Use of neutrons in biology and medicine

Jayne Lawrence
Institute of Pharmaceutical Science
King's College London
London

Neutrons, a very good probe for biological studies because……

- they penetrate deeply into biological materials
- do not damage biological materials
- they possess wavelengths ~ atomic dimensions
- they possess energies ~ atomic motions
- they exhibit a large dynamic range in space, time & energy
- they can distinguish between H & D (basis of ***contrast variation***)
- in contrast to X-rays, can detect the location of H (essential in many biological processes)

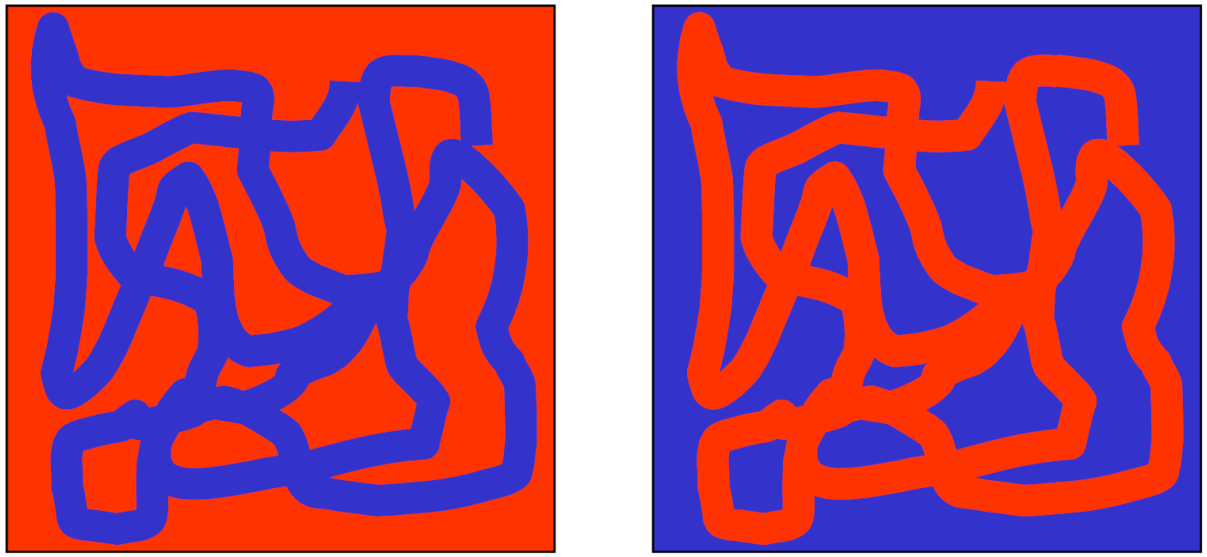
Neutrons provide structural and dynamic data……

- molecular size and shape
- membrane structure
- atomic structure
- molecular dynamics

However the technique suffers from……

- low resolution
- low flux
- longer acquisition times (than X-rays)
- requirement for relatively large samples

Contrast variation



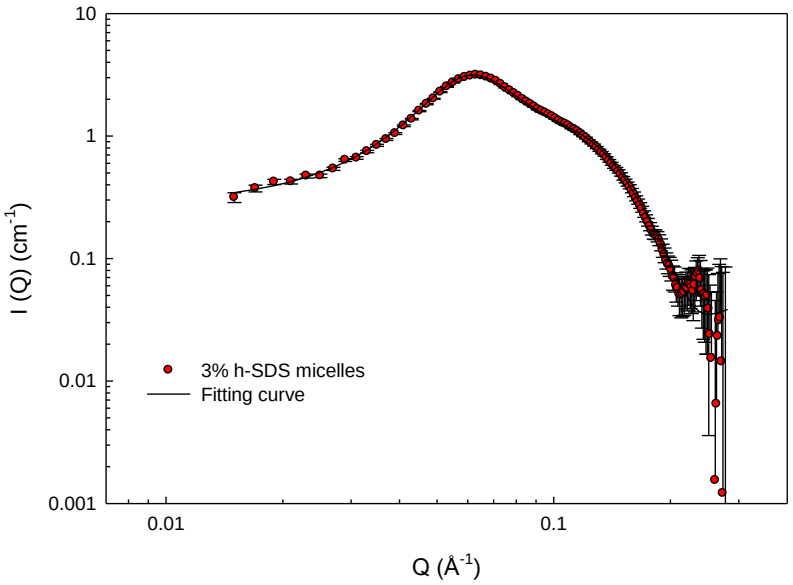
Two structures give the same scattering, although the incoherent scattering may be different

$$\frac{d\Sigma}{d\Omega}(\vec{q}) \propto (\rho_1 - \rho_2)^2$$

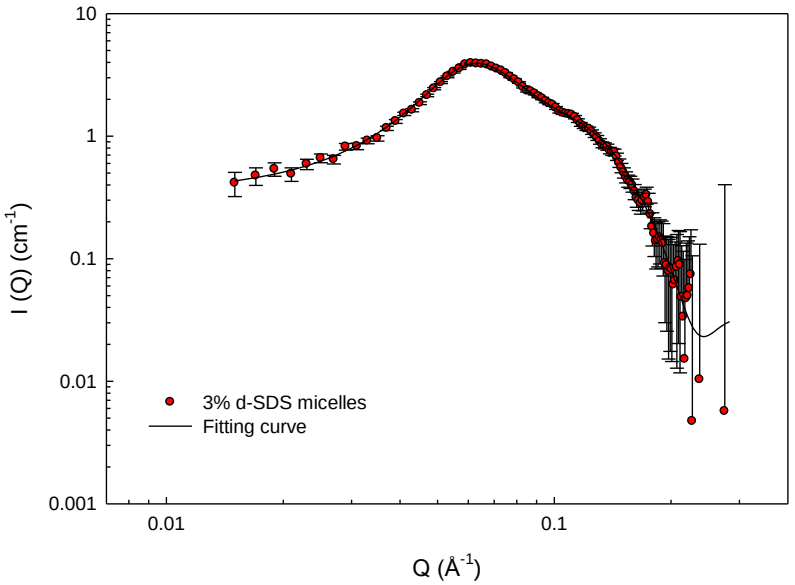
Substitution of deuterium for hydrogen

SANS profiles and corresponding fits obtained using ellipsoidal model and Hayter Penfold structure factor 3% w/v solutions of

(a) h-SDS in D_2O



(b) d-SDS in H_2O



	R_{core} (Å)	axial ratio of core	shell thickness (Å)	minor radius (Å)	major radius (Å)	axial ratio	Charge/*Z*	INV Debye/*k*
h-SDS	16.5	1.49	3.3	19.8	27.8	1.40	23.0	0.041
d-SDS	16.5	1.45	3.2	19.8	27.3	1.38	23.5	0.041

Hseih et al submitted

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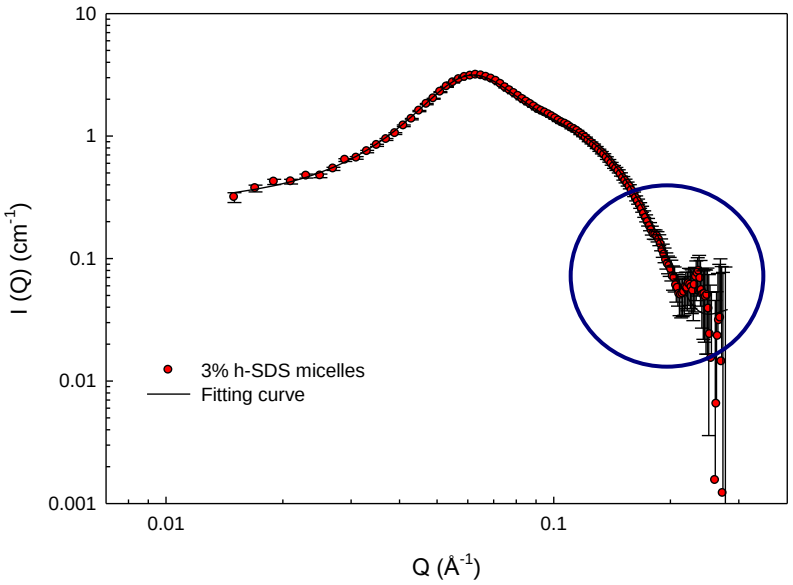

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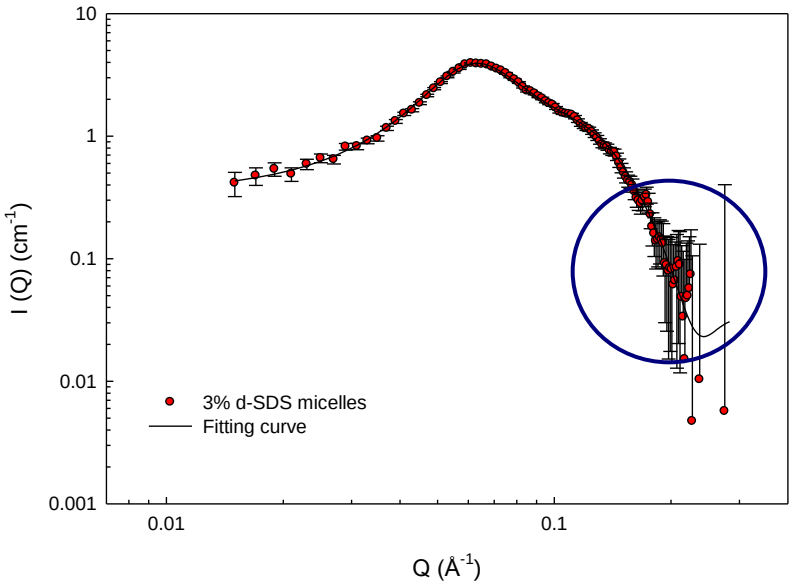

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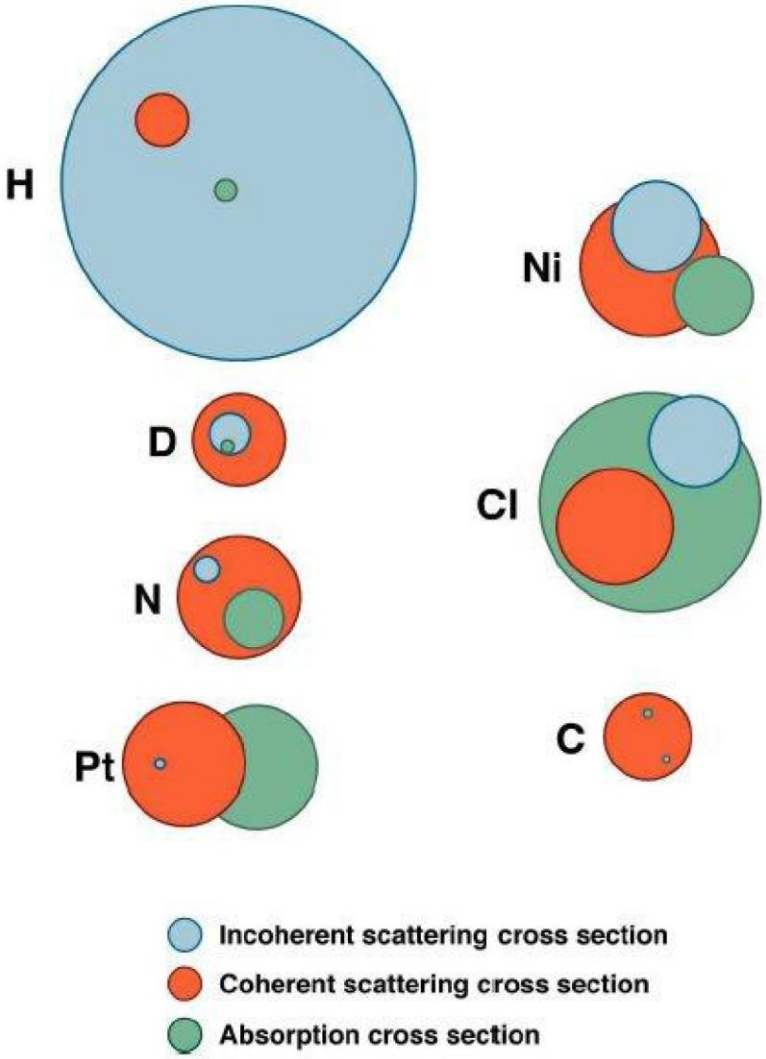
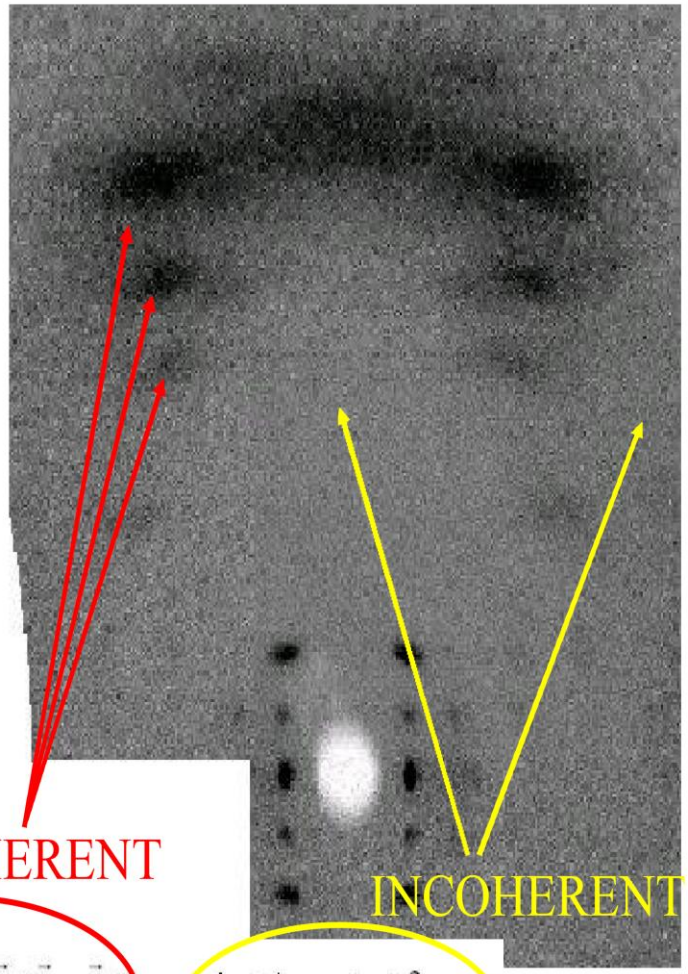
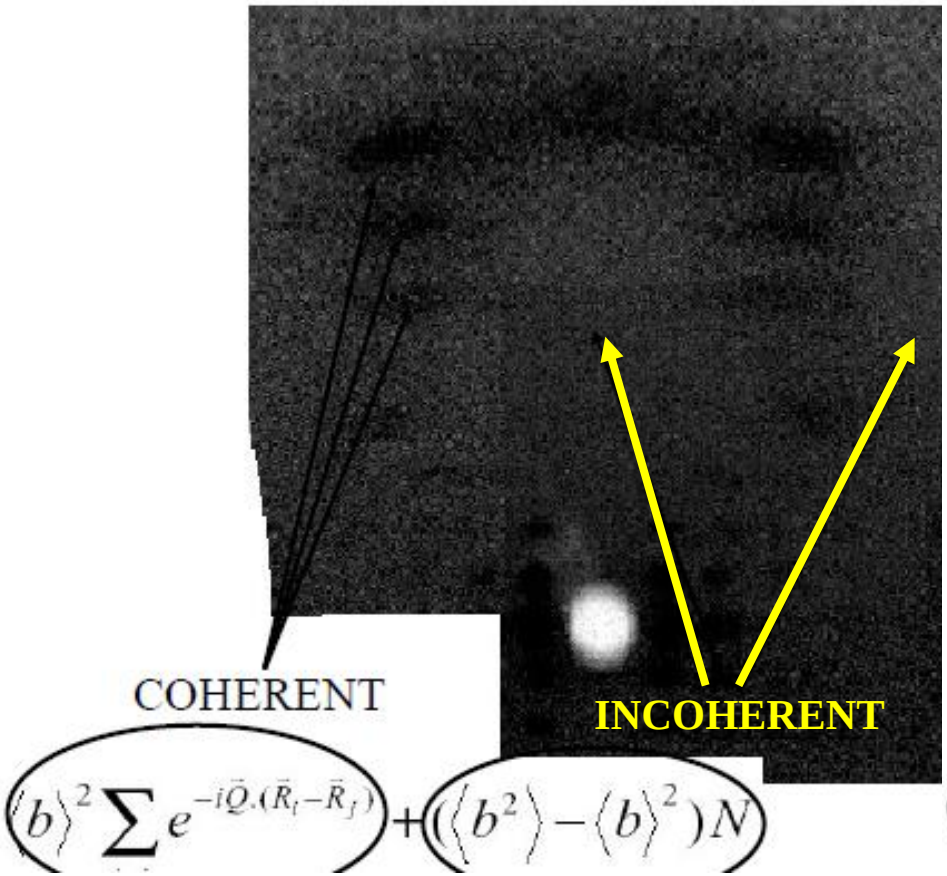
Incoherent scattering



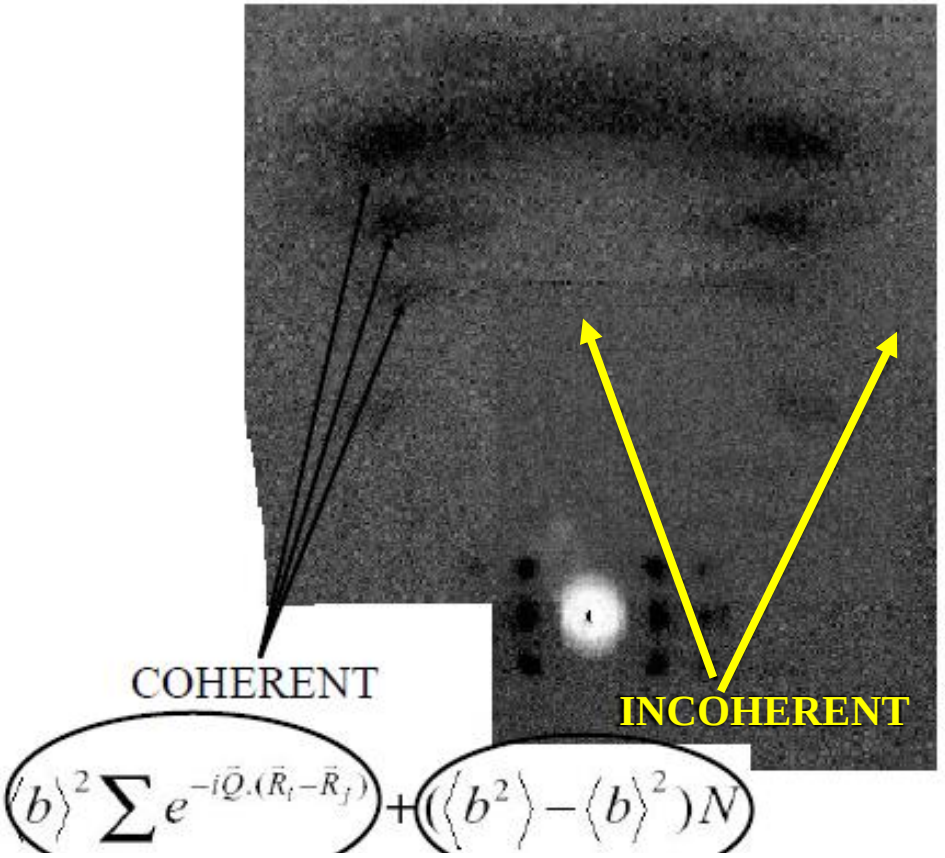


$$\frac{d\sigma}{d\Omega} = \langle b \rangle^2 \sum_{i,j} e^{-i\vec{Q}.(\vec{R}_i - \vec{R}_j)} + (\langle b^2 \rangle - \langle b \rangle^2) N$$

Comparison of incoherent scattering in different solvents



Solvent – H_2O



Solvent – D_2O

Courtesy of Prof. T Forsyth

Structure determination using neutron scattering

- small angle neutron scattering (SANS)
- low resolution crystallography
- high resolution crystallography
- (powder diffraction)
- membrane diffraction
- specular neutron reflection (SNR)

Small angle neutron scattering

- characterizes nanostructures & hierarchical structures of materials in size range 1 to 400 nm

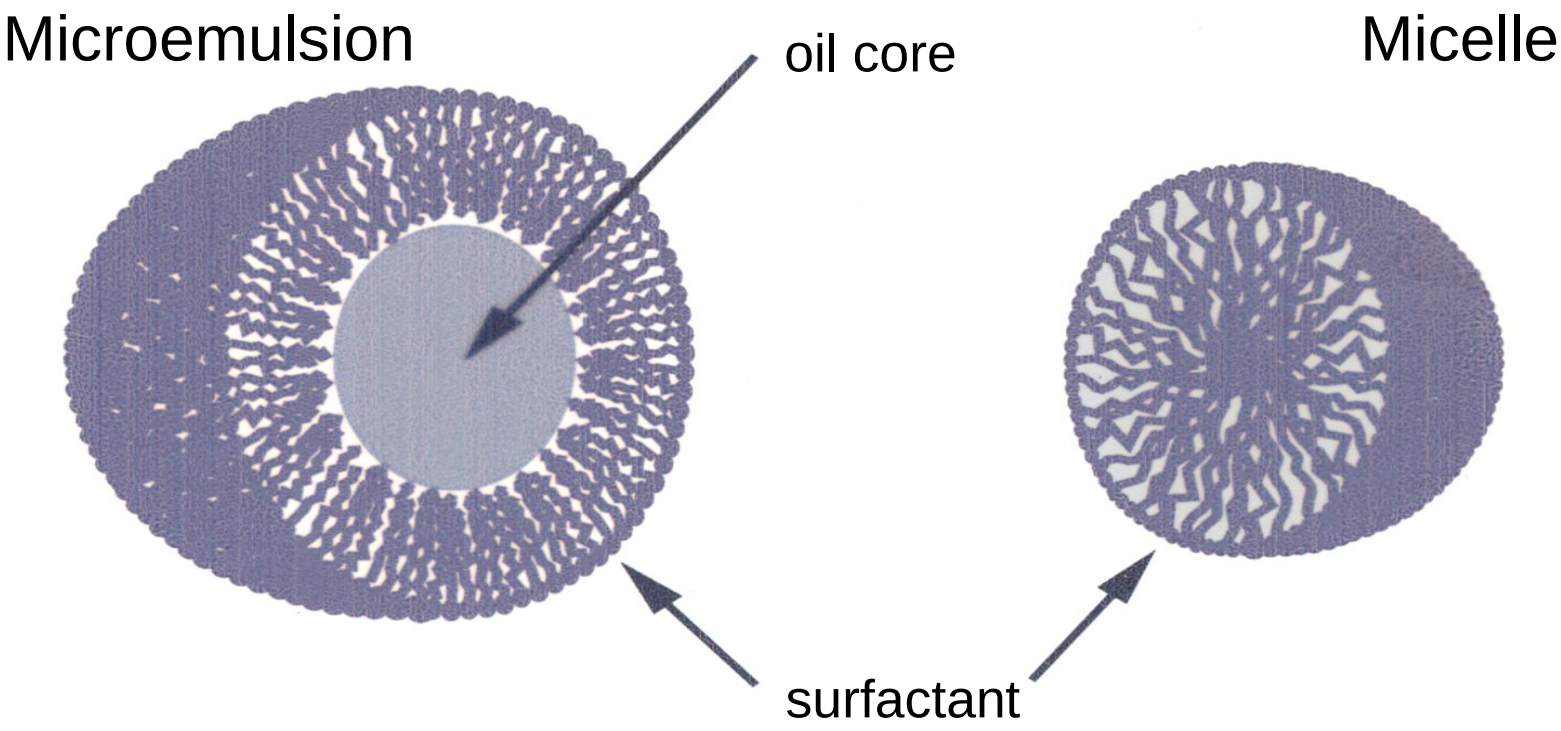
- yields low resolution information on shape

- contrast variation & labelling used to provide information on structure in multi-component systems

- numerous examples of SANS in biology & medicine including:-
  - protein-surfactant interactions,
  - light-induced structural changes in pea thylakoids,
  - the solution structure of human proliferating cell nuclear antigen biomineralization
  - gene delivery vehicles……..

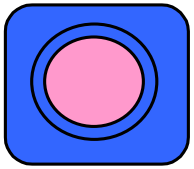
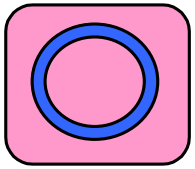
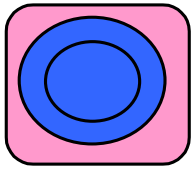
Microemulsions as drug delivery vehicles

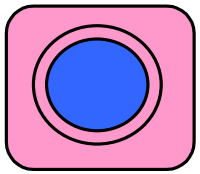
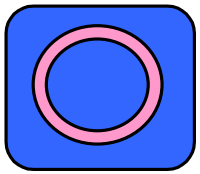
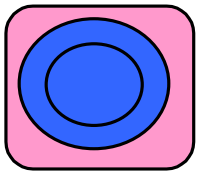
oil = extra locus of drug solubilisation?



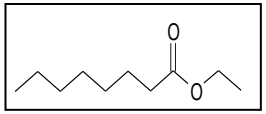
Observation – microemulsions prepared using small molecular volume oils with a good capacity for drug do not result in microemulsions with an increased capacity for drug – **but why?**

Microemulsions as drug delivery vehicles

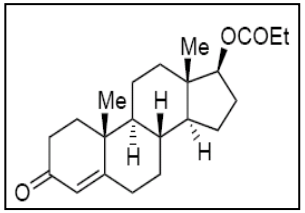
Compositions	Core	Shell	Droplet
Hydrogenated deuterated			
Oil	h-Oil	h-Oil	h-Oil
Surfactant (SAA)	d-SAA	d-SAA	h-SAA
Solvent	D_2O	H_2O	D_2O

Compositions	Core	Shell	Droplet
Hydrogenated Deuterated			
Oil	d-Oil	d-Oil	d-Oil
Surfactant (SAA)	h-SAA	h-SAA	d-SAA
Solvent	H_2O	D_2O	H_2O

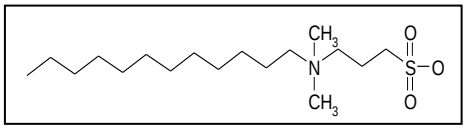
Fits to SANS data of microemulsions with & without drug



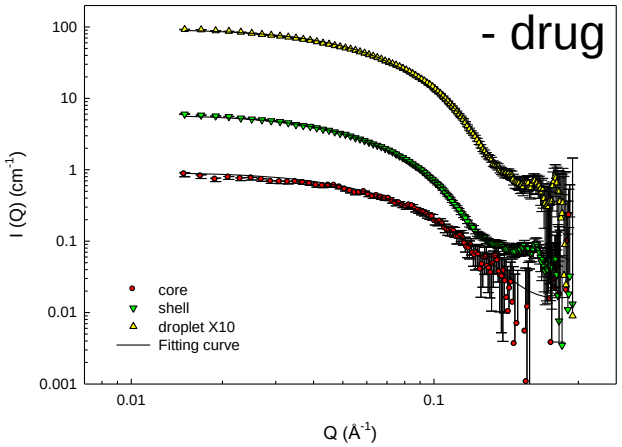
oil = ethyl caprylate



drug = testosterone propionate

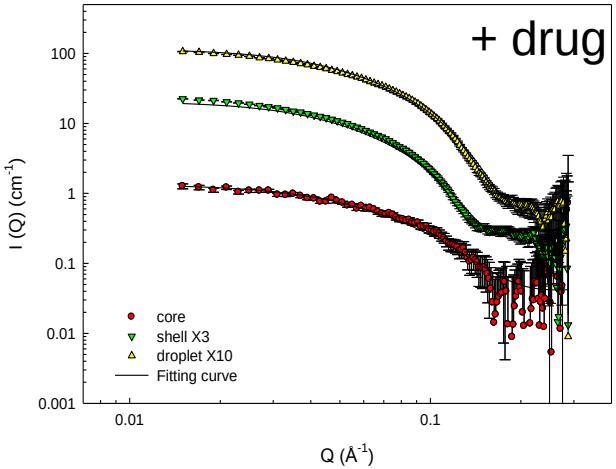


surfactant = *N,N*-dimethyldodecyl-ammoniopropanesulfonate



$$I(Q) = n \times P(Q) \times S(Q)$$

SANS data fitted using core-shell ellipsoid and hard sphere structure factor



Summary of results

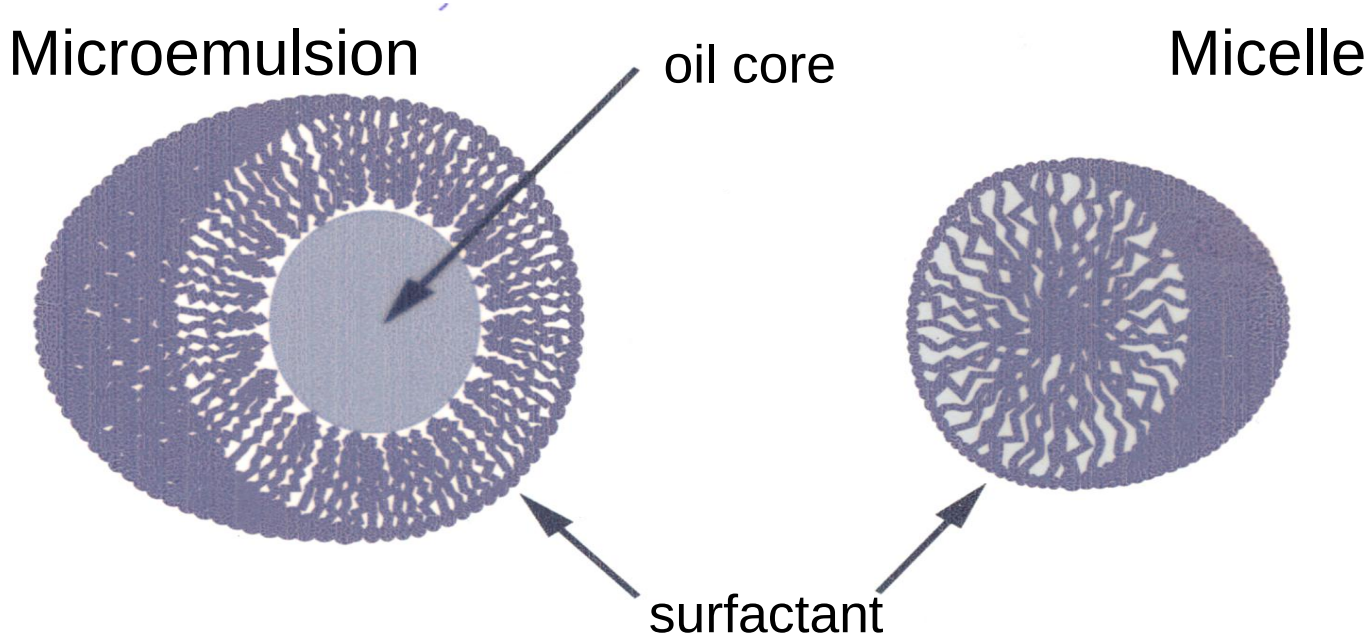
Samples	Shell thickness (Å)	Minor radius (Å)	Major radius (Å)	Axial ratio	P_{oil}	P_{drug} in shell
Microemulsion no drug	18.9	25.3	59.2	2.3	0.57	-
Microemulsion with drug	17.2	23.6	58.5	2.5	0.53	0.24

Hseih et al submitted

Effect of oil on drug solubilisation

Smaller molecular volume oils intimately mixed with the surfactant tails - did not produce core of oil

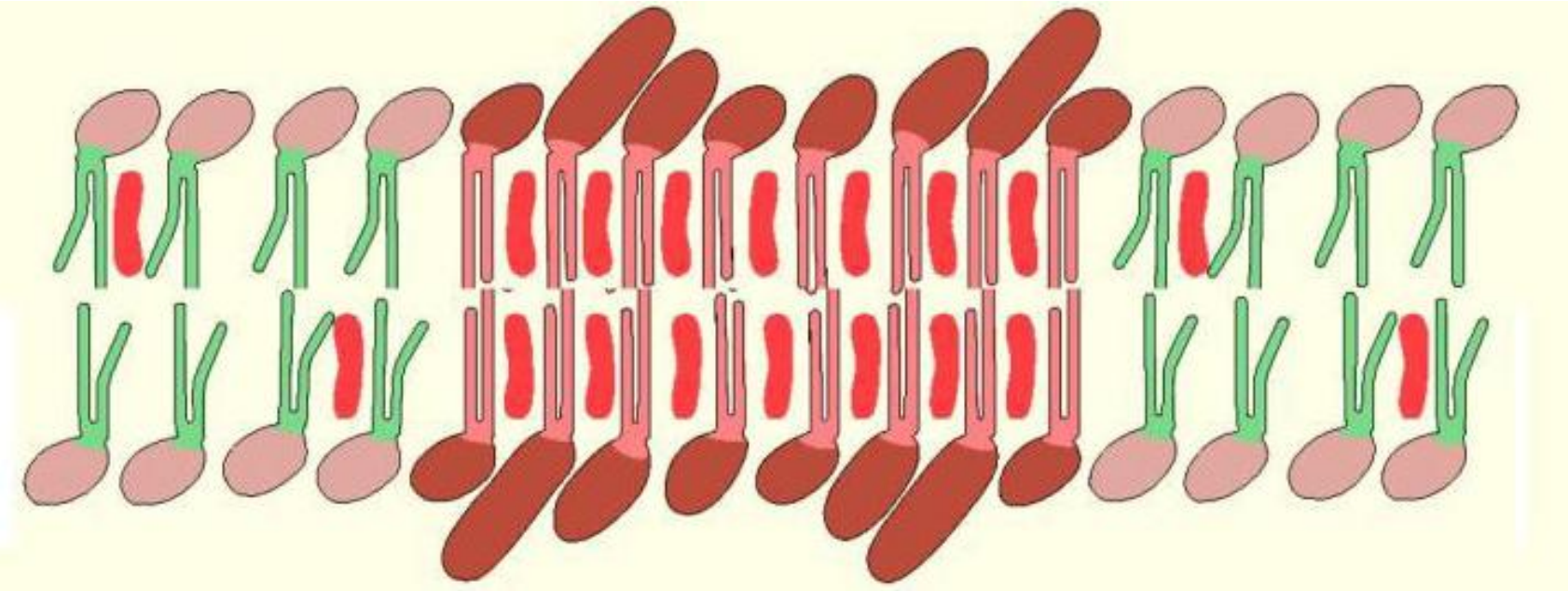
Result - no increase in drug solubilisation



Larger molecular volume oils produced oil core

Result - increase in drug solubilisation

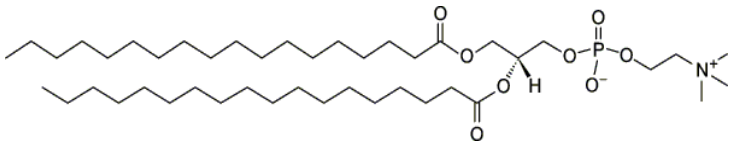
SANS studies of lipid membrane rafts



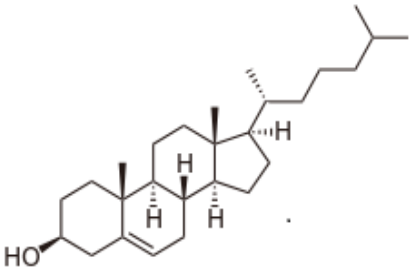
Schematic representation of a cell membrane showing a lipid raft rich in saturated lipid (dark head group) and cholesterol (small, elongated molecule), floating in a ‘sea’ of predominantly unsaturated phospholipid (light head group)

SANS studies of lipid membrane rafts

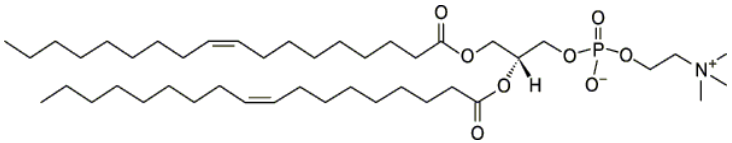
3 lipid component mixture



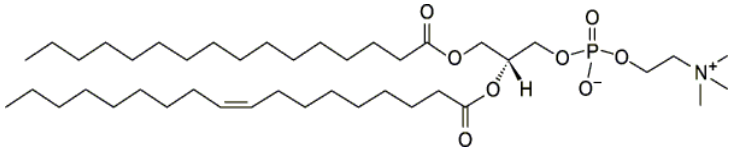
dipalmitoylphosphatidylcholine (DPPC)



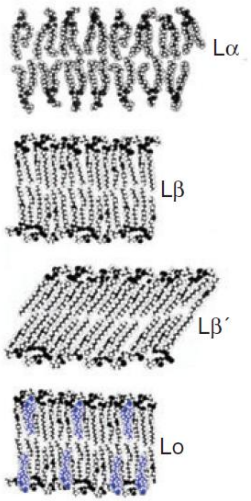
cholesterol

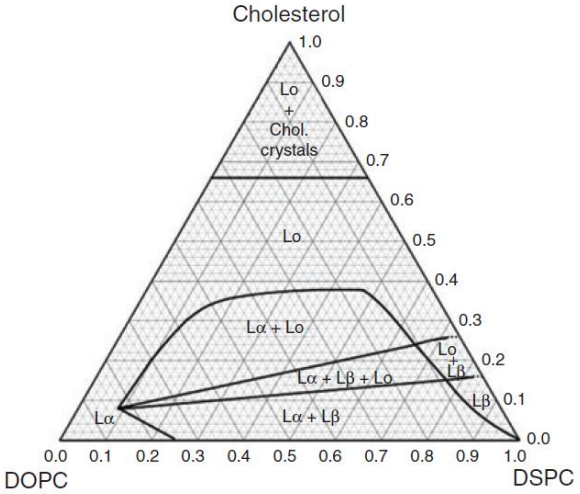


dioleoylphosphatidylcholine (DOPC)



palmitoyloleoylphosphatidylcholine (POPC)





bilayer mixtures of DSPC/DOPC/cholesterol at 23°C

cartoons represent the 4 observed phase types

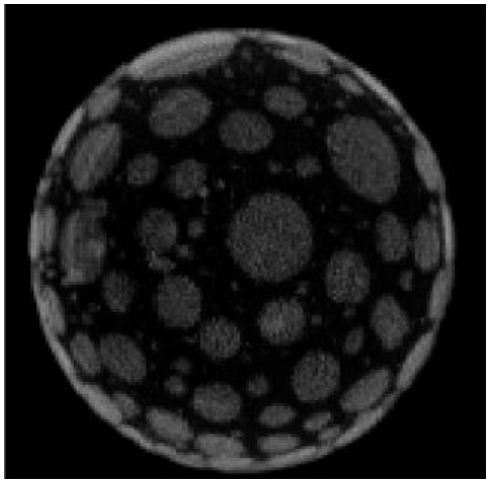
- fluid & disordered Lα phase
- rigid & ordered Lβ phase
- rigid & ordered Lβ´ phase with tilted alkyl chains
- fluid & ordered Lo phase

Note there are 4 regions of 2 phase coexistence, region of interest consists of Lα + Lβ

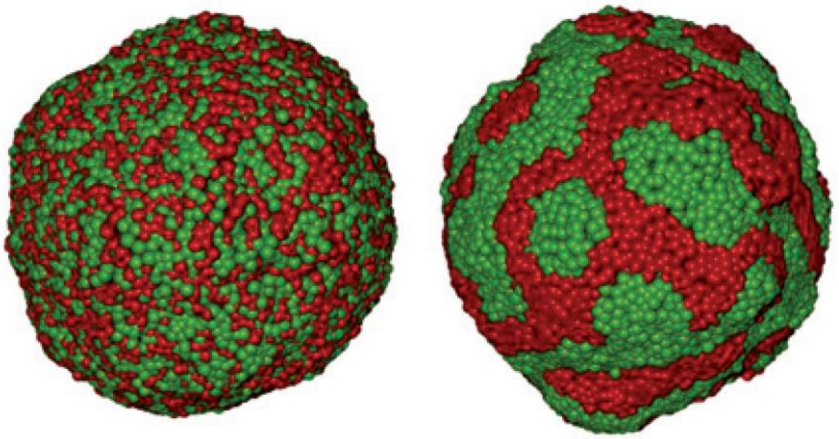
Note that there is 1 region of 3 phase coexistence, namely Lα + Lβ + Lo

Feigenson (2006) Nature Chem Biol **2** 560

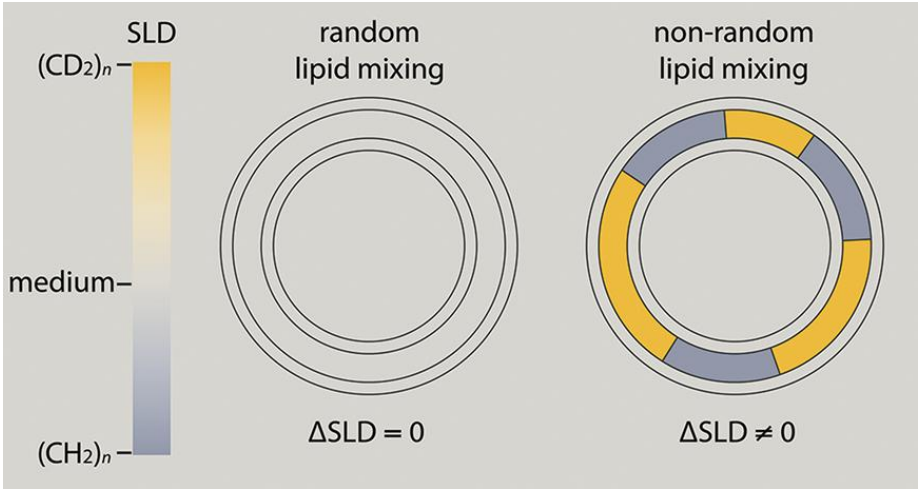
SANS studies of lipid membrane rafts



confocal image of phase separation in vesicle membranes



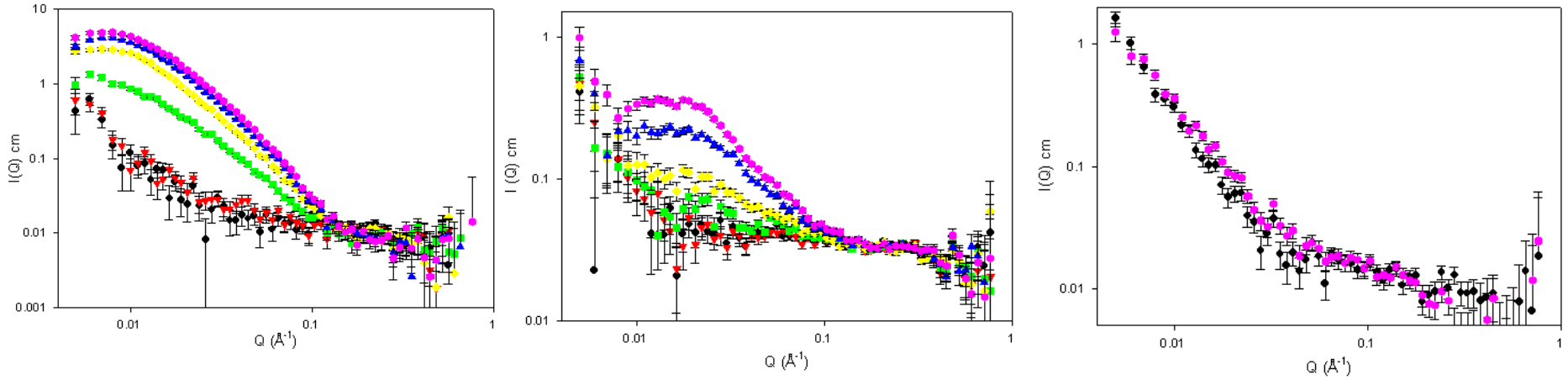
computer simulation of phase separation in vesicle membranes



schematic of a SANS contrast matching experiment

lipid phase separation is driven by immiscibility of saturated (stearyl) & unsaturated (oleyl) acyl chains

SANS studies of lipid membrane rafts



SANS data showing raft formation in vesicles comprising 10 mg ml^{-1} chain deuterated *d*-DPPC:DOPC:Chol 2:2:1 (left), 1:1:1 (middle) & 0.9:0.5:0.5 (right)

50°C (black circles),

35°C (red upside down triangles),

25°C (green squares),

20°C (yellow diamonds),

15°C (blue upright triangles)

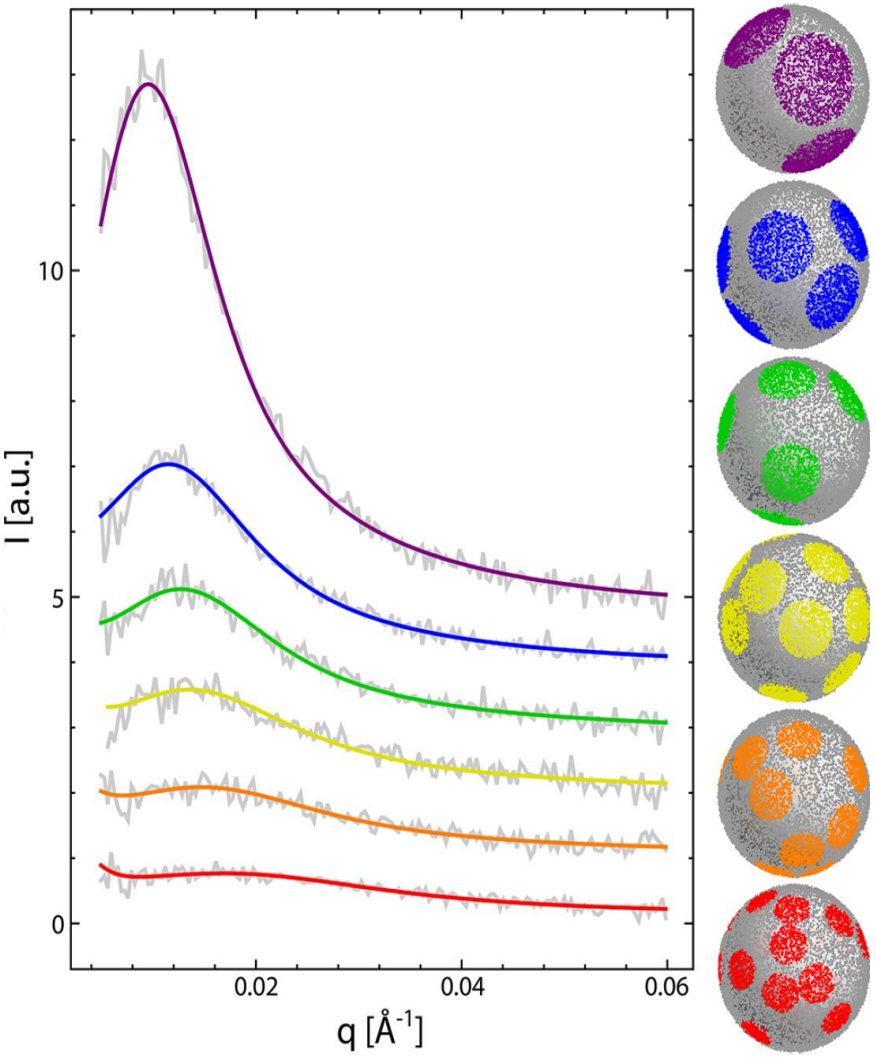
10°C (pink hexagons)

detection of raft requires a scattering length density (SLD) difference between coexisting phases

here difference (or contrast) was achieved by using chain deuterated DPPC

Lawrence et al unpublished data

SANS studies of lipid membrane rafts



SANS data at 20^{o}C (light grey lines) and best-fit curves (coloured lines)

example Monte Carlo vesicles corresponding to the best-fit parameters at each composition

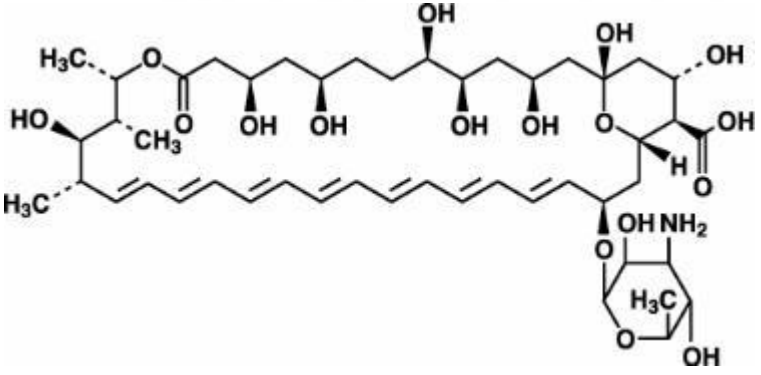
DSPC:	DOPC:	POPC:	chol
0.39:	0.39:	0.0:	0.22 (purple)
0.39:	0.14:	0.25:	0.22 (blue)
0.39:	0.08:	0.31:	0.22 (green)
0.39:	0.06:	0.33:	0.22 (yellow)
0.39:	0.04:	0.35:	0.22 (orange)
0.39:	0.02:	0.37:	0.22 (red)
0.39:	0:	0.39:	0.22 (not shown)

Herbele et al (2013) J. Am. Chem. Soc. **135** 6863

Understanding the activity of antimicrobial drugs

"Antimicrobial resistance poses a catastrophic threat"

Professor Dame Sally Davies
Chief Medical Officer
Annual Report 2013



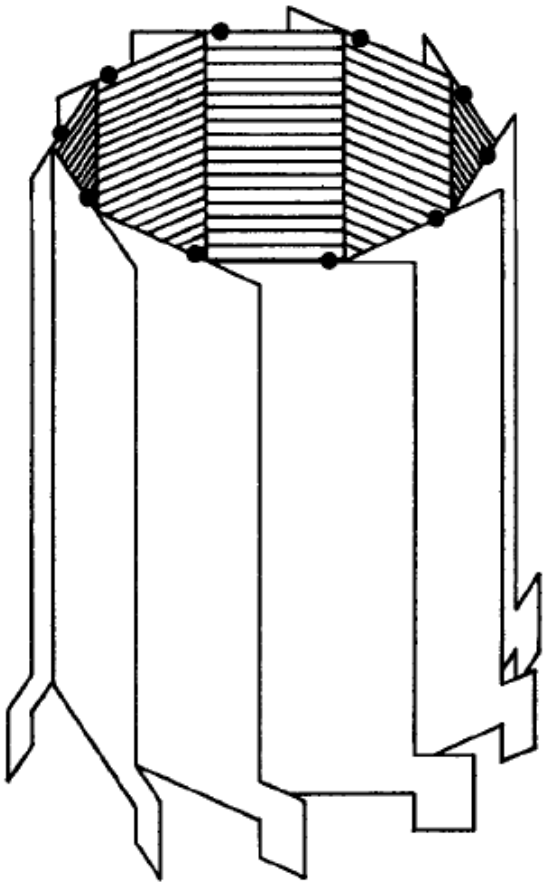
amphotericin B (AmB)

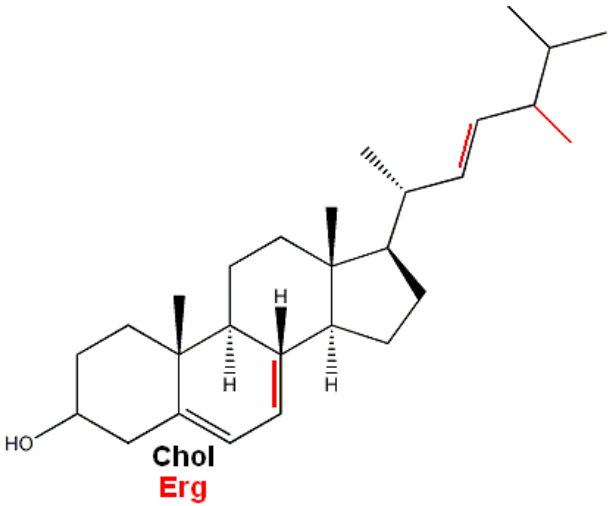
- polyene macrolide antibiotic with mycosamine head group
- first line treatment for deep-seated *systemic* fungal infections

Mechanism of action of AmB

text book mechanism is formation of channels/pores involving AmB-sterol complexes that allow passage of monovalent ions across the cell membrane

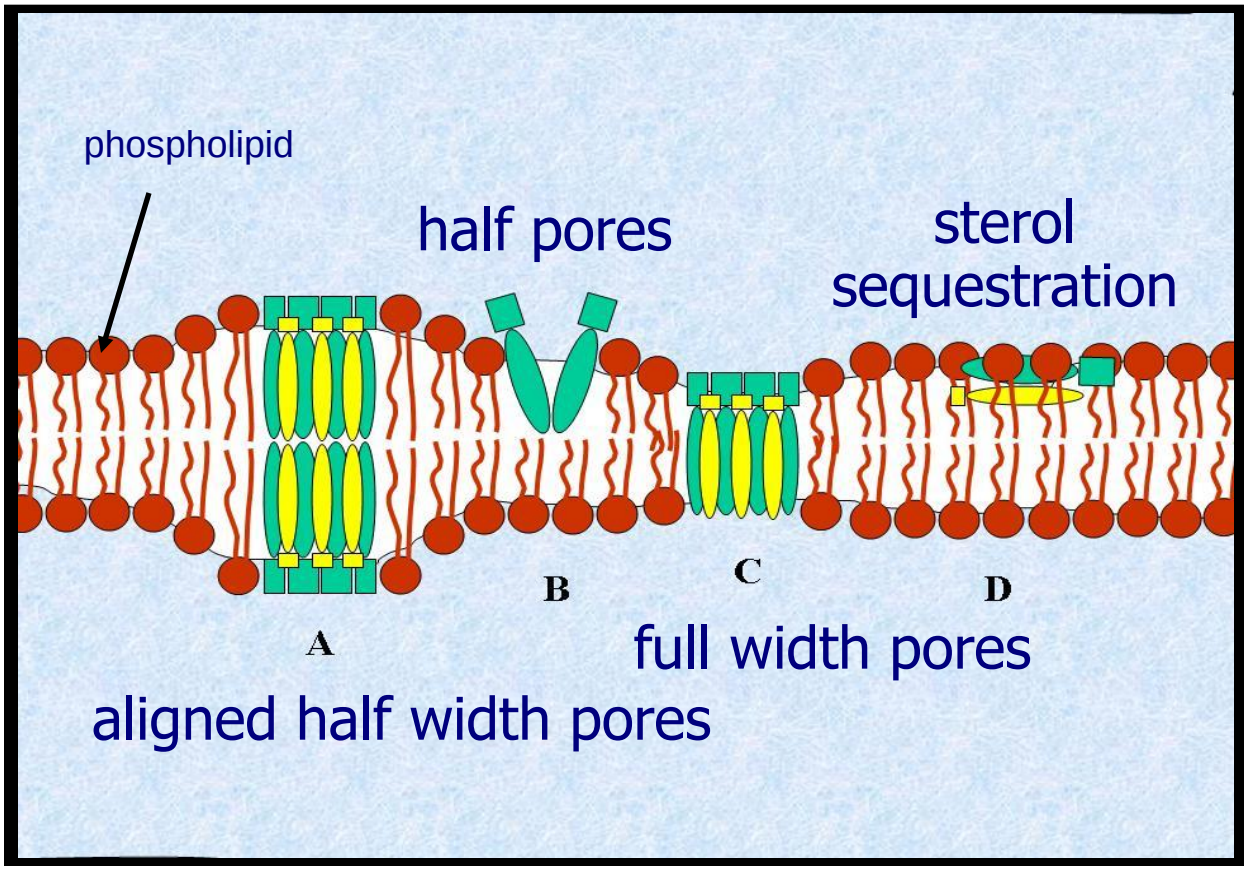
proposed that there is a preferential complexation of AmB with fungal, ergosterol containing, rather than with mammalian, cholesterol containing membranes





Finkelstein & Holz (1973) Membranes **2** 377
Hsuchen & Feingold (1973) Antimicrob. Agents Chemother. **4** 309

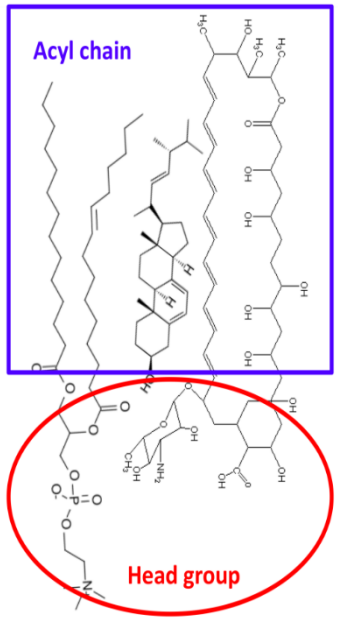
Different researchers, using different techniques, investigating different systems



…….get different results, and infer different mechanisms

SNR studies of POPC/(sterol) monolayers

		Acyl chain Layer	Head Group Layer	Percent Head Hydration
POPC	- AmB	13 Å	8 Å	51%
	+ AmB	15 Å	8 Å	51%
POPC-Cholesterol	- AmB	17 Å	8 Å	53%
	+ AmB	19 Å	9 Å	62%
POPC-Ergosterol	- AmB	17 Å	9 Å	54%
	+ AmB	19 Å	9 Å	57%



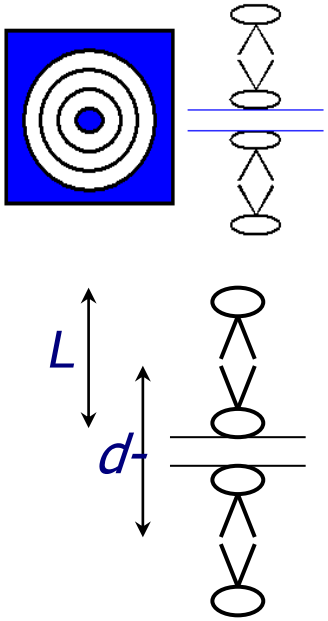
Uncertainties on layer thicknesses: ± 0.5 Å (-AmB), ± 1.0 Å (+AmB)

- sterols cause ~4 Å increase in overall monolayer thickness
- AmB causes ~2 Å increase in chain layer thickness
- no difference between effect of AmB with two sterols

Foglia et al (2014) Langmuir **30** 9147

SANS studies of POPC/(sterol) vesicles

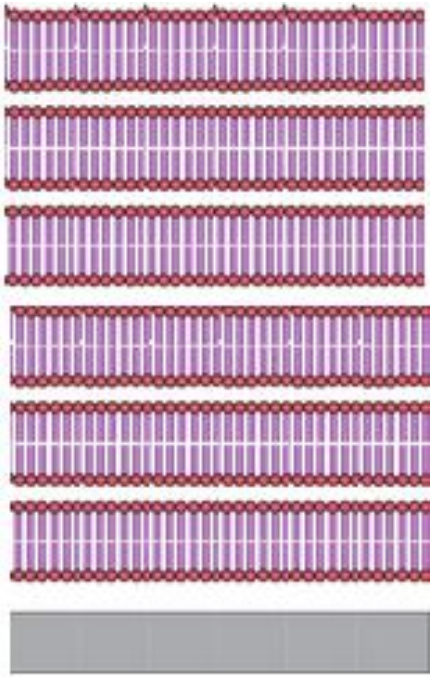
		Bilayer (*L*)	*d*-spacing (*d*-)
POPC	No AmB	37 ± 0.5 Å	56 ± 8 Å
	+ AmB	40 ± 0.5 Å	60 ± 3 Å
POPC-cholesterol	No AmB	41 ± 0.5 Å	57 ± 4 Å
	+ AmB	44 ± 0.5 Å	62 ± 4 Å
POPC-ergosterol	No AmB	41 ± 0.5 Å	56 ± 4 Å
	+ AmB	44 ± 0.5 Å	62 ± 4 Å

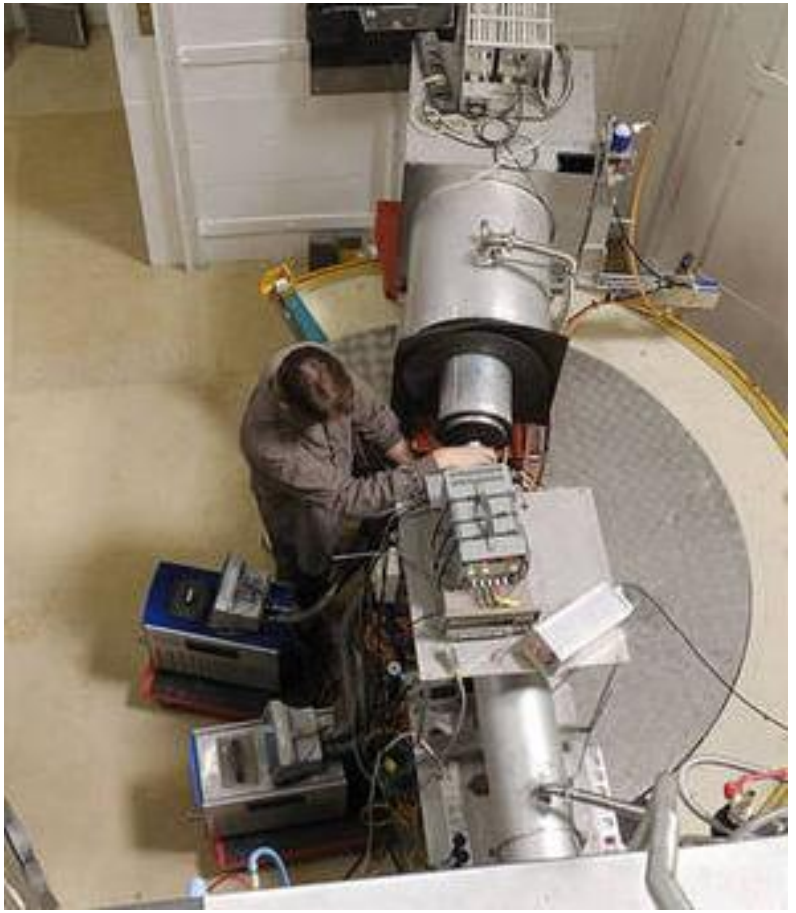


- sterols cause 4 Å increase in POPC bilayer thickness
- AmB causes 3 Å increase in POPC-cholesterol & POPC-ergosterol bilayer thickness
- no difference between effect of AmB with two sterols

Foglia et al (2011) Biochim. Biophys. Acta **1808** 1574

Neutron diffraction studies of POPC/(sterol) multilayers



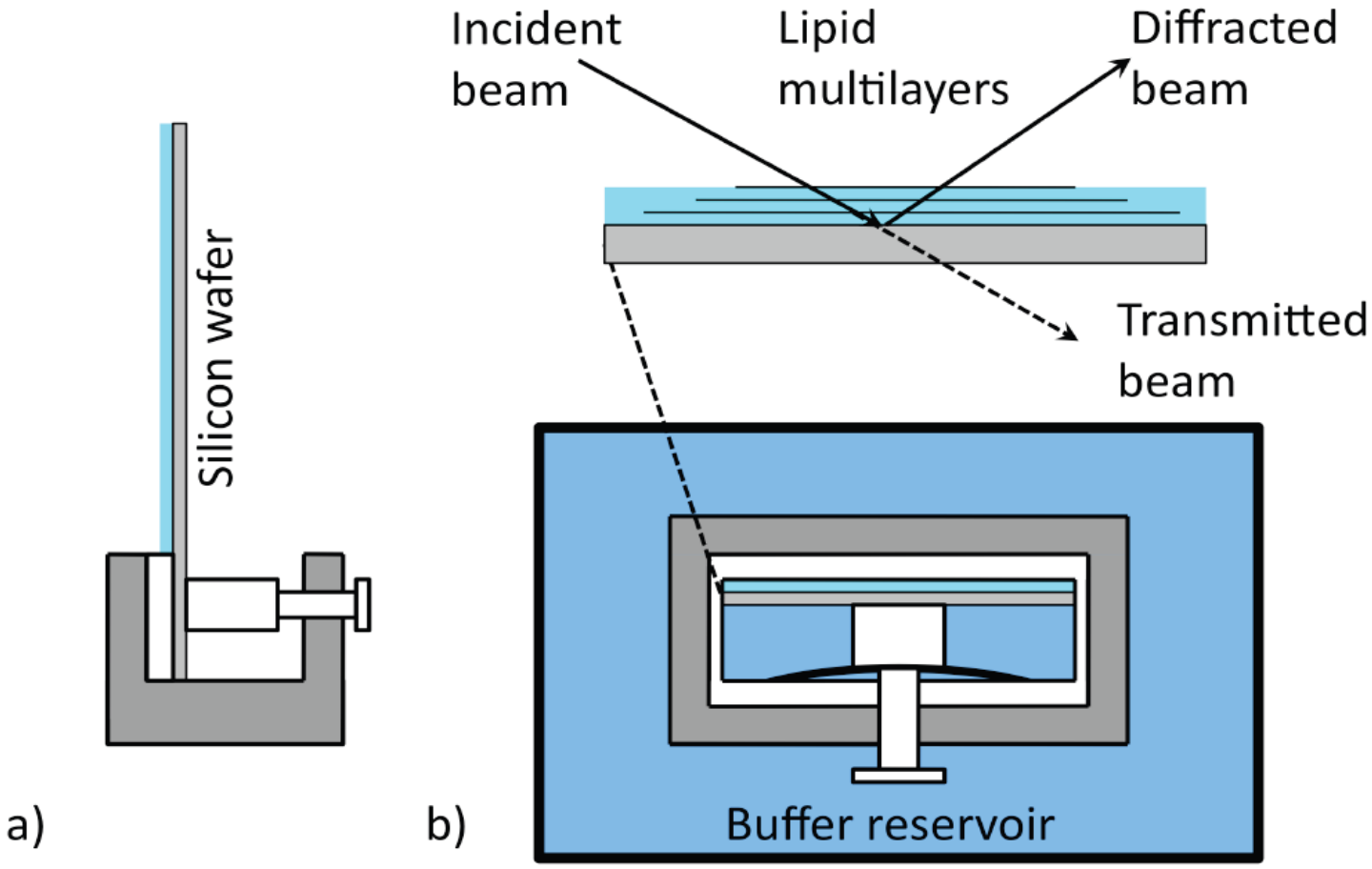


either d_{31}-POPC, d-$_{31}$POPC-cholesterol or d-$_{31}$POPC-ergosterol ± 5 mol% AmB

100% RH; 100% D2O; ambient temp

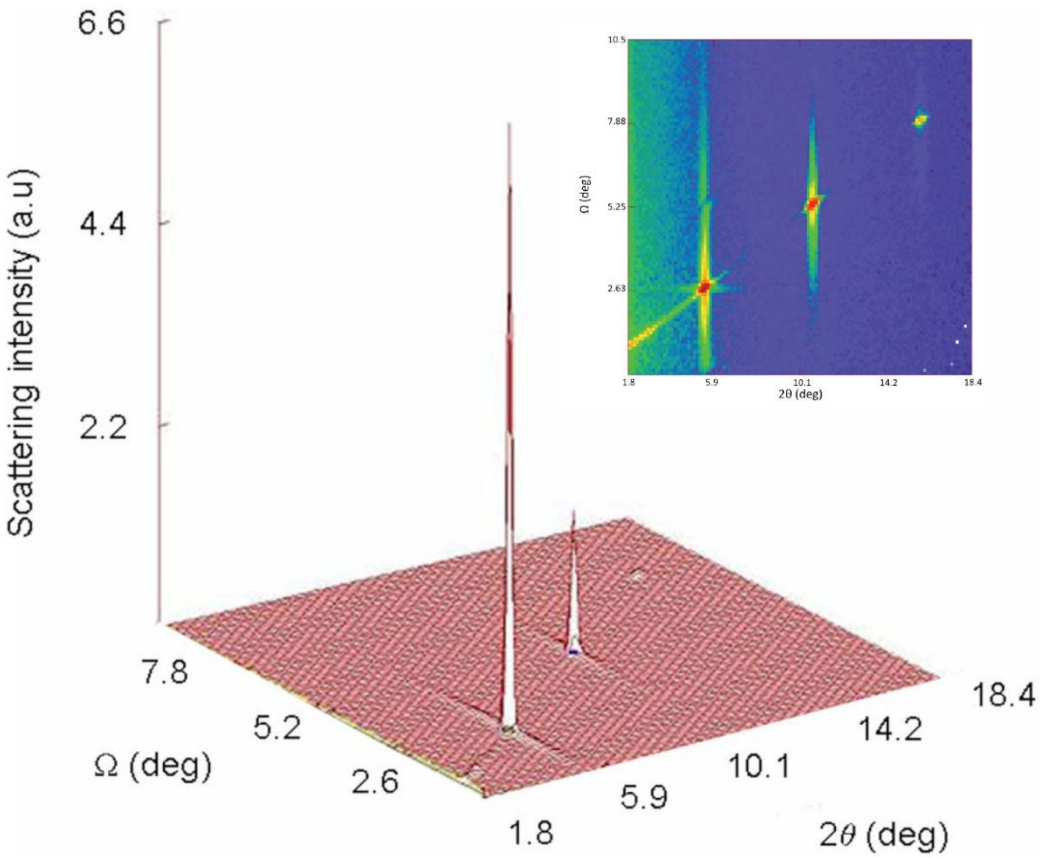
performed on D16 @ ILL

Neutron diffraction studies of POPC/(sterol) multilayers



Schematic representation of the silicon-supported multilayers and its holder in the climate chamber

Neutron diffraction pattern of multilayers of POPC/ergosterol



sharp peaks, indicating well ordered samples

insert shows the first 3 orders of diffraction (*2θ* = 5.3 deg; 10.6 deg; 15.9 deg*)*

3D plot shows the scattering intensity as function of

Ω (i.e. angle between the incident beam & the sample plane – sample rotation) &

2θ (i.e. angle between the incident & scattered beams)

Foglia et al (2012) Scientific Reports **2** 778

Sterols influence POPC bilayers differently

	POPC-cholesterol		POPC-ergosterol	
	− AmB	+ AmB	− AmB	+ AmB
d_B	37	44	37	44
L_{POPC}	20	23	23	26
L_{Sterol}	17	12	17	15
L_{AmB}	-	12	-	15

Bilayer thickness, d_B, and end-to-end lengths, L_i, of bilayer components, i; all dimensions given in Å.

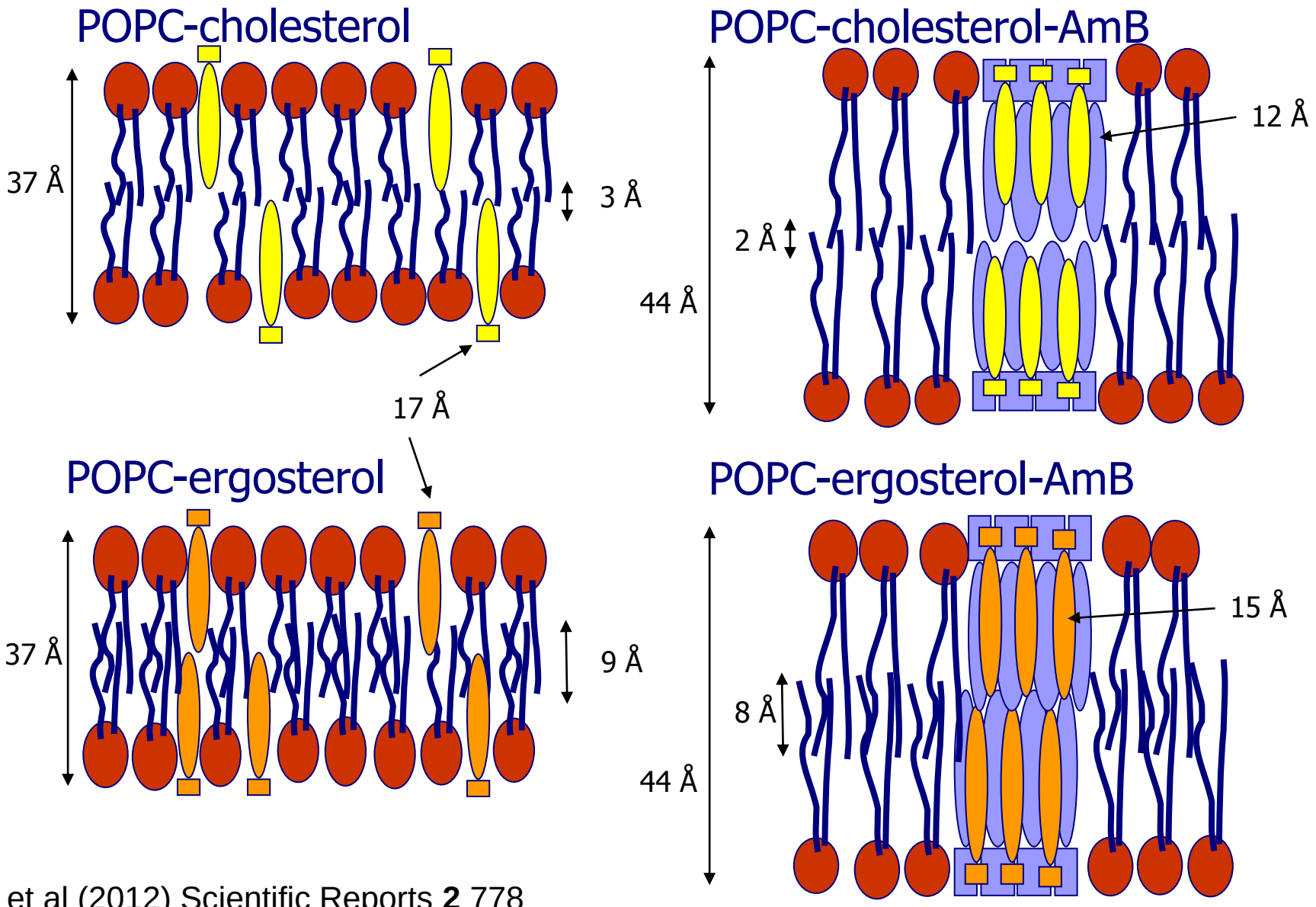
POPC chains more extended when ergosterol present (L_{POPC} = 23 vs 20 Å) & exhibit greater degree of overlap (9 vs 3 Å)

Regardless of sterol, AmB increases bilayer thickness by ~ 7 Å but POPC chains more extended when ergosterol present (L_{POPC} = 26 vs 23 Å) & exhibit greater degree of overlap (8 vs 2 Å)

AmB causes sterols to tilt wrt the normal, 30^o for cholesterol and 45^o for ergosterol

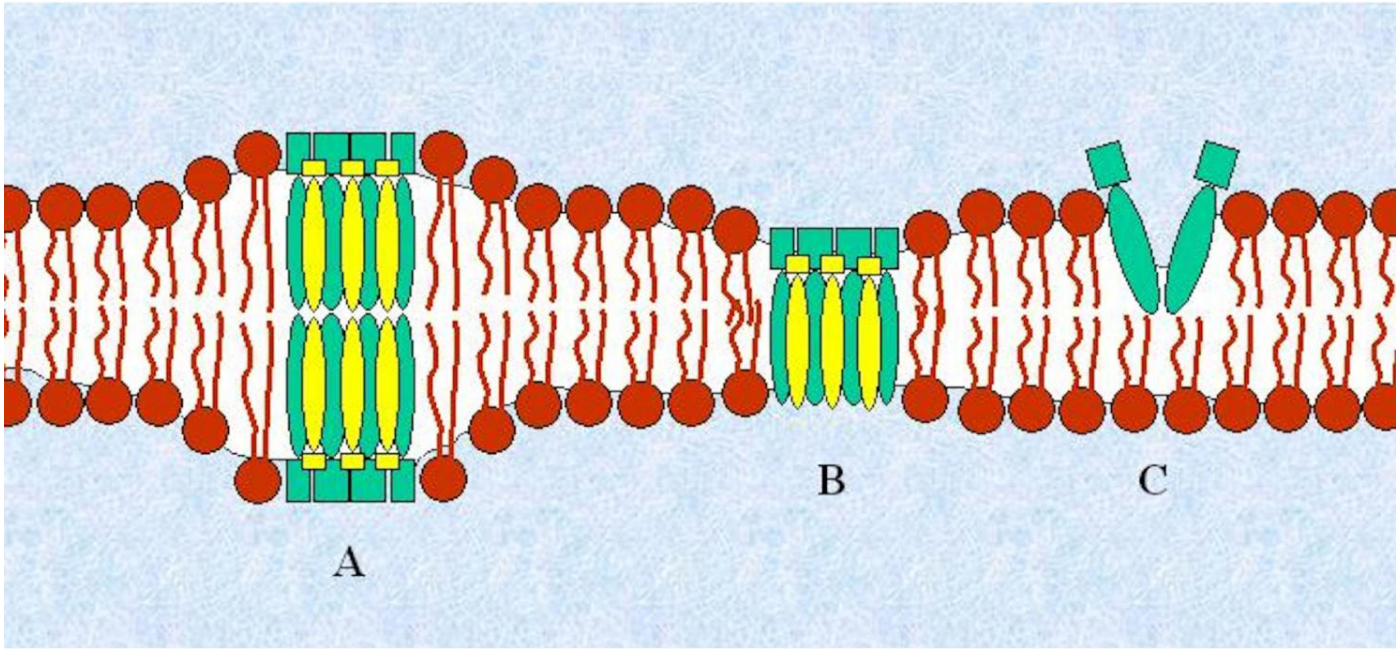
Foglia et al (2012) Scientific Reports **2** 778

Sterols influence POPC bilayers differently



Foglia et al (2012) Scientific Reports **2** 778

Mechanism of action of AmB



AmB - green,
sterols - yellow,
phospholipids - red

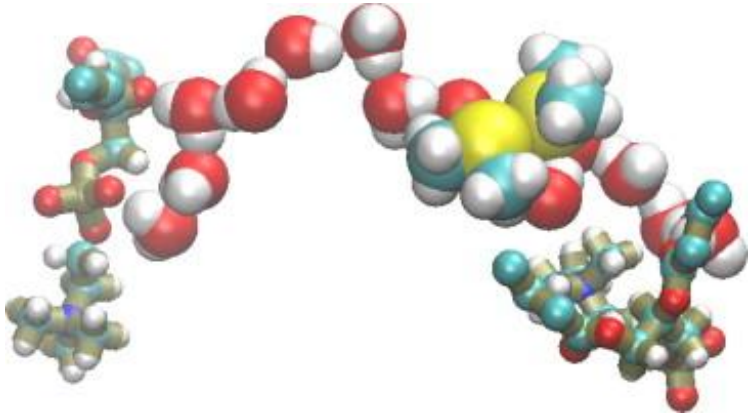
only in ergosterol & AmB containing bilayers does AmB & ergosterol intrude into other leaflet

- possibly leading to stabilisation of transmembrane ion channels

AmB perturbs the location of ergosterol by 0.5 Å as opposed to 3 Å in the case of cholesterol

- may be an enthalpic contribution therefore to account (at least in part) for AmB's preferred into action with ergosterol containing membranes

Local structural neutron diffraction (LSND) enhanced with isotopic substitution



LSND coupled with EPSR analysis allowed a very detailed description of the binding of water (and DMSO) molecules to the short alkyl chain lipid head groups

other groups investigated water structure around sugars, amino acids & peptides

Foglia et al (2010) J Chem Phys 113 145103
Dabkowska et al (2013) Chem Phys 410 31

Low resolution neutron crystallography

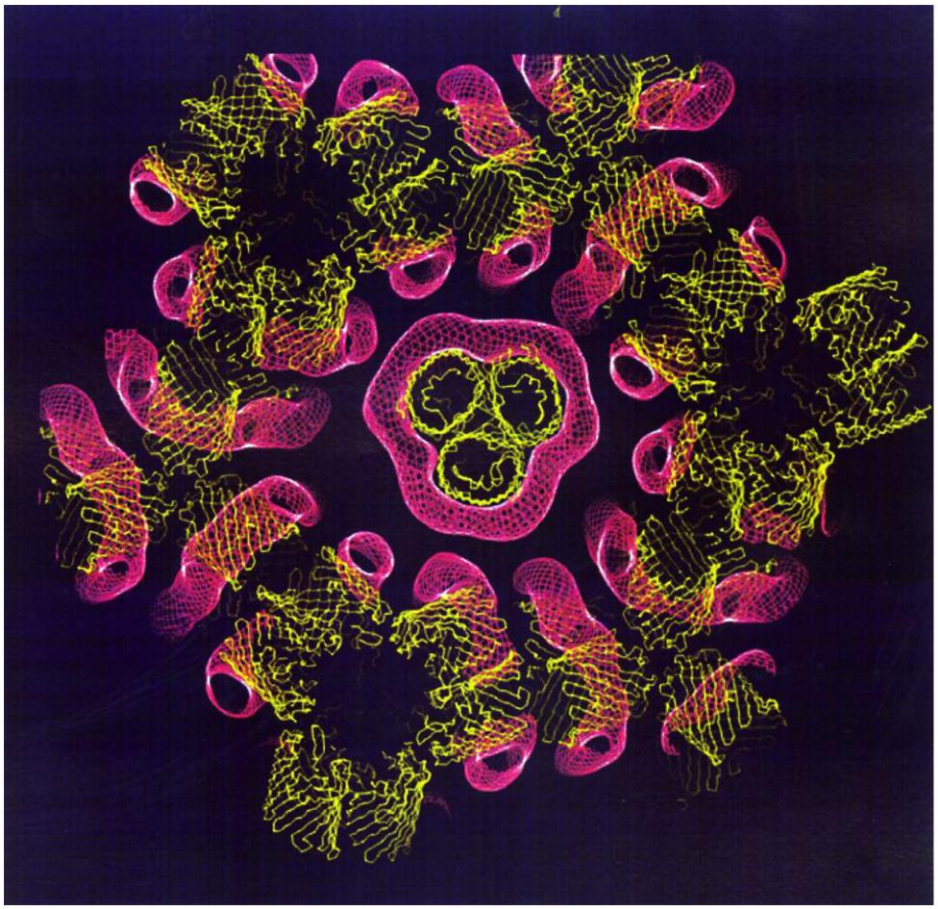
- powerful technique for visualizing disordered regions in crystals of biomolecules (eg membrane proteins, viruses) and biomolecule complexes (eg DNA/protein complexes or lipoproteins)

- crystals can be less than 0.1 mm^3 in size, although if sample is deuterium-labelled, crystals can be as small as 0.01 mm^3 due to better signal-to-noise ratio

- possible to highlight different components within a complex by complex matching using a crystallisation buffer of differing amounts of D_2O

Timmins et al. (2006) Neutrons in Biology, Springer Berlin Heidelberg

Low resolution neutron crystallography in combination with X-ray crystallography



X-ray structure of protein in yellow

neutron structure of deuterated detergent molecules in purple

membrane protein (here OmpF porin) crystallised in 40% D_2O & therefore protein is contrast matched to neutrons & 'invisible'

detergent molecules (used for crystallisation are 'invisible' to X-rays) but neutrons show are bound to 'hydrophobic zone ' surrounding each protein trimer & which is exposed to lipid *in vivo*

E. Pebay-Peyroula et al. Structure (1995) 3, 1051-1059; Biochimie (1998) 80, 543-551

High resolution neutron crystallography

- imaging of positions of water molecules (not easy using X-ray crystallography and NMR spectroscopy) but very important in biology
- determination of hydrogen bonding networks
- both are critical to protein function as water mediates many biological functions

However high resolution protein crystallography has tended to be a minority interest due to requirement for large crystals & very long collection times (i.e. weeks-months)

LADI (& VIVALDI) Laue-based single crystal diffractometers

- illuminates sample with all available neutrons
- maximal flux at sample
- large number of reflections at all incident neutron λ
- routine for a protein crystal of 1-5 mm^3 to be used - recently small crystals of perdeuterated proteins of ~ 0.15 mm^3 have been successfully measured
- collection times now hours-days, e.g. data collected in 3.5 days at 2.0 Å resolution from a 1.4 mm^3 thaumatin crystal and 3 weeks to 1.85 Å resolution from a perdeuterated antifreeze protein (AFP) only 0.13 mm^3 in volume
- upper limit of 50 kDa realisable

Blakeley et al Curr. Opin. Struct. Biol (2008) **18** 593

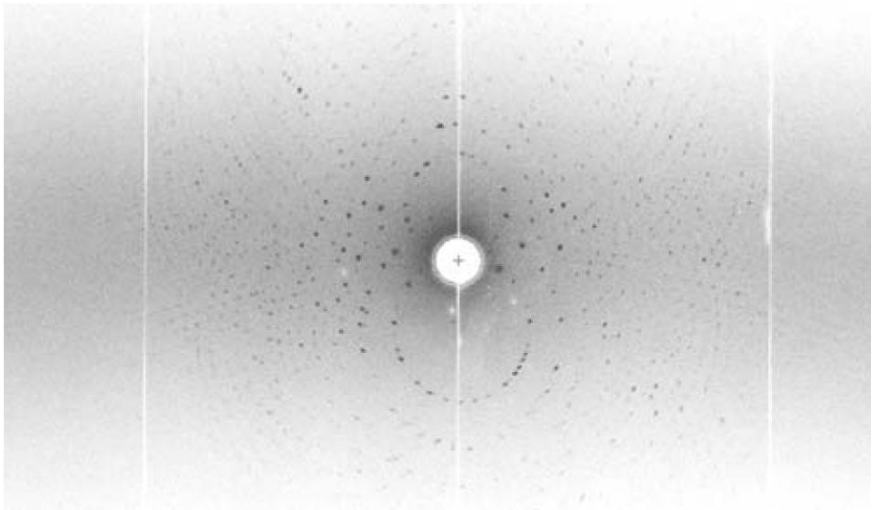
Blakeley et al Acta Cryst. (2010) **D66** 1198

High resolution neutron crystallography

D-xylose isomerase:- 43 kDa enzyme that catalyses the first reaction in the catabolism of D-xylose

0.95Å X-ray structure but NO direct observation of H although the refined model suggested that the site of ring opening was suitable for proton donation by His53.

Neutron Laue diffraction data at 2.2Å enabled direct observation of His53 protonation supporting the above mechanism



Fenn et al Biochem. (2004) **43** 6464
Meilleur et al Eur. Biophys. J. (2006) **35** 601

Neutron powder diffraction

- determination of the real-space structure of materials at the atomic/micro-structural level
- determination of long-range structure in polycrystalline materials, short-range atomic structure in disordered or amorphous materials, structural distortions, & any strain & crystal size induced changes to the structure
- complimentary to X-rays
- high neutron penetration of neutrons allows use of complex sample environments including low & high temperatures, in electric & magnetic fields, & under varying pressure
- most pharmaceuticals contain H, D, C, N & O - the neutron's sensitivity to such elements & the difference in scattering between isotopes - means neutron powder diffraction has a role in structure determination

Useful reading

Chapter 23 Neutron crystallography of proteins and Chapter 24 Molecular Biology in Methods of Experimental Physics Volume 23 Part C Neutron Scattering (1987) Academic Press Inc (London)

Neutron scattering in biology – techniques and applications (2006) Springer-Verlag Berlin Heidelberg, (New York)

Neutrons for biologists: a beginners guide, or why you should consider using neutrons JH Lakey J. R. Soc. Interface (2009) 6 S567-S573.

http://www.strubi.ox.ac.uk/people/gilbert/neutrons.html#spins