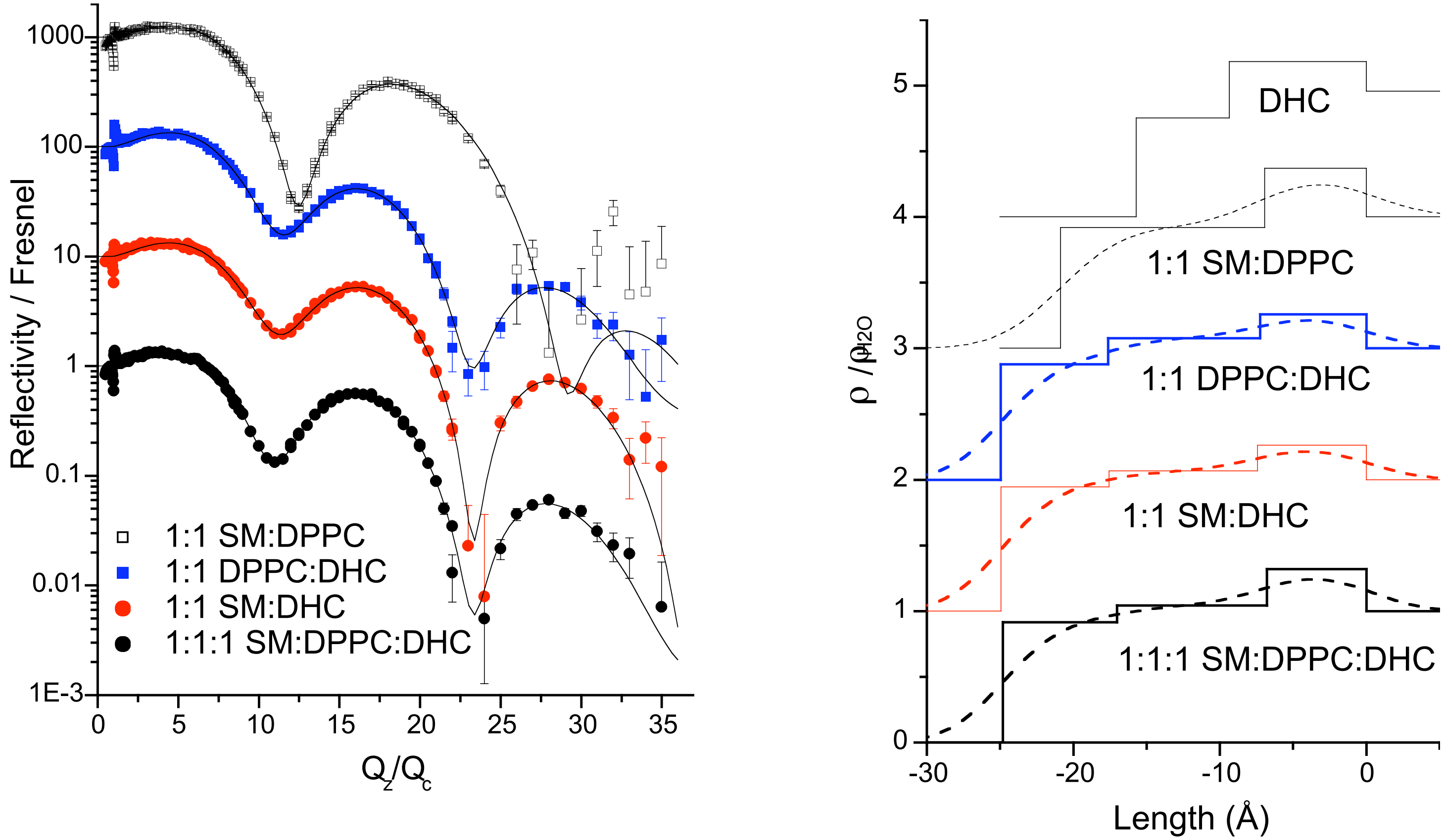
- Phase diagrams
- Cholesterol desorption by cyclodextrin (CD)
- X-ray diffraction and x-ray reflectivity
- Effect of "inhibiting" and "promoting" sterols on cholesterol activity in RBC

# X-ray Reflectivity



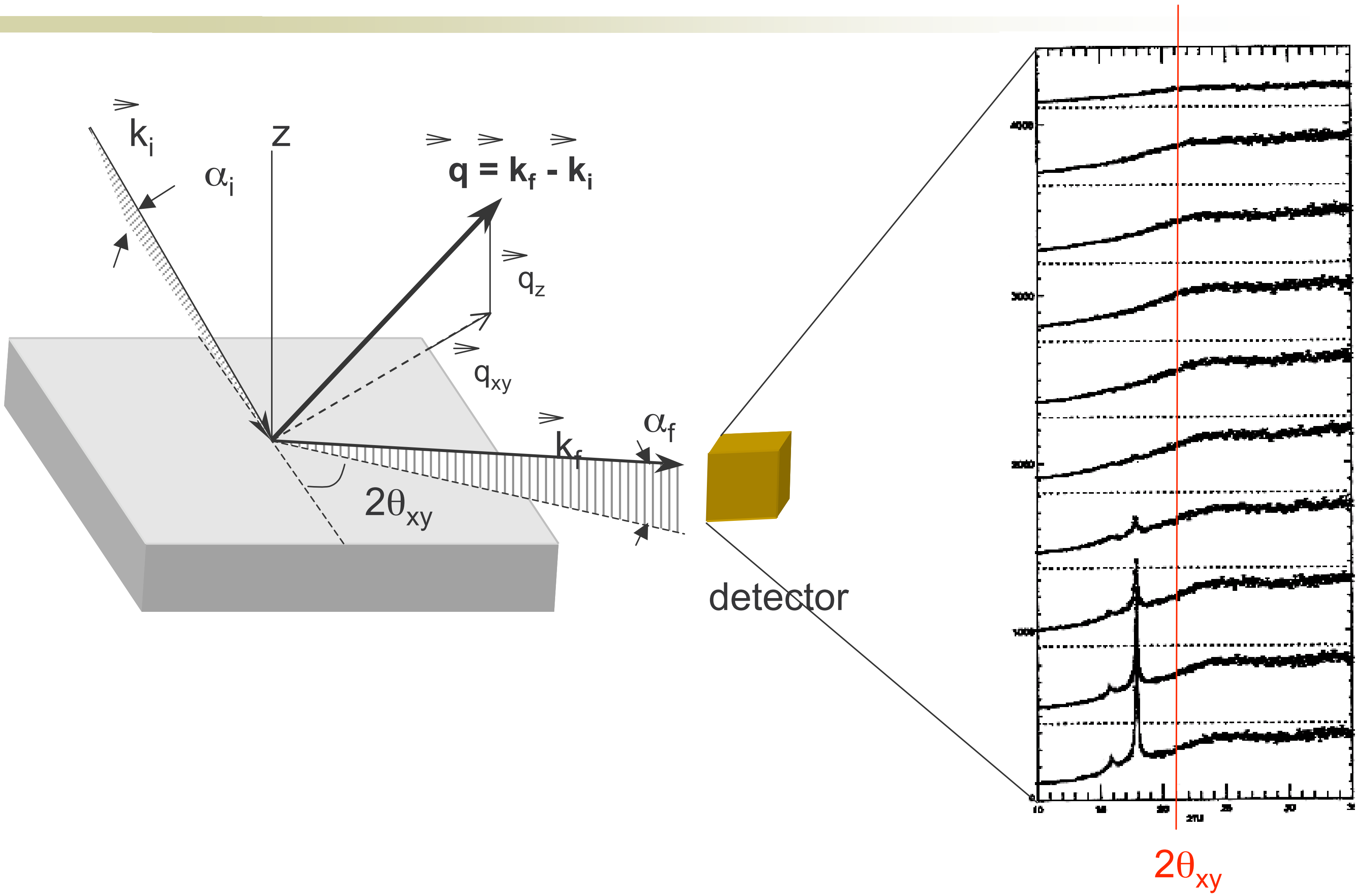



# XR Data on Mixtures

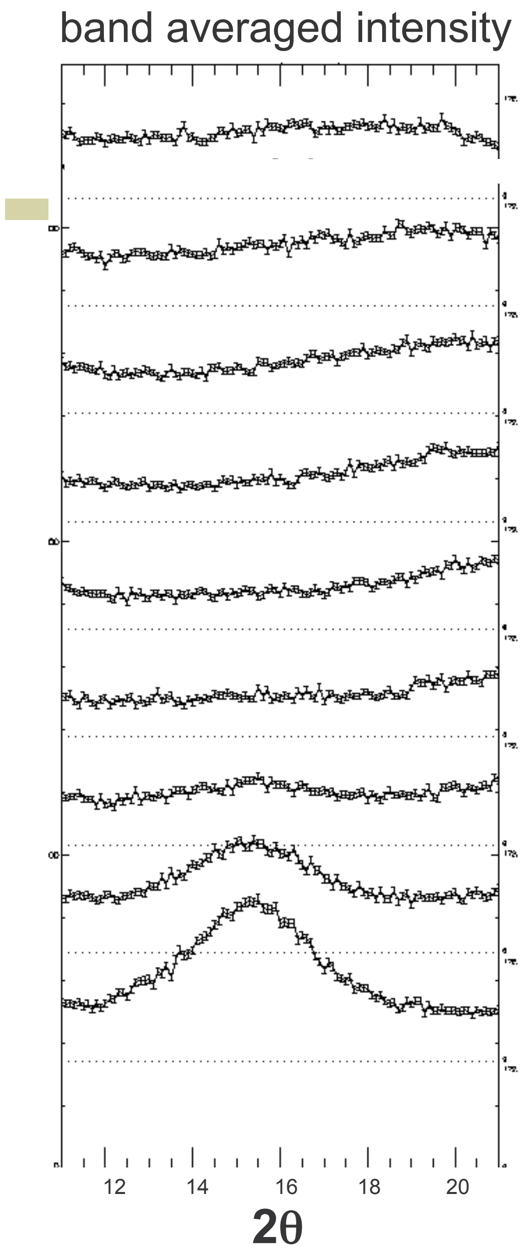


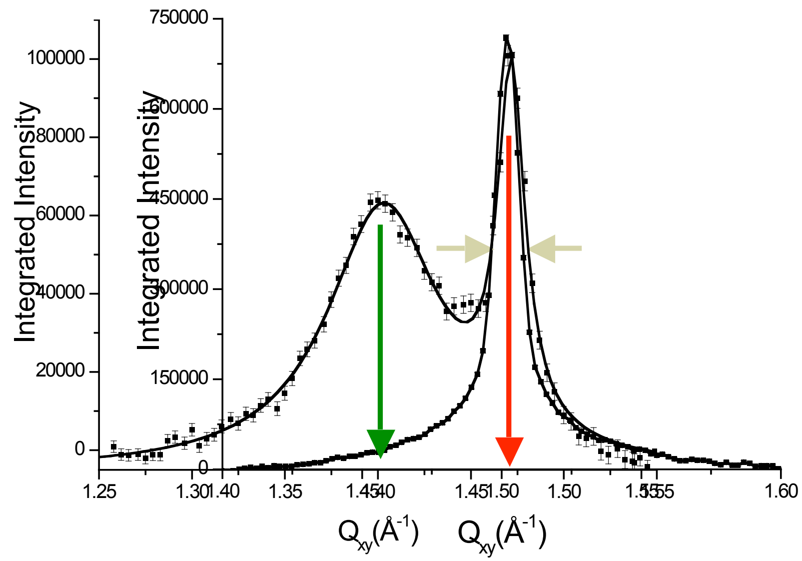

- DChol intercalates into acyl chain region (10 Å) - this layer is also responsible for Bragg scattering observed in GIXD)
- Reduces the tilt of phospholipids (cholesterol condensation effects)

# Grazing Incidence X-ray Diffraction (GIXD)



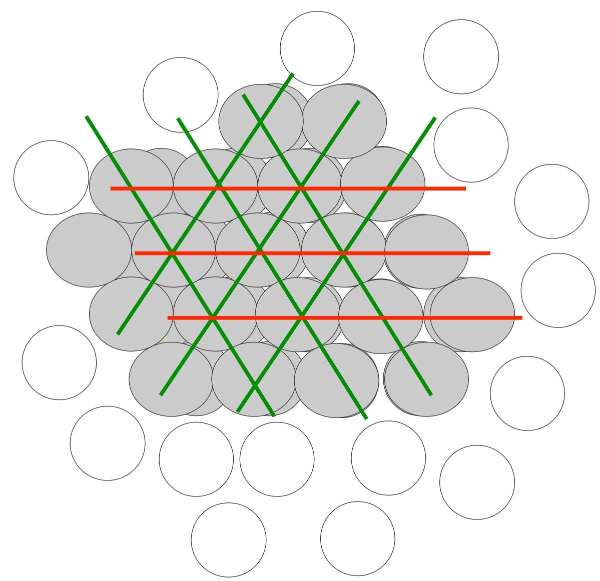
# GIXD



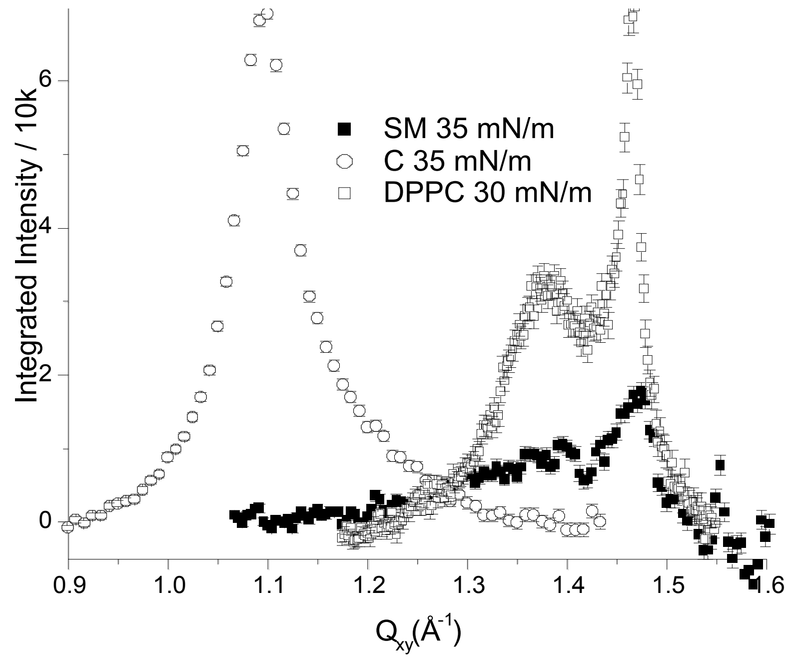




$q_{xy}$ ~ 1 / d-spacing

FWHM ~ 1 / coh. length

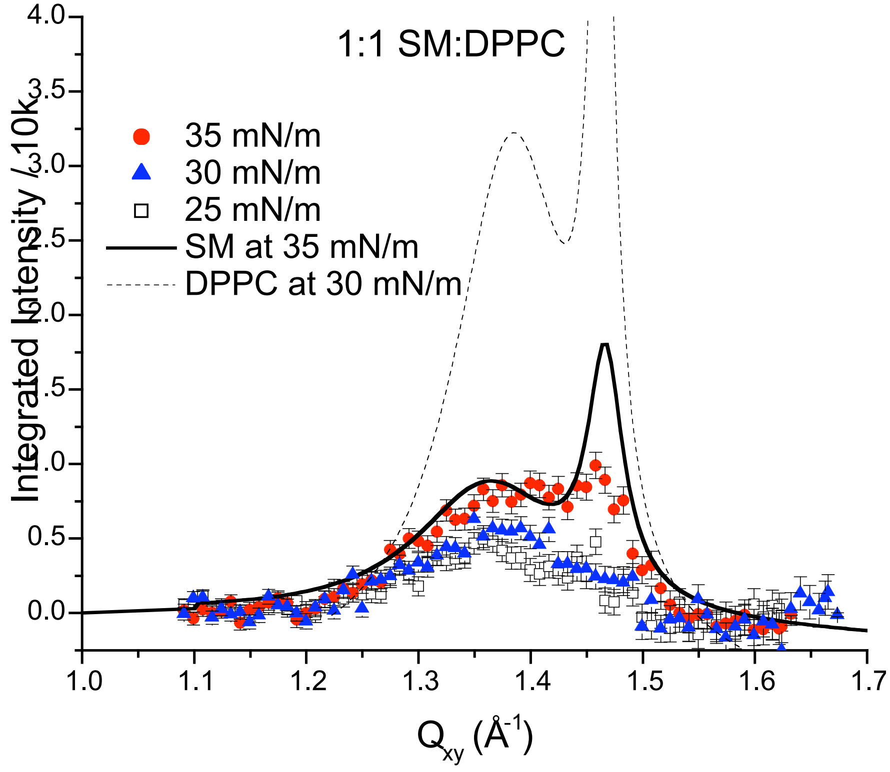


# GIXD of Pure Components




| | d-spacing(s) (Å) | Coherence Lengths (Å) L11, L10 01 | Integrated Intensity |
|---|---|---|---|
| SM (35 mN/m) | 4.29, 4.61 | 173,40 | 0.5 |
| DChol (35 mN/m) | 5.72 | 74.6 | 1.5 |
| DPPC (30 mN/m) | 4.29, 4.56 | 242, 47 | 1.3 |

# Pressure Dependence of Phospholipid Systems

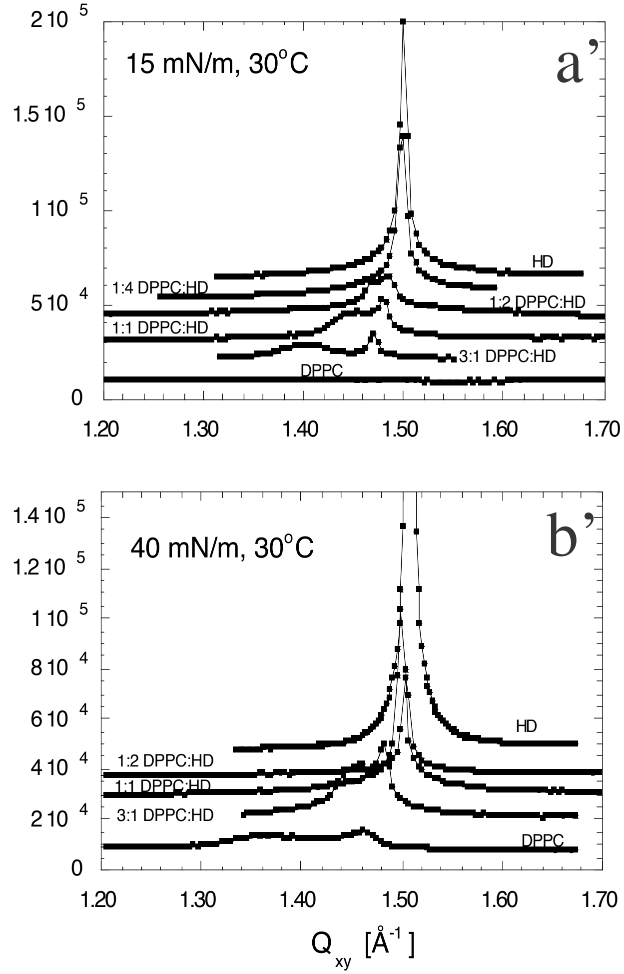



d-spacing, scattering intensity, coherence length (memory of crystallinity) are pressure-dependent

# Effects of C16 on Lipid Structure

Low pressure

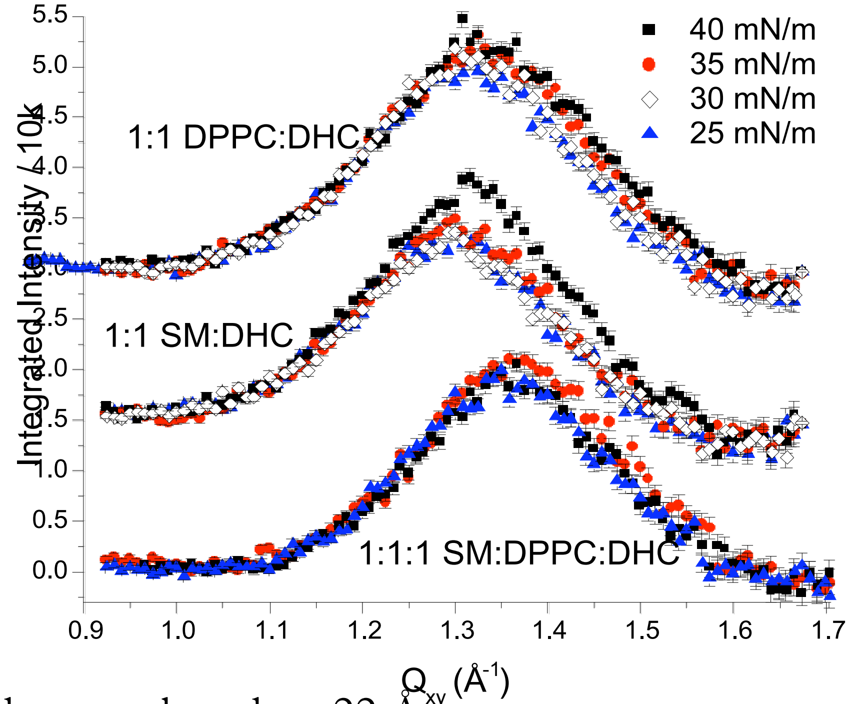
High pressure




*KYCL et al.*
*J. Chem. Phys.*
*116 (2002) 774-783*

- d-spacing, scattering intensity, coherence length (memory of crystallinity) are pressure-dependent
- C16 induces lipid packing at a lower pressure

# Presence of DChol Leads to Very Different Scattering Behavior




- coherence length ~ 22 Å
- d-spacing ≠ f (surface pressure)
- coherence length ≠ f (surface pressure)
- amt of scattering entities ≠ f (surface pressure)

- DChol = DHC

*CE, MKR, JM, KK, KYCL, BJ 91 (2006) L01-L03*

### 1:1 DPPC:DChol

| Π | d spacing | coherence length | integrated intensity |
|---|---|---|---|
| 25 | 4.79 | 22.37 | 1.1 |
| 30 | 4.79 | 22.61 | 1.2 |
| 35 | 4.75 | 21.46 | 1.2 |
| 40 | 4.73 | 21.01 | 1.4 |

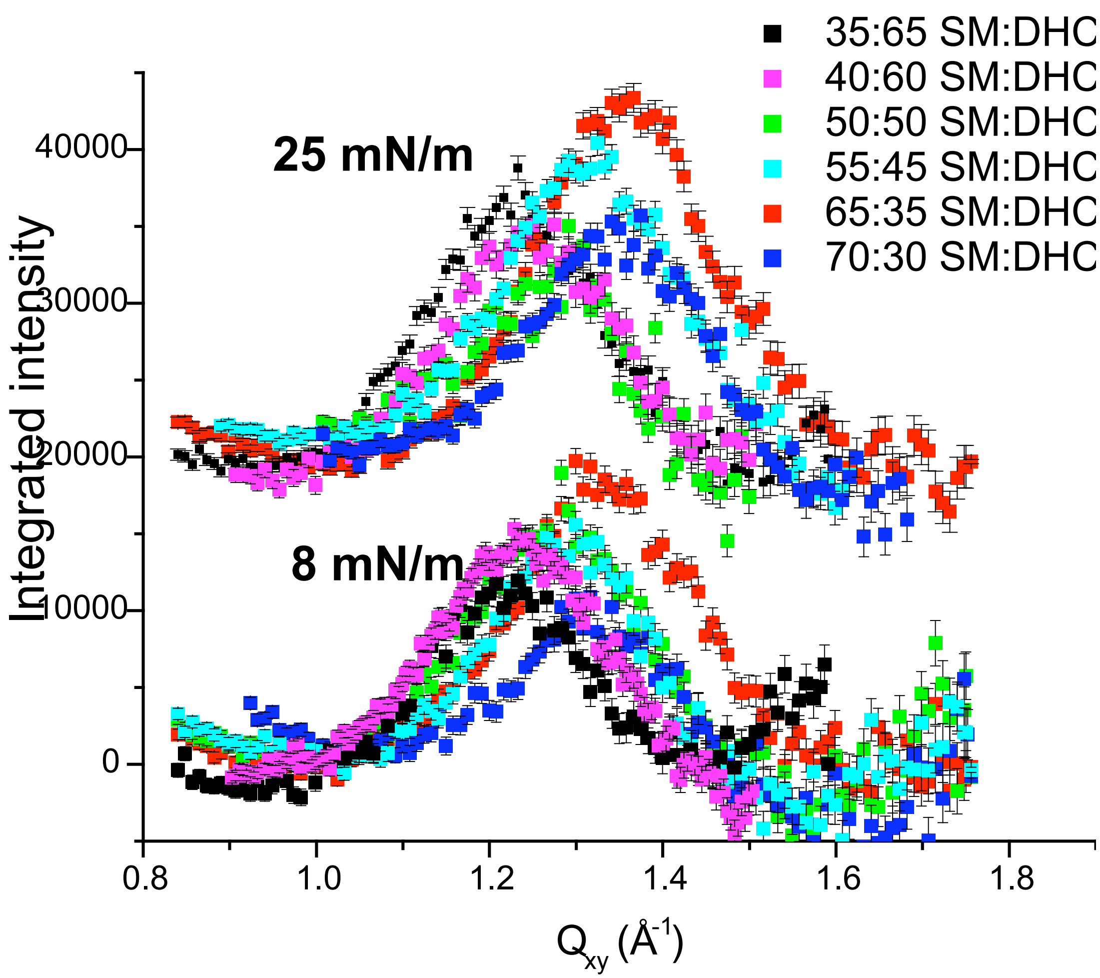
### 1:1 SM:DChol

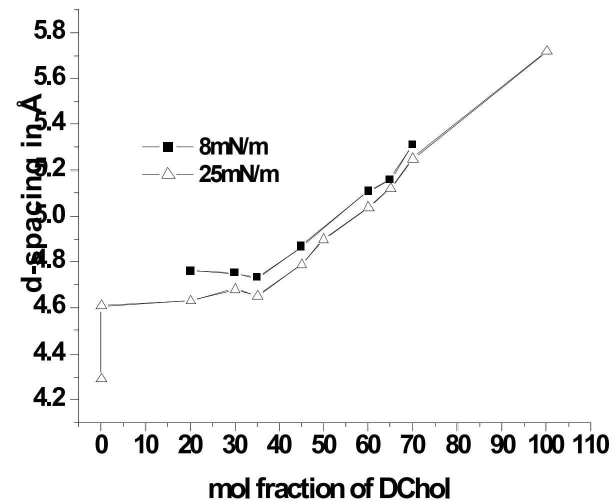
| Pressure | d spacing | coherence length | integrated intesnity |
|---|---|---|---|
| 25 | 4.86 | 24.39 | 1.0 |
| 30 | 4.85 | 23.61 | 1.0 |
| 35 | 4.84 | 23.38 | 1.1 |
| 40 | 4.80 | 22.25 | 1.3 |

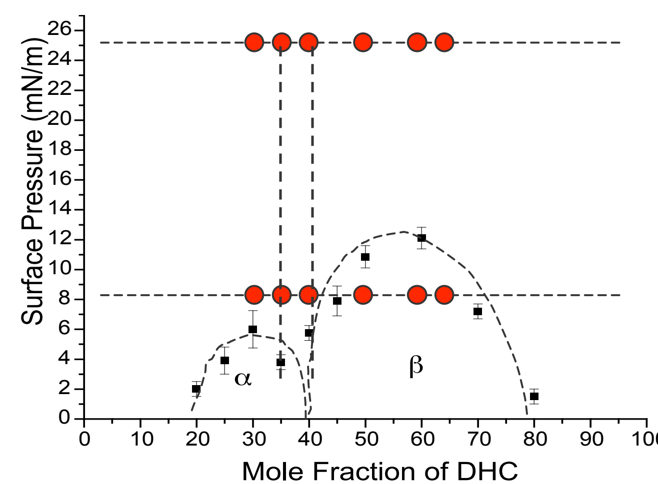
### 1:1:1 SM:DPPC:DChol

| Pressure | d spacing | coherence length | integrated intesnity |
|---|---|---|---|
| 25 | 4.70 | 22.94 | 1.0 |
| 35 | 4.60 | 21.85 | 1.0 |
| 40 | 4.62 | 23.03 | 1.0 |

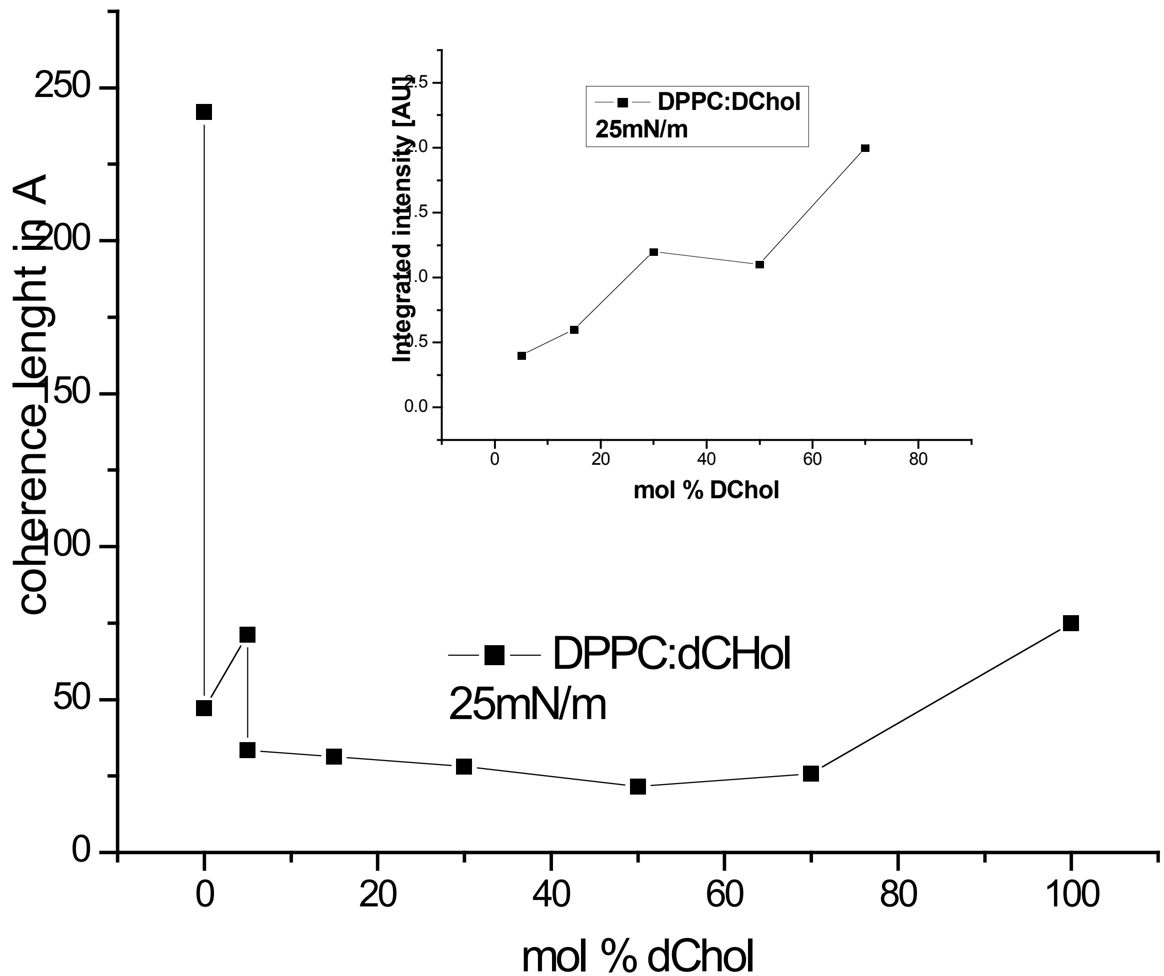
# Structures Exist Below and Above Liquid-Liquid Immiscibility Line



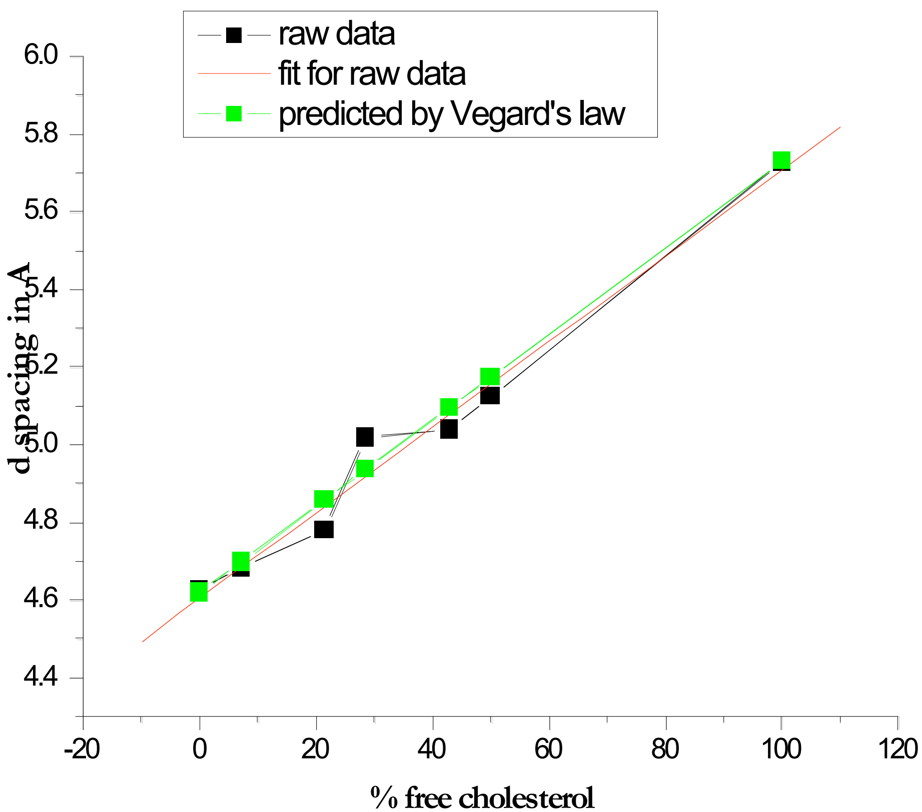



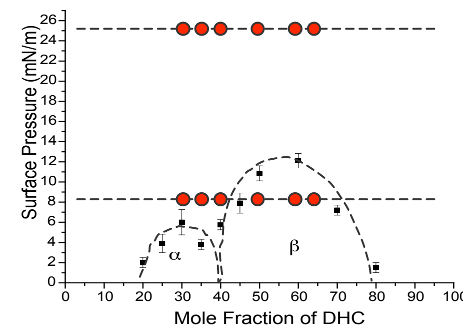



# Coherence Dependence on Chol Content in DPPC:Chol Systems



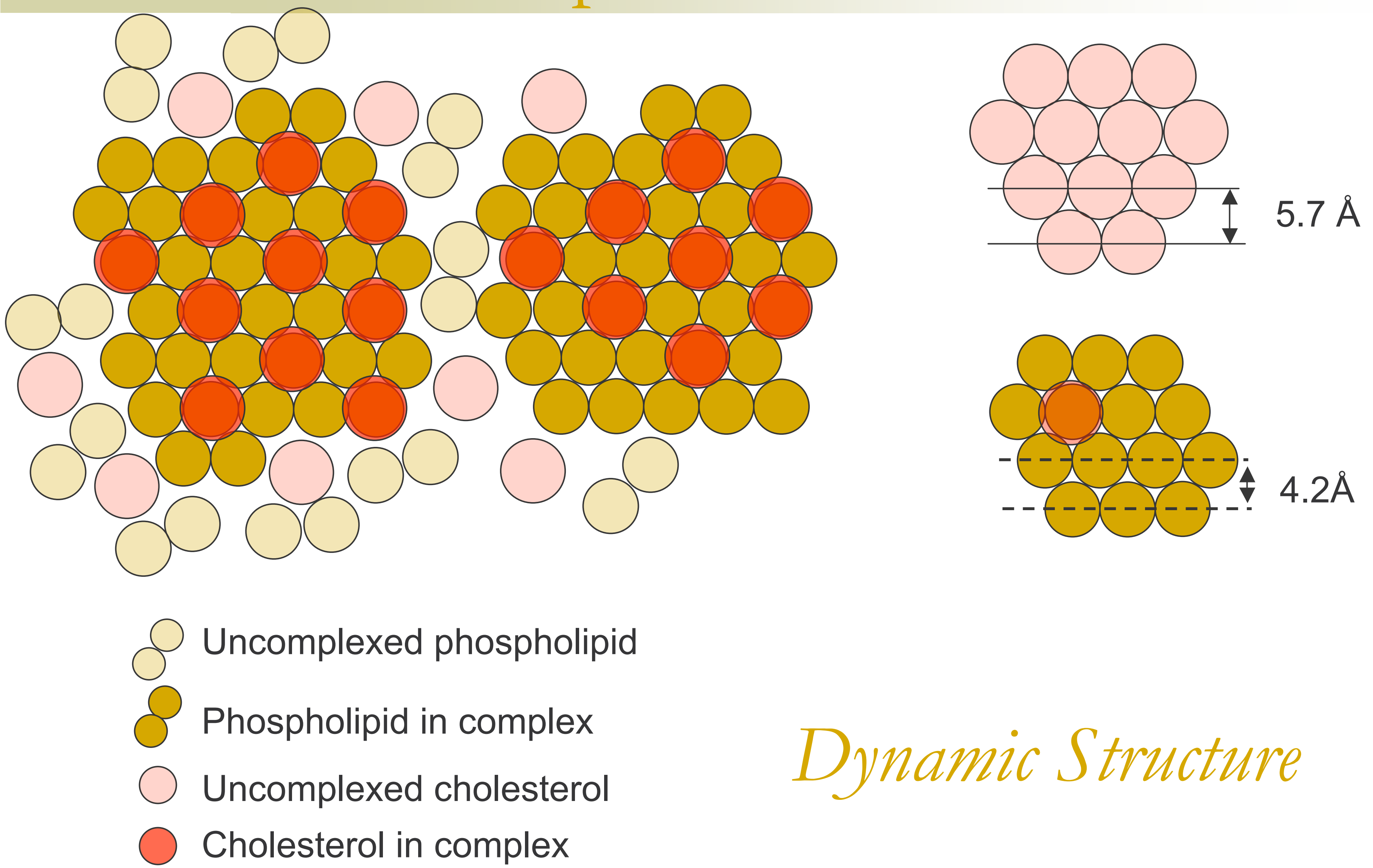
# Vegard's Law




*MR, JM, KK, KYCL, unpublished data (2007)*

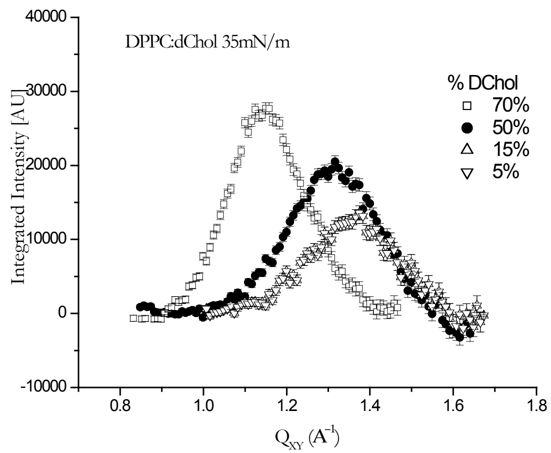
# Proposed Model



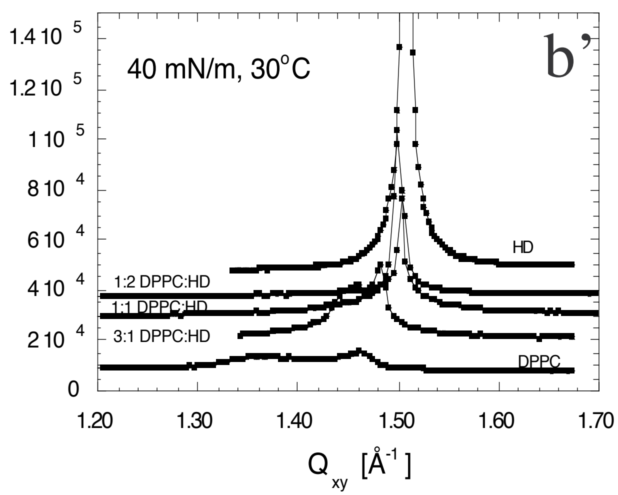

*Dynamic Structure*

# Structural Signature of Displacement Effects

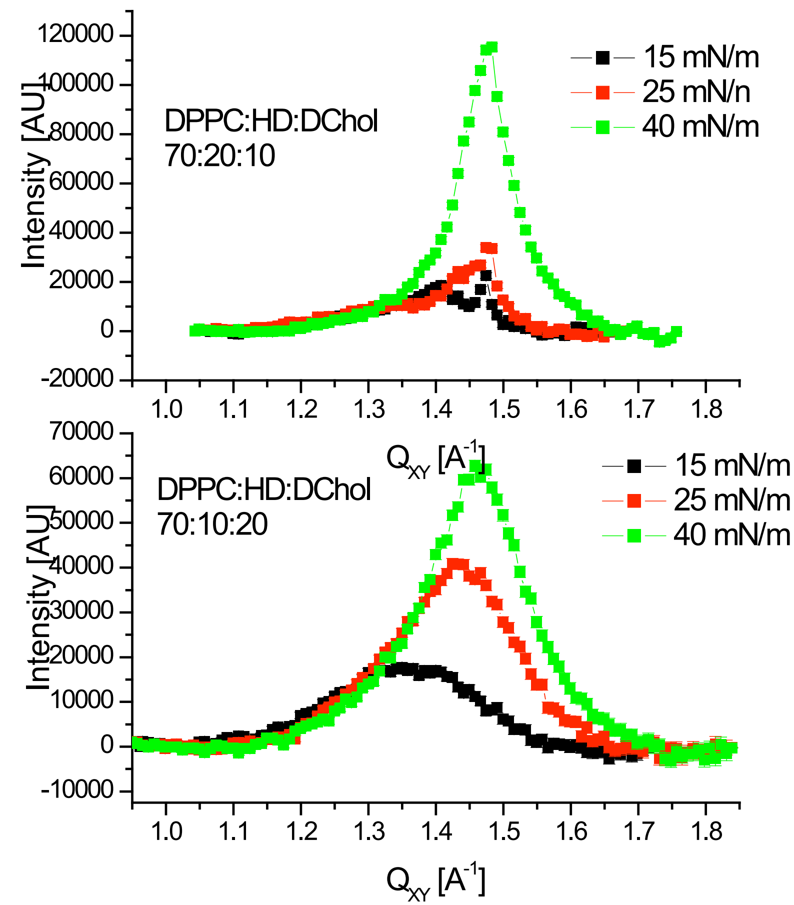
DPPC:DChol




DPPC:HD




DPPC:HD:DChol



# Presence of DPPC:HD and DPPC:DChol peaks

| DPPC:HD:DChol | Pressure (mN/m) | d-spacing (Å) | Coherence length (Å) | Integrated intensity (a.u) |
|---|---|---|---|---|
| 100:0:0 | 15 | - | - | - |
| | 40 | 4.31, 4.57 | 150, 50 | ? |
| 0:100:0 | 15 | 4.19 | 850 | ? |
| | 40 | 4.17 | 620 | ? |
| 0:0:100 | 25 | 5.71 | 63.1 | 1.7 |
| | 35 | 5.72 | 75.5 | 1.5 |
| 70:10:20 | 15 | 4.65 | 27.0 | 0.9 |
| | 25 | 4.37, 4.88 | 35.4, 43.3 | 1.6, 0.3 |
| | 40 | 4.30 | 33.3 | 3.5 |
| 70:20:10 | 15 | 4.27, 4.46, 4.80 | 311.6, 71.1, 35.4 | 0.1, 0.4, 0.4 |
| | 25 | 4.25, 4.36, 4.76 | 311.6, 81.4, 30.0 | 0.2, 0.4, 0.6 |
| | 40 | 4.25, 4.42 | 71.1, 56.8 | 2.8, 0.3 |
| 50:30:20 | 15 | 4.26, 4.42, 4.76 | 569, 78.4, 39.1 | 0.1, 0.5, 0.4 |
| | 25 | 4.27, 4.49, 4.77 | 68.6, 56.7, 33.0 | 0.9, 0.4, 0.5 |
| | 40 | 4.25, 4.52 | 56.7, 33.3 | 1.7, 0.9 |
| 50:40:10 | 15 | 4.25, 4.39 | 504.9, 130.9 | 0.5, 1.0 |
| | 25 | 4.25, 4.30, 4.62 | 1042.2, 114.7, 51.6 | 0.3, 2.0, 0.4 |
| | 40 | 4.19, 4.22 | 1042.2, 63.1 | 0.6, 3.0 |

# Conclusions from X-ray Scattering Study

- Intermediate d-spacing provides evidence for cholesterol:phospholipid intermediate structures

- Reduced coherence lengths
  => nanoscale domains
  => dynamic structures

- Signatures of PC/HD and PC/DChol complexes observed in ternary mixtures

# Four Different Approaches

- Phase diagrams
- Cholesterol desorption by cyclodextrin (CD)
- X-ray diffraction and x-ray reflectivity
- Effect of "inhibiting" and "promoting" sterols on cholesterol activity in RBC

# Probing Free Cholesterol in RBC

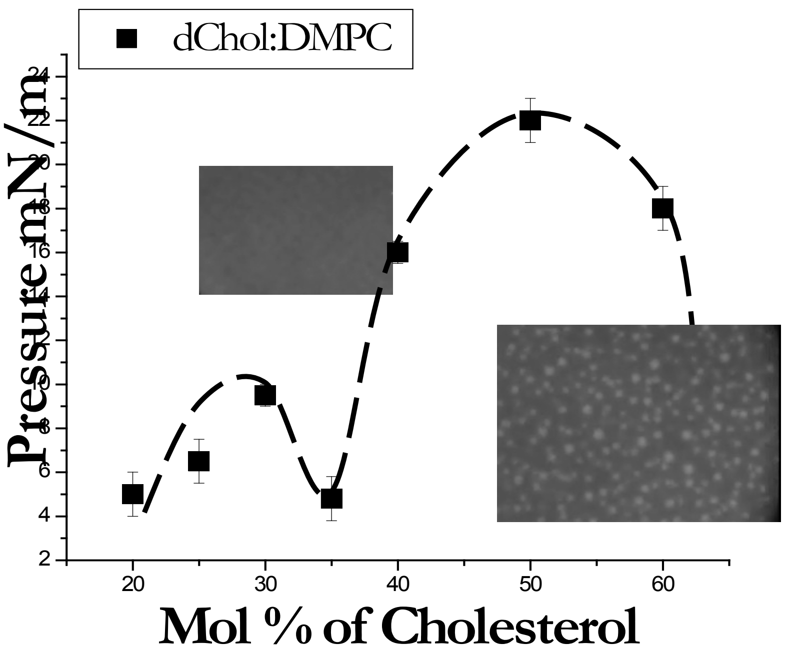
- Cholesterol oxidase can oxidize cholesterol

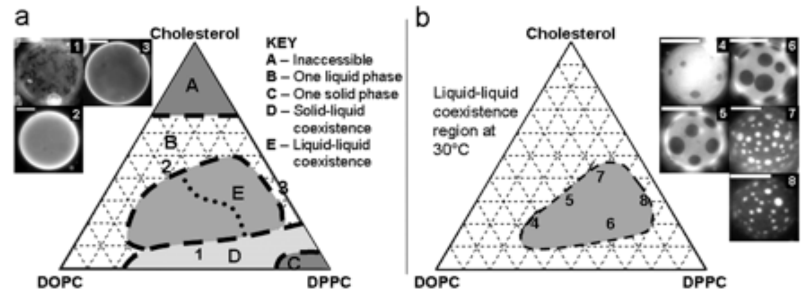
  –> causes the production of cholestenone only if there is excess (free) cholesterol available

  - Cholesterol oxidase depends on chemical activity of cholesterol

- The amount of cholestenone can be quantified by GC-FID

# “Inhibiting” and “Promoting” Sterols



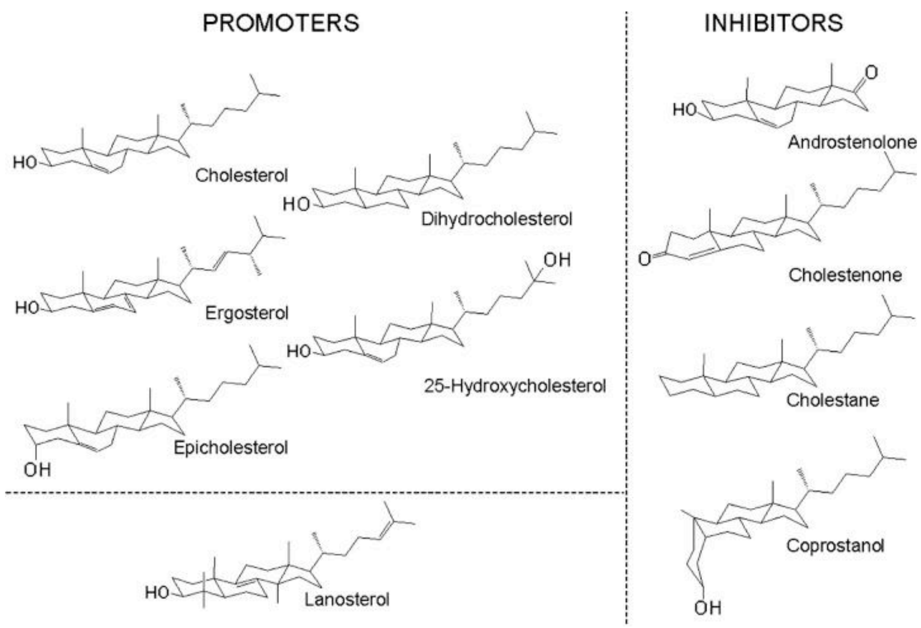



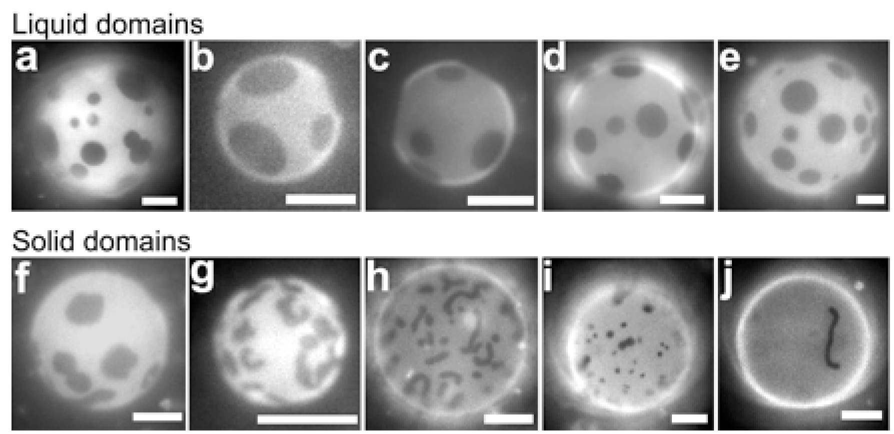

Observed phase diagram of micron-scale liquid immiscibility region in GUVs at 30°C.

**Separation of Liquid Phases in Giant Vesicles of Ternary Mixtures of Phospholipids and Cholesterol**
**Sarah L. Veatch and Sarah L. Keller**

**Phase Behavior of Lipid Monolayers Containing DPPC and Cholesterol Analogs**
**Benjamin L. Stottrup and Sarah L. Keller**

# “Inhibiting” and “Promoting” Sterols



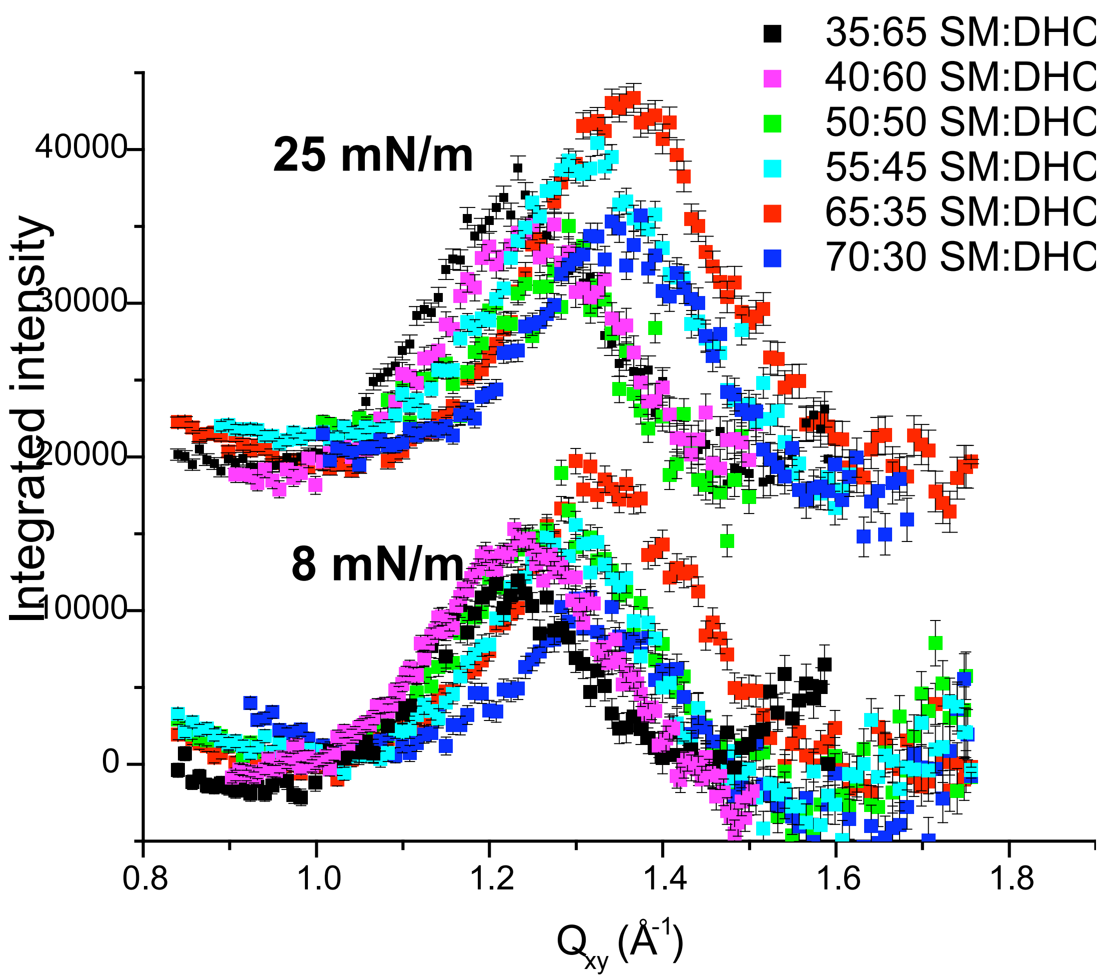



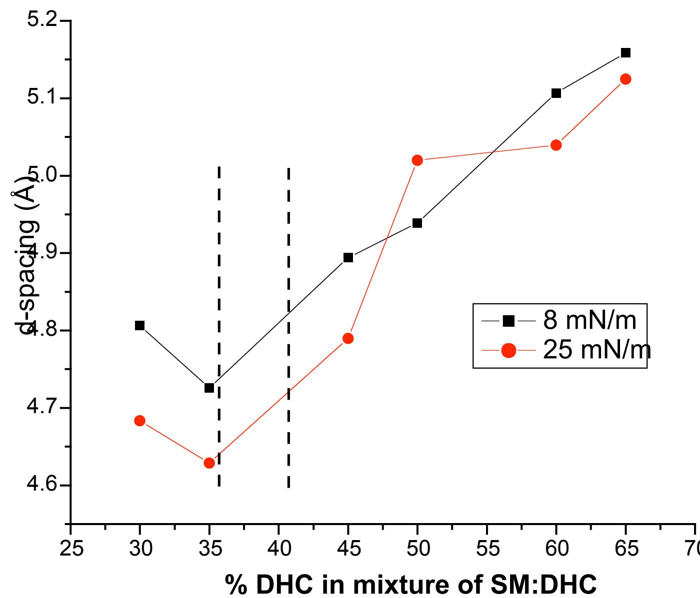

Veatch, Keller et al. 2005

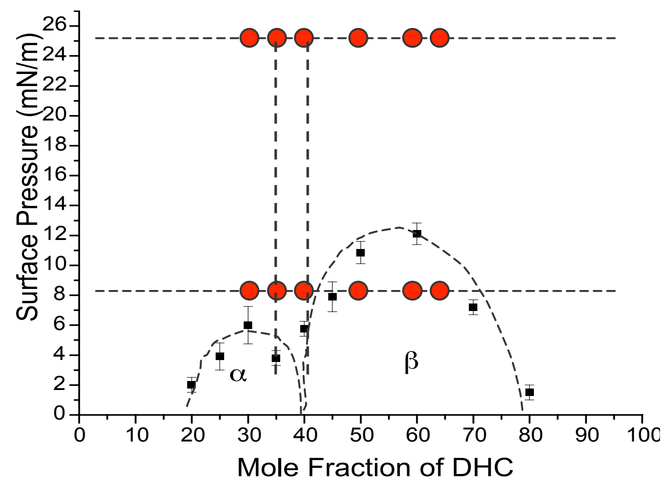
# How does immiscibility correlates with active cholesterol?

- Hypotheses:
  - Domains imply/require complex
  - Lack of domains <u>does not imply</u> no complex
  - Complex is necessary but not sufficient for domain phase separation
  - Displacement of cholesterol by intercalators signifies complexation of intercalator with phospholipids, giving rise to free cholesterol
    *intercalators* <==> cholesterol mimics

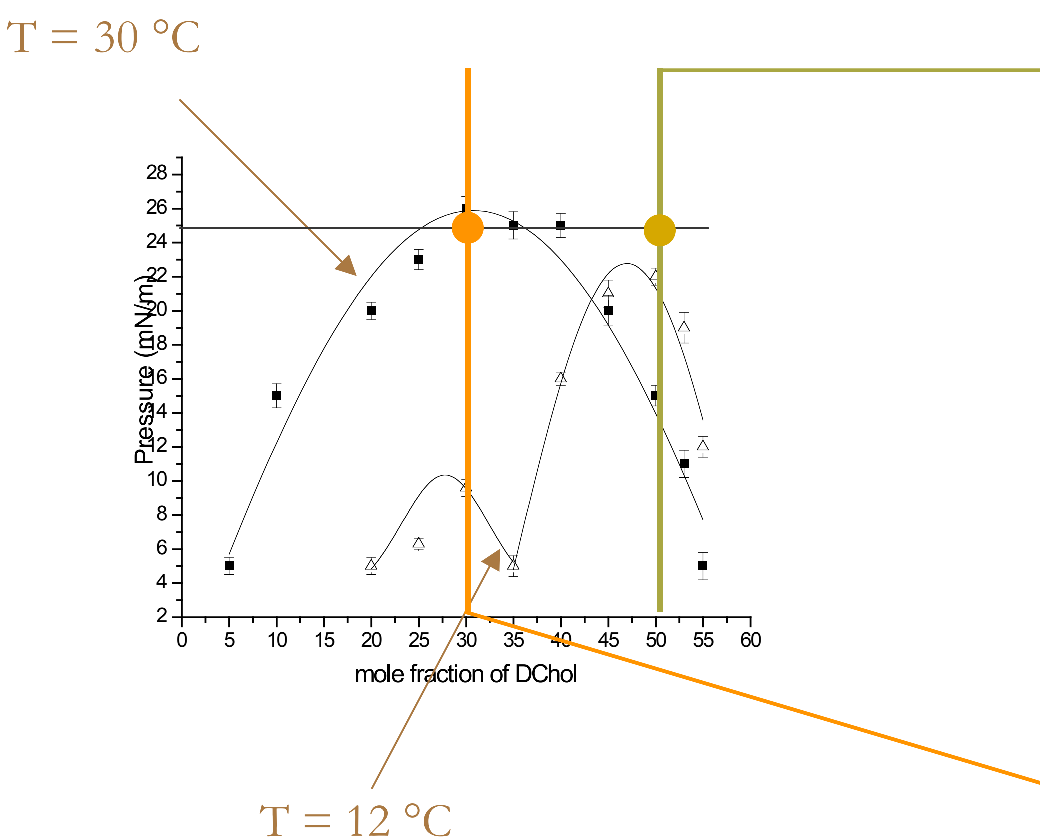
# Structures Exist Below and Above Liquid-Liquid Immiscibility Line

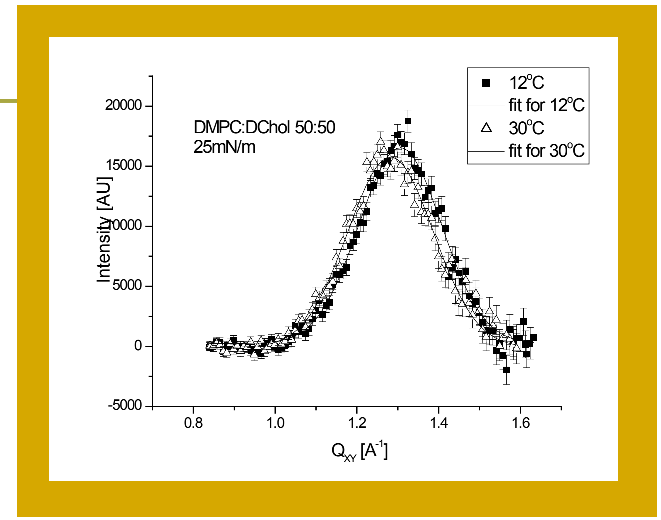


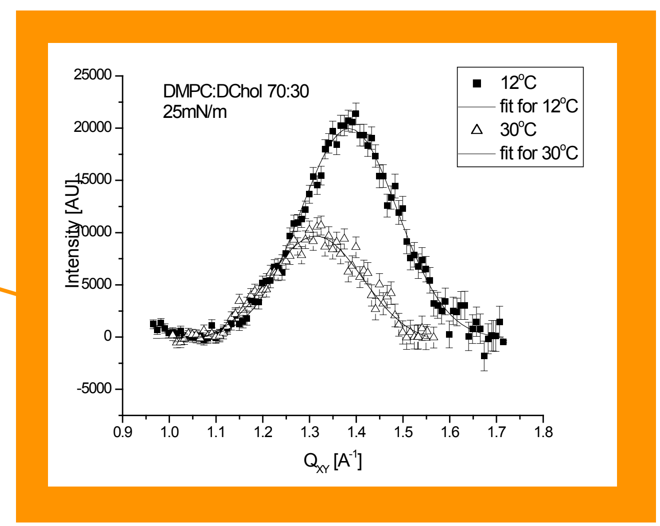



# Temperature Dependence



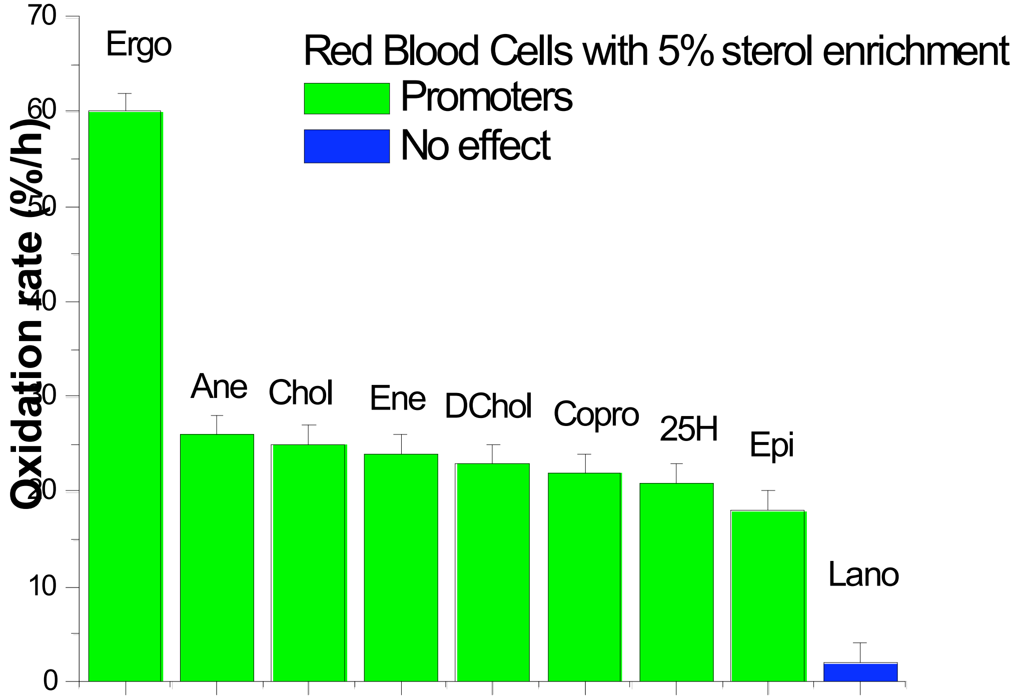




**DMPC/DChol Binary Mixture**

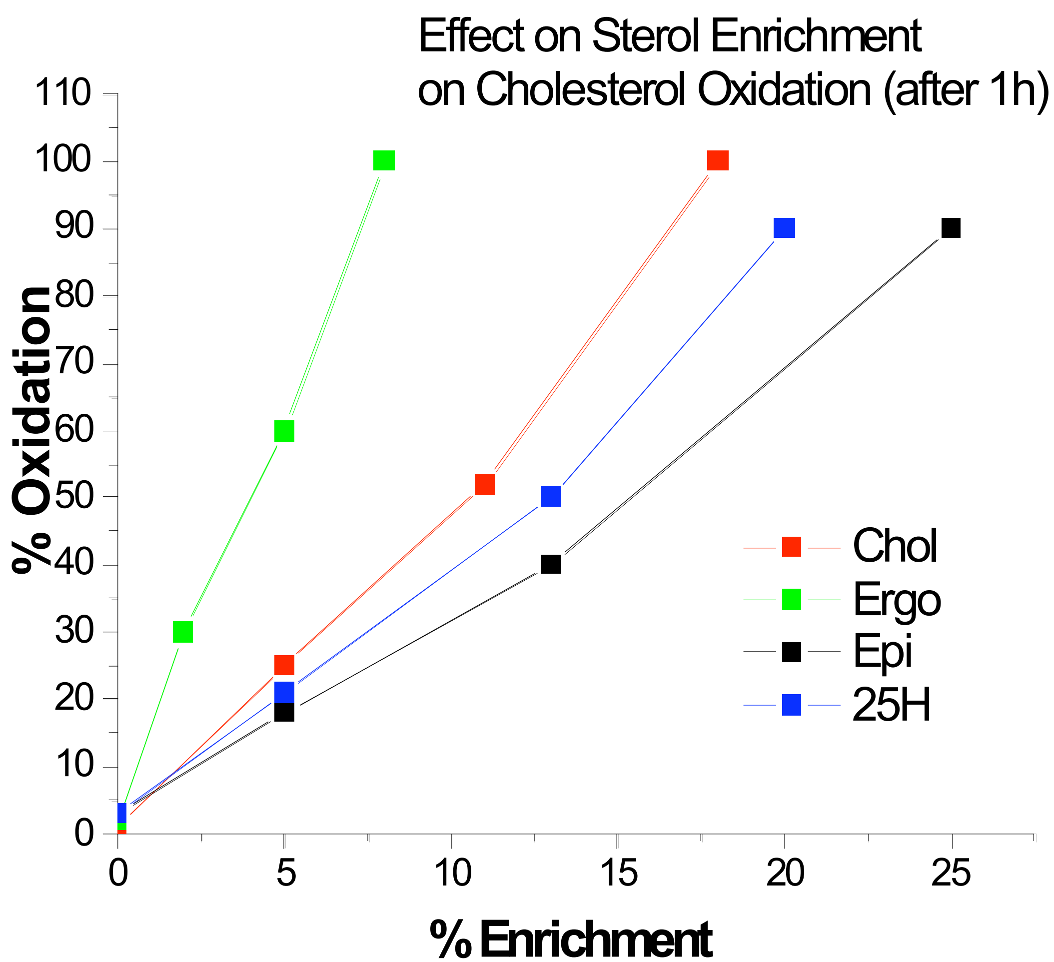
# “Inhibiting” and “Promoting” Sterols

- If our hypotheses are correct:
  - should always observe active cholesterol with promoter sterols
  - could still observe active cholesterol even with inhibitor sterols
- What would disprove our hypotheses?
  - If we observe no active cholesterol in case of promoter sterols
  - This would mean that domains do not imply/depend upon complex formation

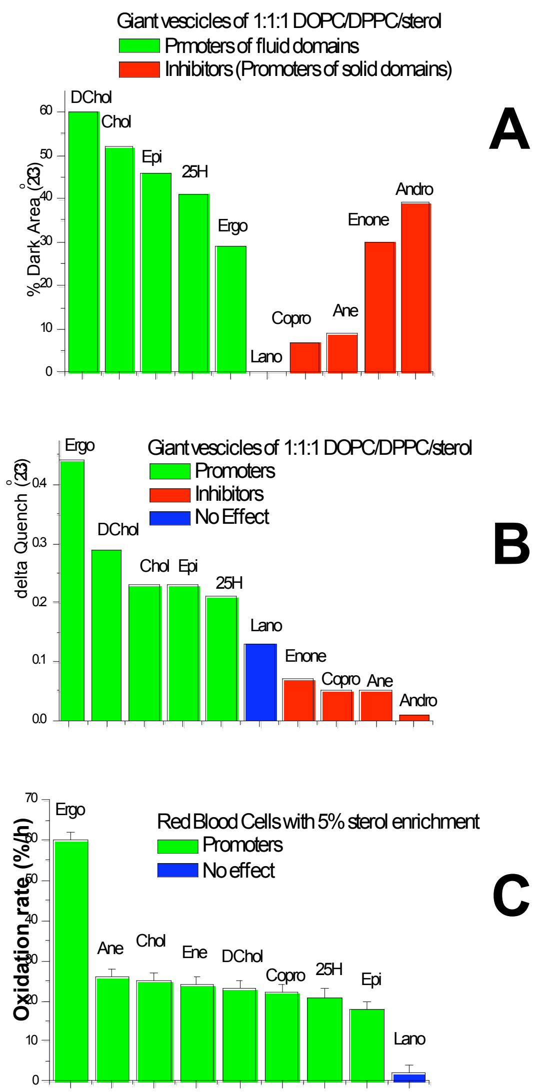
# Testing for Cholestenone



# Effect of Sterol Enrichment

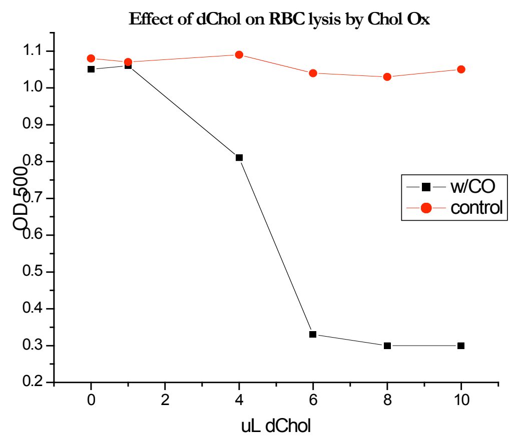


# Displacement Observed for "Promoting"and "Inhibiting" Sterols

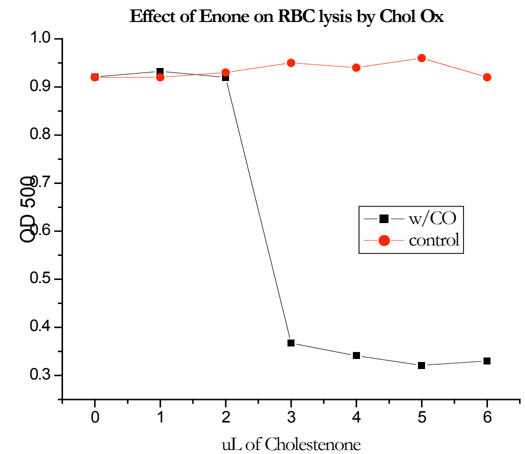


# “Inhibiting” and “Promoting” Sterols Promoting Cell Lysis

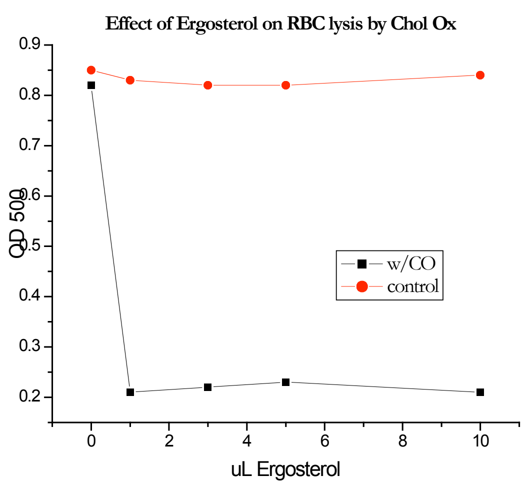
## Dihydrocholesterol (P)



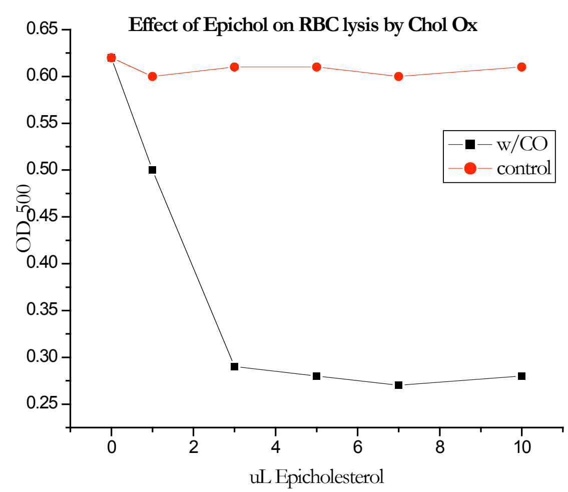

## Cholestenone (I)




## Ergosterol (P)




## Epicholesterol (P)



# Conclusions from RBC Study

- Tested all "promoting" sterols
  - All promoted cholestenone production and cell lysis by cholesterol oxidase
    - All displace cholesterol: produced free cholesterol available for cholesterol oxidase
- Cholesterol can exist in two states: bound to phospholipids and free

# Conclusions

- 2 different states of cholesterol when present in lipid mixtures:
  - inactive (bound to lipid)
  - active (free)
- Production of active (free) cholesterol is possible even when actual cholesterol content is low but with alcohol present
- Alcohol displaces cholesterol from complexes and makes it available for cyclodextrin even with cholesterol content in the *alpha region*
- No cholesterol: quasi-long range order
- With cholesterol: very short-range order (coherence length ~ 22 Å); no pressure dependence
- Packing of phospholipid/cholesterol "complexes" depends on cholesterol content
- "Inhibiting" and "Promoting" sterols both can displace cholesterol

# Acknowledgments

Canay Maria + Chris (in UK)


- Ted Steck (U of Chicago) and Yvonne Lange (Rush)
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- Kristian Kjaer (Niels Bohr Inst.)
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- YTCK:Trinity College, Cambridge, the Oxbridge Society of Hong Kong, and the Jardines Scholarship
- NSF-MCB

  Packard Foundation

  NSF-CRIF

  BW1 beamline at Hasylab, DESY, Germany

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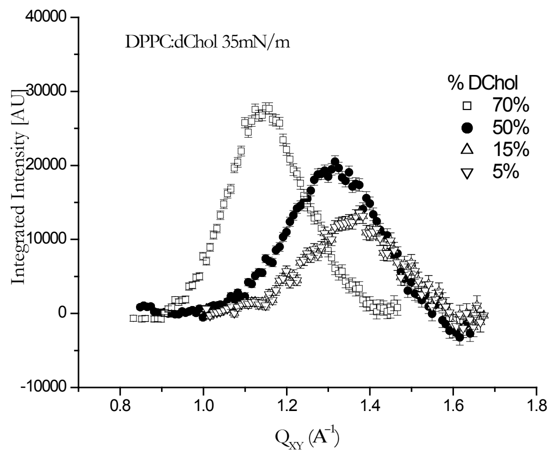
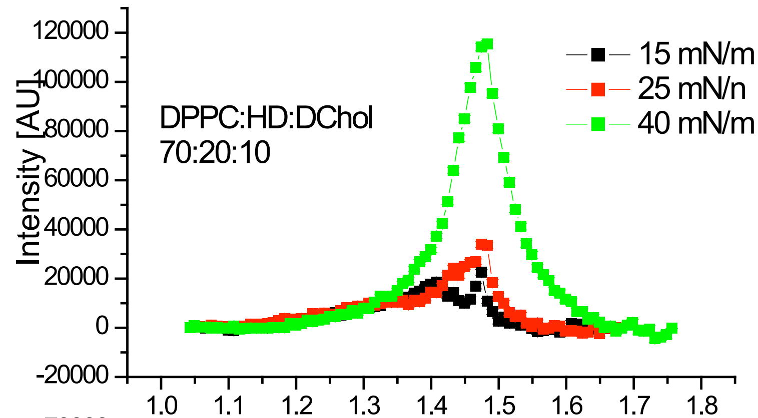
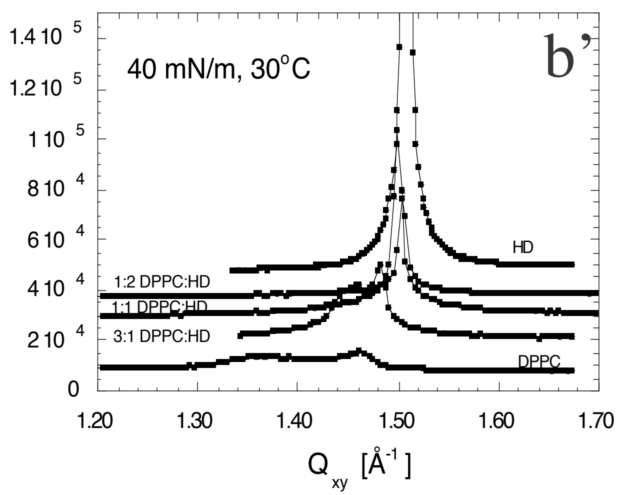
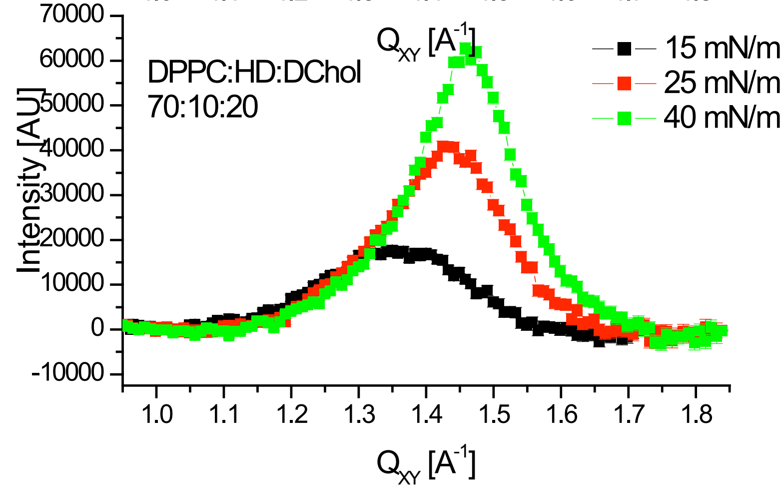



DPPC:HD:DChol




DPPC:HD



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