Method Development in Bio-Deuteration: Towards Purification of Deuterated Lipopolysaccharides (LPS)

Luke Stephen Broughan, MBio Graduate

Student Seminar

30/07/2024

Neutron structural biology:

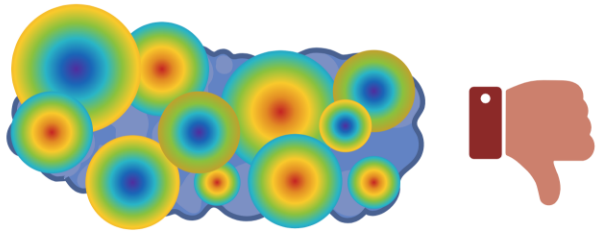
- X-ray scattering has been the workhorse of structural biology since the 1940s.
- Today, **neutron scattering** offers complementary advantages for biology…
  - **Deeply penetrative** of bulk samples of very large molecules (e.g. proteins).
  - **Non-destructive** – can be combined with HPLC, AF4, etc. *in situ*.
  - **Sensitive to light elements** – in biomolecules, H coordinates structure/function.
  - **H/D contrast** – **bio-deuteration** reduces background, allows contrast variation techniques.

Hémonnot & Köster (2017) *ACS Nano*
Haertlein *et al.* (2016) *Methods in Enzymology*
Ankner *et al.* (2013) *Current Protocols in Protein Science*

Deuteration for biological neutron scattering:

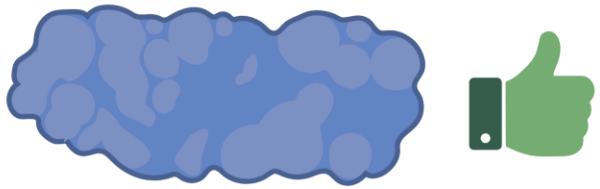
Deuteration for **crystallography**:

H-Molecule:



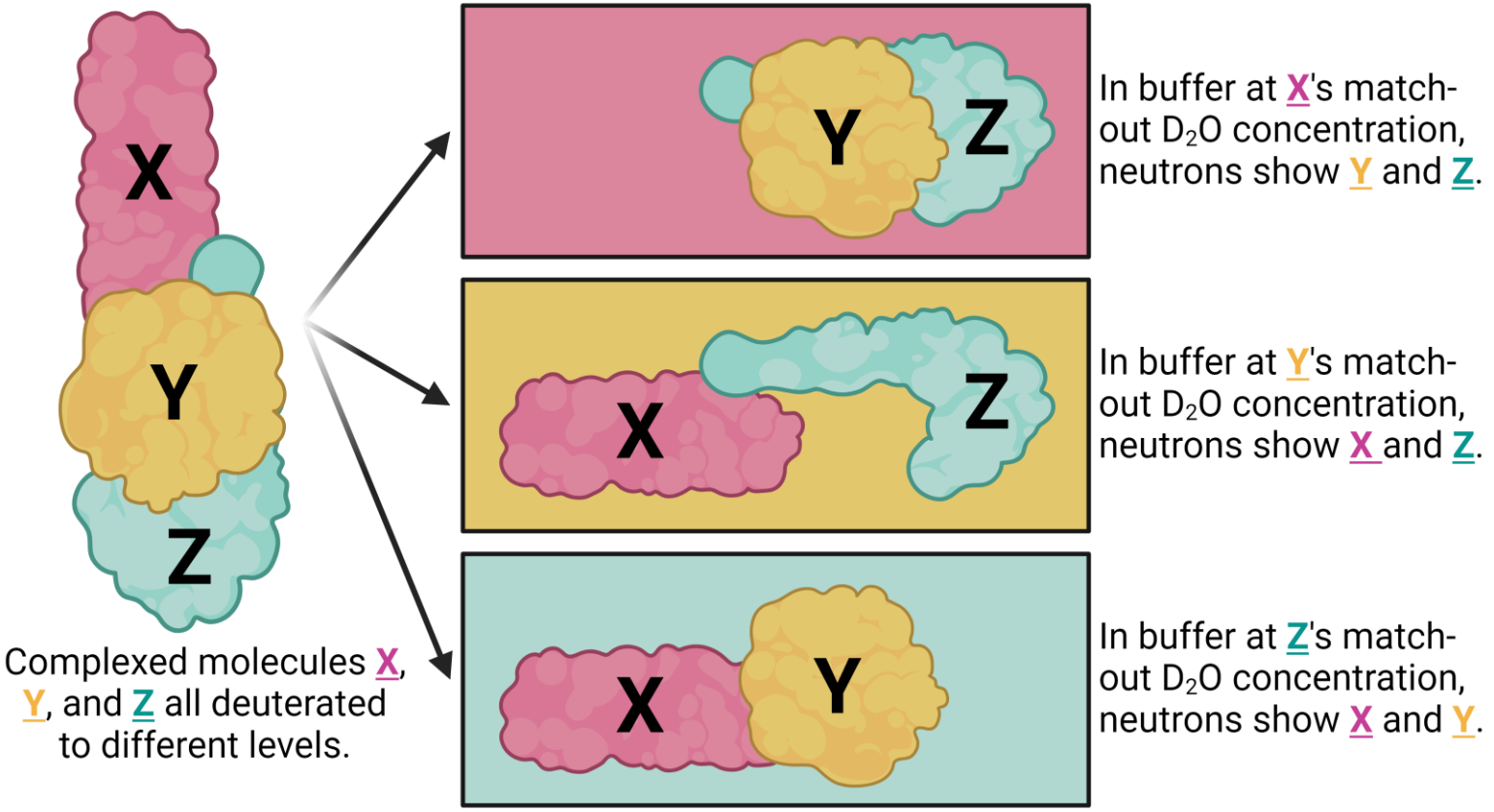
H scatters neutrons incoherently, obscuring informative scattering.

D-Molecule:



Replacing H with D eliminates incoherent background, restores detail.

Deuteration for **NR and SANS**:



Complexed molecules X, Y, and Z all deuterated to different levels.

In buffer at X's match-out D_2O concentration, neutrons show Y and Z.

In buffer at Y's match-out D_2O concentration, neutrons show X and Z.

In buffer at Z's match-out D_2O concentration, neutrons show X and Y.

Koruza *et al.* (2018) *Archives of Biochemistry and Biophysics*

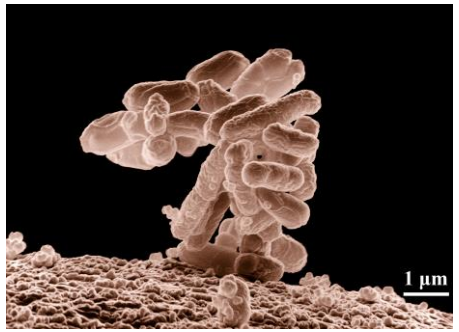
Created with BioRender.com.

Bio-deuteration and efficiency:

- Cells on D-media produce D-biomolecules much more efficiently than chemical approaches…
- …but D and D_2O are inhibitory *in vivo* – D-cell culture is challenging and poorly characterised.
- D-cell culture needs large amounts of D_2O, but we **recycle filtered D_2O** for certain biomass:

Bacterial deuteration
Escherichia coli

Fresh D_2O



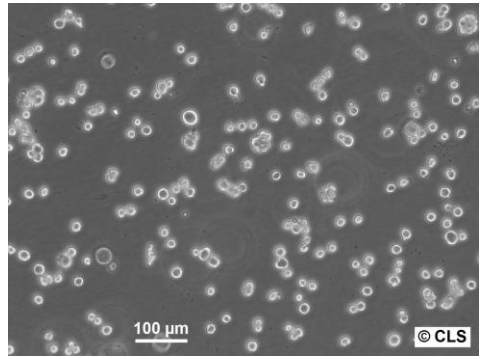
Membrane studies, protein production

Used D_2O

Algal deuteration
Botryococcus braunii

D-algal autolysate

Insect cell deuteration
Spodoptera frugiperda



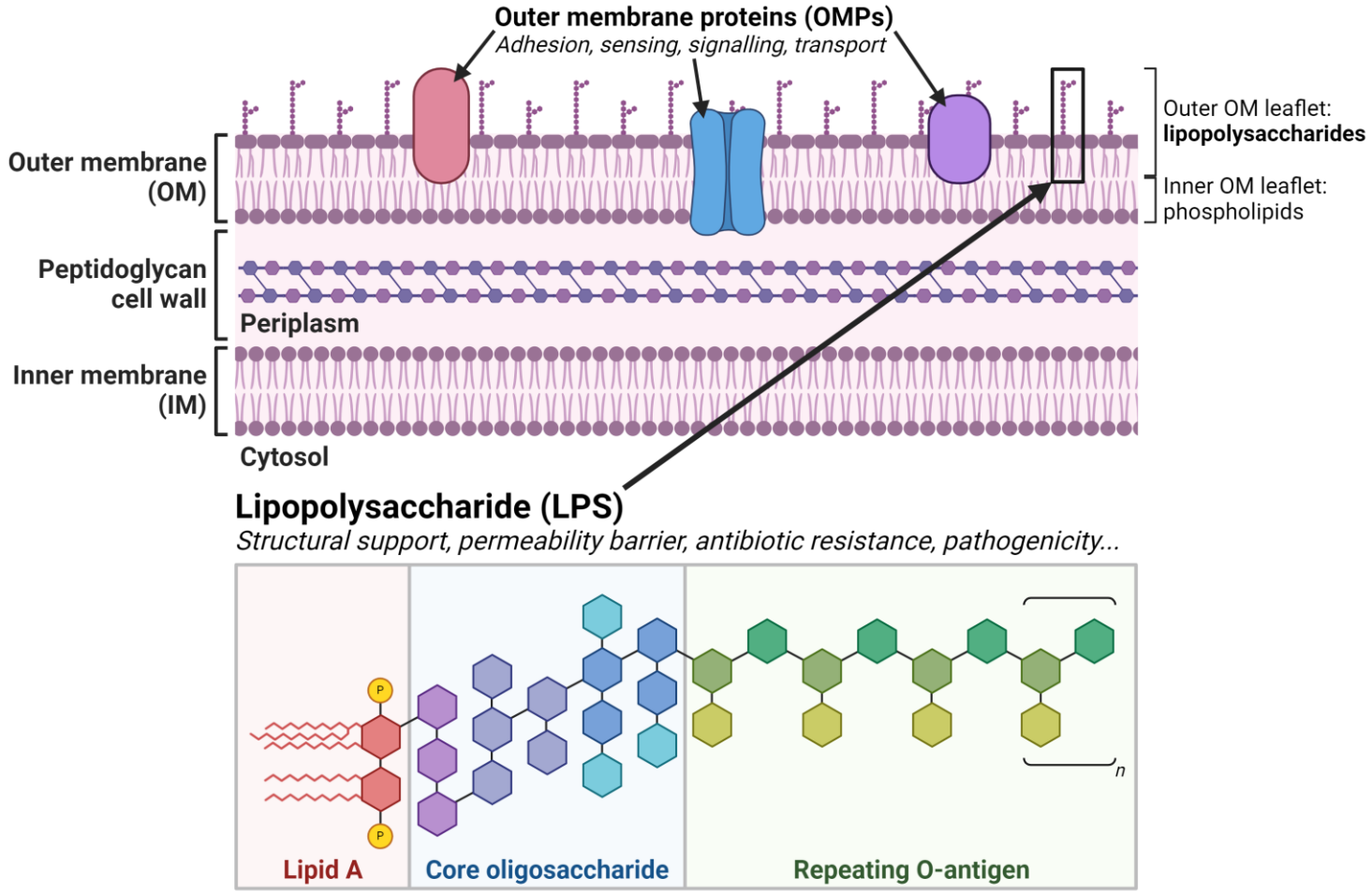
Protein production (baculovirus system)

D-algal autolysate

Ramaraj *et al.* (2016) *Emergent Life Sciences Research*

Bacterial membranes – what and why?

- Gram-negative bacteria are surrounded by an **inner membrane** (IM) and **outer membrane** (OM).
- The OM, with its outer leaflet of **lipopolysaccharides** (LPS), provides many survival and virulence functions.
- Structural studies of the Gram-negative cell surface (e.g. with cryo-EM, AFM) can answer fundamental biological questions as well as reveal interactions with antibiotics and the immune system.



Science and Technology Facilities Council
ISIS Neutron and Muon Source

www.isis.stfc.ac.uk
@isisneutronmuon
uk.linkedin.com/showcase/isis-neutron-and-muon-source

Le Brun *et al.* (2013) *Biomacromolecules*
Created with BioRender.com.

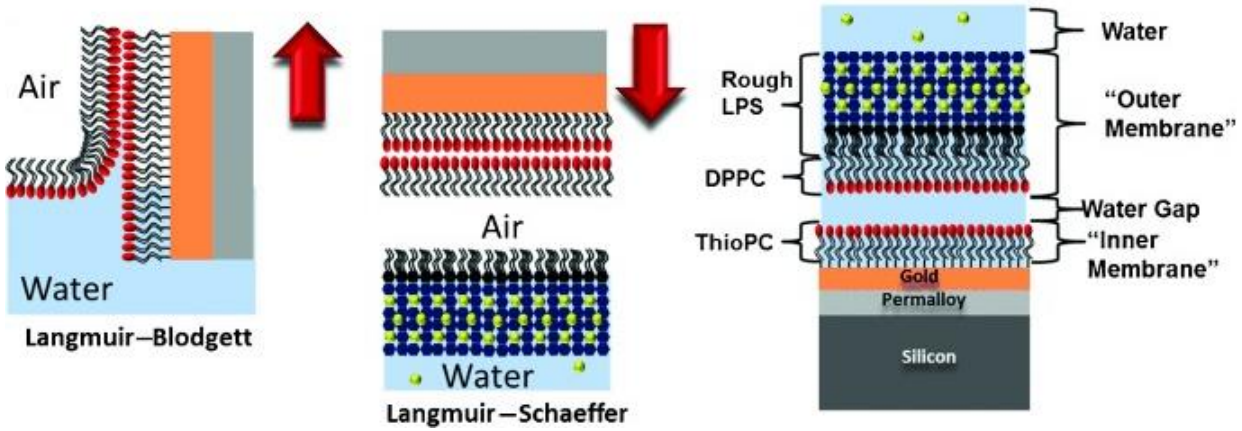
Adapted from Clifton *et al.* (2015) *Angewandte Chemie International Edition*

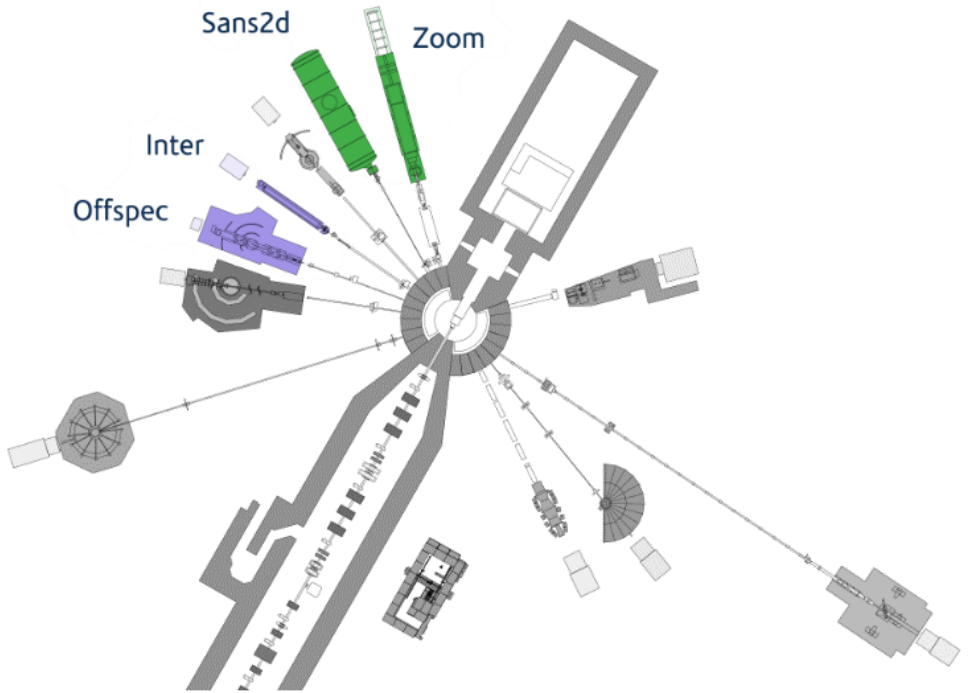
Scope of my project:

- **D-LPS** are of interest for contrast variation NR and SANS experiments on **supported lipid bilayers** that simulate the bacterial cell surface.

- This work began **bacterial bio-deuteration** at ISIS:

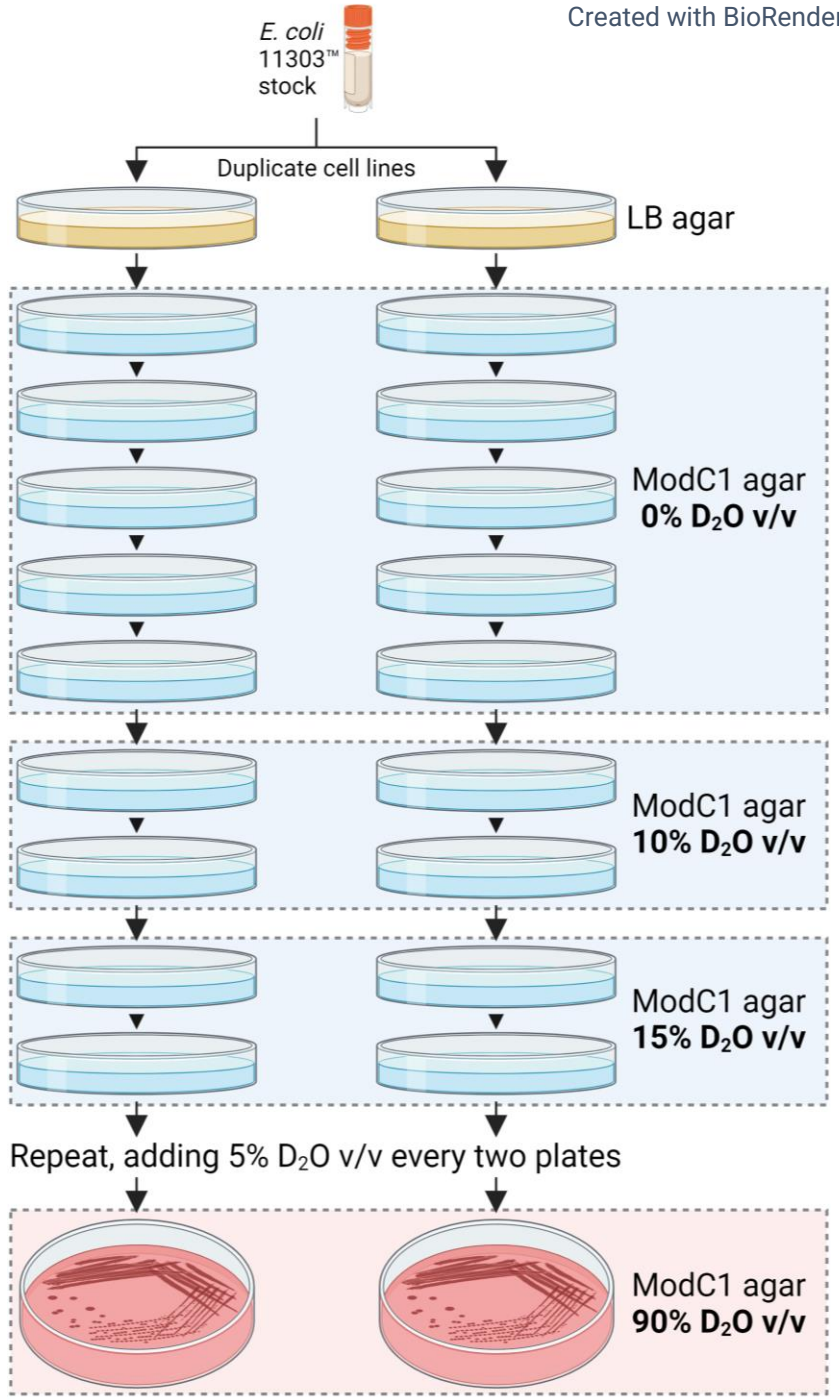
  1. Adapt *E. coli* to grow under deuterated conditions (live bio-deuteration).
  2. Measure growth and biochemistry of D-*E. coli* to optimise deuterated cell culture.
  3. Develop protocol to purify *E. coli* membranes and separate out the LPS-rich OMs.



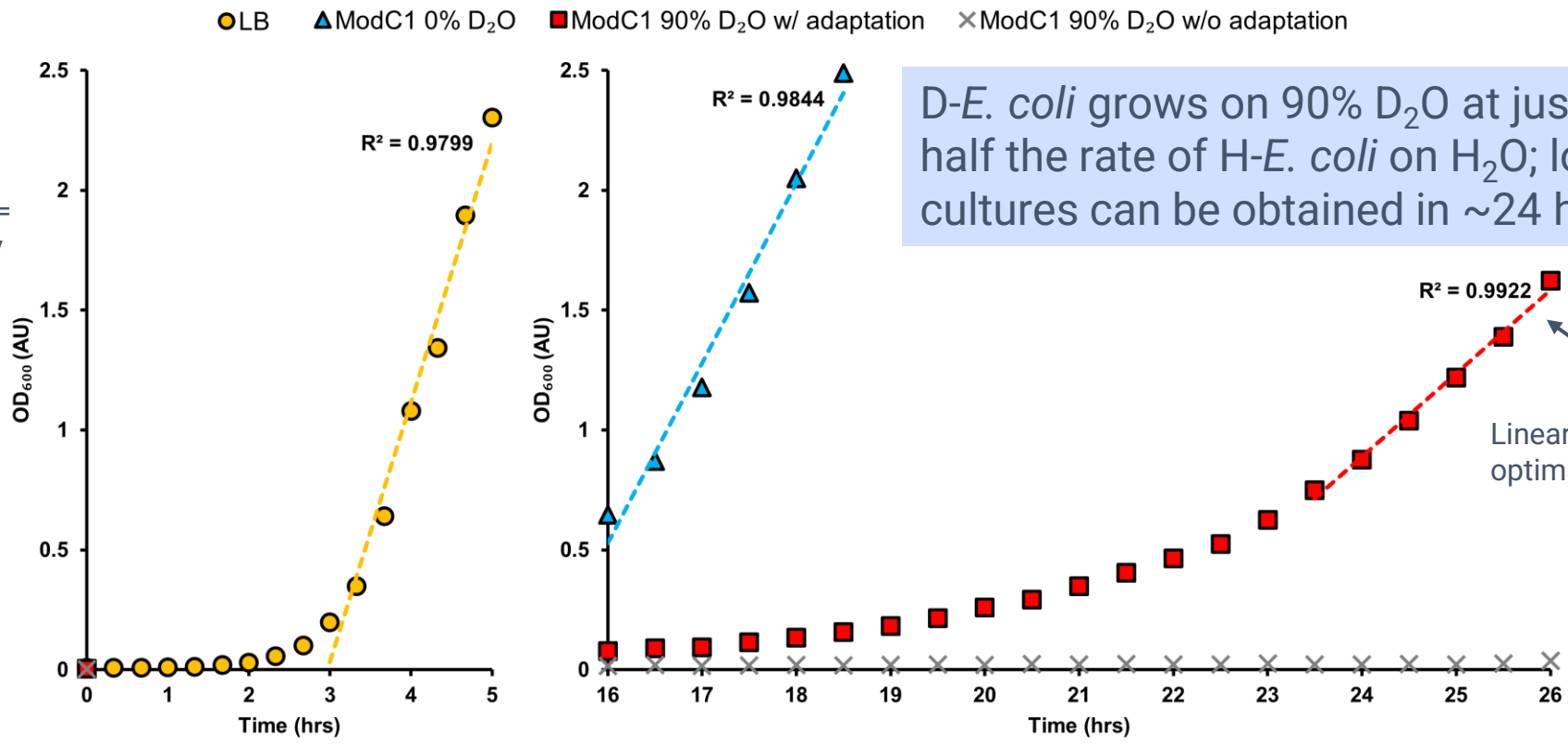


Bio-deuteration of *E. coli:*

- D/D_2O toxicity requires an **adaptation protocol** be devised and performed – stepping up D_2O exposure.
- *E. coli* (ATCC 11303™) was subcultured through a series of agar plates containing a minimal nutrient medium (ModC1) formulated with stepwise greater proportions of D_2O.
- **Selection pressure** for D tolerance – by the end, clones surviving on ModC1 **90% D_2O** were obtained.
- **"D-*E. coli*"** were harvested and preserved.



Characterising kinetics of D-*E. coli:*



Absorbance at 600 nm = bacterial culture density

D-*E. coli* grows on 90% D_2O at just over half the rate of H-*E. coli* on H_2O; log phase cultures can be obtained in ~24 hrs.

Linear region = log phase, optimal for LPS extraction

	Lag (hrs)	Growth rate (hrs^{-1})	Doubling time (mins)
H-*E. coli* on LB (rich media)	2.98	1.23	33.75
H-*E. coli* on ModC1, no D_2O	15.3	0.55	75.82
D-*E. coli* on ModC1, 90% D_2O	**24.43**	**0.31**	**134.72**
H-*E. coli* on ModC1, 90% D_2O	☠	☠	☠

Science and Technology Facilities Council

ISIS Neutron and Muon Source

Characterising biochemistry of D-*E. coli:*

- **API 20E** = biochemical assay panel designed for enterobacterial metabolic characterisation.
- Characterised *E. coli* grown on 0/30/60/90% D_2O, under both H_2O and D_2O assay conditions.

In H_2O:

In D_2O:

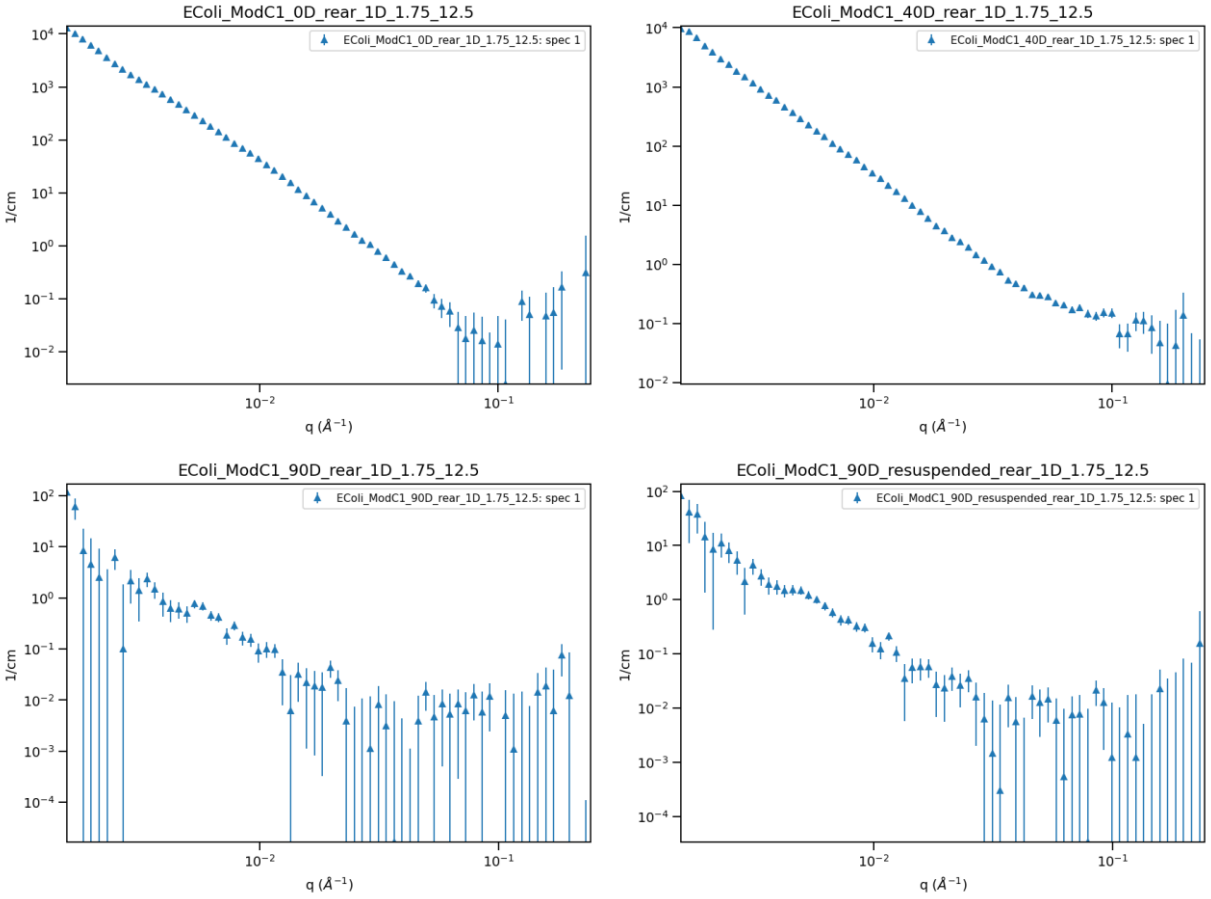
β-galactosidase **Lysine decarboxylase**

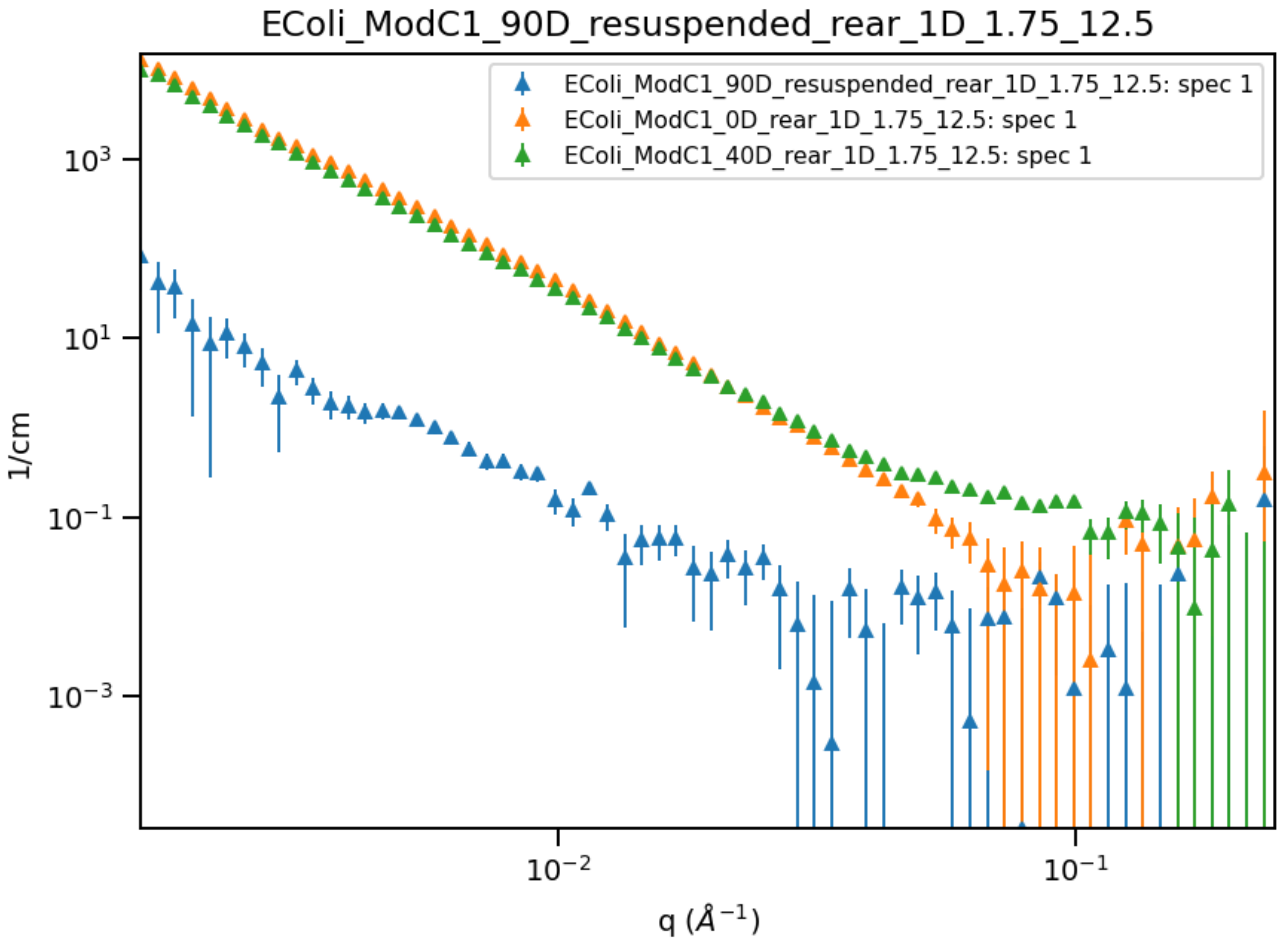
- LDC may be unable to utilise D due to greater C-D bond strength, disrupting pH/pD homeostasis.
- Likely causes denaturation of ONPG – big implications for recombinant protein production, and a strategy to further support D-cell culture…?

Juers *et al.* (2012) *Protein Science*
Chen (2022) *Deuterium Oxide and Deuteration in Biosciences*
Tomizawa *et al.* (2016) *Biochimie Open*

SANS2D Xpress measurements of D-*E. coli:*

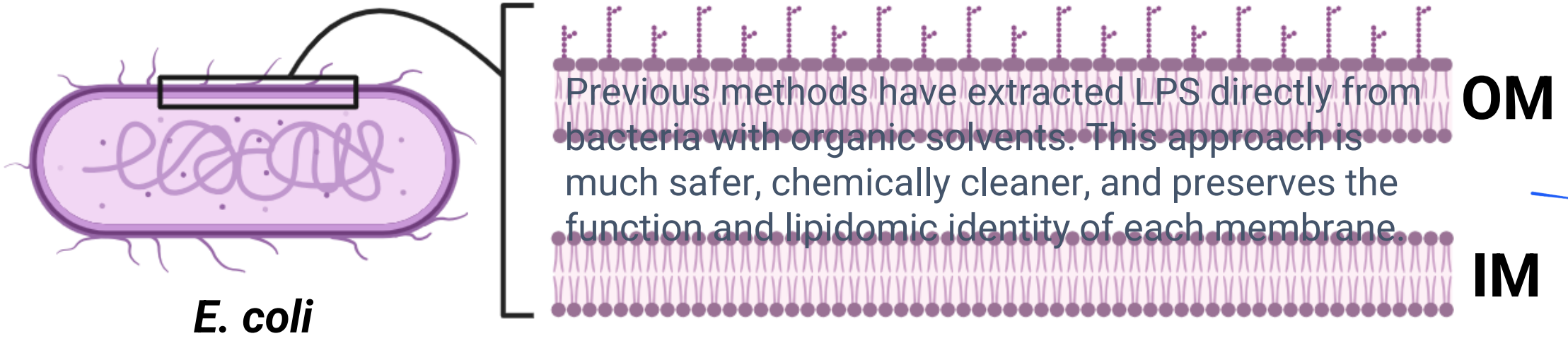
- Measured *E. coli* cultured on ModC1 0/40/90% D_2O at very high OD (>2.5, stationary phase).
- SANS2D with rotating rack, max RPM, ambient temperature, detectors at 12 m.





A new protocol for bacterial membrane fractionation:

Reminder! The aim was to purify total membranes (OMs + IMs), then OMs and IMs from each other…

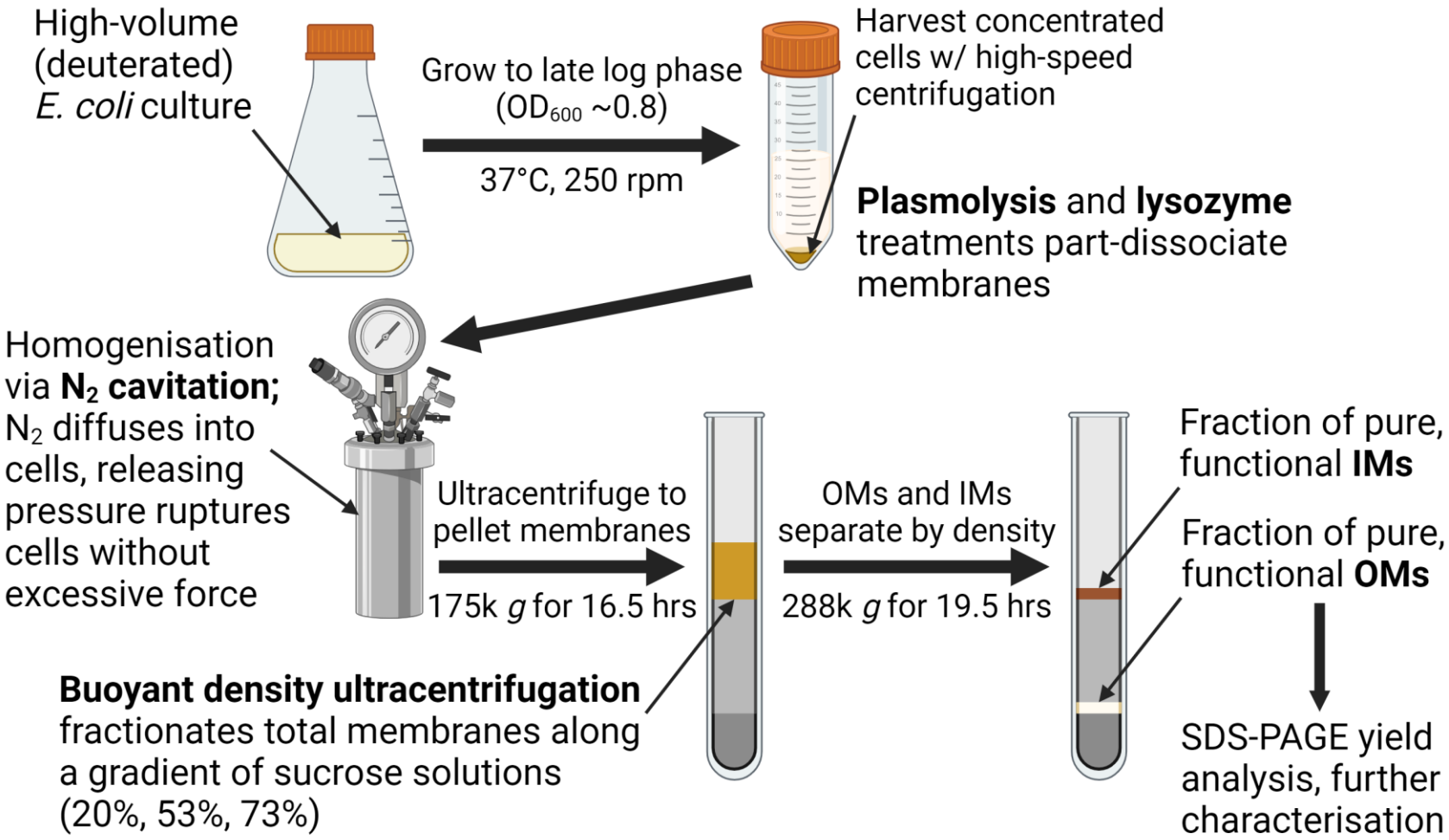


Rezania *et al.* (2011) *Avicenna Journal of Medical Biotechnology*
Ahamad & Katti (2016) *Journal of Microbiological Methods*
Shu & Mi (2023) *Bio-Protocol*

Created with BioRender.com.

A new protocol for bacterial membrane fractionation:

- Based on Cian *et al.* (2020).
- Modified for compatibility with this *E. coli* strain and available BioLab equipment (Parr cell disruption vessel).
- Trialled with H-*E. coli* on LB, now applying to D-*E. coli* on ModC1 90% D_2O.
- Up to 3 L processed per run.
- 3-5 days end-to-end.

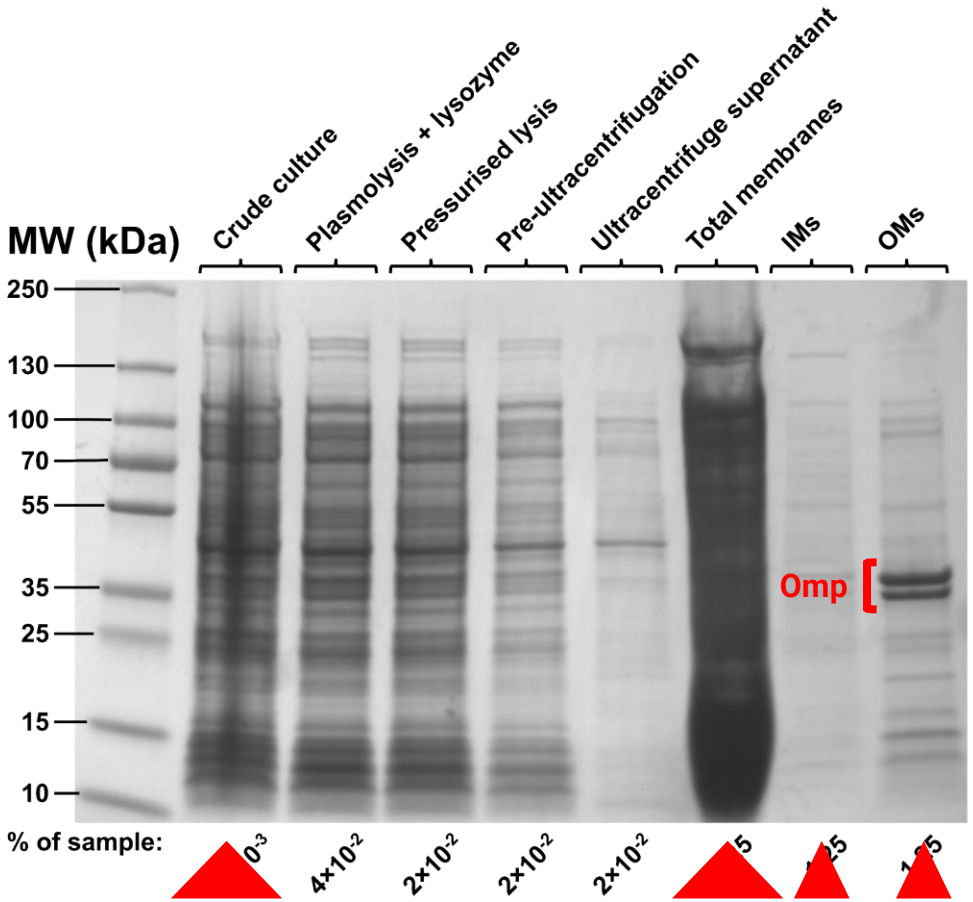


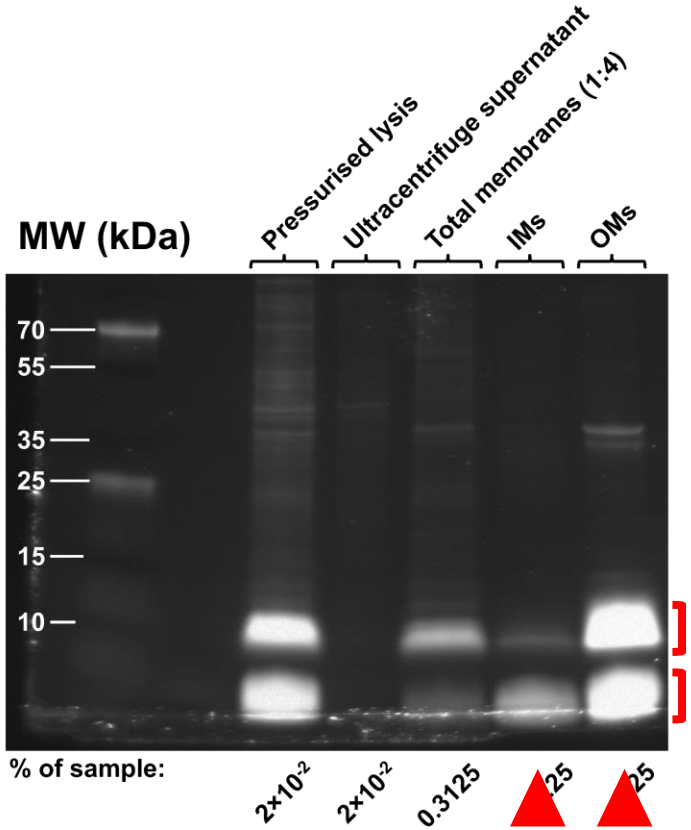
Science and Technology Facilities Council
ISIS Neutron and Muon Source
www.isis.stfc.ac.uk
@isisneutronmuon
uk.linkedin.com/showcase/isis-neutron-and-muon-source

Cian *et al.* (2020) *Journal of Visualised Experiments*
Created with BioRender.com.

Characterising membrane yields:

SDS-PAGE to visualise **proteins (left)** and **LPS (right)** from samples throughout purification:





Progressive clarification until co
of proteins in total membrane e

Distinct
discrete

Clean prep of highly concentrated LPS in OMs, ready for reconstitution into synthetic membranes on beamline.

Nakao *et al.* (2013) *PLoS One*
Pupo *et al.* (2020) *The FEBS Journal*

Further steps:

- Characterisation of D-*E. coli* and products on SANS2D – preliminary Xpress completed.
- Now applying scaled-up membrane prep to D-*E. coli* and preparing D-membranes – further characterisation with SDS-PAGE and HPLC.
- Potential to deuterate other bacterial strains via this method to expand the range of purified bacterial D-biomolecules available.
- Further development of the bio-deuteration production lines in the ISIS BioLabs.

Luke Stephen Broughan
Bio-Deuteration Support Scientist
Luke.Broughan@stfc.ac.uk

Peixun Li
Science Lead, D-Lab
Peixun.Li@stfc.ac.uk

Ludmila Mee
Manager, BioLabs
Ludmila.Mee@stfc.ac.uk

Sophie Ayscough (ESS/ISIS), Yao Chen, and Kun Ma for LPS guidance; Zoë Fisher (ESS), Hanna Wacklin-Knecht (ESS), and Karyn Wilde (ANSTO) for deuteration guidance; Sarah Langham and Rachel Pearce for technical support; Mingrui Liao (Manchester) for methodological advice; Rob Lillywhite (Warwick), Marion Patel (Warwick), and Sarah Youngs for administrative support; Najet Mahmoudi for SANS2D access and expertise; the Research Complex at Harwell for use of their facilities; James Taylor for risk assessment guidance; Robin Thorn (UWE) for microbiology advice.