USE OF NEUTRONS IN BIOLOGY AND MEDICINE

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USEFUL READING

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

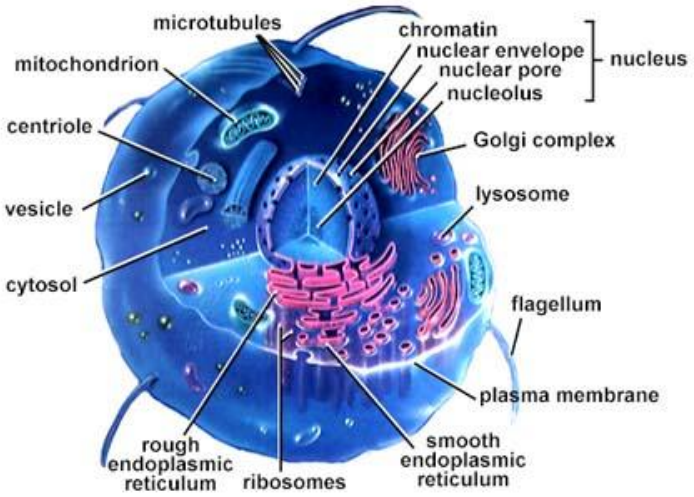
- Neutron Scattering in Biology – Techniques and Applications (2006) Springer-Verlag Berlin Heidelberg, (New York)

- Neutrons for Biologists: A Beginners Guide, or Why You Should Consider Using Neutrons JH Lakey Journal of the Royal Society Interface (2009) 6 S567-S573.

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

BIOLOGY - STUDY OF LIVING ORGANISMS

- structure, function, growth, origin, evolution, distribution and classification of living things

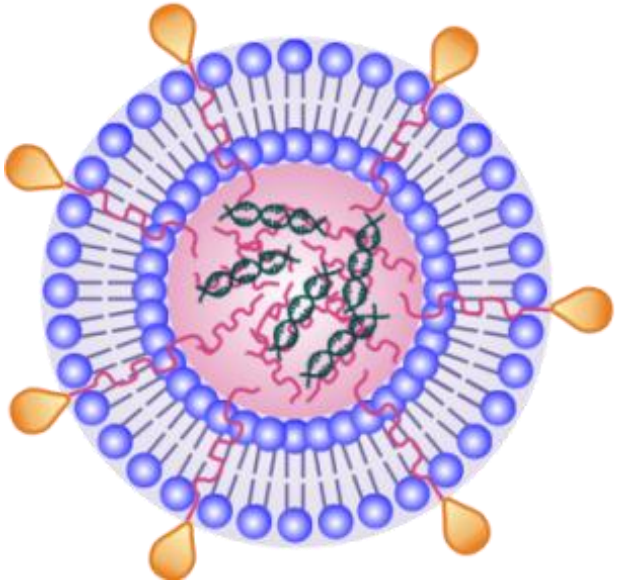


- neutrons particularly useful in the study of ***structural biology***

MEDICINE - SCIENCE OF HEALING

- any substance that is used to treat diseases and promote health

- neutrons particularly useful in the ***study of medicines*** including developing improved delivery systems (drugs and genes)



NEUTRONS ARE A GOOD PROBE FOR BIOLOGICAL & MEDICAL STUDIES BECAUSE THEY……

- can penetrate deeply into materials
- do not damage biological materials
- posses wavelengths ~ atomic dimensions
- possess energies ~ atomic motions
- possess a large dynamic range in space, time and energy
- can distinguish between H and D (basis of ***CONTRAST VARIATION***) and therefore can be used to study complex biological & medical systems
- can detect the location of protons (essential in many biological processes)

NEUTRONS CAN PROVIDE STRUCTURAL AND DYNAMIC DATA ON……

- the size, shape and internal structure of molecules or aggregates
- membrane structure
- atomic structure
- molecular dynamics

However the technique suffers from……

- low resolution
- low flux
- long acquisition times
- the requirement for relatively large samples

SCATTERING LENGTHS

Element	Neutrons	X Rays
	$b \times 10^{13}$ (cm)	$b \times 10^{13}$ (cm)
H	-3.74	3.8
D	6.67	2.8
C	6.65	16.9
N	9.40	19.7
O	5.80	22.5
P	5.10	42.3
S	2.85	45.0
Mn	-3.60	70.0
Fe	9.51	73.0
Pt	9.50	220.0

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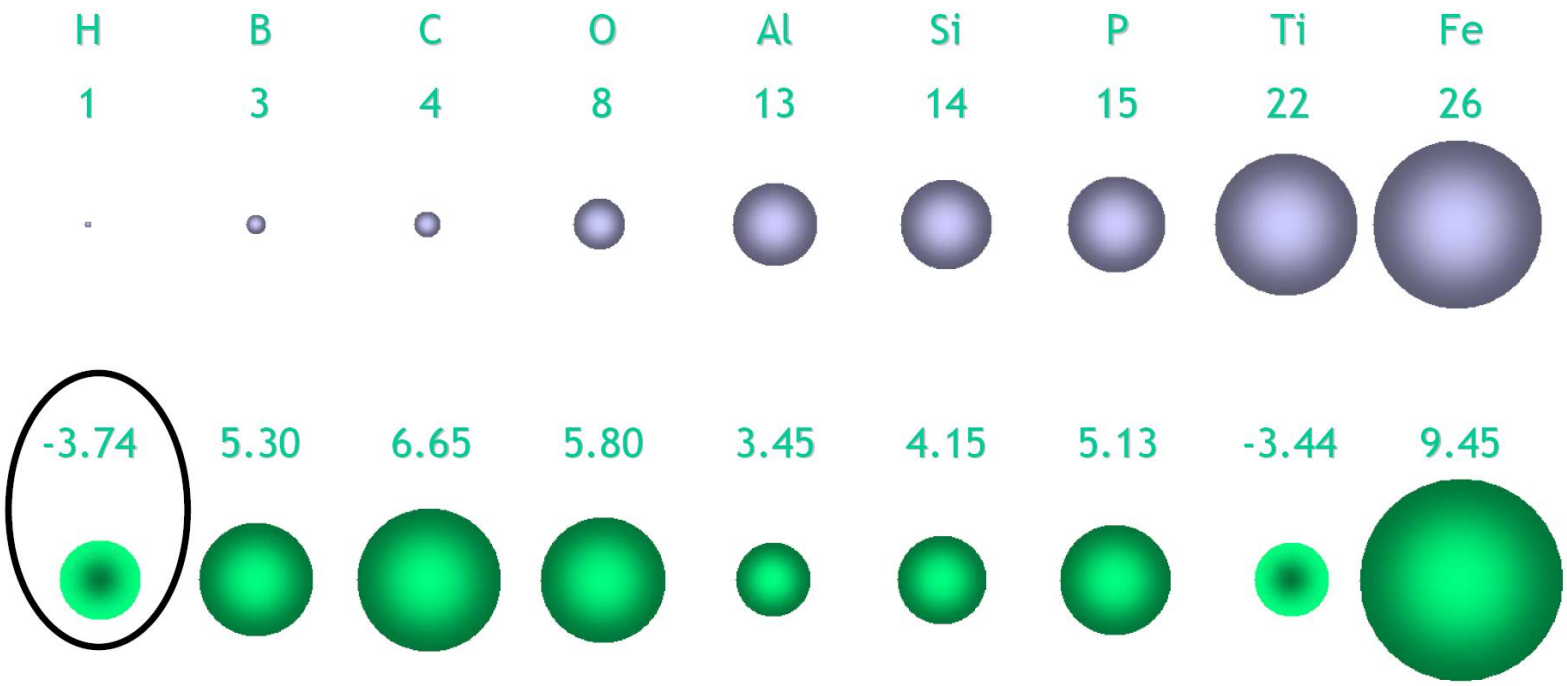
X-ray and neutron scattering

X-ray

Scattering proportional to Z

Neutron

Scattering not proportional to Z



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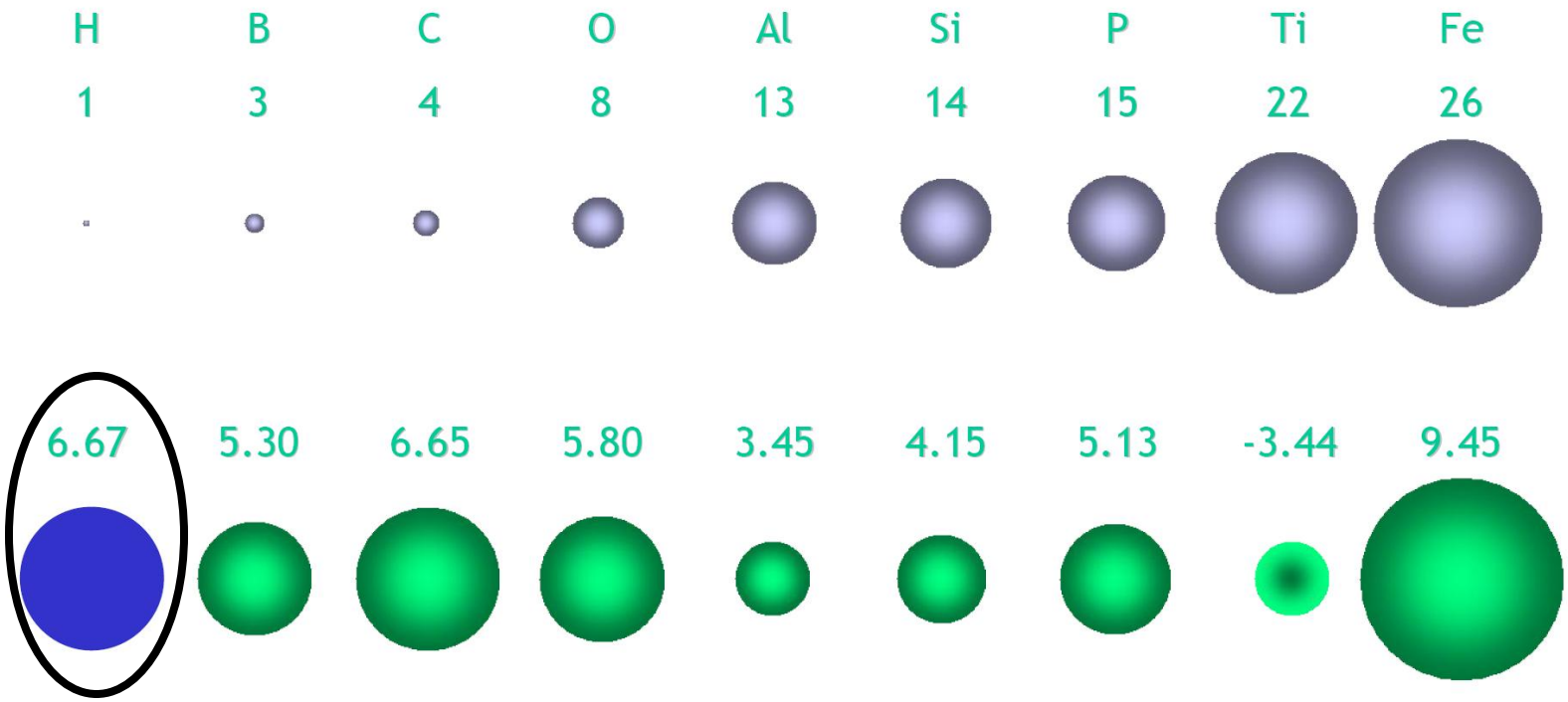
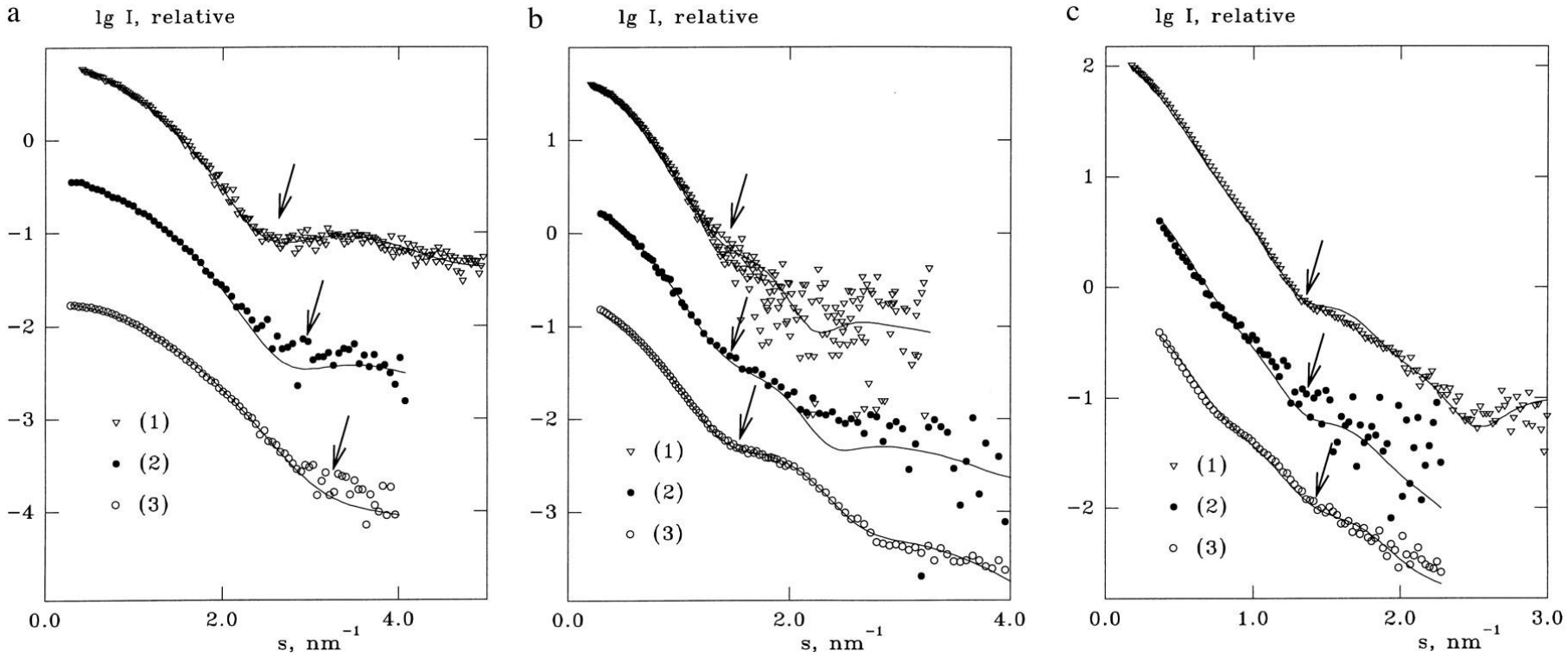


CONTRAST VARIATION

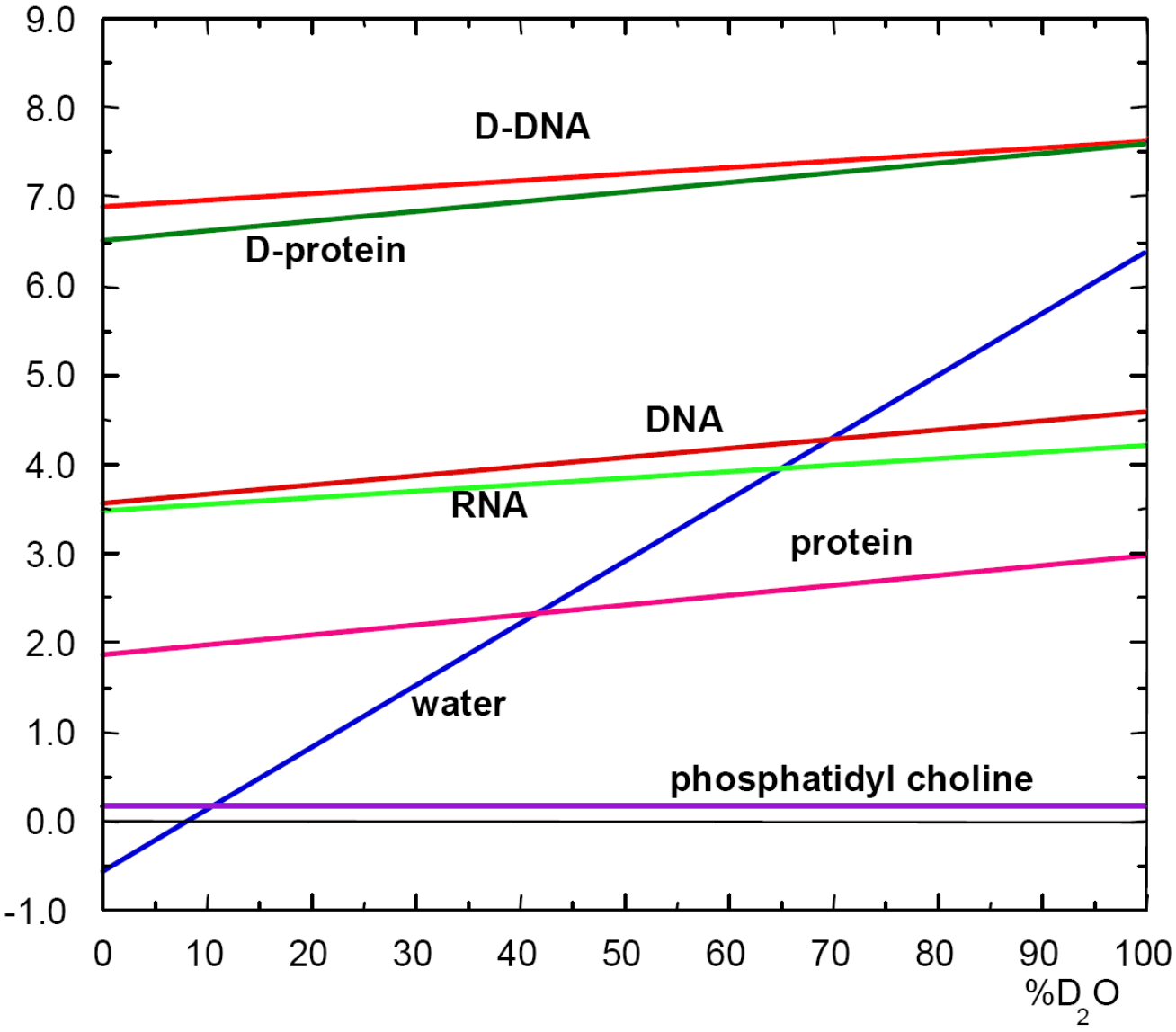
- In the absence of any specific isotope labelling, large range of contrasts possible through the use of mixture of D_2O/H_2O



SANS profiles for (a) lysozyme (b) thioredoxine reductase and (c) ribonucleotide reductase. Sets of data (1-3) correspond to sample measured by x-rays, neutrons (sample dispersed in H_2O) and neutrons, (sample dispersed in D_2O).

D.I. Svergun et al. PNAS (1998) 95, 2267-2272

VARIATION IN SCATTERING LENGTH DENSITY WITH PERCENT D_2O



CONTRAST VARIATION

- Remember not all protons exchangeable (some may be buried)
  – time maybe required for exchange to occur
  – scattering length density changes with solvent (D_2O/H_2O) composition.

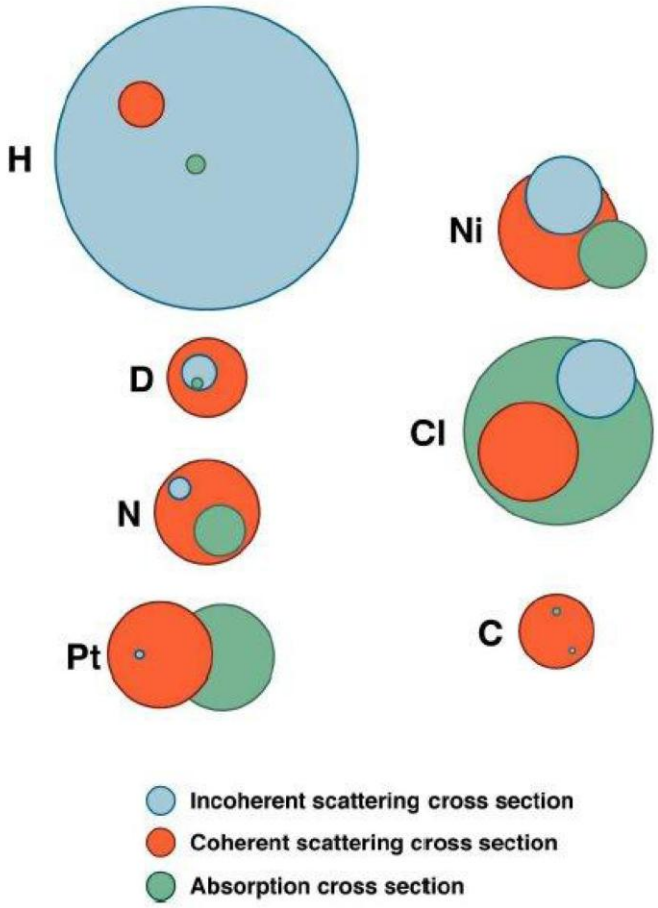
- To distinguish between the components within a system in which the scattering length densities are all similar require selective labelling of specific components
  - normally with D

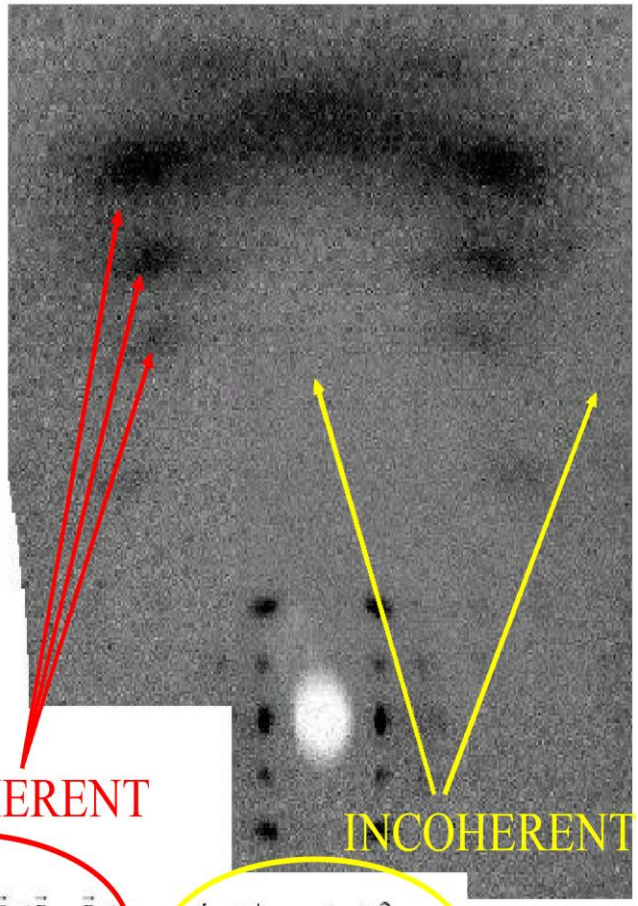
CONTRAST VARIATION

- Contrast variation first exploited in experiments on the 30S sub-unit of the ribosome (**M. S. Capel et al Science (1987) 238, 1403-1406**) and σ-factor and core enzyme of *E. coli* RNA polymerse (**Lederer et al J. Mol. Biol. (1991(219, 747-755**) where labelling was exploited to determine by triangulation the distances between the protein components.

- When using contrast variation it is essential to ensure that replacement of H with D does not alter the properties of the system under study
  - bond lengths different
  - alter phase transition

INCOHERENT SCATTERING





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

If possible perform experiments with as little H in solvent as possible, thereby reduce the incoherent scattering/counting time

STRUCTURE DETERMINATION IN BIOLOGY & MEDICINE USING NEUTRON SCATTERING

- Small angle neutron scattering
- Low resolution crystallography
- High resolution crystallography
- (Fibre diffraction)

- Membrane diffraction
- Reflectometry

SMALL ANGLE NEUTRON SCATTERING

- Characterize nanostructures & hierarchical structures of materials ranging from 1 to 100-400 nm (depending upon SANS instrument)
- Yields low resolution information on shape (in solution)
- Contrast variation & labelling can be used to provide important information on structure in multi-component systems
- Numerous examples of SANS being used for biological and medical questions including understanding:-

  nature of protein-surfactant interactions,

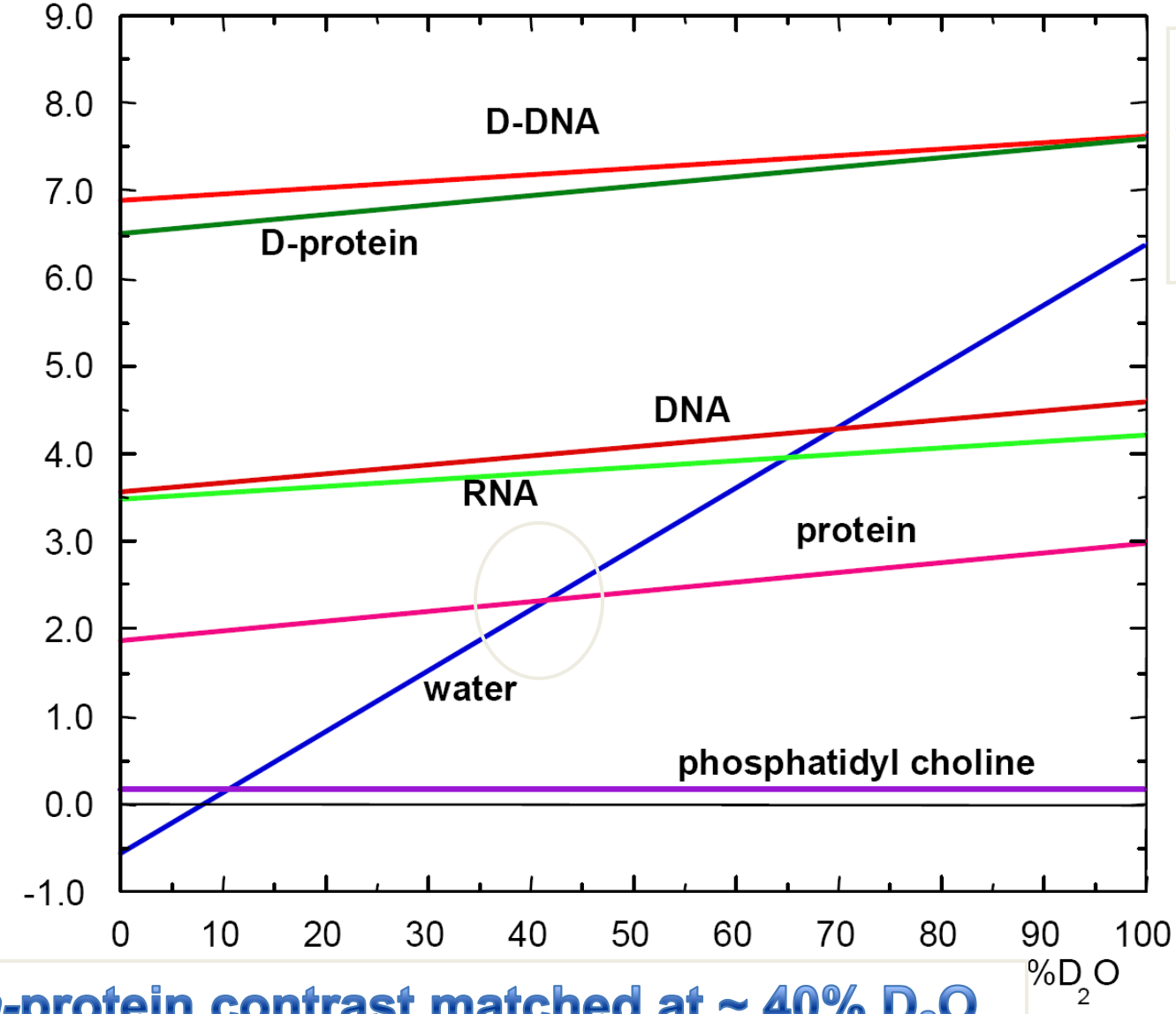
  how light induces structural changes in pea thylakoids,

  solution structure of human proliferating cell nuclear antigen

  biomineralization

  internal architecture of gene delivery vehicles……..

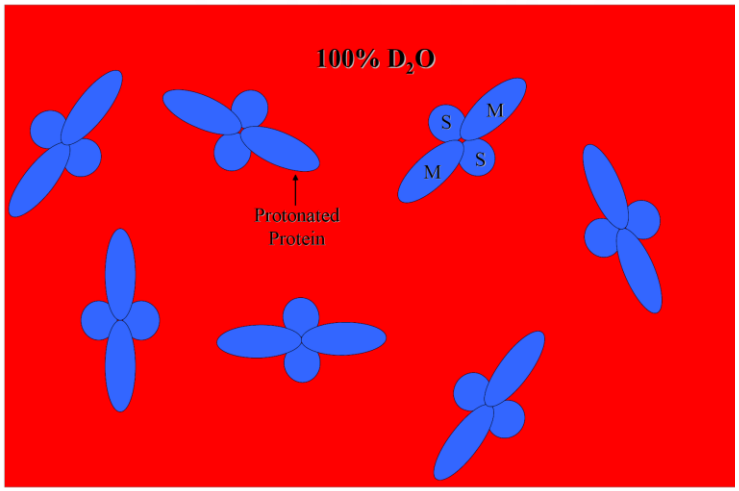
VARIATION IN SCATTERING LENGTH DENSITY WITH PERCENT D_2O



NB Proteins studied at 80% rather than 100% deuteration

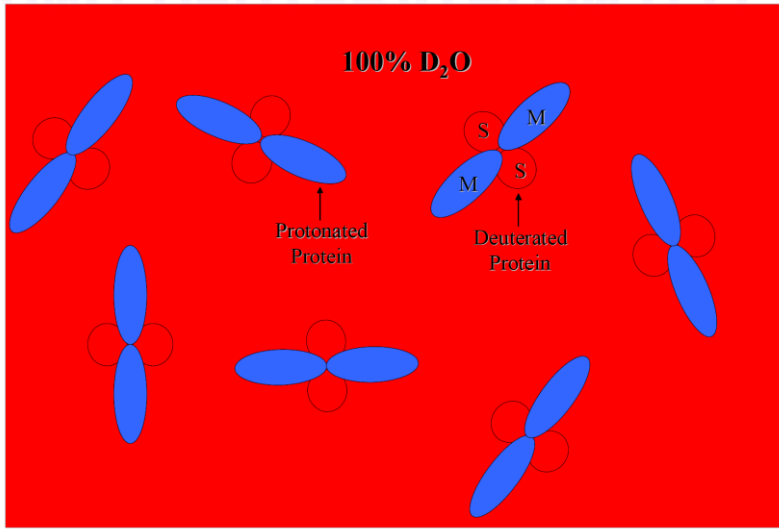
***h*-protein contrast matched at ~ 40% D_2O**

CONTRAST VARIATION PROTEIN CONSISTING OF 2 SUB-UNITS

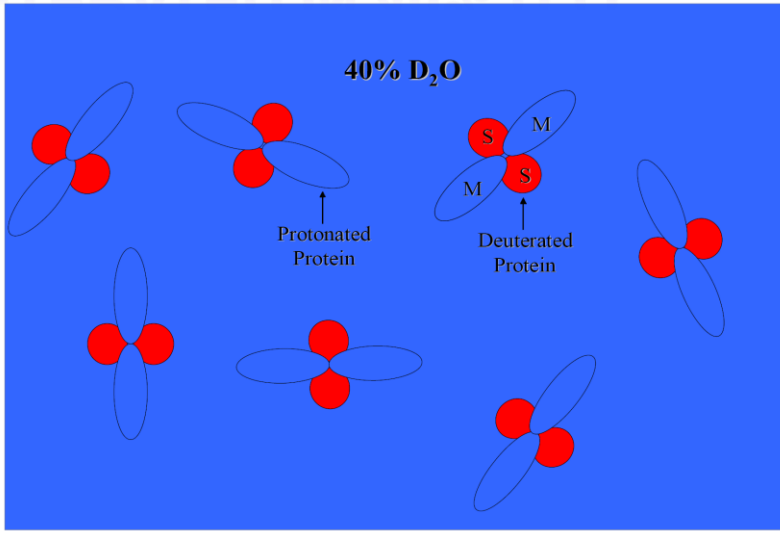


HYDROGENOUS PROTEIN IN D_2O

PROTEIN WITH ONE SUB-UNIT DEUTERATED IN D_2O

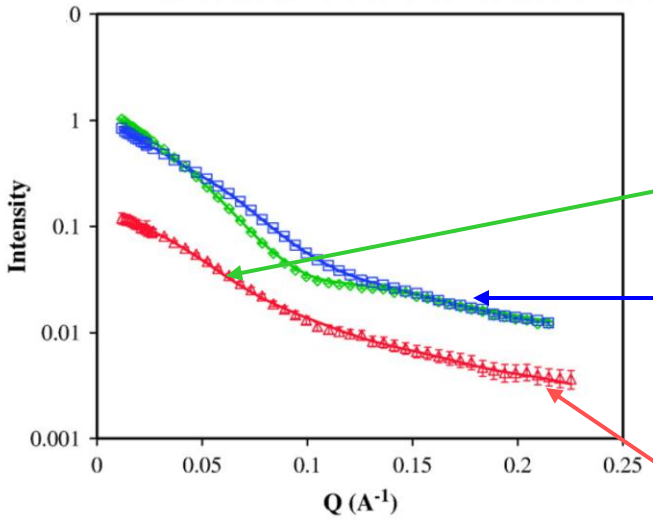


PROTEIN WITH ONE SUB-UNIT DEUTERATED IN 40% D_2O



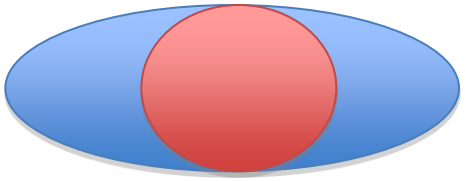
SHAPE AND SUB-UNIT ORGANISATION OF THE DNA METHYLTRANSFERASE M.AHDI BY SANS

SANS DATA AND *AB INITIO* FIT (SVERGUN ET AL. 1999)

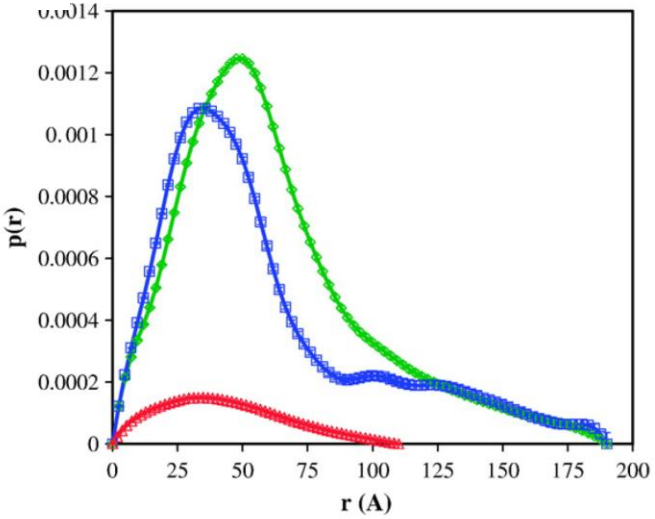


h-M.Ahdl in D_2O

d-S sub-unit & *h*-M sub-unit in D_2O



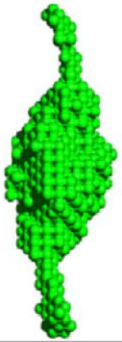
d-S sub-unit & *h*-M sub-unit in 40% D_2O

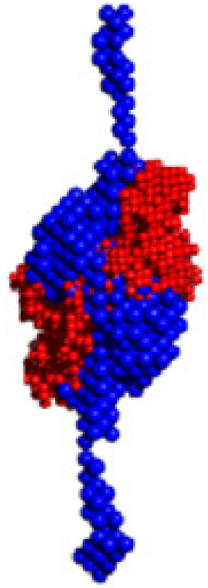


DISTANCE DISTRIBUTION FUNCTIONS

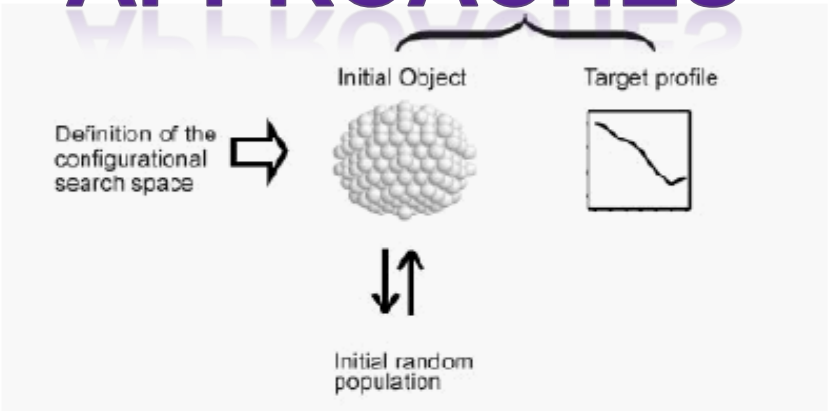
P. Callow et al. J. Mol. Biol. (2007) 369, 177–185

MODEL OF M.AHDL

M.AhdI Complex	
M Subunits	
S Subunits	

S Subunits and M Subunits	

AB INITIO MODELLING: AN OBJECTIVE ALTERNATIVE TO SUBJECTIVE APPROACHES

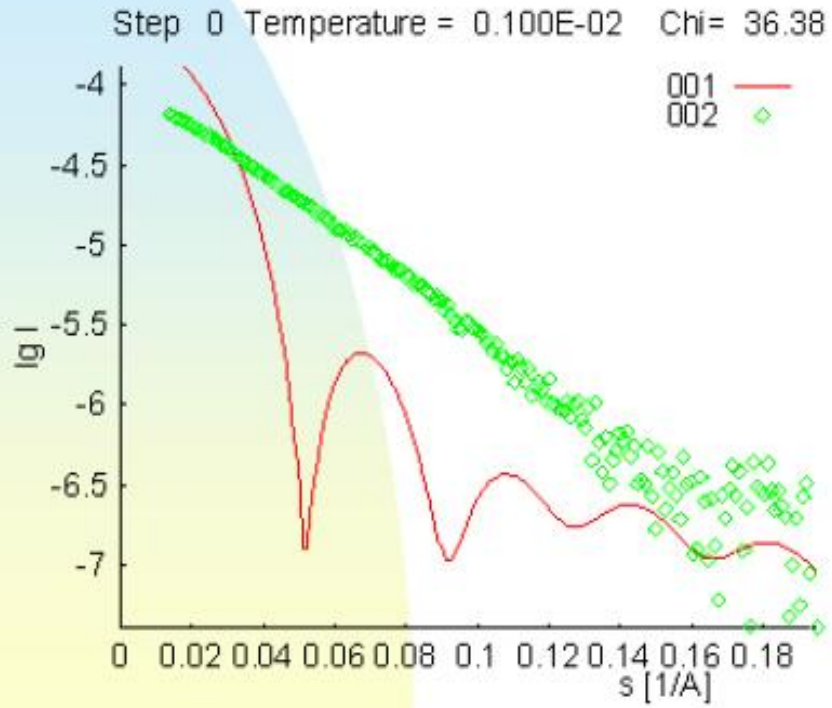


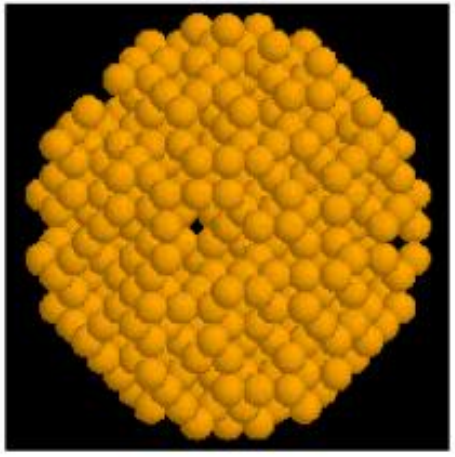
AB INITIO SHAPE DETERMINATION BY SIMULATED ANNEALING USING A SINGLE PHASE DUMMY ATOM MODEL

DAMMIN

D. I. Svergun Biophys. J. (1999) 76, 2879-2886

S1 shape reconstruction





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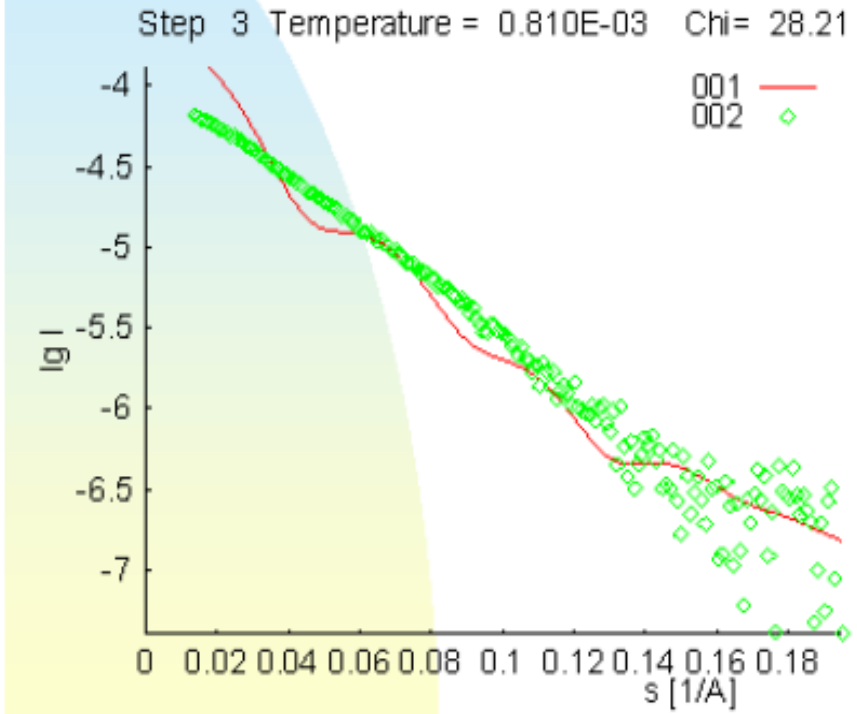

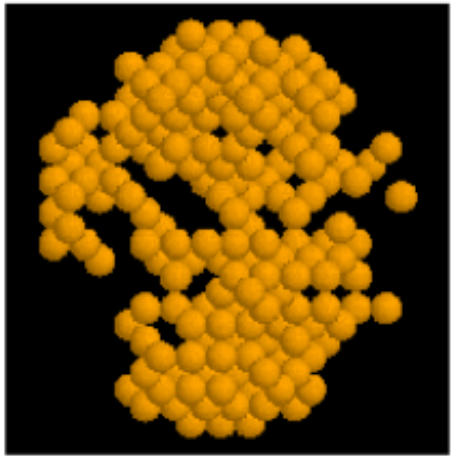

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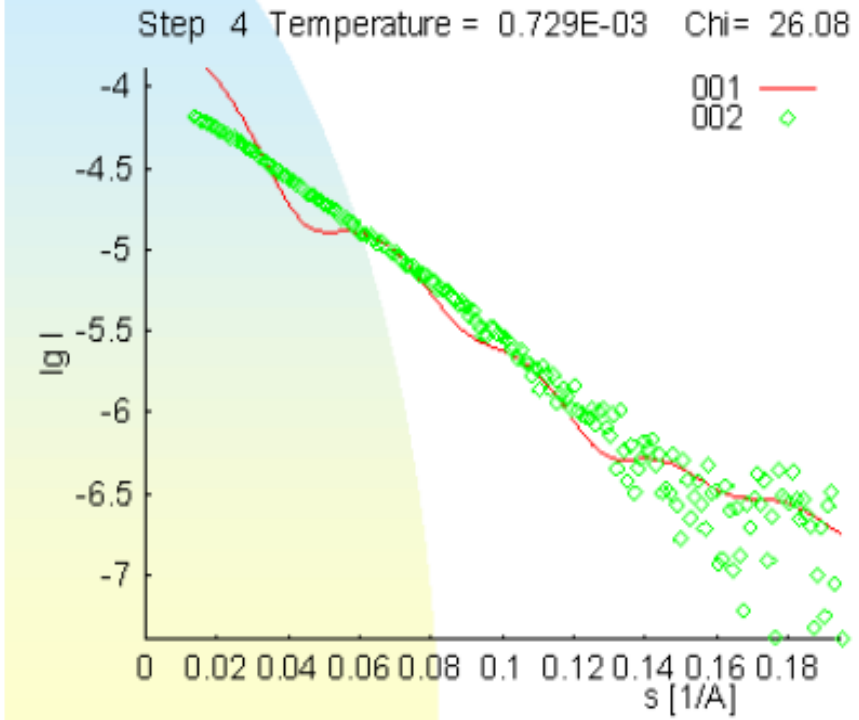

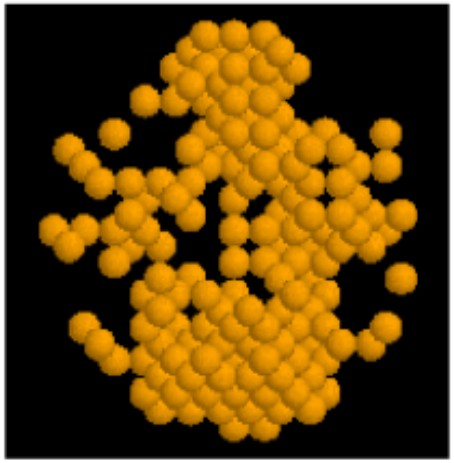

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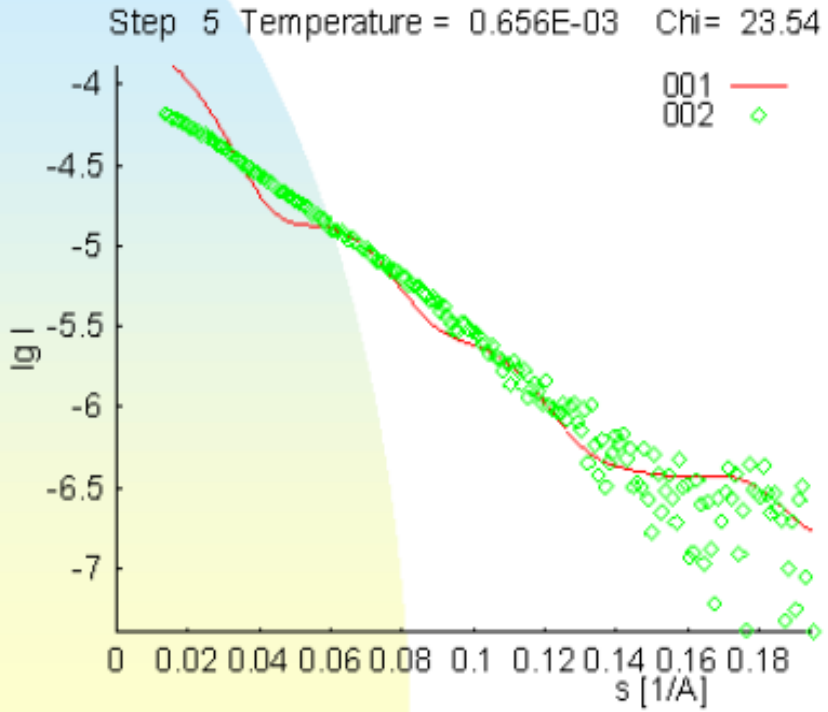

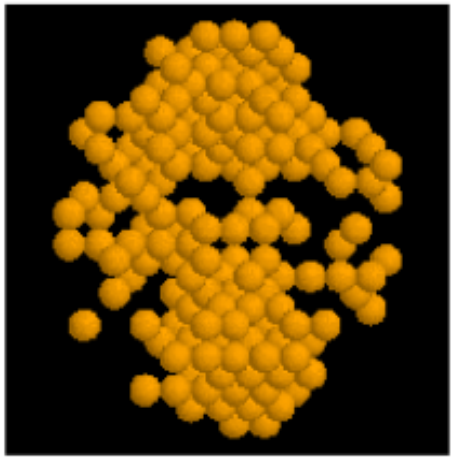

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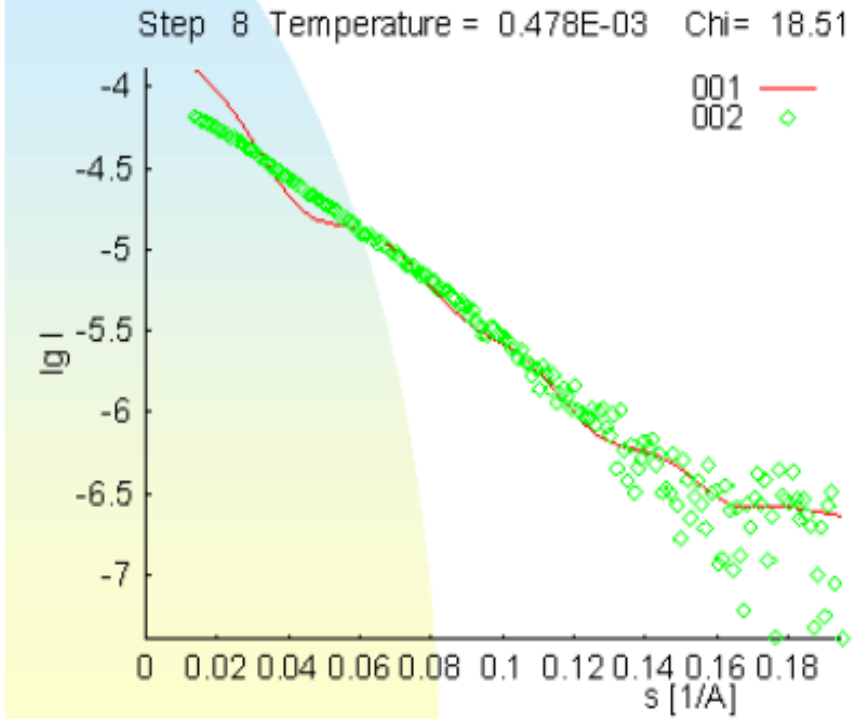

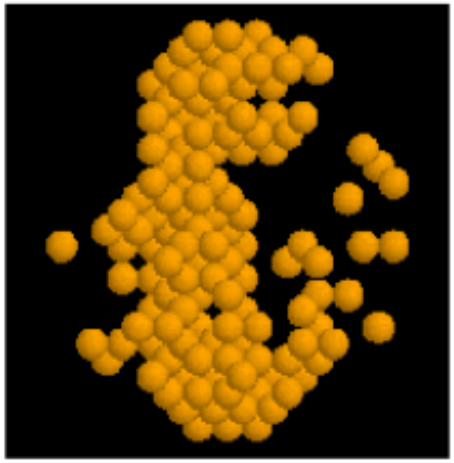

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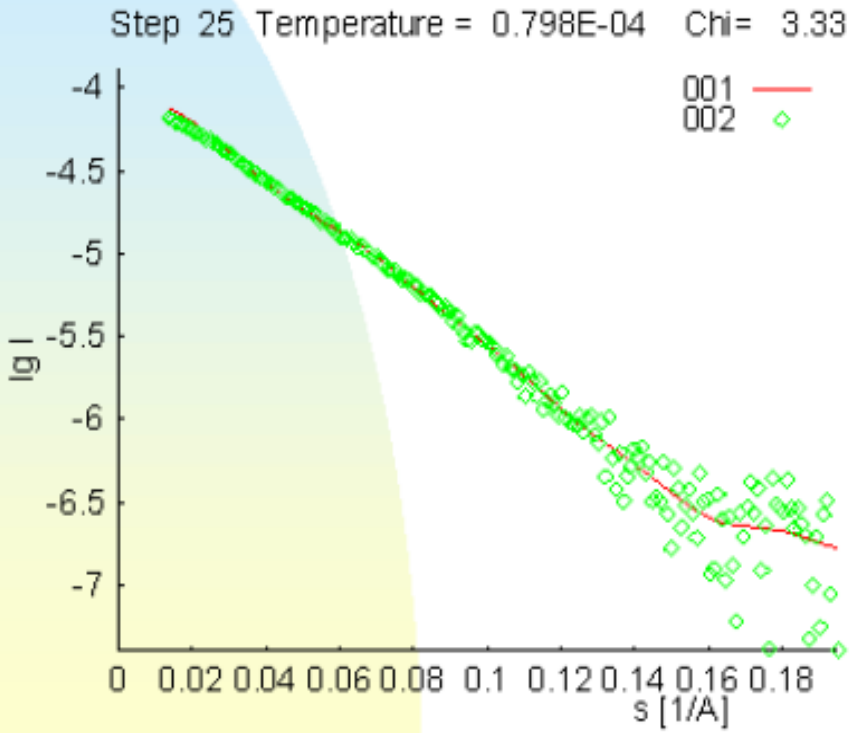

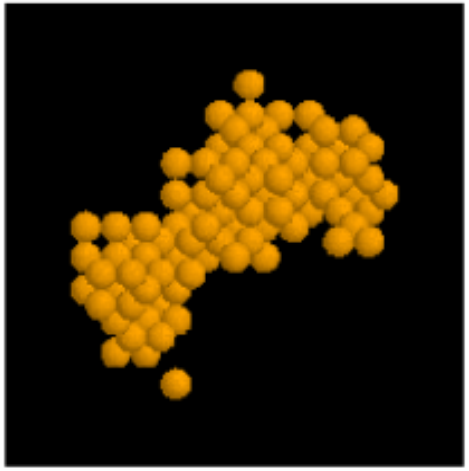

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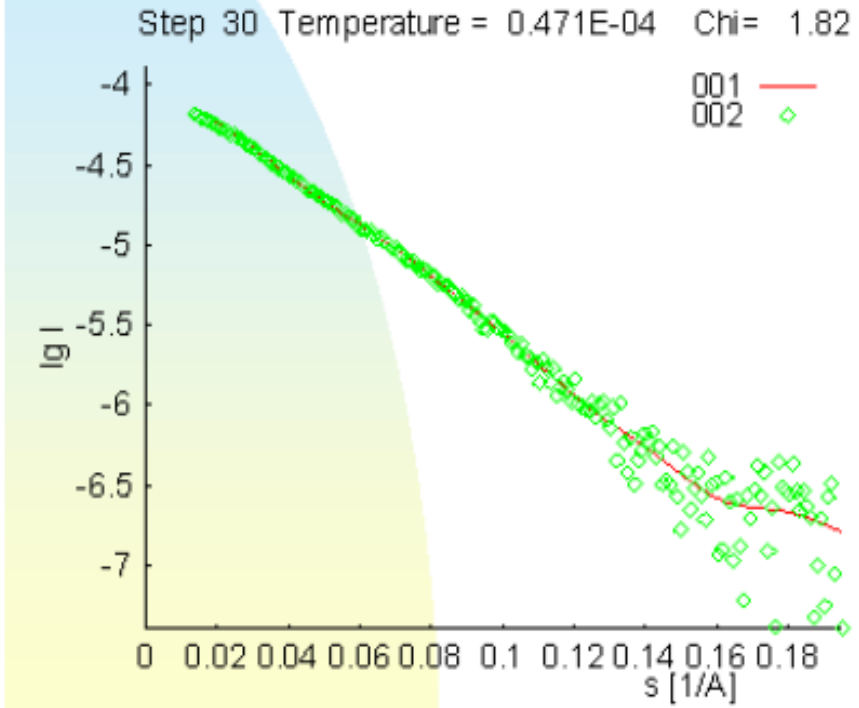

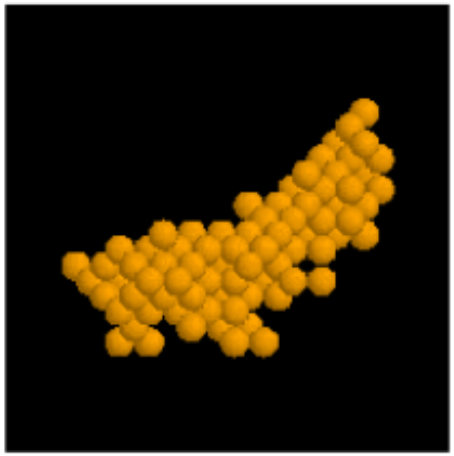

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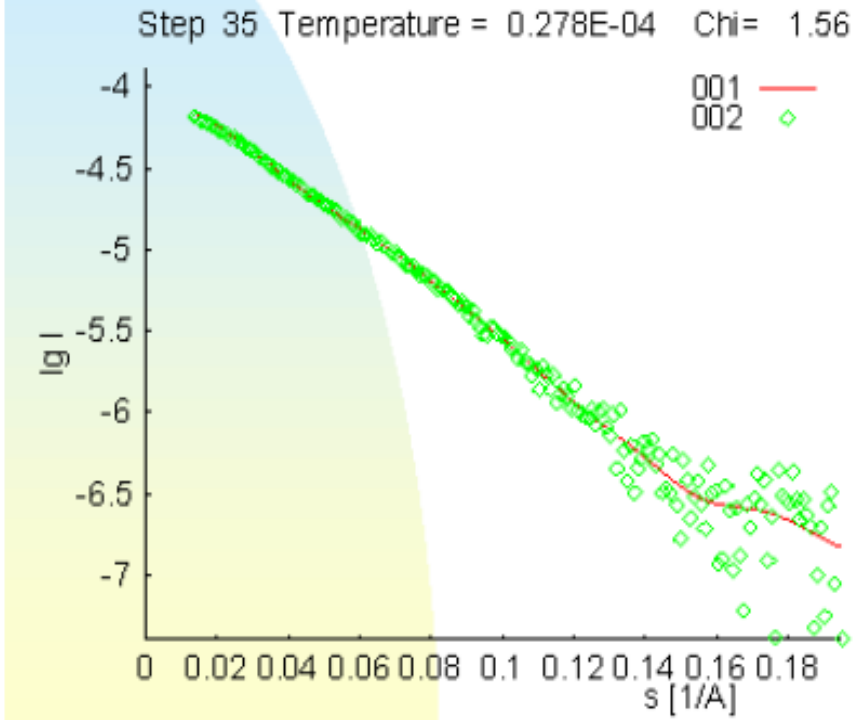

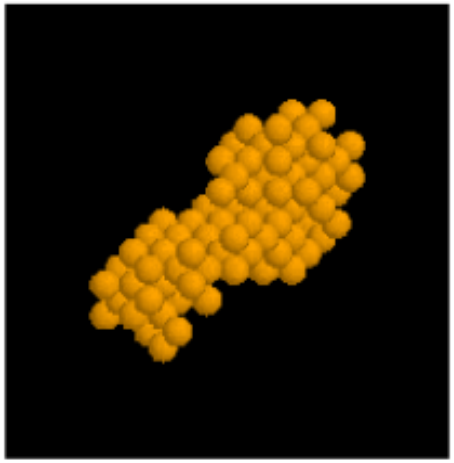

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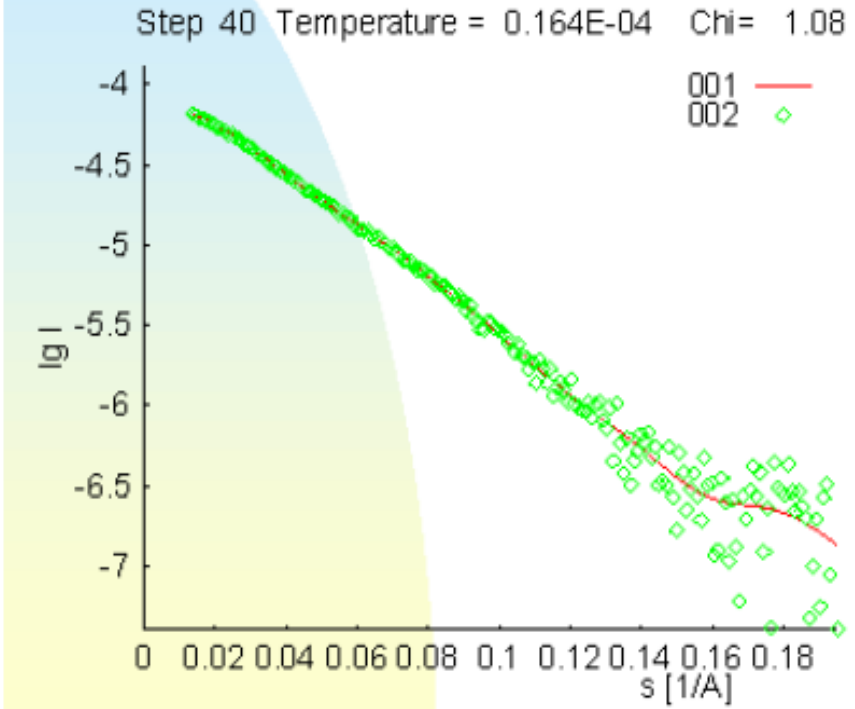

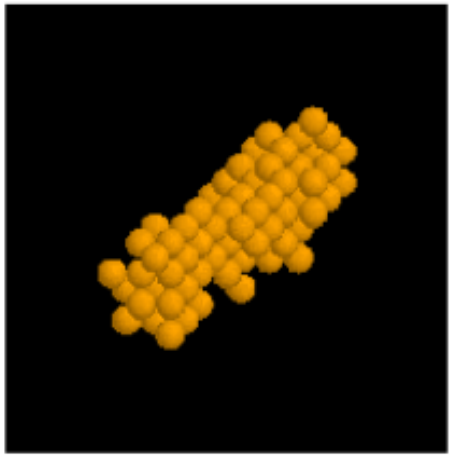

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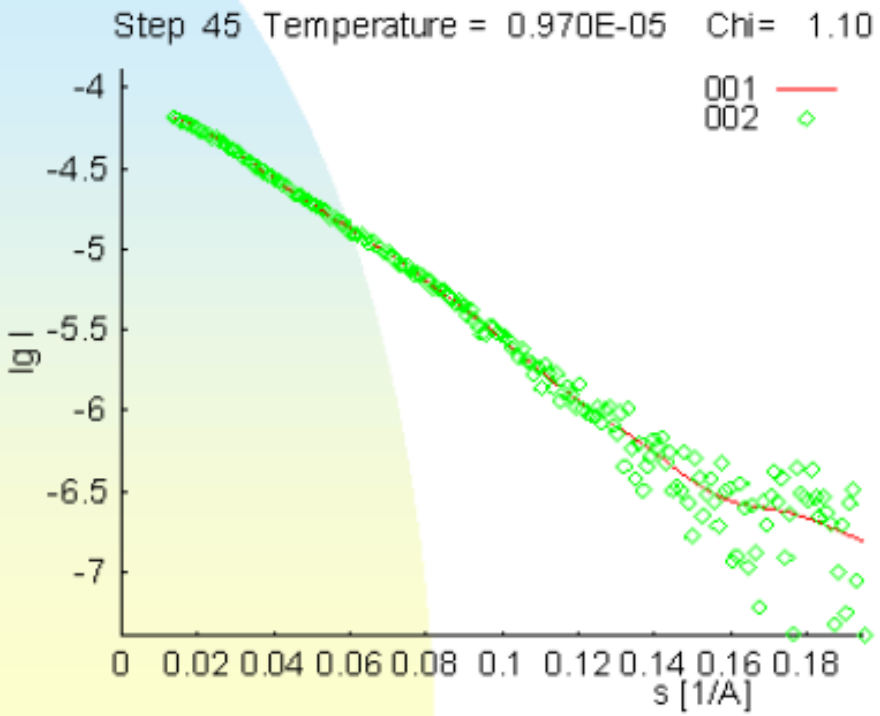

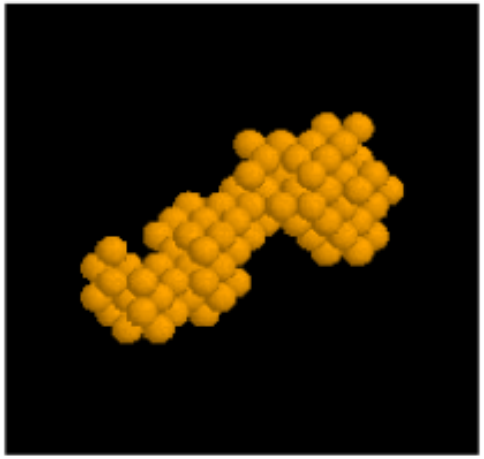

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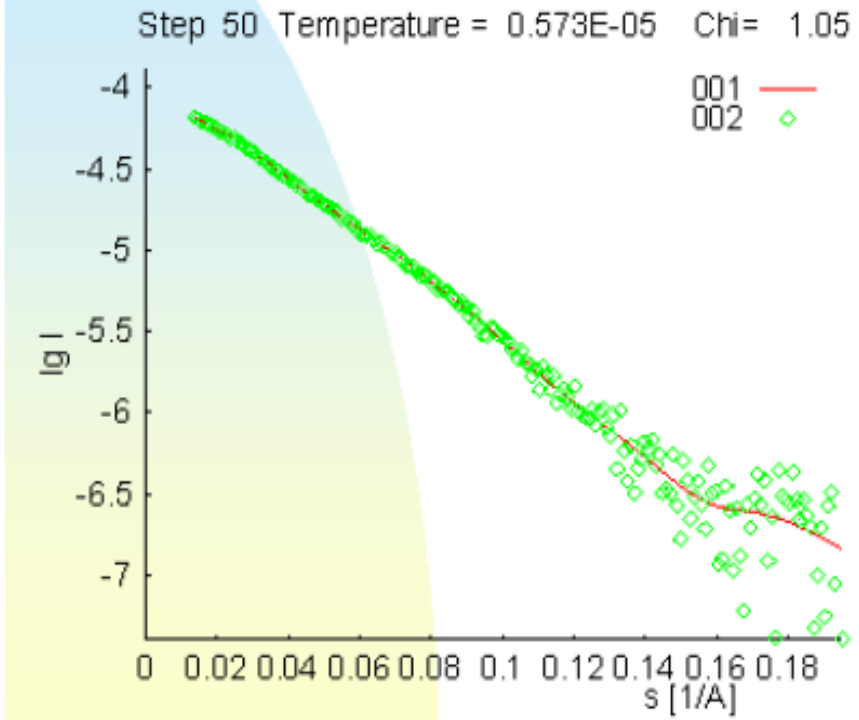
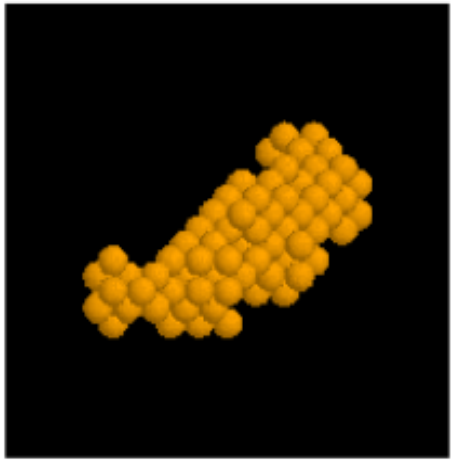
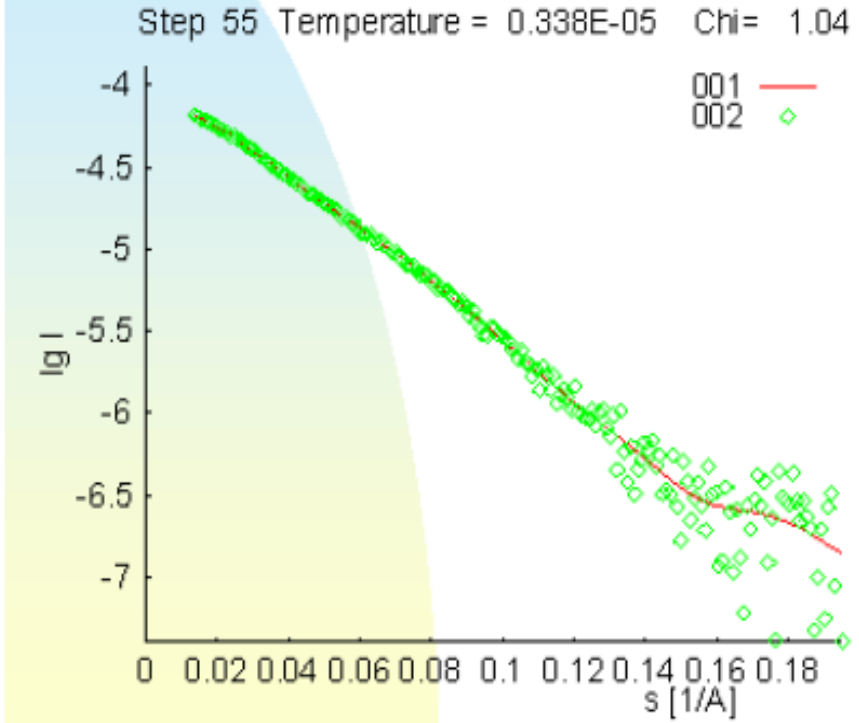
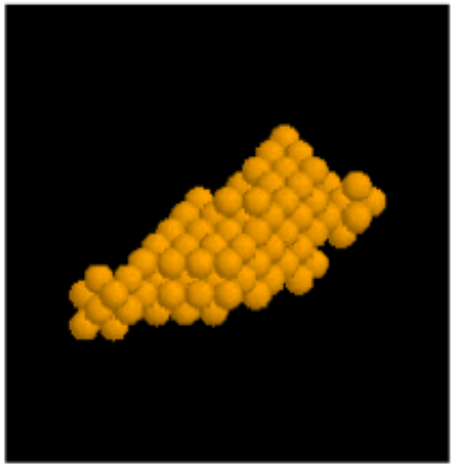




http://www.embl-hamburg.de/ExternalInfo/Research/Sax/

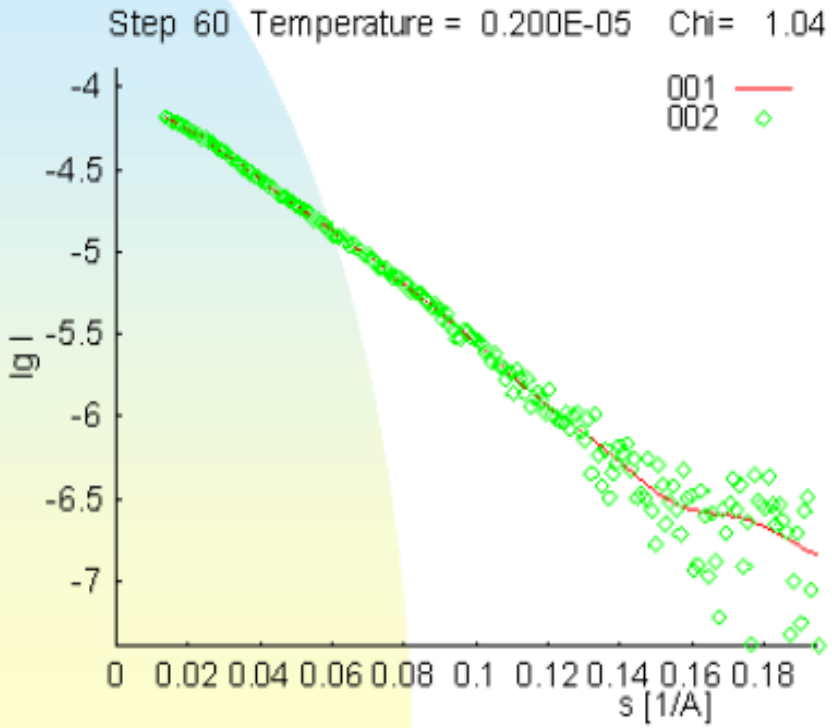
S1 shape reconstruction

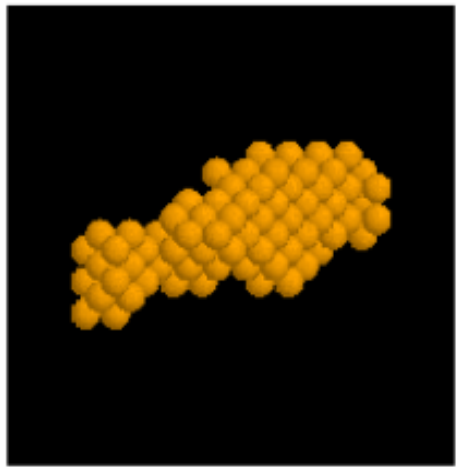




http://www.embl-hamburg.de/ExternalInfo/Research/Sax/

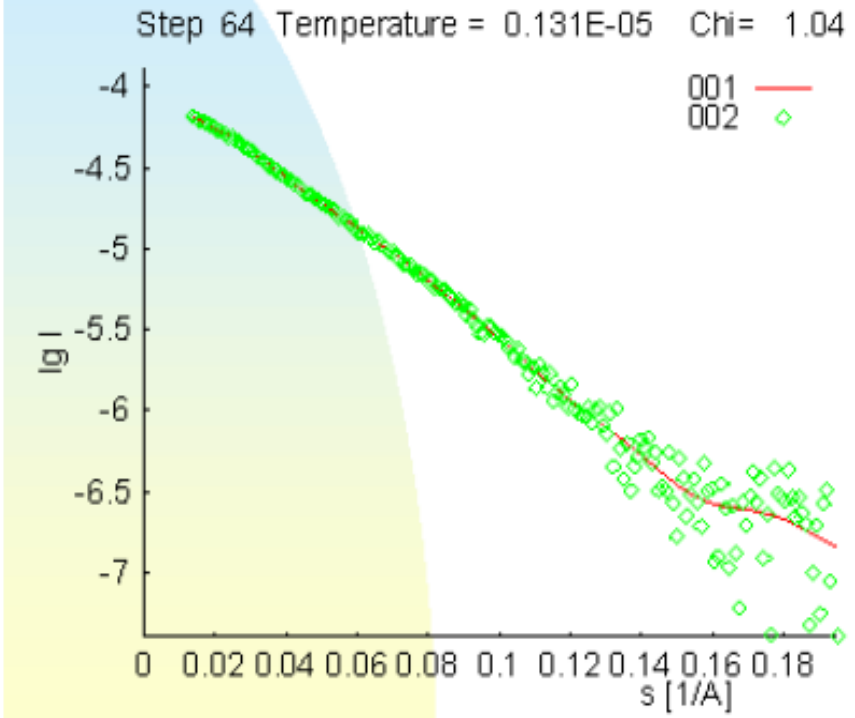
S1 shape reconstruction

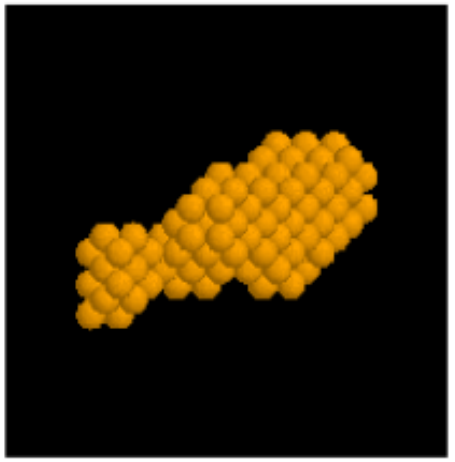




http://www.embl-hamburg.de/ExternalInfo/Research/Sax/

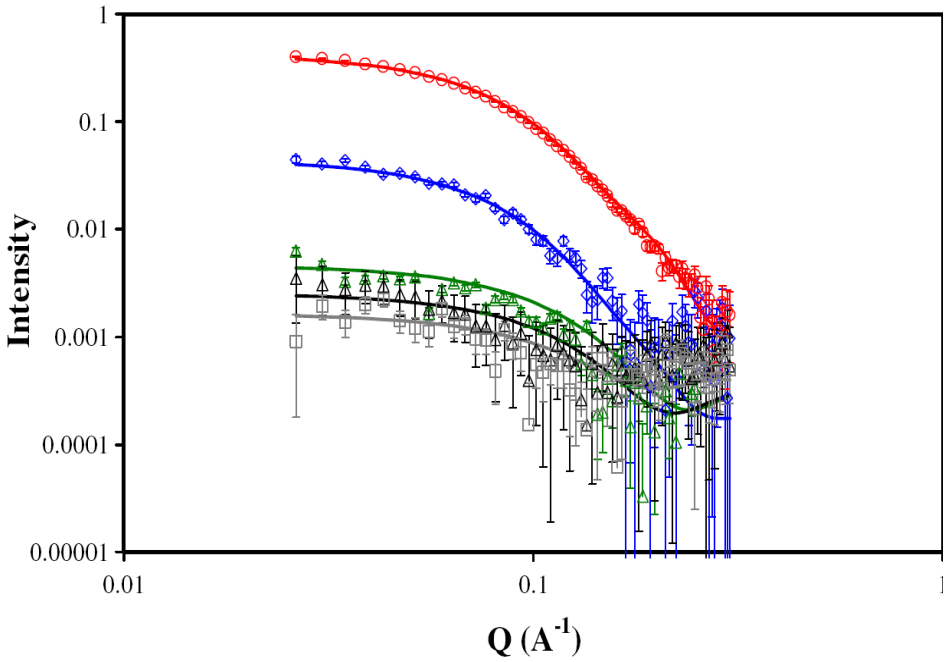
S1 shape reconstruction





http://www.embl-hamburg.de/ExternalInfo/Research/Sax/

SELECTIVE DEUTERATION OF TRYPTOPHAN & METHIONINE IN MALTOSE BINDING PROTEIN (MBP)



Perdeuterated MBP in H_2O

Unmodified (h-)MBP in H_2O

Doubly labelled MBP trp. & met. residues deuterated (d-trp/met MBP) in 40% D_2O

Singly labelled MBP with trp. residues deuterated (d-trp MBP) in 40% D_2O,

Singly labelled MBP with met. residues deuterated (d-met MBP) in 40% D_2O

trp. and met. classified as very hydrophobic amino acid residues

8 trp. (d_3) residues and 8 met (d_5) out of a total of 370

V. Laux et al. Eur. Biophys. J. (2008) 37, 815–822

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

- if the sample is deuterium-labelled, crystals can be as small as 0.01 mm^3 due to better signal-to-noise ratios

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

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

LOW RESOLUTION NEUTRON CRYSTALLOGRAPHY

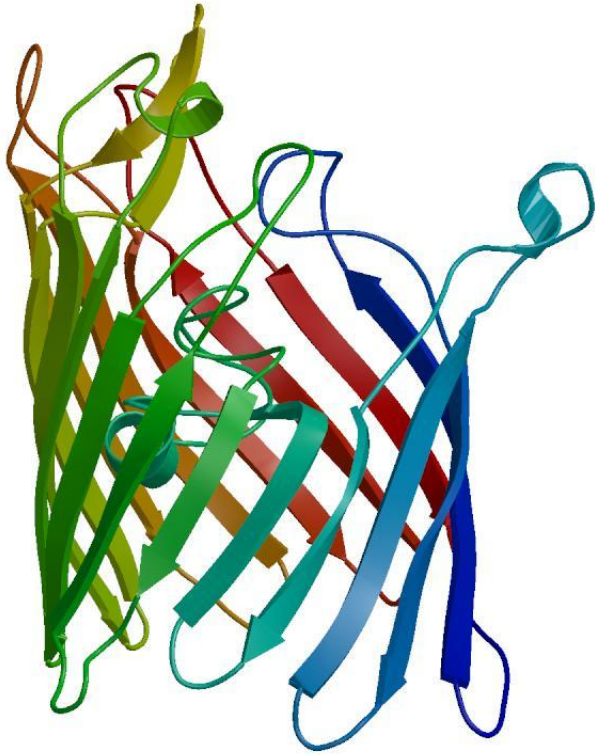
- provides important information about membrane proteins

- ***membrane proteins*** are usually removed from the cell membrane and crystallised using detergents which act to replace the lipid found *in vivo*

- information on the binding of these detergent molecules to the protein gives *indirect* information on the lipid binding in the membrane

- the detergent molecules are invisible in high resolution X-ray crystallographic electron density map due to their fluidity but can be visualized in low resolution neutron crystallography

LOW RESOLUTION NEUTRON CRYSTALLOGRAPHY

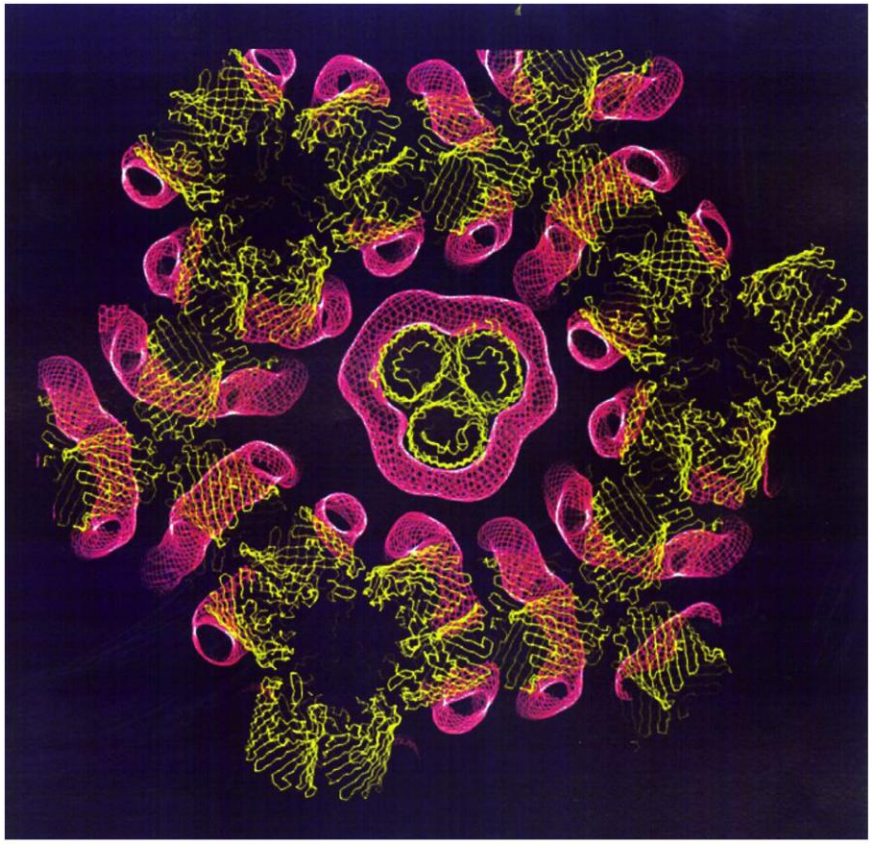


OmpF porin - integral membrane channel-forming protein which spans the outer membrane of Gram-negative bacteria (eg *E. coli*)

Allows the selective diffusion of small hydrophilic molecules across the membrane

Active pore trimer ~75 x 55Å

LOW RESOLUTION NEUTRON CRYSTALLOGRAPHY



View parallel to threefold axis of trimer

OmpF porin crystallised in 40% D_2O & therefore is contrast matched & 'invisible'

X-ray structure shown in yellow

deuterated detergent molecules are shown in purple

detergent molecules bound to 'hydrophobic zone ' surrounding the trimer & which is exposed to lipid *in vivo*

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

LOW RESOLUTION NEUTRON CRYSTALLOGRAPHY

Results of investigations of membrane protein structures have demonstrated that:-

- not only are protein-protein interactions important in the formation of crystals
- but that in some cases protein-detergent and even detergent-detergent interactions are involved

HIGH RESOLUTION NEUTRON CRYSTALLOGRAPHY - ADVANTAGES

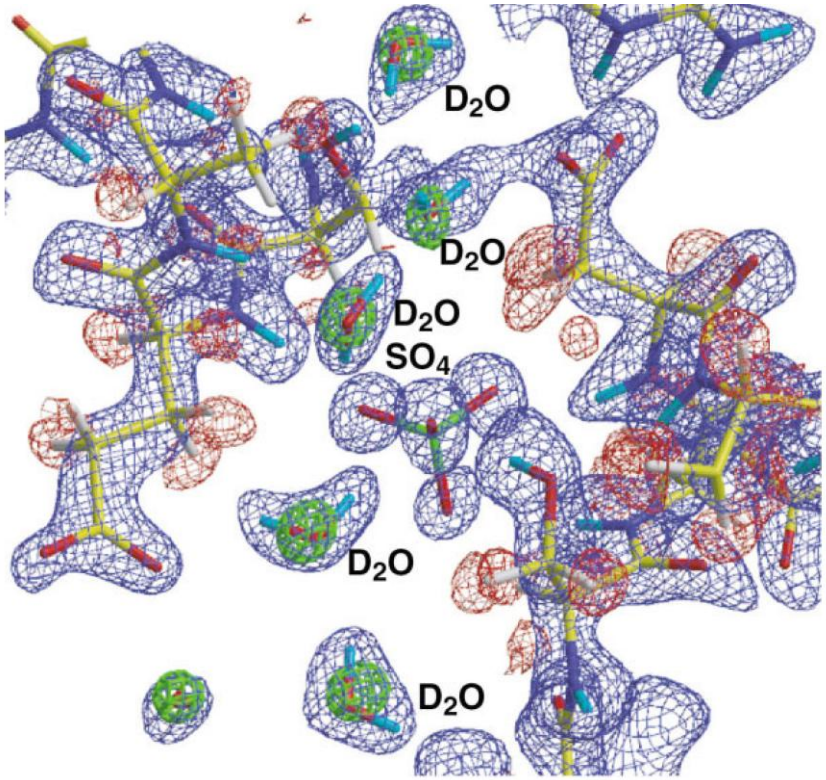
- Imaging of positions of water molecules bound to protein
- Determination of hydrogen bonding networks
- Both are critical to protein function as water mediates many biological functions

- Although X-ray crystallography and NMR spectroscopy are powerful methods for determining the 3D structure of proteins, it is difficult for such techniques to determine the positions of all the hydrogen atoms accurately

HIGH RESOLUTION NEUTRON CRYSTALLOGRAPHY - DISADVANTAGES

- Conventional high resolution neutron crystallography sees a low flux (until recently neutron crystallographic studies have been confined to monochromatic beam lines)
- High resolution protein crystallography was a minority interest due to the need for large crystals because of the low flux of neutron sources
- Very long collection times: weeks-months!
- Large crystals needed
- Problems with cryo-cooling

HIGH RESOLUTION NEUTRON CRYSTALLOGRAPHY - MYOGLOBIN



Myoglobin-myoglobin contact region

(*2* | *Fo*| - | *F*c |) neutron density map contoured at 1.5σ in blue and 2.0σ in red (note that hydrogen atom, ^1H, appears as a negative contour level due to its negative scattering length). The (*2* | *Fo*| - | *F*c |) X-ray map for the water molecules is shown in green (the σ - levels for the water molecule density were individually set between 2.0 and 3.5 σ). The triangular neutron contours correspond to D_2O molecules.

High-resolution neutron protein crystallography (@ 1.5Å resolution) provided information about

a) the location of hydrogens including those present in water

b) how the hydrogen in water is bound to neighbouring atoms and

c) how the hydrogen in water is oriented (i.e., whether it packs in an ordered or disordered fashion)

T. Chatake et al. Proteins: Struct. Funct.Gene. (2003) 50, 516–523

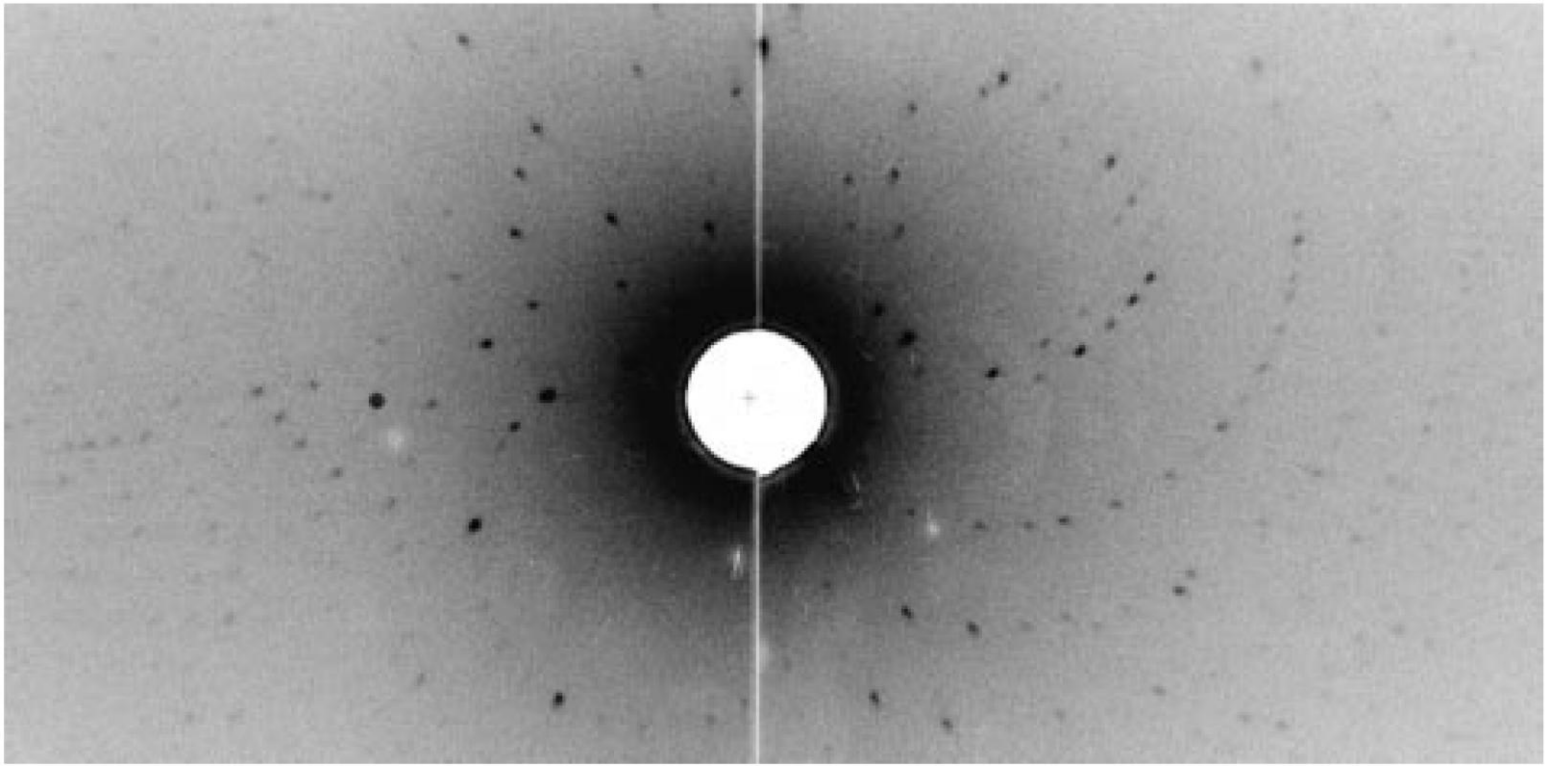
LADI NEUTRON LAUE DIFFRACTOMETER AT THE ILL

- Illuminates sample with all available neutrons
- Maximal flux @ sample
- Large number of reflections at all incident neutron λ
- It is now routine for a protein crystal of 1-5 mm^3 to be studied, although crystals as small as 0.15 mm^3 have been recently measured on perdeuterated proteins
- Collection times reduced to hours-days. Data sets have been collected in 3.5 days to 2.0 Å resolution from a 1.4 mm^3 thaumatin crystal and over several days to 2Å resolution from a perdeuterated antifreeze protein (AFP) only 0.13 mm^3 in volume
- Upper limit of 50 kDa realisable

M. Blakeley et al. Curr. Opin. Struct. Biol (2008) 18 593-600

HIGH RESOLUTION NEUTRON CRYSTALLOGRAPHY – CONCANAVALIN A @ 15K*

Concanavalin A is a saccharide-binding protein which belongs to the legume lectin family



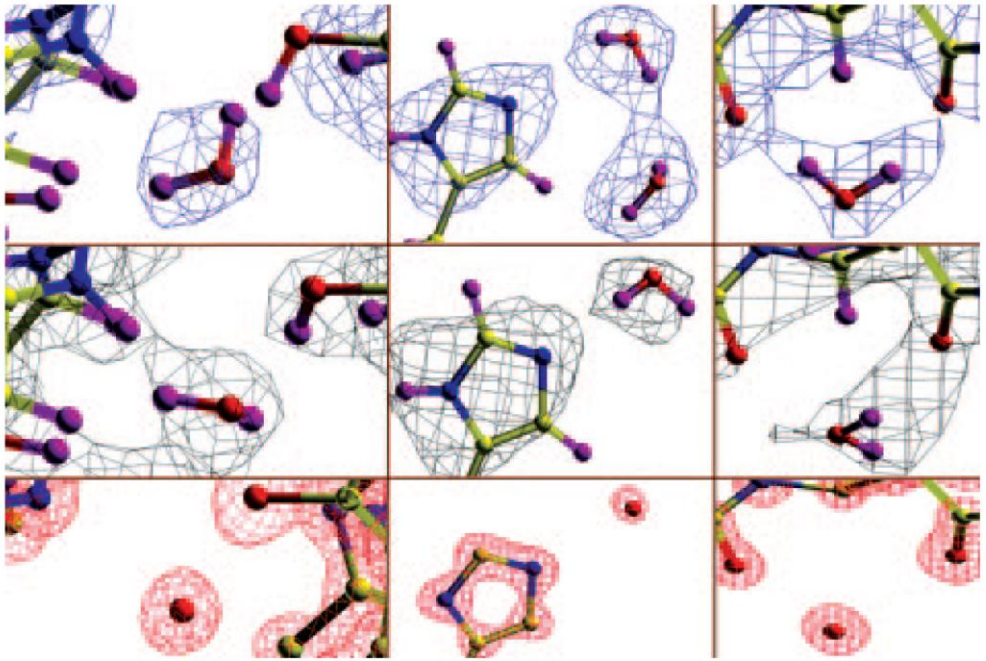
1.5 mm^3

Xystals

* to increase resolution

M. Blakeley et al. PNAS (2004) 101, 16405-16410

HIGH RESOLUTION NEUTRON CRYSTALLOGRAPHY – CONCANAVALIN A @ 15K



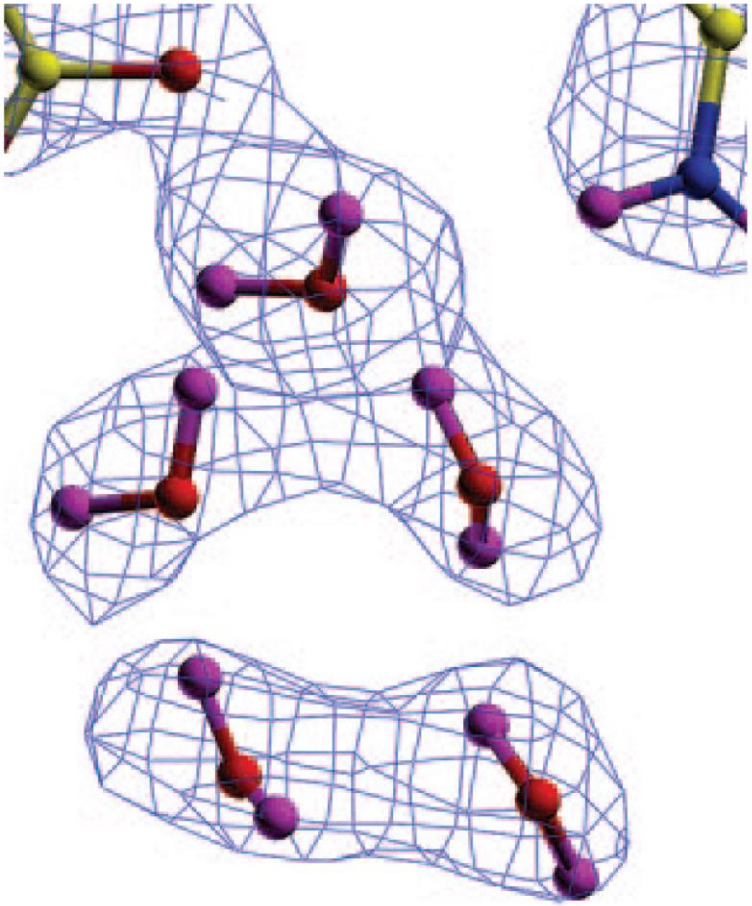
Neutron crystallography
Neutron crystallography at 293K

X-ray crystallography at 111K

shows structure contains 2x bound water molecules when compared to data obtained at 293 K

M. Blakeley et al. PNAS (2004) 101, 16405-16410

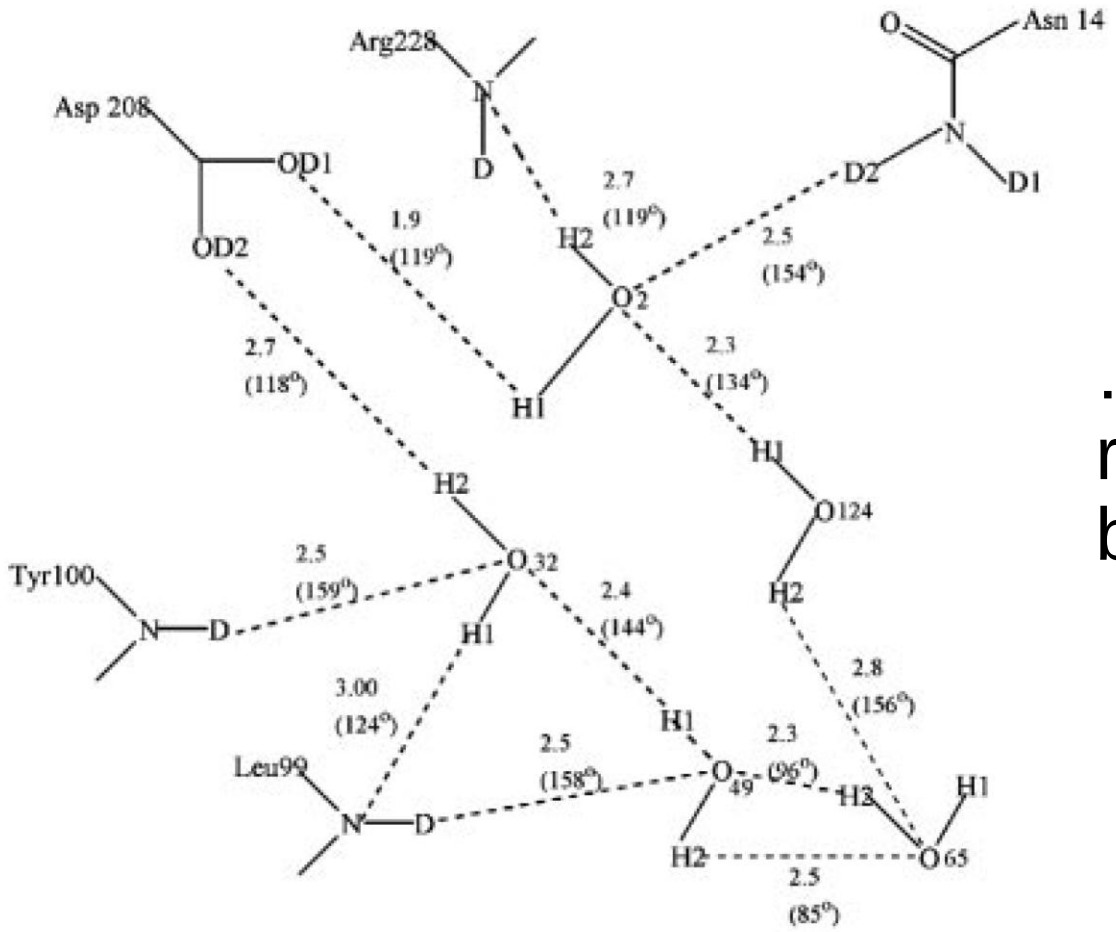
HIGH RESOLUTION NEUTRON CRYSTALLOGRAPHY – CONCANAVALIN A @ 15K



and the positions of 5 waters in the saccharide-binding site…

M. Blakeley et al. PNAS (2004) 101, 16405-16410

HIGH RESOLUTION NEUTRON CRYSTALLOGRAPHY – CONCANAVALIN A @ 15K



…and enables resolution of the H-bonding network

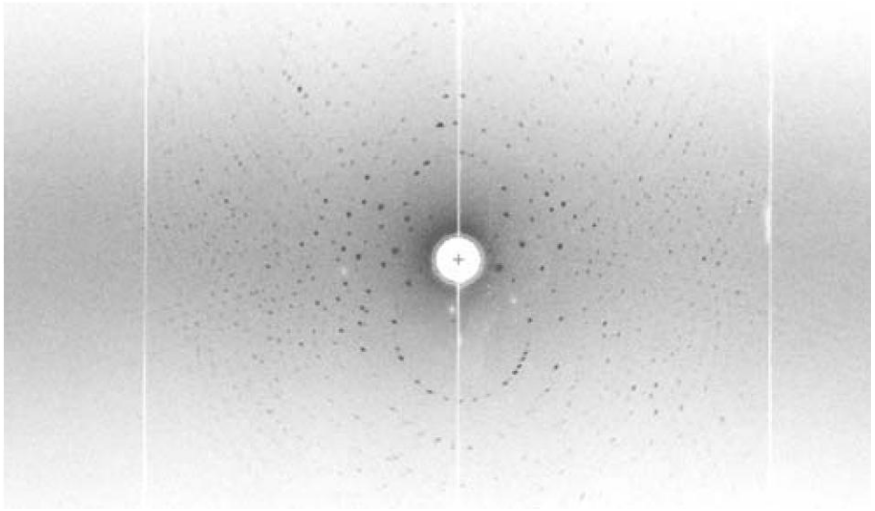
M. Blakeley et al. PNAS (2004) 101, 16405-16410

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 hydrogen although the refined model suggested that the site of ring opening was suitable for proton donation by His53.

Neutron laue diffraction data @ 2.2Å enabled direct observation of His53 protonation supporting the above mechanism



T.D. Fenn et al Biochem. (2004) 43, 6464-6474
F. Meilleur et al Eur. Biophys. J. (2006) 35:601–609

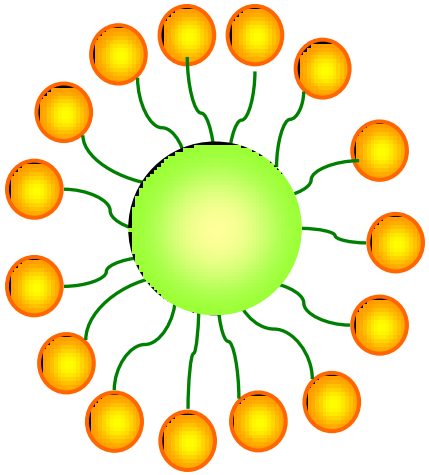
NEUTRON POWDER DIFFRACTION CRYSTALLOGRAPHY

- allows the real-space structure of materials to be determined at the atomic and micro-structural level
- allows determination of: long-range structure in polycrystalline materials, short-range atomic structure in disordered or amorphous materials, structural distortions, and any strain and crystal size induced changes to the structure
- it is complimentary to X-rays due to the neutrons‘ penetrative ability, light element sensitivity, isotope dependent scattering, and its magnetic interaction
- the ability of a neutron to penetrate materials allows the use of sophisticated sample environments - low and high temperatures, in electric and magnetic fields, and under varying pressure

NEUTRON POWDER DIFFRACTION CRYSTALLOGRAPHY

- Many pharmaceuticals contain predominately light elements such as H, D, C, N and O - the neutron's sensitivity to such elements and the difference in scattering between isotopes means neutron powder diffraction plays an important role in determining the structural features of such compounds

UNDERSTANDING MICROEMULSIONS AS DRUG DELIVERY VEHICLES

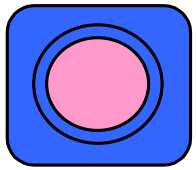
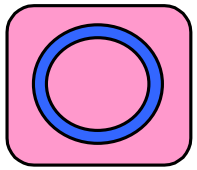
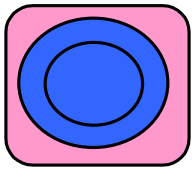


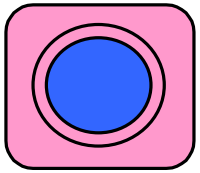
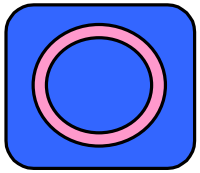
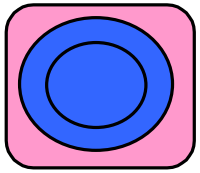
HYPOTHESIS – oil core acts as an additional locus of solubilisation of drug in the aggregate

OBSERVATION – small molecular volume oils exhibiting a good capacity for drug do not results in microemulsions with increased capacity for drug

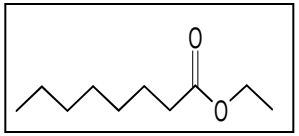
UNDERSTANDING – provided by SANS

MICROEMULSIONS AS DRUG DELIVERY VEHICLES

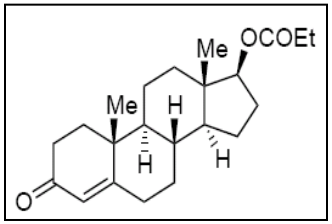
Compositions	Core	Shell	Droplet
Hydrogenated deuterated			
Oil	h-Oil	h-Oil	h-Oil
Surfactant (SAA)	d-SAA	d-SAA	h-SAA
Solvent	D_2O	H_2O	D_2O

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

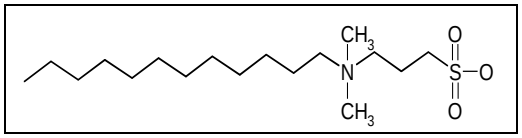
SANS DATA AND FITS OF OIL-IN-WATER MICROEMULSIONS WITH & WITHOUT DRUG



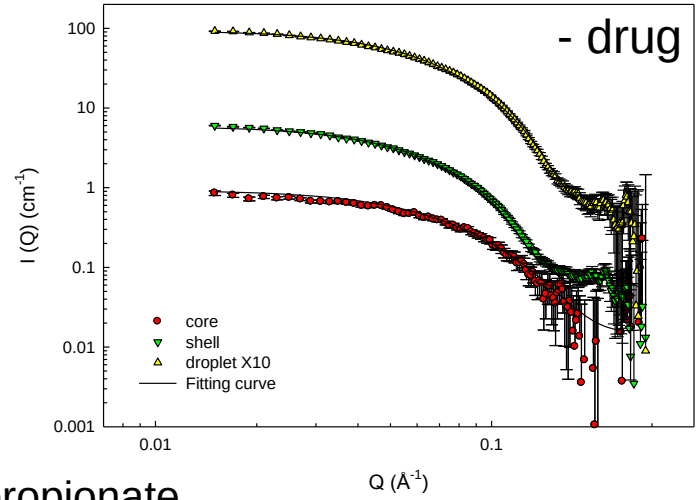
oil = ethyl caprylate



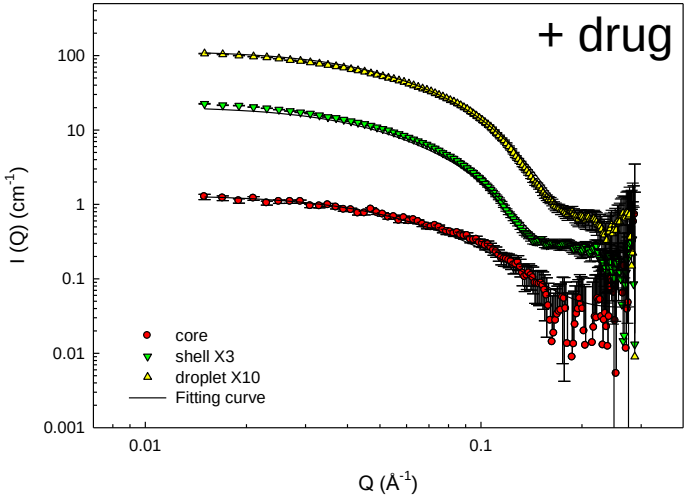
drug = testosterone propionate



surfactant = *N,N*-dimethyldodecyl-ammoniopropanesulfonate (DDAPS)



Data fitted using core-shell ellipsoid and hard sphere structure factor



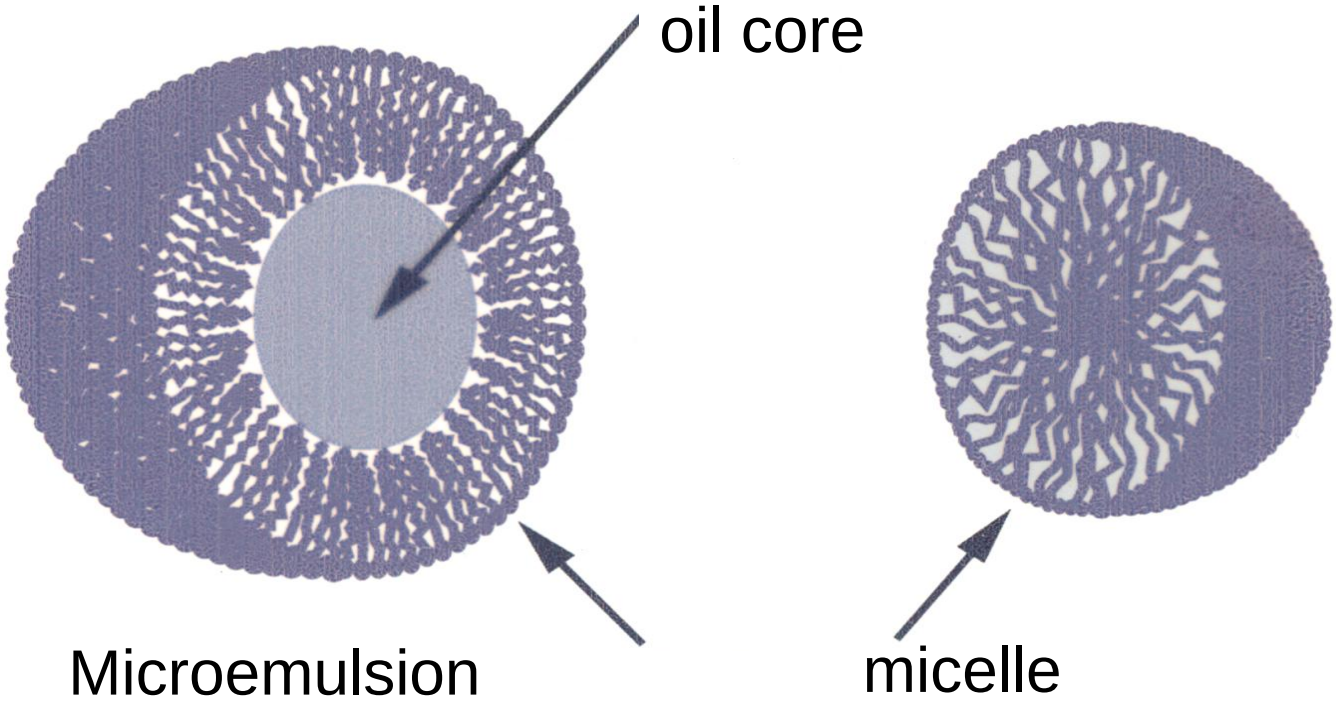
$$I(Q) = n \times P(Q) \times S(Q)$$

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

EFFECT OF OIL ON DRUG SOLUBILISATION

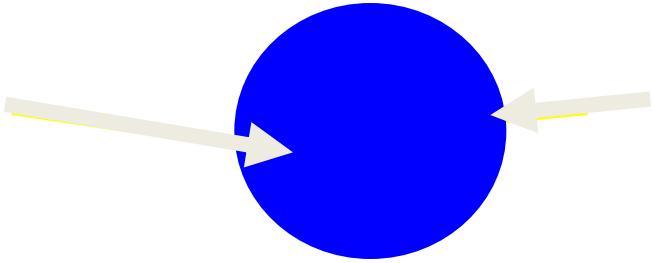
Larger molecular volume oils formed a distinct core in the centre of the microemulsion droplet, whereas smaller oils mixed intimately with the surfactant tails



oil = extra locus of drug solubilisation?

POLYMER-STABILISED DRUG NANOPARTICLES

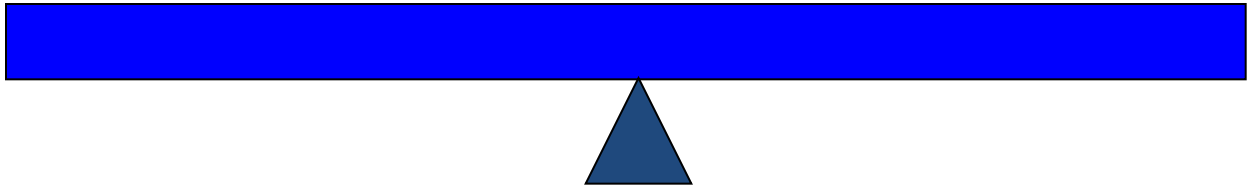
Crystalline drug nanoparticle



Hydrophilic polymeric stabiliser

low molecular weight

high molecular weight



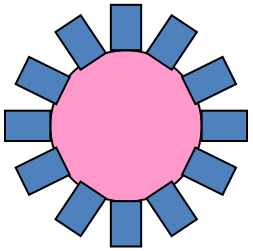
high drug loading

particle stability

NATURE OF ADSORPTION OF HYDROPHILIC POLYMER WITH MOLECULAR WEIGHT

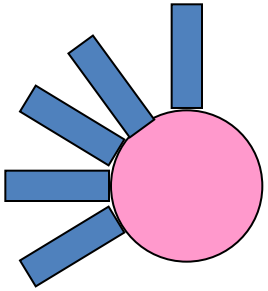
low molecular weight

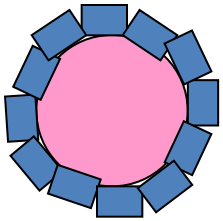
high molecular weight



End-on ‘brush’ conformation

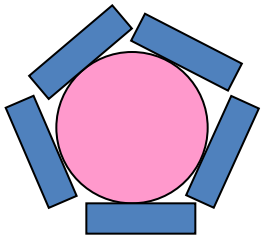
molecular weight dependent adsorption



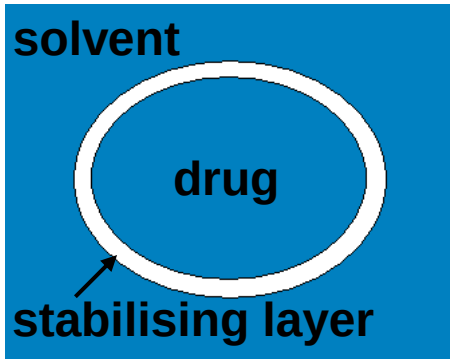


flat conformation

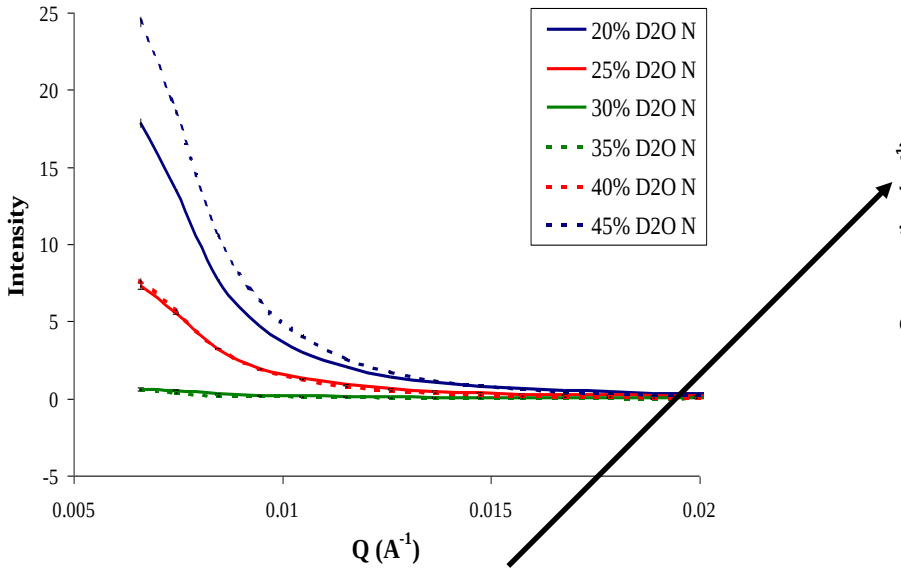
molecular weight in-dependent adsorption

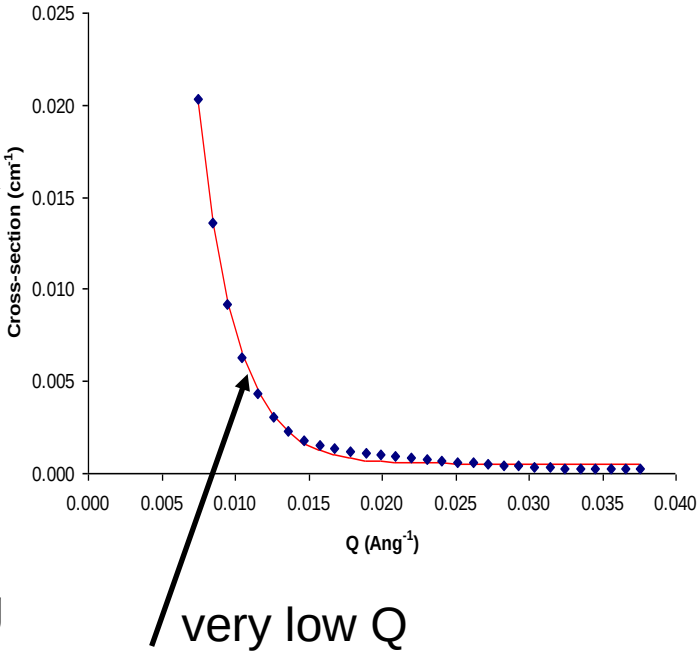


CONTRAST MATCHING OF DRUG NANOPARTICLES



- nanoparticles dispersed in H_2O/D_2O mix that makes the nanoparticles 'invisible' to neutrons
- (very weak) scattering seen only from stabilising layer



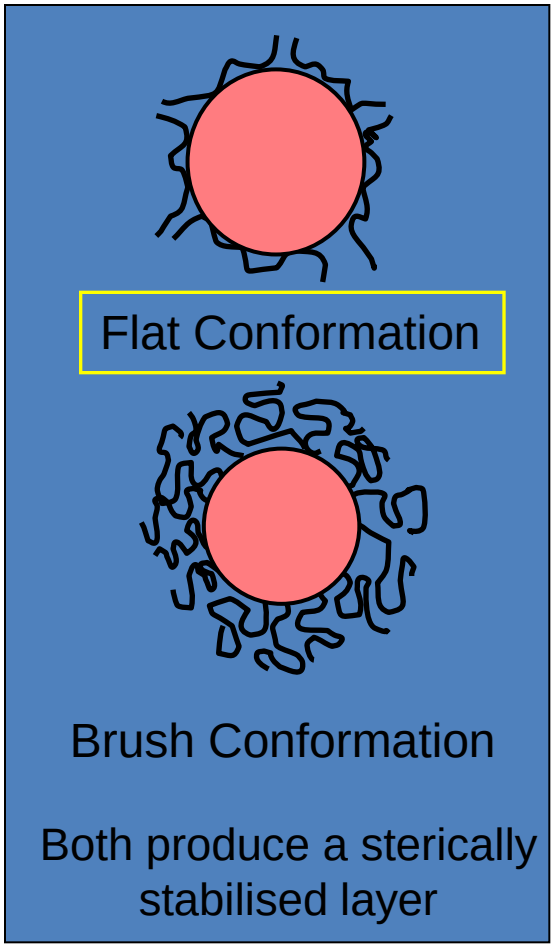


SANS RESULTS – NABUMETONE NANOPARTICLES

Polymer and Molecular Weight M_v (kg/mol)	σ (Å)	Γ (mg m^{-2})
HPC 110	80.4	11.4
HPC 95	76.0	11.6
HPC 80	78.9	11.6
HPC 65	76.5	11.0
HPC 55	78.3	11.0
HPC 45	80.3	11.3
HPMC 7	76.8	10.5
HPMC 5	80.0	10.8

σ second moment of the adsorbed layer thickness

Γ adsorbed amount of polymer



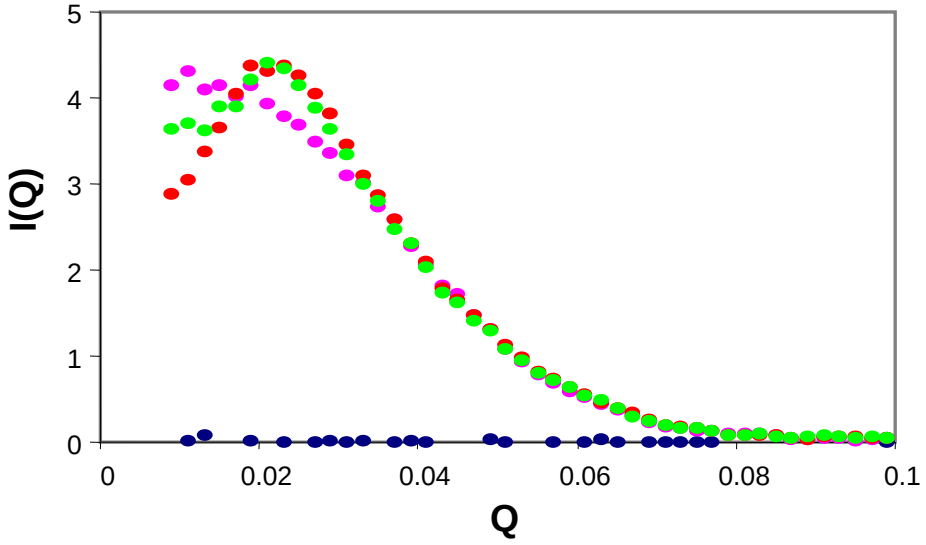
NANOSUSPOMICROEMULSIONS

- Contain both drug nanoparticles (**~ 300 NM**) and microemulsions (**~ 18 NM**): able to deliver two different drugs in one formulation
- *'Stable'* version of a suspoemulsion (currently used in agrichemical industry)
- Prepared from simple mixing of nanoparticles & microemulsions
- *In situ* measurement of microemulsion stability was possible using neutrons, preparation cloudy in appearance

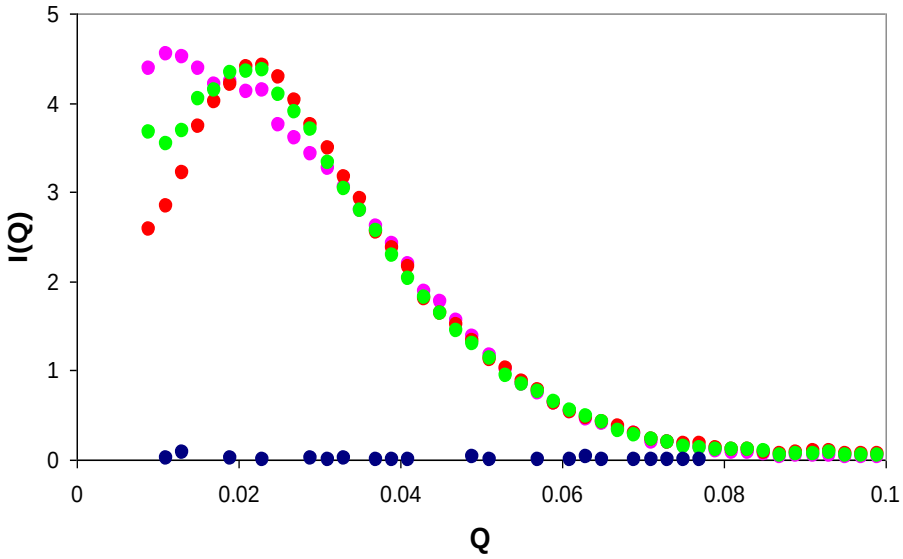
NANOSUSPOMICROEMULSIONS

2.4 vol% of Brij 97 m/e & 2.4 vol% of SDS stabilised griseofulvin nanoparticles

no testosterone propionate



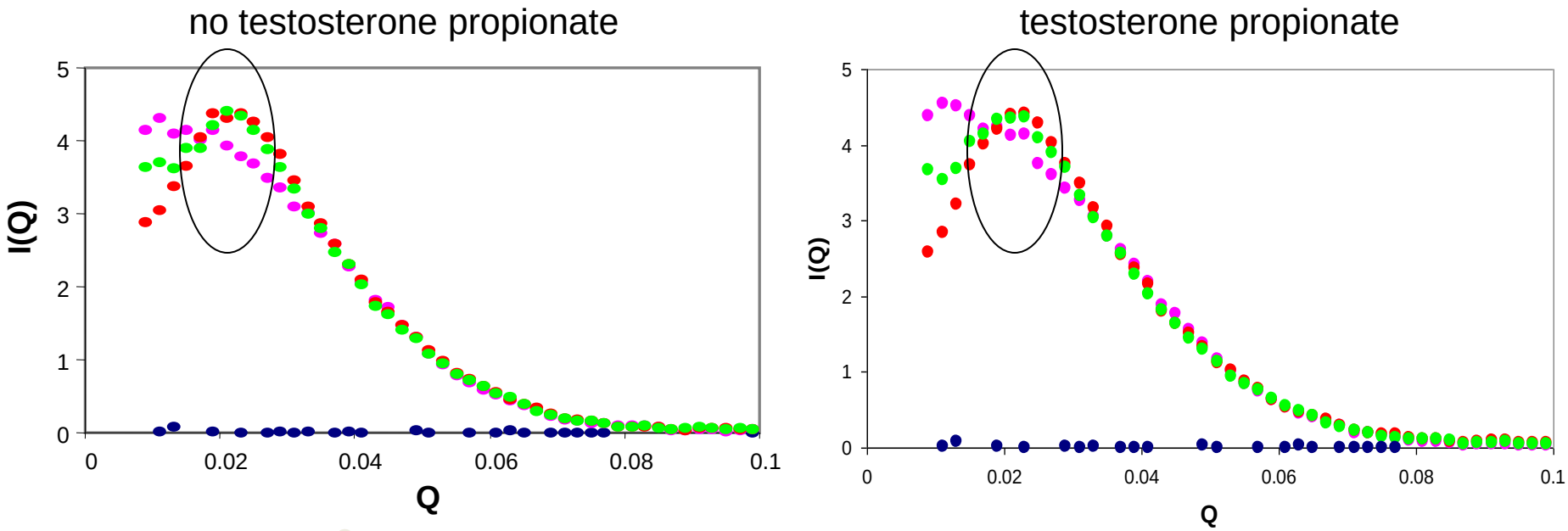
testosterone propionate



- nanoparticles
- microemulsions before contact with nanoparticles
- microemulsions immediately after contact with nanoparticles
- microemulsions after 24 h contact with nanoparticles

NANOSUSPOMICROEMULSIONS

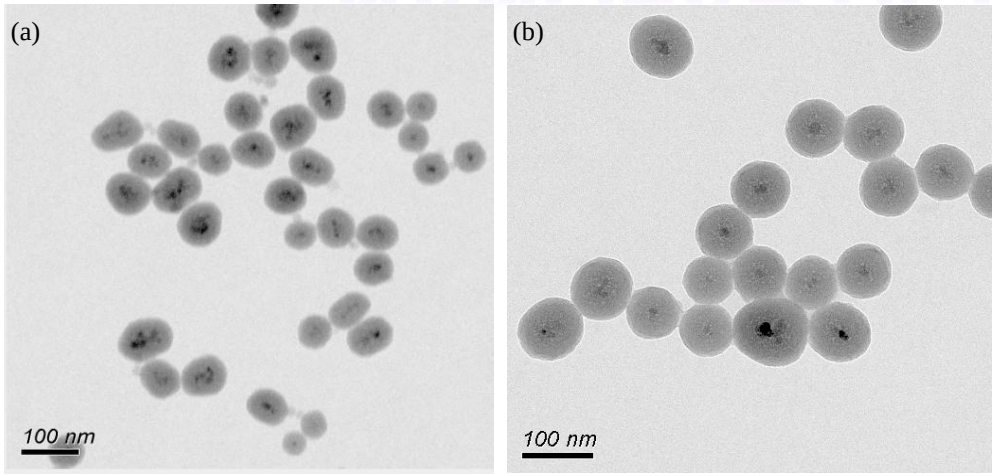
2.4 vol% of Brij 97 m/e & 2.4 vol% of SDS stabilised griseofulvin nanoparticles



- nanoparticles
- microemulsions before contact with nanoparticles
- microemulsions immediately after contact with nanoparticles
- microemulsions after 24 h contact with nanoparticles

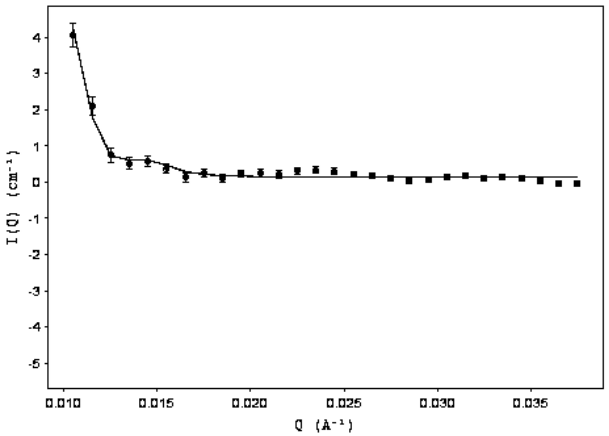
S(Q), interaction peak due to exchange of charged SDS from nanoparticles to m/e

MAGNETITE SILICA NANOPARTICLES

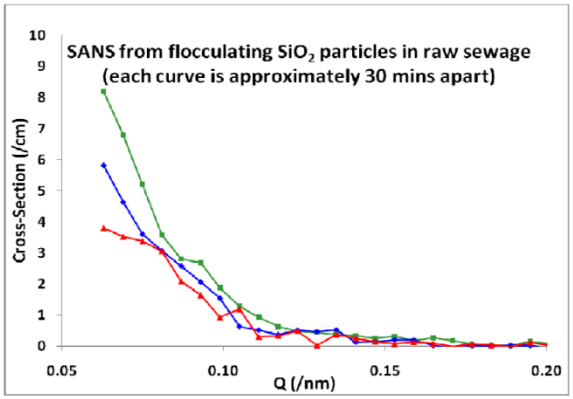


iron core allowed nanoparticles to be observed using SANS in the presence of biological material (raw sewage)

effect of surfactant stabilisers (e.g. Tween 80) on flocculation could be observed as a function of time



Static SANS measurement



Time resolved SANS measurement