SAXS and SANS investigations of membrane proteins in solution

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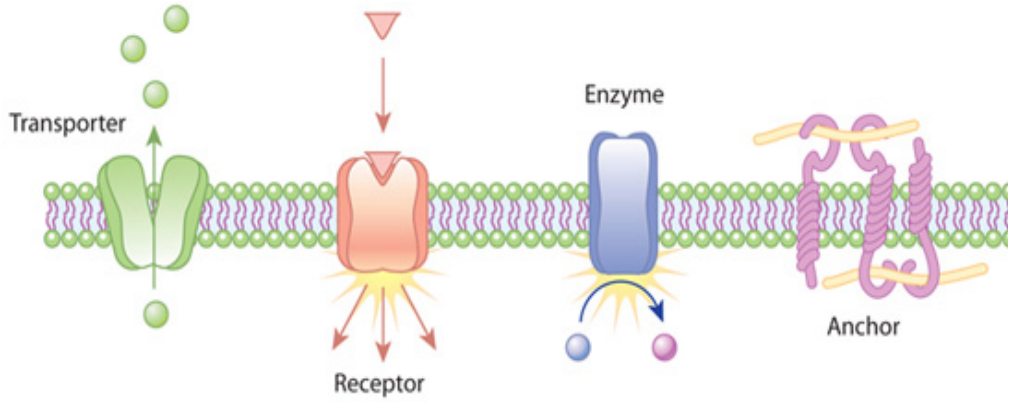
Membrane Proteins



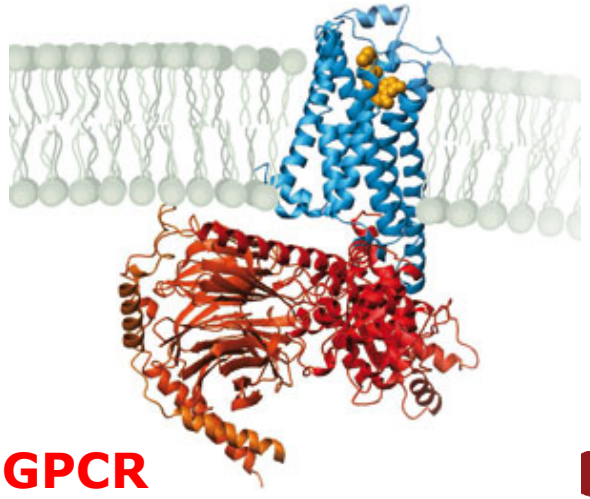
- ~**30%** of the proteins in the cell
- ~**50%** of all drug targets today
- Extremely challenging to study
- **80 000** protein structures in PDB

  ~**366** Unique membrane proteins

(2012-11-19)

The Nobel Prize in Chemistry 2012

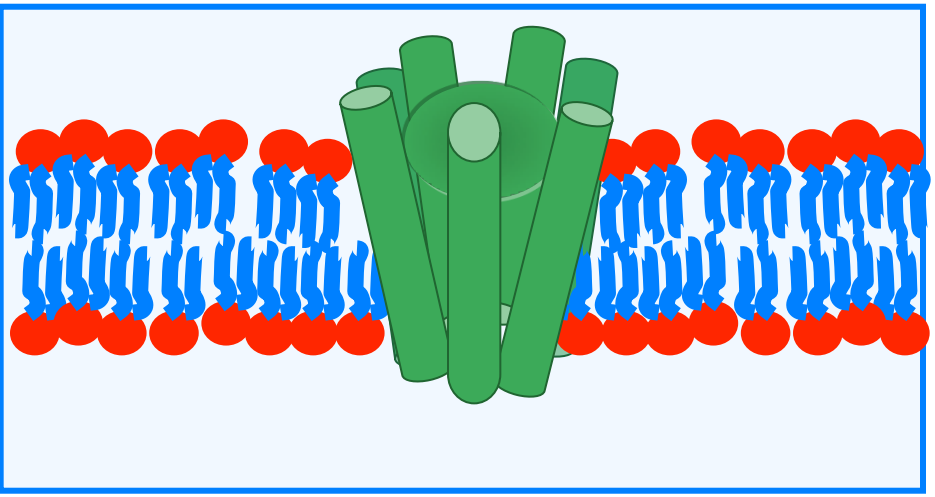
Robert Lefkowitz and Brian Kobilka



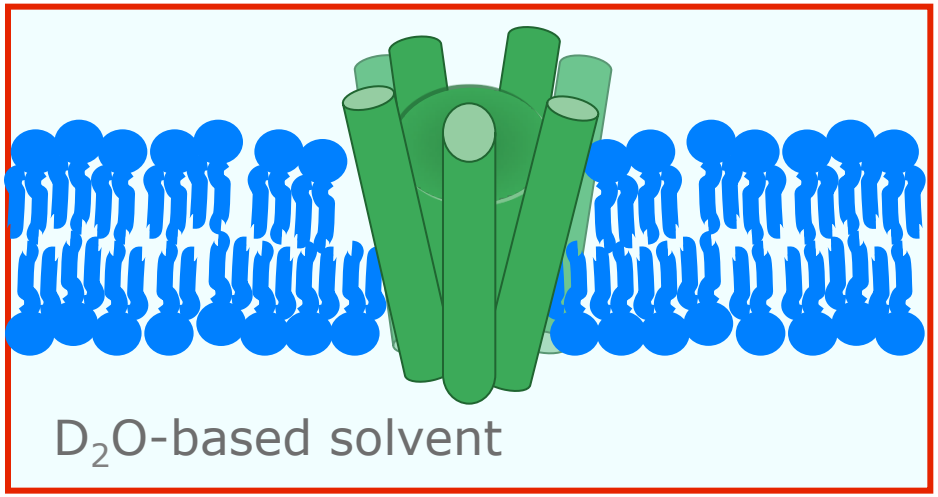
GPCR

Membrane proteins, neutrons and X-rays

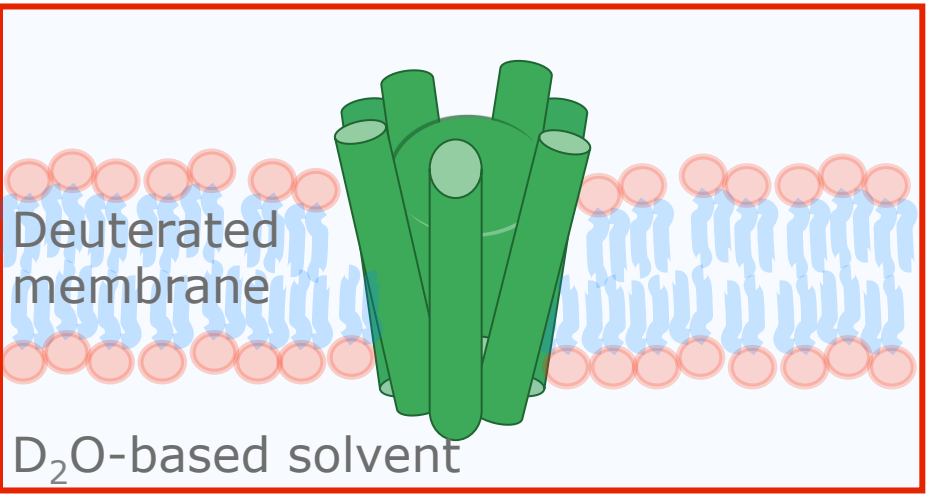
X-ray contrast



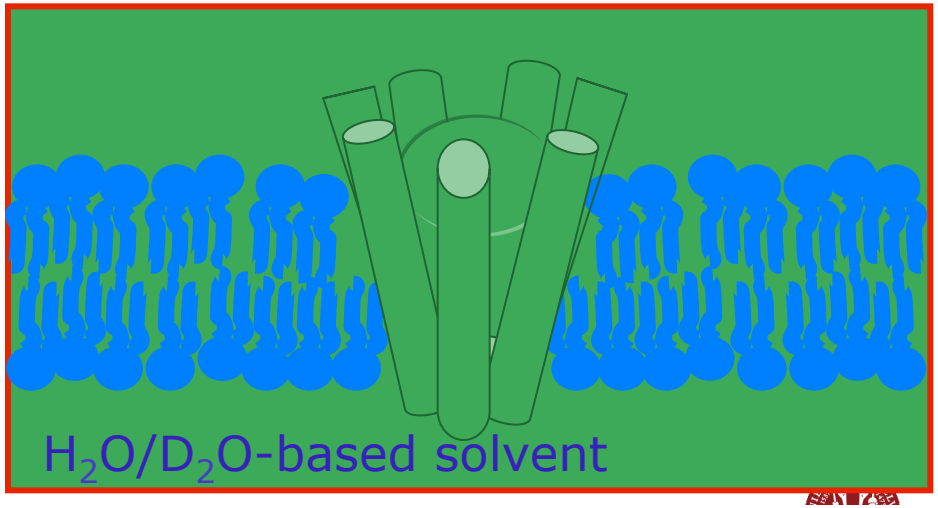
Neutron contrast 1



Neutron contrast 2



Neutron contrast 3

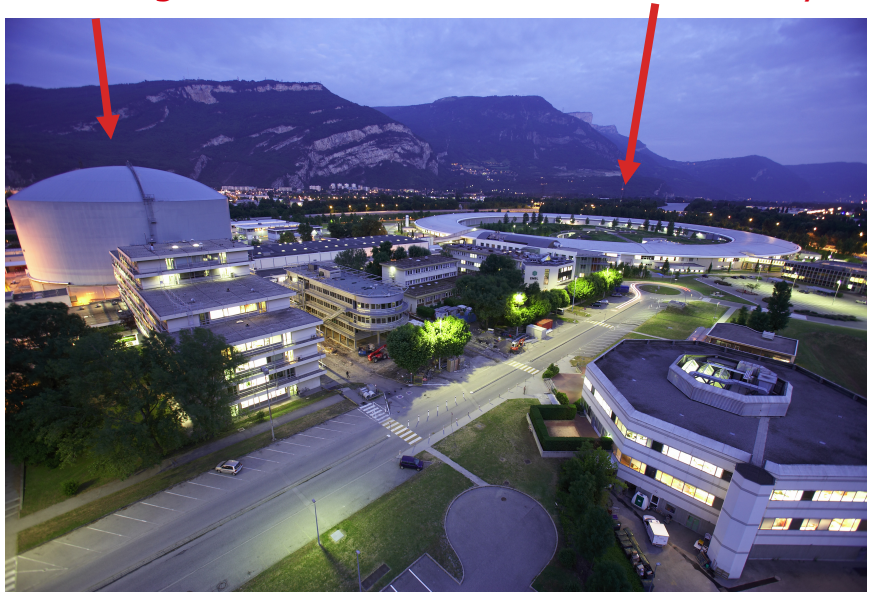


Neutrons give the possibility of **not** seing everything at the same time…. X-rays help assembling the whole picture

Frequent users at large scale facilities

ILL: Institut Laue Langevin

ESRF: European Synchrotron Radiation Facility



Grenoble, France

ESRF/ILL: Allows for investigating the same samples with both neutrons and X-rays, and at the same time!

But we also go to:
Max-Lab, Lund (SAXS)
Paul Scherrer Institute, Zürich (SANS and SAXS)
EMBL, Hamburg (SAXS)
Soleil, Paris (SAXS)
FRM-II, Munich (SANS)

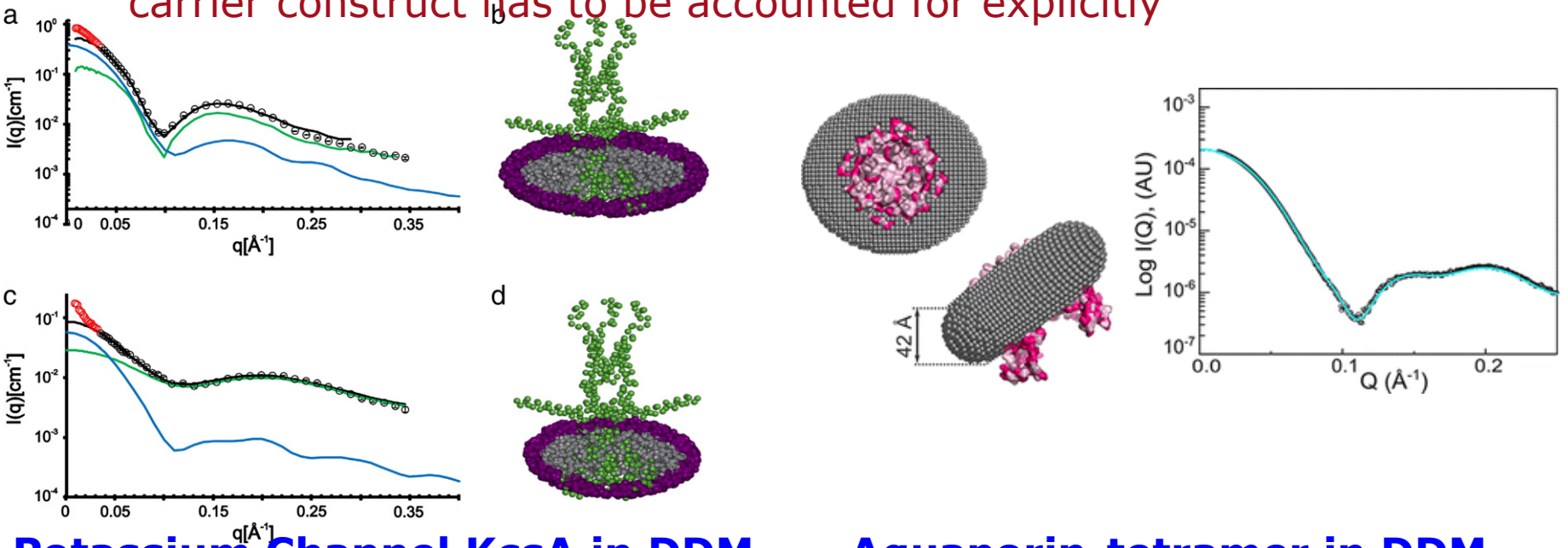
+Measure at in-house SAXS instrument

Grenoble, May 2013: *Just finished SAXS(24h)/ SANS(24h) experiment in Grenoble. From lunch right after successful termination of SANS experiment*

Challenges associated with SAXS/SANS studies of membrane proteins

-Sample handling is more difficult than for water soluble proteins

-SAXS/SANS data analysis is non-trivial because the entire MP + carrier construct has to be accounted for explicitly



Potassium Channel KcsA in DDM micelles.

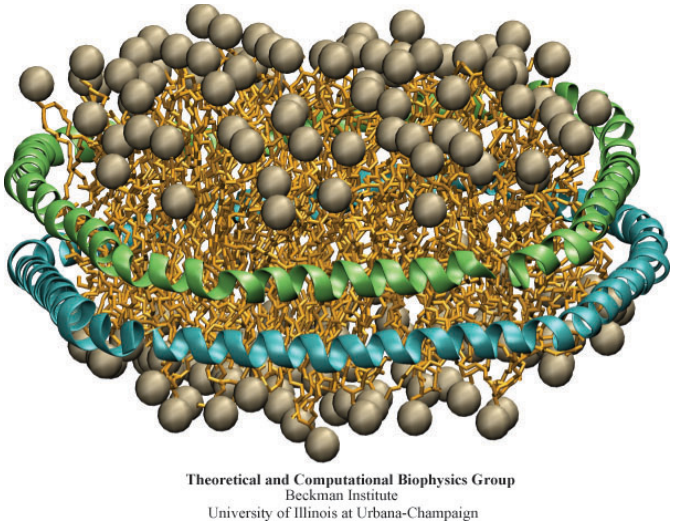
Calcutta, Jessen, Behrens, Oliveira, Pedersen et al, Biophysica Biochem Acta, 1818, 2290, ***2012***

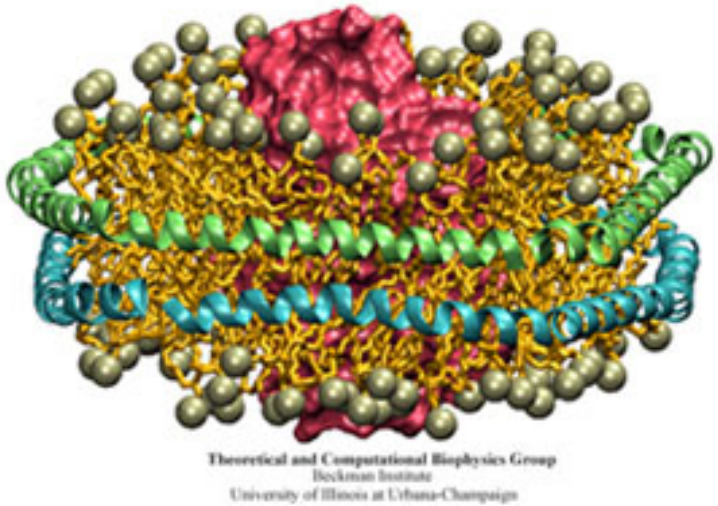
Aquaporin-tetramer in DDM micelles.

Berthaud, Manzi, Perez, Mangenot, JACS, 134(24), 10080, ***2012***

See recent review on SANS and membrane proteins in Breyton et al, Eur Phys. J. E, 36(71), **2013**.

Nanodiscs – Empty and membrane protein loaded:





What: Phospholipid ”disc” stabilized by two amphipatic protein belts *(His-tagged 220 AA 8-alpha helical structure)*

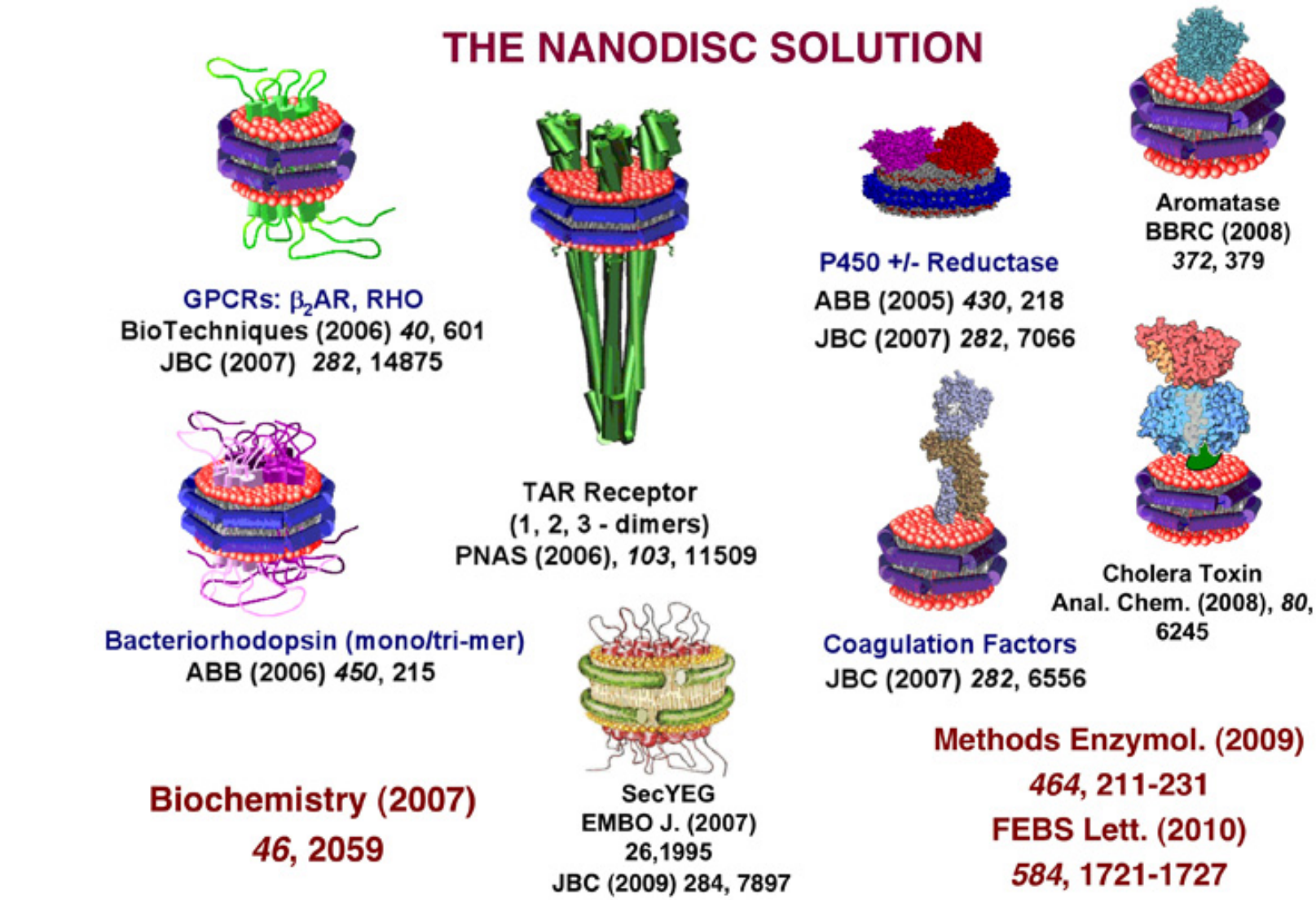
-Nanodiscs are very interesting in their own right and from a membrane physics point of view

-But **really** interesting as a ”sample-holder” for structural and functional studies of membrane proteins

System developed by Sligar *et al*, based on the Human Apo-A1 system (HDL). *[Bayburt, Grinkova, Sligar, Nano Lett, 2002]*

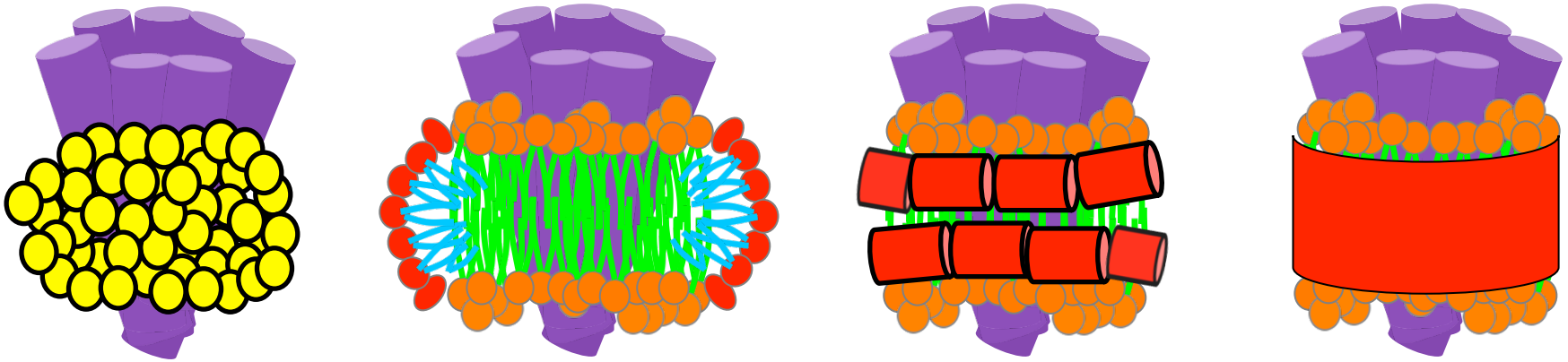
From the Sligar lab homepage:

http://sligarlab.life.uiuc.edu/nanodisc.html



NB: Almost all the studies are functional studies.
Important… but also a lot easier than structural studies

Different means of reconstituting membrane proteins



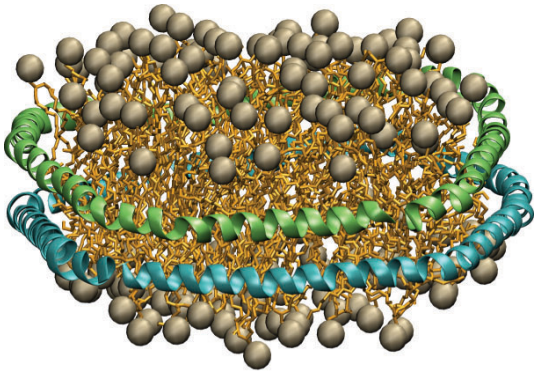
-Simple reconstitution
-Non-native lipid environment
-Polydisperse =>Less optimal for SAXS/SANS analysis

-Complicated reconstitution
-More native-like lipid environment
-Monodisperse => Optimal for SAXS/SANS analysis

The monodispersity of Nanodiscs and their native-like lipid environment make them ideal as molecular sample holders of membrane proteins for structural characterization *(In theory)*

Research goal: Develop phospholipid nanodiscs into a platform for Small-angle scattering based low-resolution structural studies of membrane proteins

Main targets:



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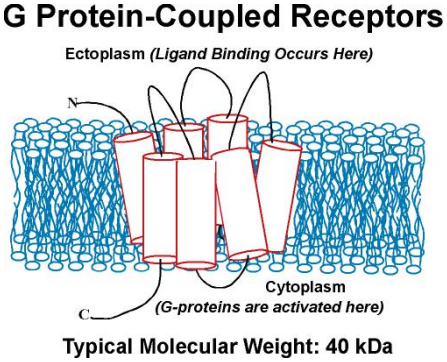
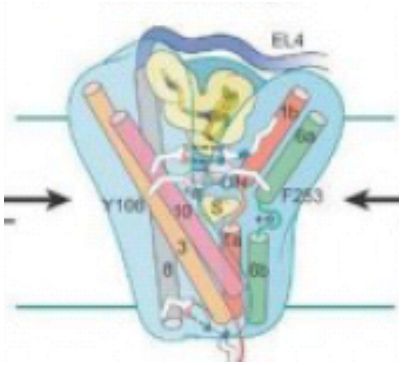
Central challenges:
Incorporation of Membrane proteins into Nanodiscs: Obtaining sufficiently "pure and well-defined" samples.

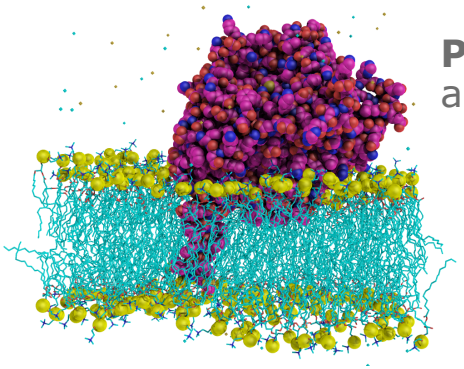
Data analysis: Development of experimental and computational methods to extract relevant structural information about the membrane proteins.



GPCR's: 7 TM's (Use Bacteriorhodopsin in the development phase)

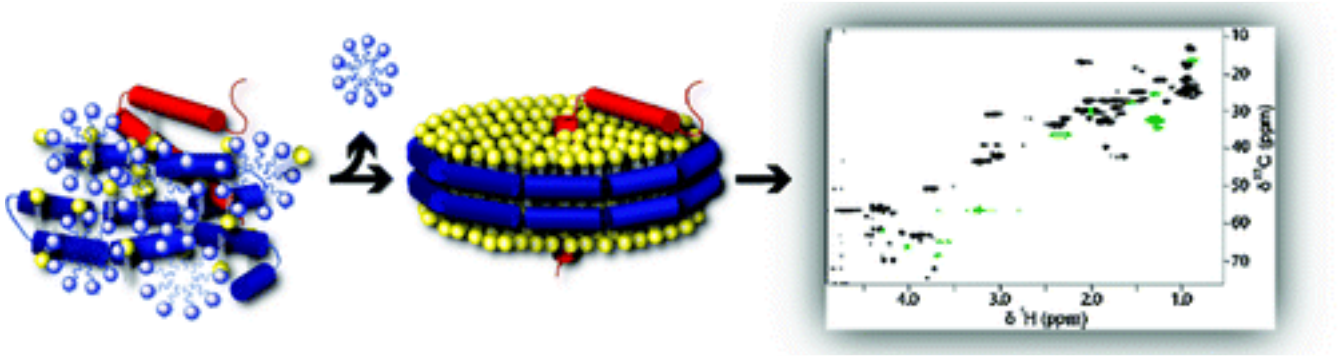


Leucine transporter: 12 TM's

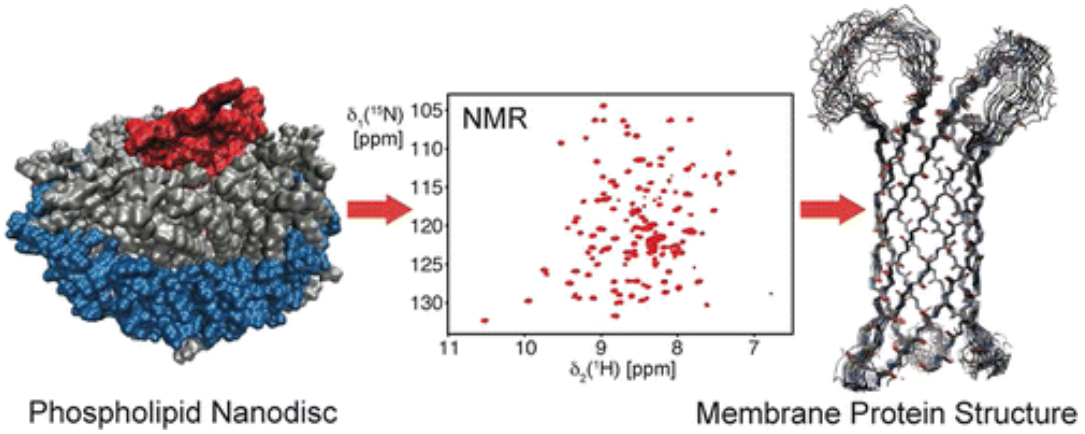


P450: Membrane anchored protein's

Parallel development of the nanodisc system for NMR based studies of membrane proteins *(not covered here)*



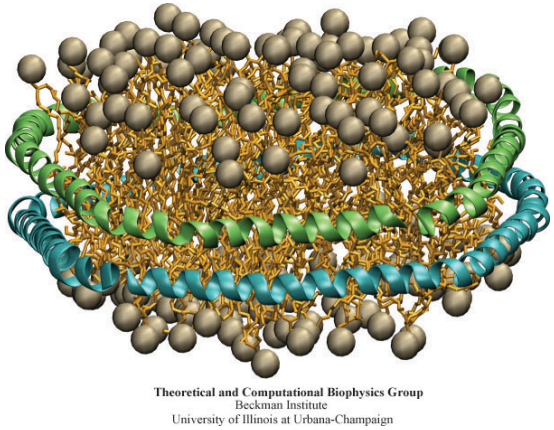
Glück et al, JACS, 2009, 131, pp 12060–12061
Proof of concept 1: *Solution NMR (^{1}H-^{13}C) study to determine the structure of a single alpha-helical MP.*



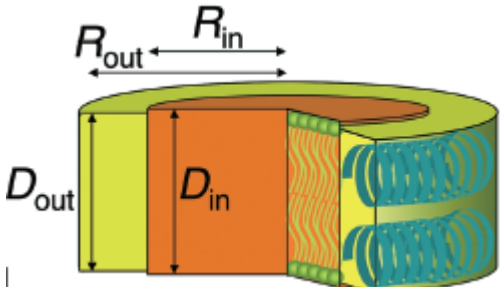
Hagn et al, JACS, 2013, 135 pp 1919–11925
Proof of concept 2: *Development of NMR-optimized smaller nanodiscs.*
=> OmpX (beta-barrel) and Bacteriorhodopsin (7TM) in nanodiscs.

Structure and control of the empty nanodisc system

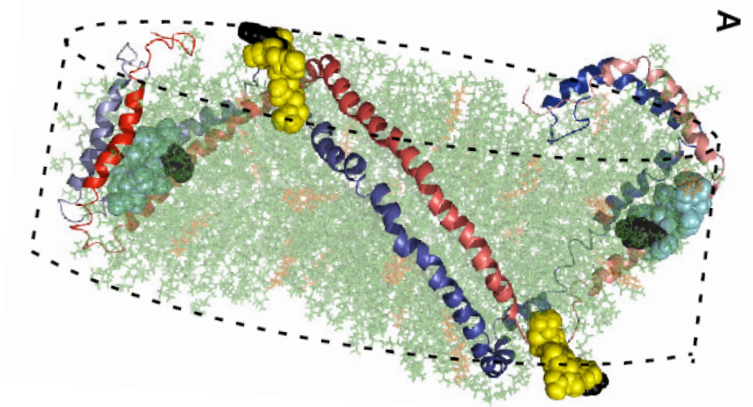
At project start in 2009: Not full consensus in the literature about what nanodisc's/HDL's looked like:



MD Simulation (Shih, Sligar, Schulten et al, Biophys. J 2005)
=>Circular discs. *But model did not agree well with SAXS data*



Small-Angle Neutron Scattering study of DMPC-APO-A1 (Nakano et al, JACS, 2009)
Geometrical based modelling
=> Circular discs. *But very low res. data*



SANS and SAXS study of cholesterol containing POPC-APO-A1 (Wu et al, JBC, 2009)
=> Double super helix model

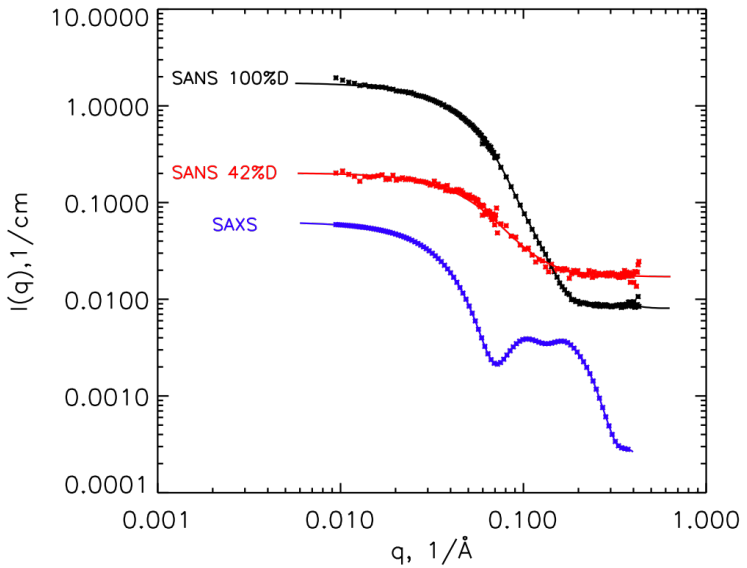
The first steps: SAXS and SANS measurements to determine the structure of the empty nanodiscs:

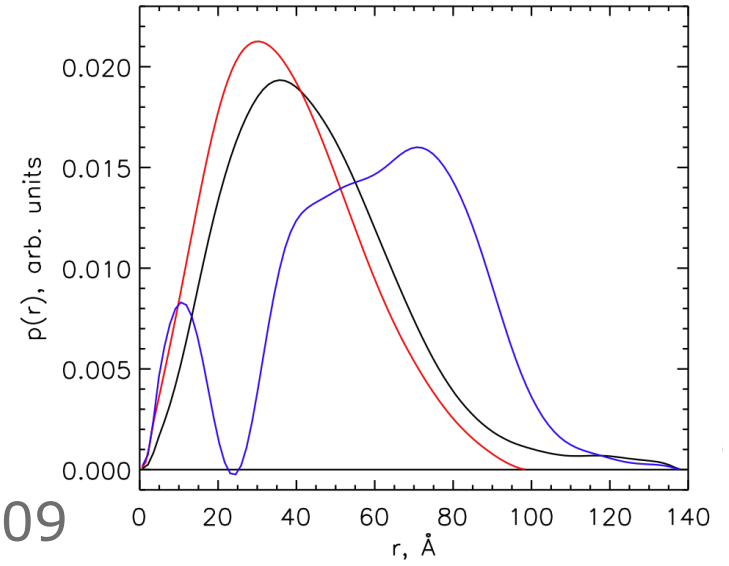
DLPC Nanodiscs
Belt: MSP1D1 (His-tagged membrane scaffold protein belt)
Lipid: DLPC (Di-Lauryl-Phosphatidyl choline)(+ DMPC and POPC)

SANS in 100% D_2O:
Bulk-contrast of entire ND

SANS in 42% D2O:
Protein belt matched out. Bulk-contrast of lipid core

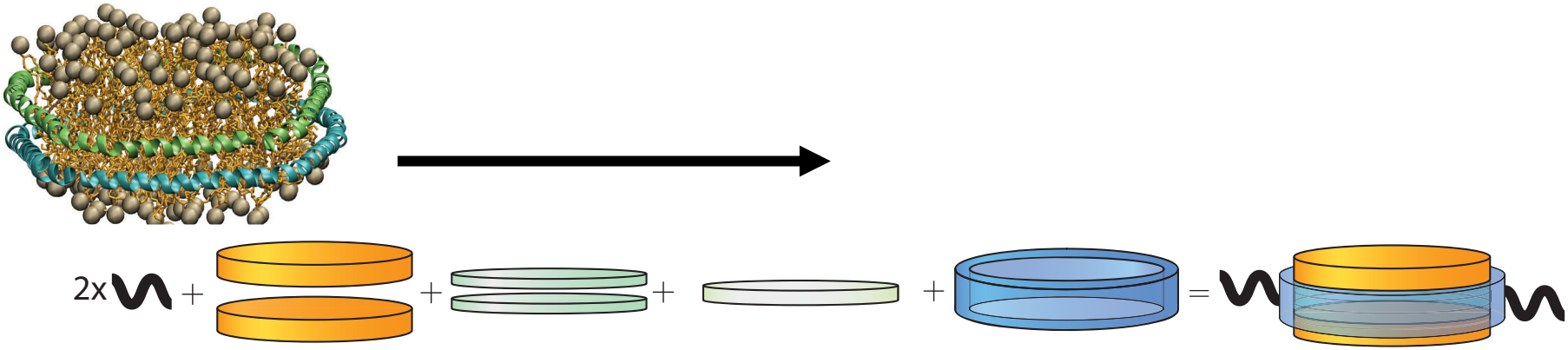
SAXS contrast:
Complex contrast situation: Different contrasts of protein belts, of hydrophobic alkyl chains of lipid and of hydrophilic headgroups.





Data from D11-ILL/ID14.3-ESRF, Nov 2009

Mathematical model for the ND's to interpret SAXS data



$$A_{tags} + A_{cap} + A_{tails} + A_{meth} + A_{belt} = A_{disc}$$

1. Derive analytical expressions to describe the scattering from nanodiscs (trivial but very long equations …)

2. Incorporate molecular constraints:

Exploit that Nanodiscs are build by well-known building blocks of well-known chemistry and scattering length:

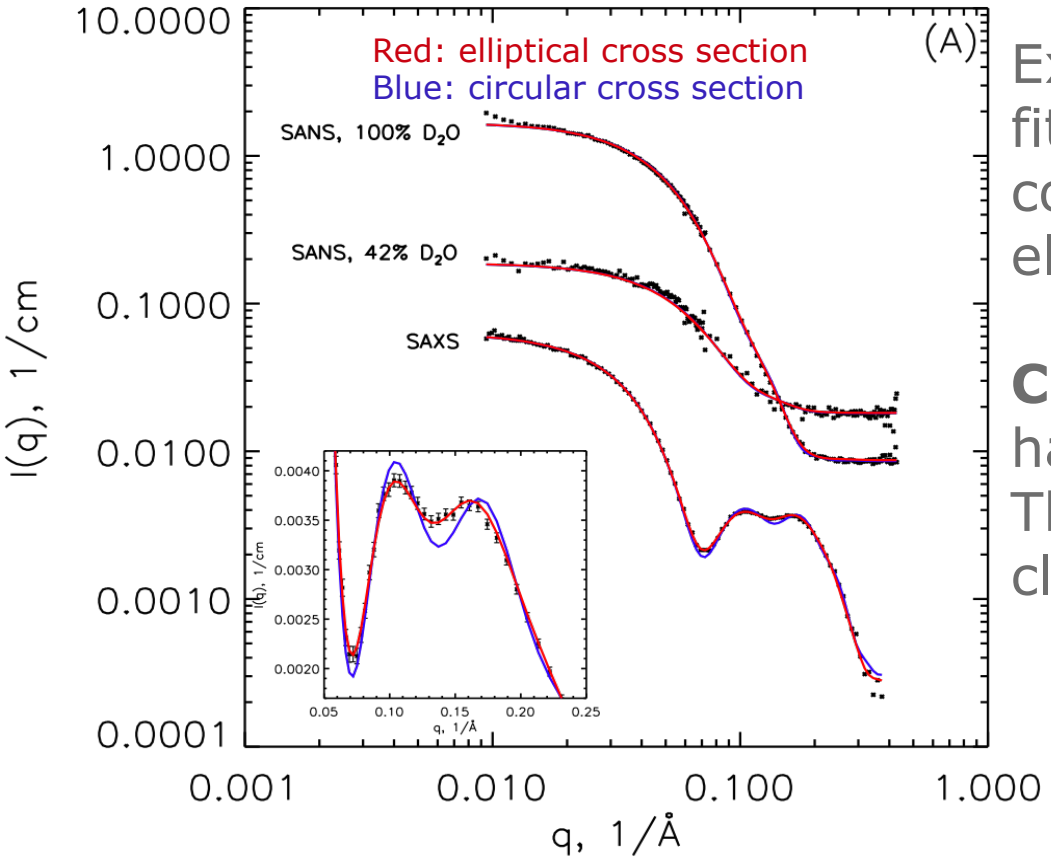
-Hydrophobic core consists of alkyl-chains

-Hydrophilic caps consist of PC headgroups and hydration water

-Belts consist of MSP

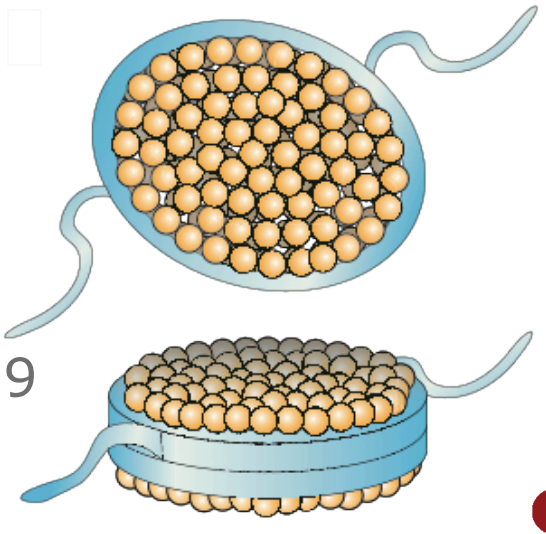
All information about concentrations and chemical composition along with estimates for the partial specific molecular volumes is build into the mathematical model to secure self-consistency.

Simultaneous fits to SANS/SAXS data => detailed structure of Nanodiscs:



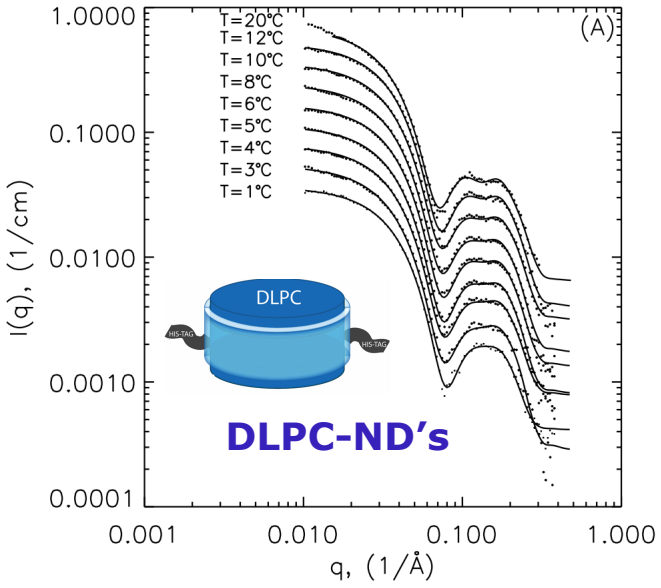
Excellent simultaneous model fits when using a fully molecular constrained analytical model for elliptical, His-tagged nanodiscs!

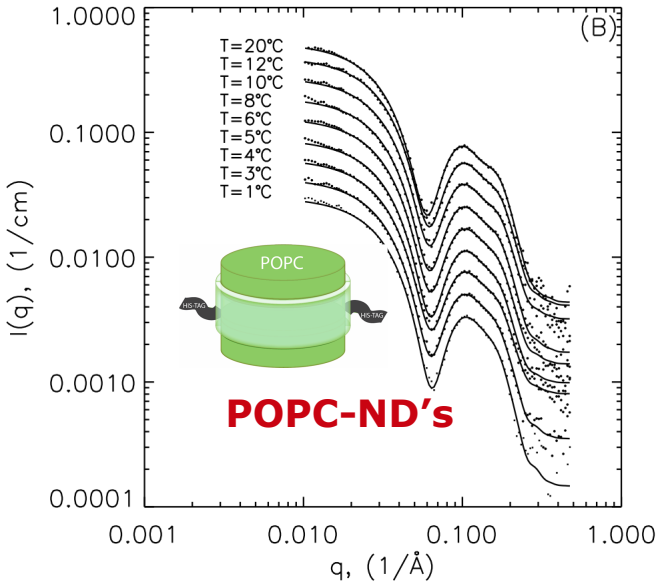
Conclusion: The Nanodiscs have a flat elliptical disc shape. The His-tags are protruding and clearly visible!

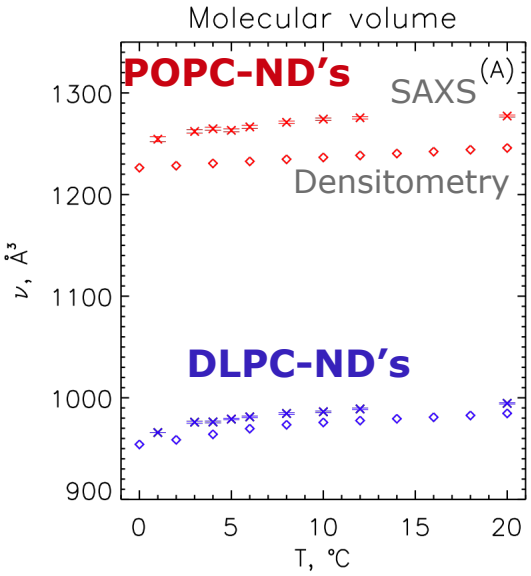


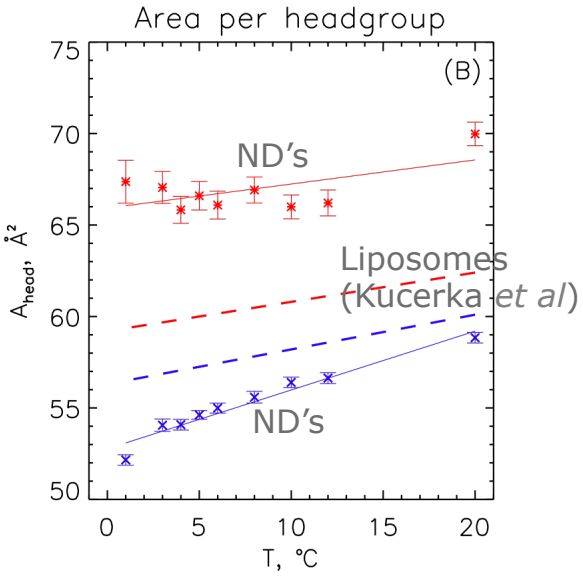
Data from D11-ILL/ID14.3 ESRF, Nov 2009

Temperature dependence T=1°C - 20°C







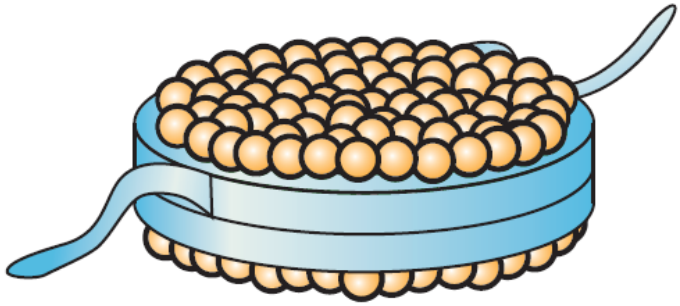


Self-consistent results: Partial specific molecular lipid volumes from SAXS data agree with values from densitometry.

New Results: Lipid packing is clearly perturbed in ND's
=> POPC is laterally stretched (thinned) and DLPC is laterally compressed (thickened) when located in nanodiscs

Conclusions from SAXS/SANS study of empty nanodiscs

-Combined SAXS and SANS gives the detailed structure (and temperature dependence) of empty nanodiscs
-The nanodiscs are disc-shaped and structurally very homogeneous with an *elliptical* cross-section ($\varepsilon \sim 1.4$)
-POPC is laterally stretched and DLPC is laterally compressed when located in nanodiscs



Stretching and compression of lipids can be understood as minimization of the "hydrophobic mismatch" between protein belt and lipid bilayer

POPC DLPC

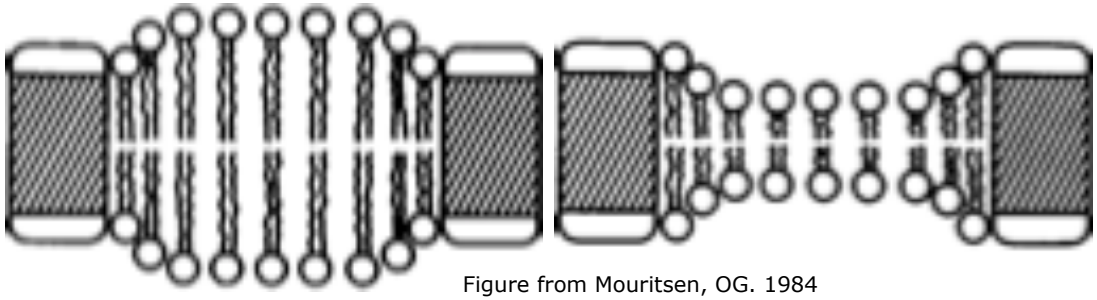
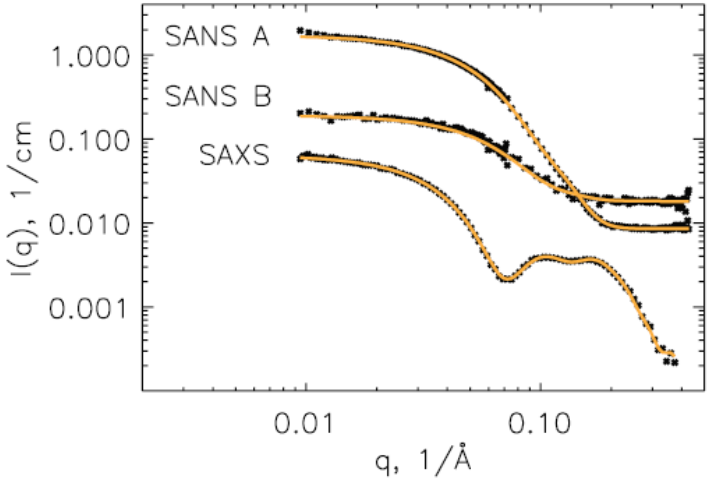


Figure from Mouritsen, OG. 1984

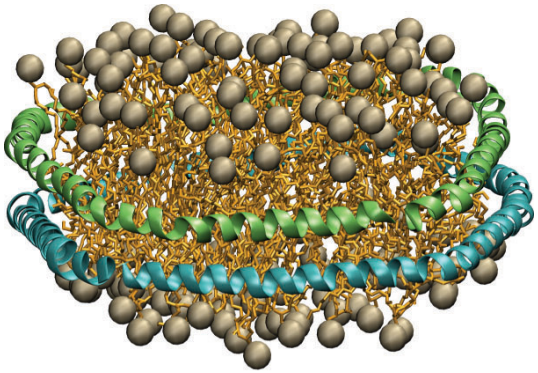


Publications:

-Skar-Gislinge, Simonsen, Mortensen, Feidenhans'l, Sligar, Lindberg Møller, Bjørnholm and Arleth, *J. Am. Chem. Soc, 2010.*
-Skar-Gislinge and Arleth, *Phys. Chem. Chem. Phys, 2011.*

Research goal: Develop phospholipid nanodiscs into a platform for Small-angle scattering based low-resolution structural studies of membrane proteins

Main targets:



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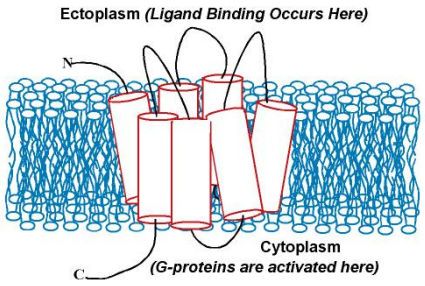
Central challenges:
Incorporation of Membrane proteins into Nanodiscs: Obtaining sufficiently "pure and well-defined" samples.

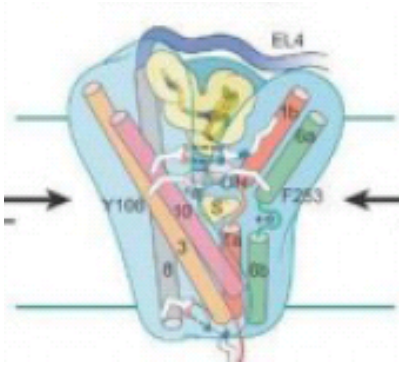
Data analysis: Development of experimental and computational methods to extract relevant structural information about the membrane proteins.

G Protein-Coupled Receptors

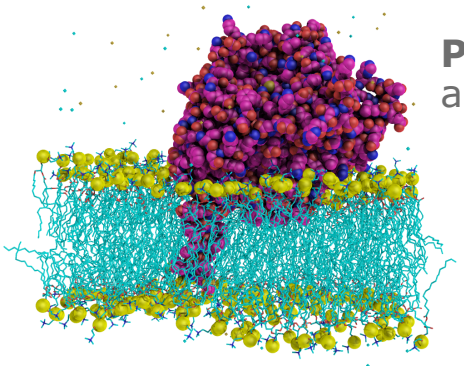


Typical Molecular Weight: 40 kDa

GPCR's: 7 TM's (Use Bacteriorhodopsin in the development phase)



Leucine transporter: 12 TM's

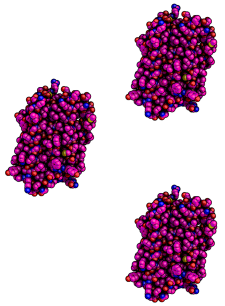


P450: Membrane anchored protein's

Bacteriorhodopsin in nanodiscs – Sample preparation

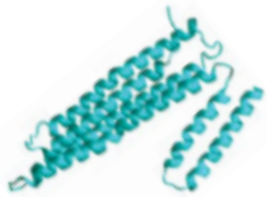
Expression of Membrane Protein:

- bR expressed according to standard procedures (Oesterhelt and Stoekenius, Methods Enzymology, 1974) by in-occulation of H-salinarium in a medium with (among other) 250 g NaCl in 1L H2O
- Monomeric bR was purified and reconstituted in Octyl Glycoside (OG) and biotinylated to have a biotin-anchor on the bR



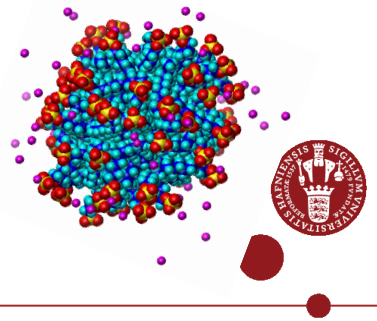
Membrane Scaffold protein:

- Membrane scaffold protein was expressed in E-coli and purified (Sligar et al, Methods Enzymology, 2009), His-tag was cleaved of by using a TEV-protease



Lipid Mixture:

- 2 POPC : 1 POPG, obtained from Avanti Polar Lipids mixed with cholate in organic solvent and dried



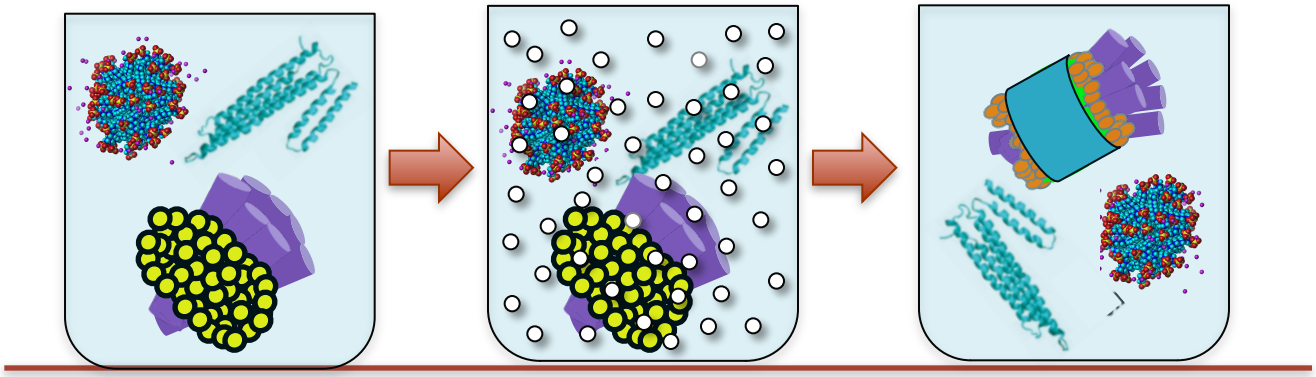
Søren Roi Midtgaard and Jens Bæk-Simonsen

Reconstitution of Bacteriorhodopsin into nanodiscs

1. Mix detergent solubilized membrane protein, phospholipids, reconstitution detergent and MSP

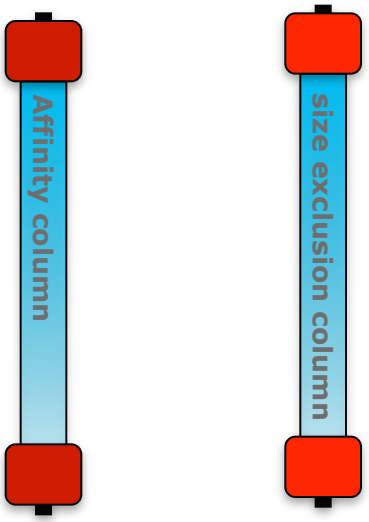
2. Add biobeads to remove reconstitution detergent (or remove detergent by dialysis)

3. Remove Biobeads and start purifying

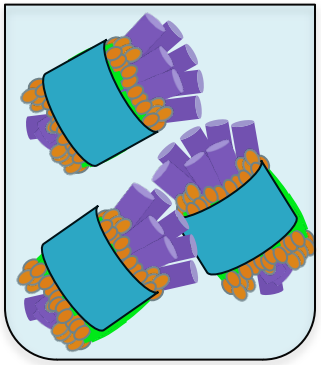


Purify loaded Nanodiscs

Purify good Nanodiscs



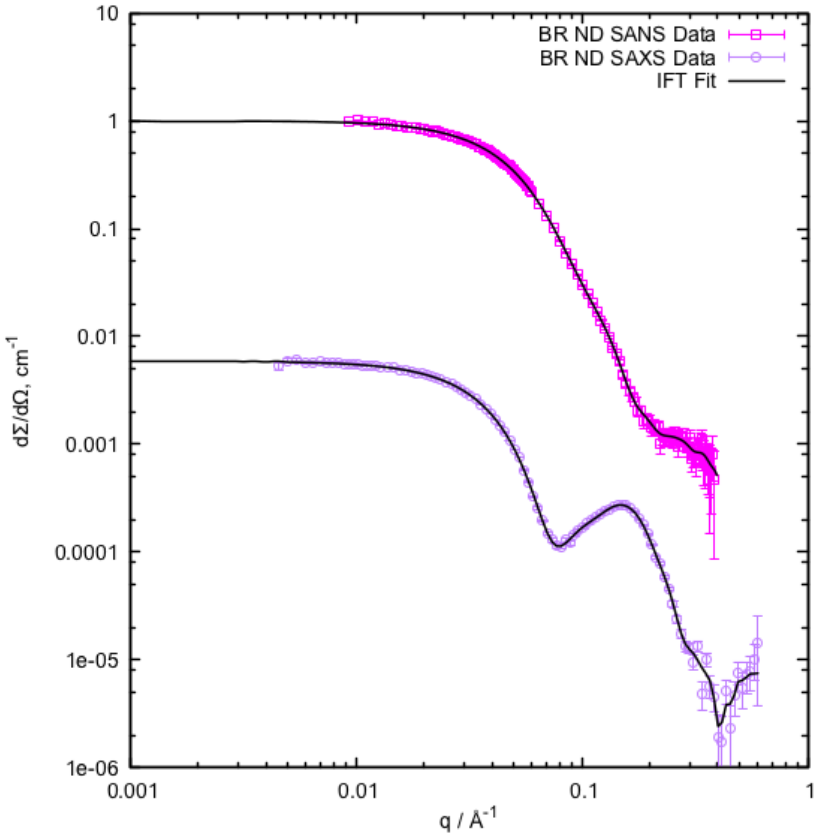
Immobilized mono-meric avidin resin

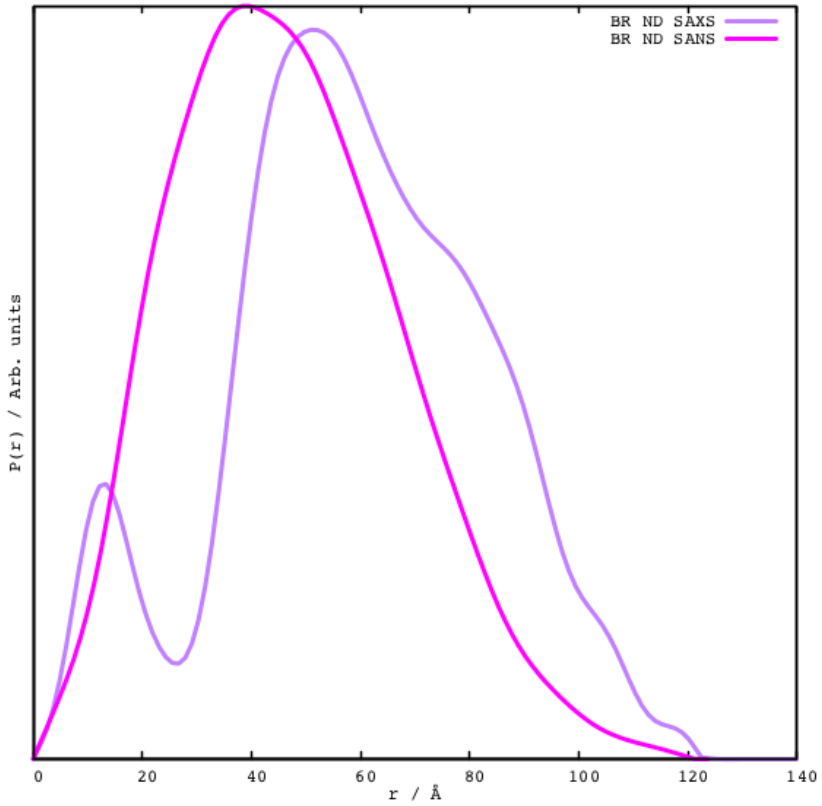


See more description e.g. in Denisov *et al*, JBC, VOL.282(10), pp.7066–7076, 2007

Note: Reconstitution of Membrane proteins into ND's is difficult and still only poorly understood. Field in need of good physical chemists (with access to SAXS).

SAXS and SANS data from bR incorporated into POPC:POPG 2:1 nanodiscs





D_{max} of ~120Å

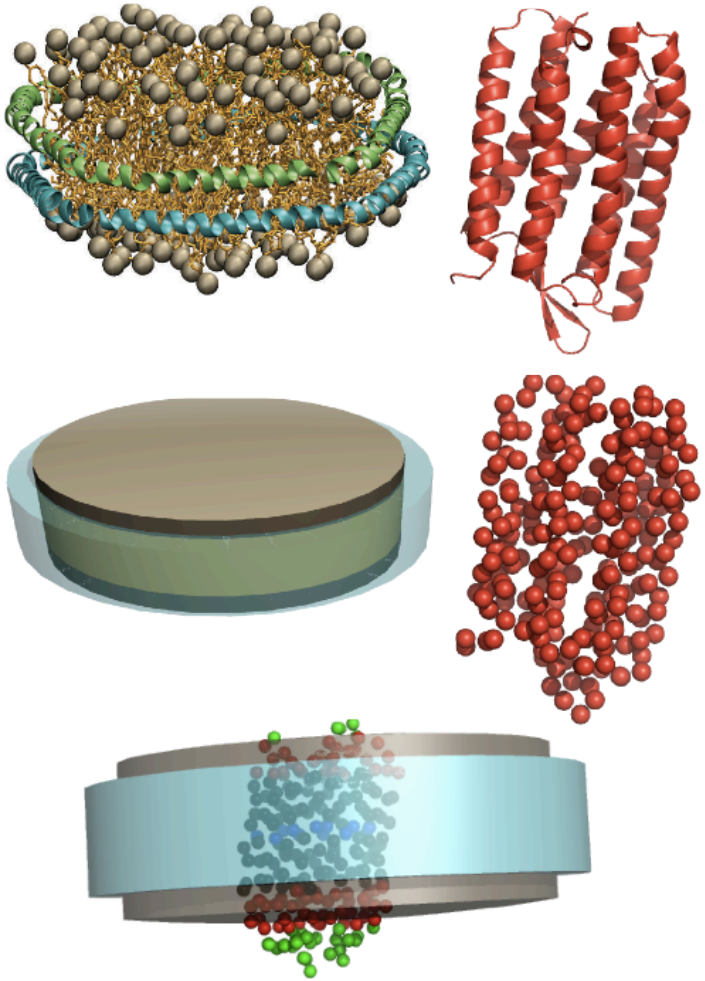
SANS/SAXS data from D11-ILL/ID14.3-ESRF (2011)

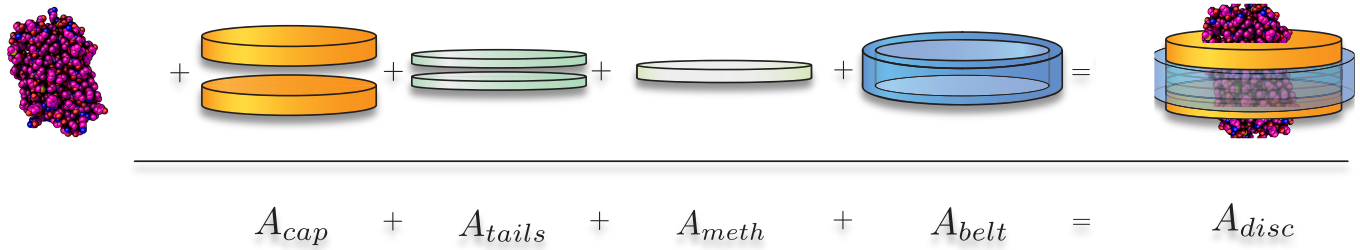
Hybrid modelling approach

-Geometrical approach to describe the nanodiscs

-Bead modelling approach (Svergun type) to describe the incorporated membrane proteins.

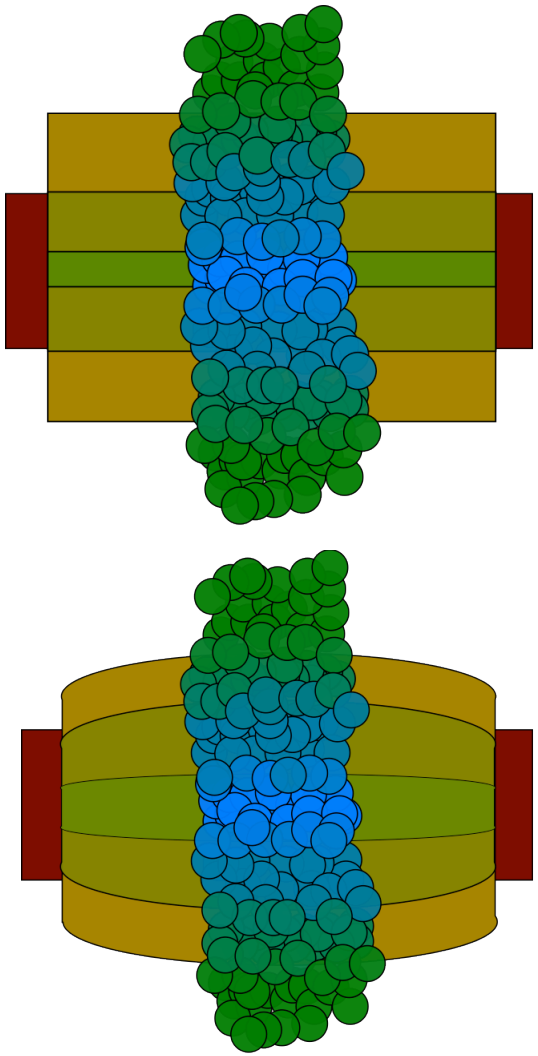
NB: Full bead modelling approach also tested, but without success so far.



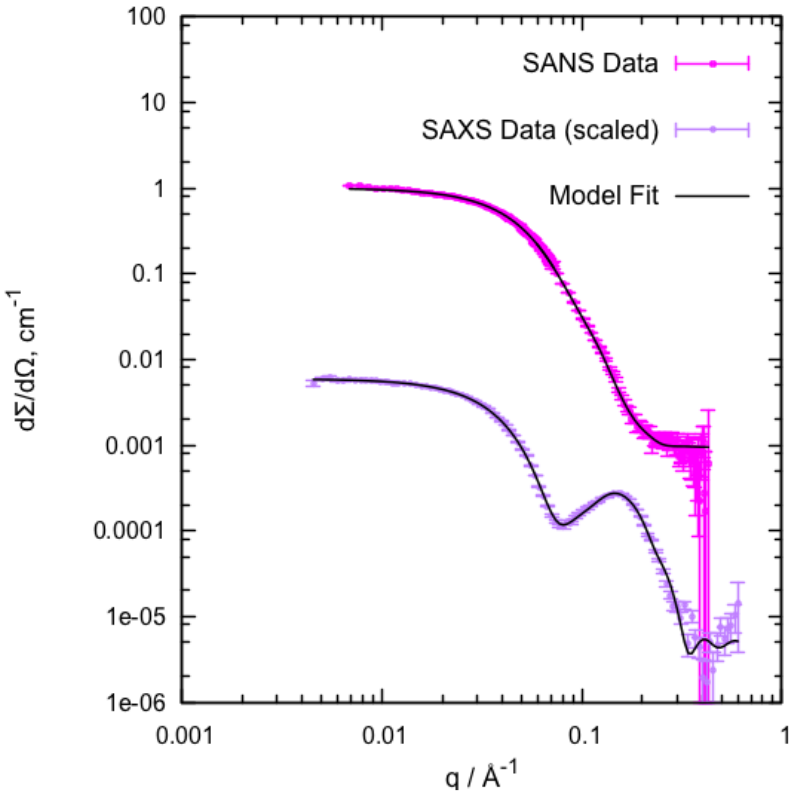


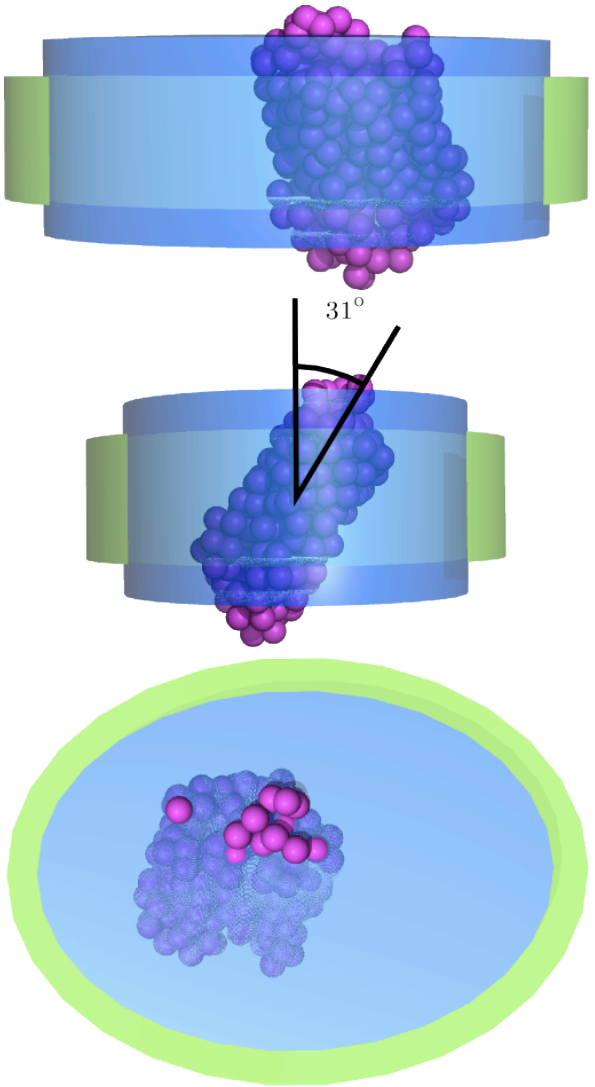
Details of the modelling approach

- Exploit that the crystal structure of bR is known. Only the position and orientation of the membrane protein is fitted in relation to the surrounding nanodisc.
- The excess scattering length of the different subdomains of the membrane protein are adapted as to which medium (water, lipid) the subdomain has excluded.
- The structure of the surrounding nanodisc is explicitly fitted and allowed to adapt to a lense shape.
- Molecular constraints are fully incorporated.
- To gain computational speed, the amplitudes from both the geometrical nanodisc and the MP are expanded in terms of spherical harmonics
- Software code implemented in a combination of C and Python. Equations checked for correctness by point based modelling *(see Pedersen, Oliveira et al, Biophys J, 2012)*



bR in Nanodiscs – Model fit results





bR slightly de-centered in the disc and slightly tilted. Tilt is in accordance with expectations from surface analysis and crystallography

Model gives information on both membrane protein and lipid environment

NB: Information contents is generally significantly higher in SAXS data that in SANS data.

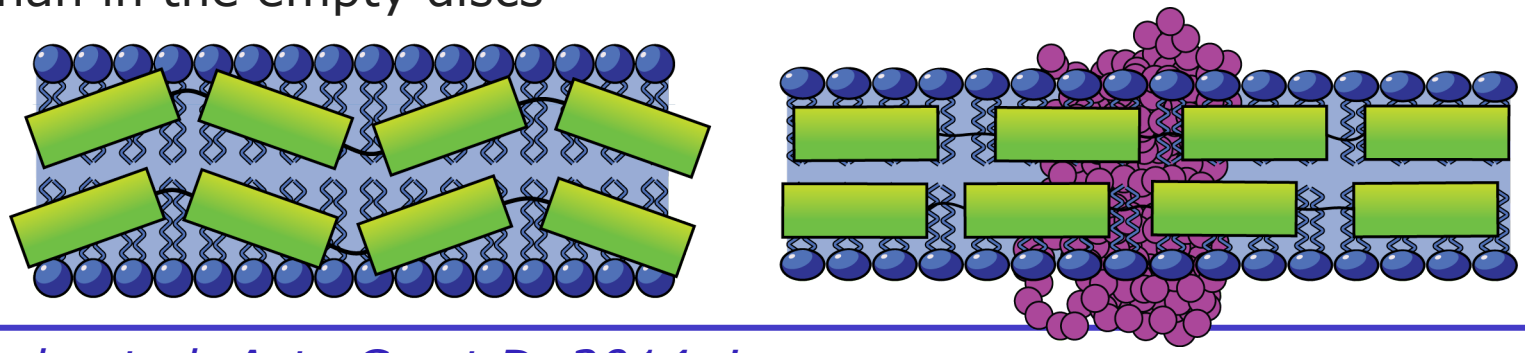
Insight into the perturbation of the lipid environment

Comparison of lipid environment in bR-loaded and empty POPC/POPG discs:

Parameter	Empty pc/pg	Br Loaded
N lipids	126	131
Area per lipid (Å^2)	63	77
Axis ratio	1.66	1.44

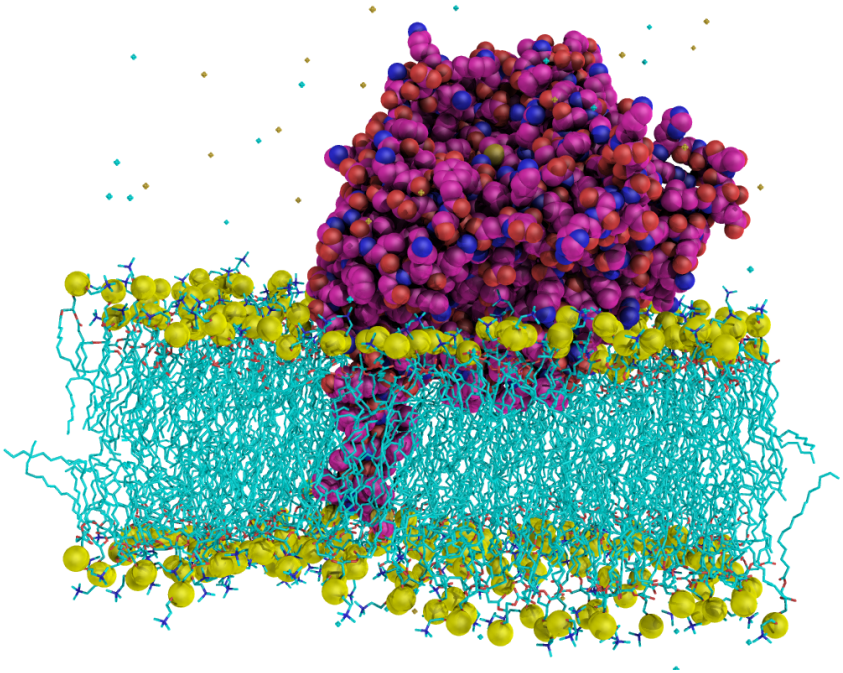
-The loaded disc is significantly more expanded and has a larger circumference

-Lipids are laterally more stretched in the loaded disc than in the empty discs



Kynde et al, Acta Cryst D, 2014, in press
-See also Pedersen et al, J. Appl. Cryst, 2013, 46(6), 1894-1898 for details on fitting approach and download of "WillItFit" software

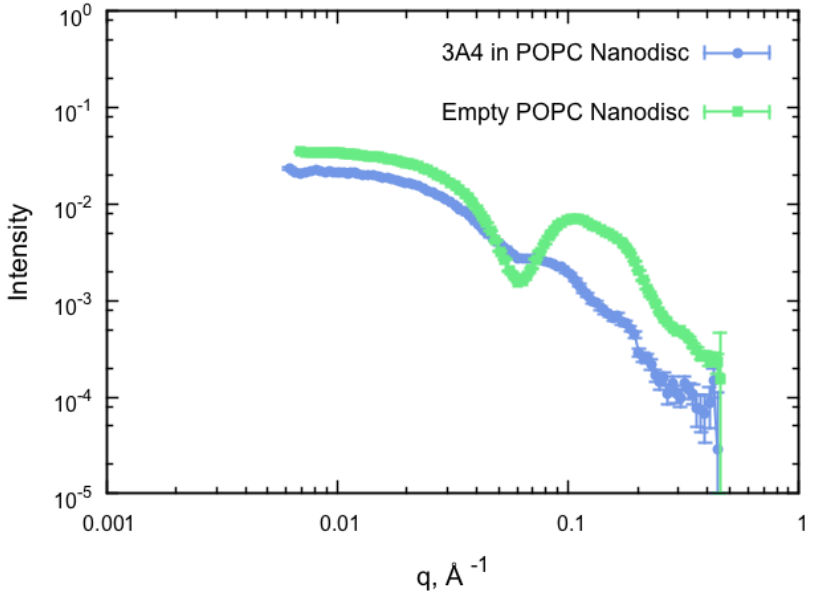
Cytochrome P450 3a4 in Nanodiscs – Experimental data

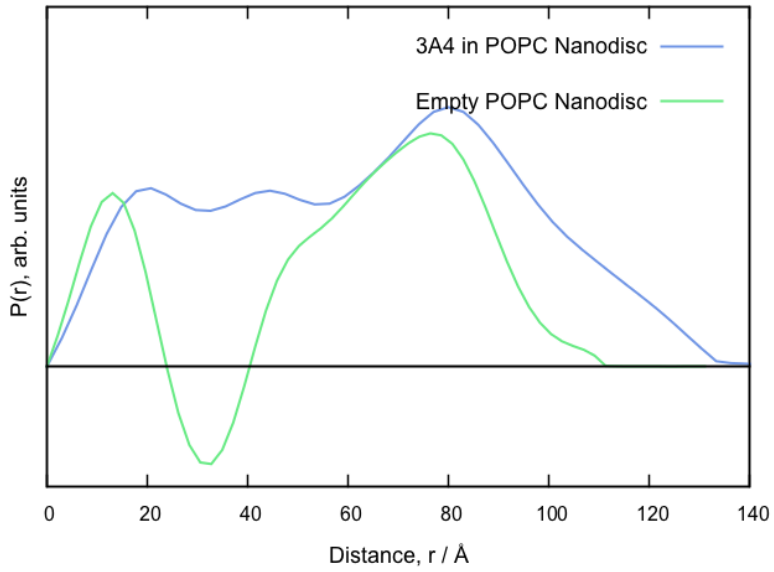


MD simulation of P450 in lipid bilayer J Inorg Chem, (2012) 108, 150

Project in collaboration with Steve Sligar, Ilya Denisov and Xin Ye, U Illinois

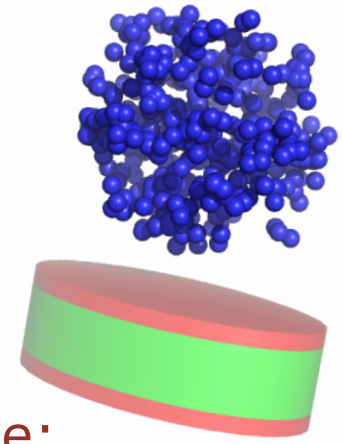
SAXS data from BM29/ESRF



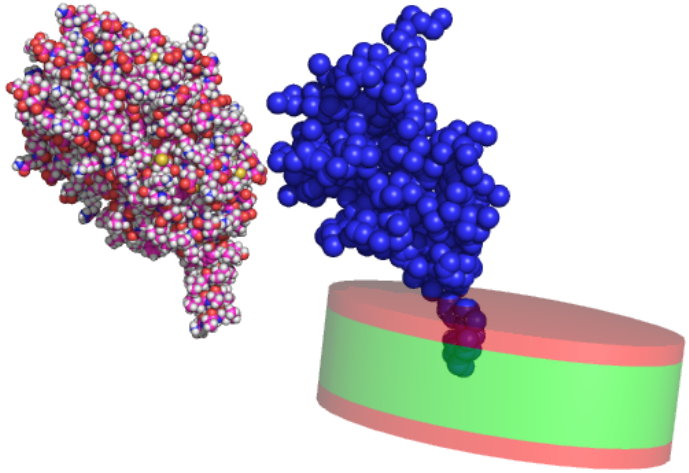


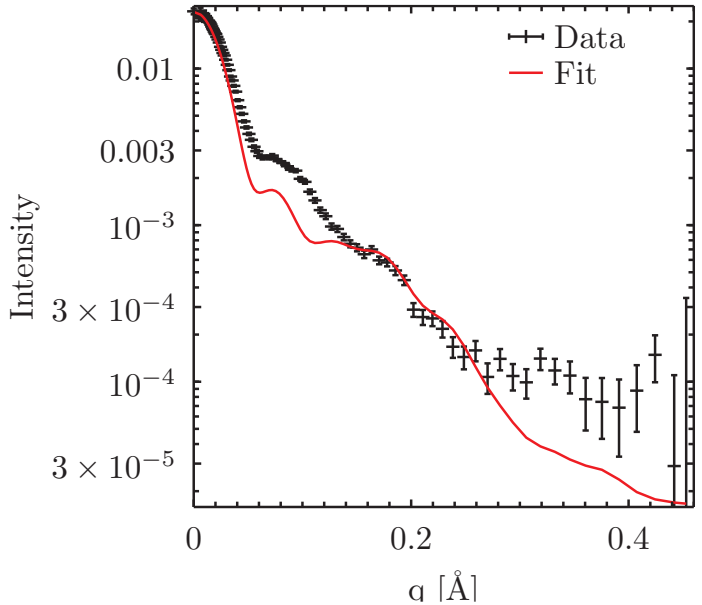
P450 in Nanodiscs – Fit results - Bead modelling

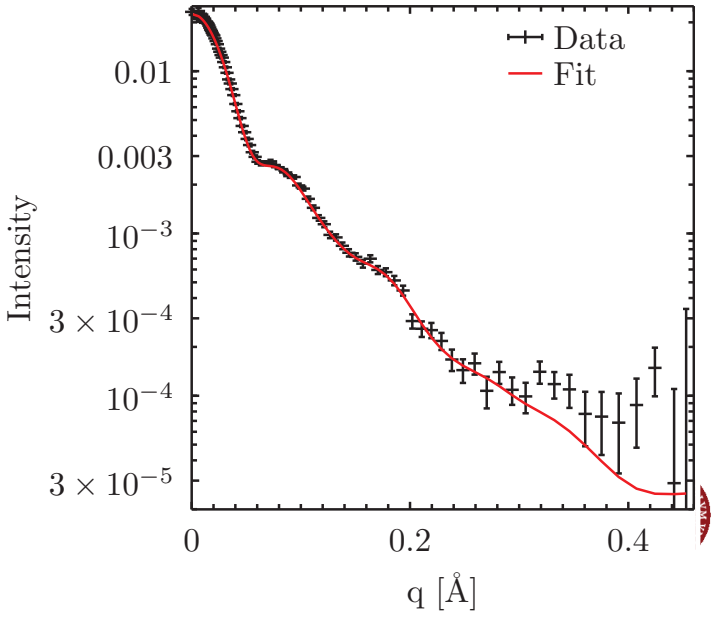
Initial condition:



After convergence:



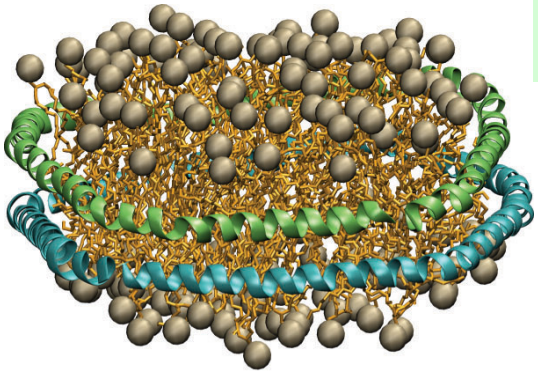




Nicholas Skar-Gislinge, Søren Kynde, Martin Cramer Pedersen et al, *pub. in prep.*

Research goal: Develop phospholipid nanodiscs into a platform for Small-angle scattering based low-resolution structural studies of membrane proteins

Main targets:



✔

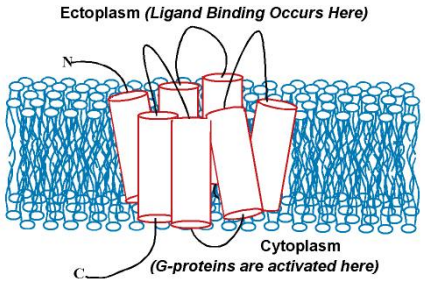
+

Central challenges:

Incorporation of Membrane proteins into Nanodiscs: Obtaining sufficiently "pure and well-defined" samples. (✔)

Data analysis: Development of experimental and computational methods to extract relevant structural information about the membrane proteins. ✔

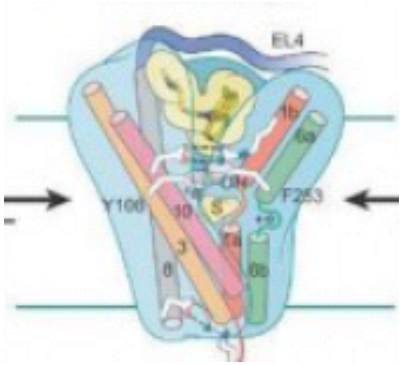
G Protein-Coupled Receptors



Typical Molecular Weight: 40 kDa

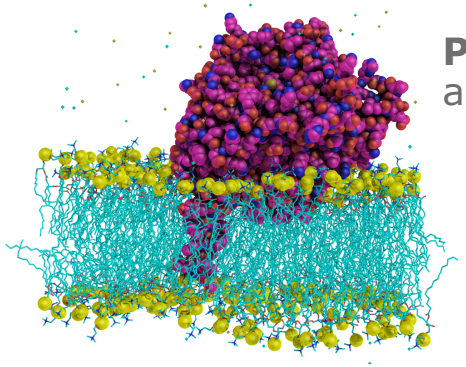
Bacteriorhodopsin
7TM

✔



Leucine transporter:
12 TM's

÷



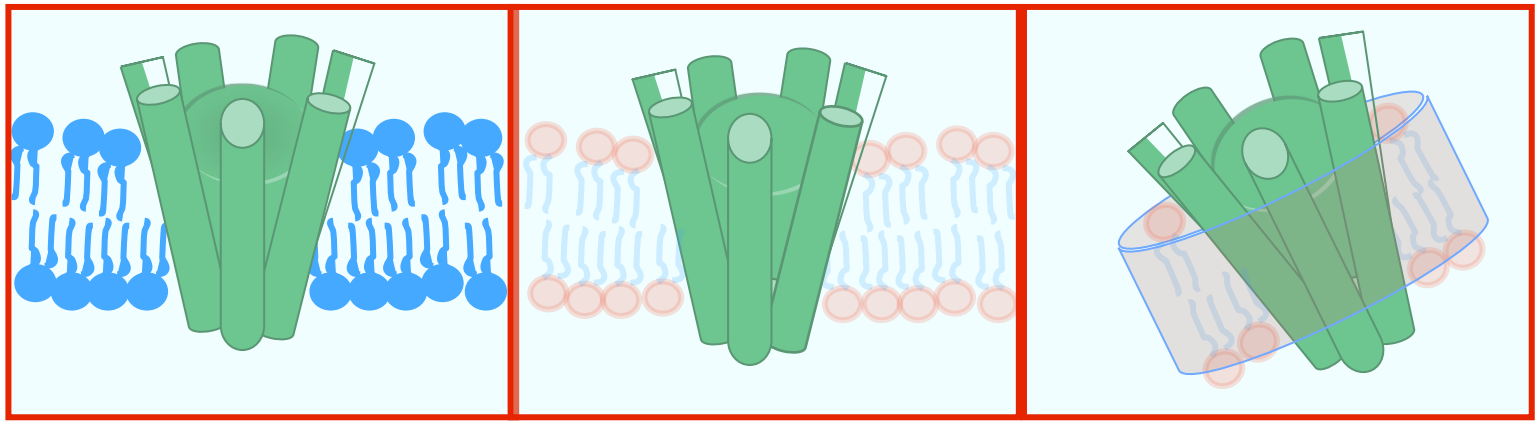
P450: Membrane anchored protein's

✔

Improving the conditions for the structural analysis:

Idea: Use specific deuteration of bio-building blocks and obtain ”stealth nanodiscs”, i.e. Nanodiscs that are invisible to neutrons so that only the membrane protein is seen.

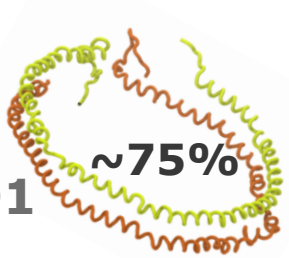
Collaboration with the D-lab at ILL, Grenoble

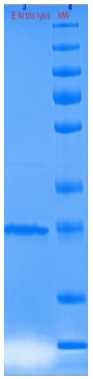


Selma Maric and Søren Roi Midtgaard

Requirements:

Specific deuteration levels for the Stealth Nanodisc model





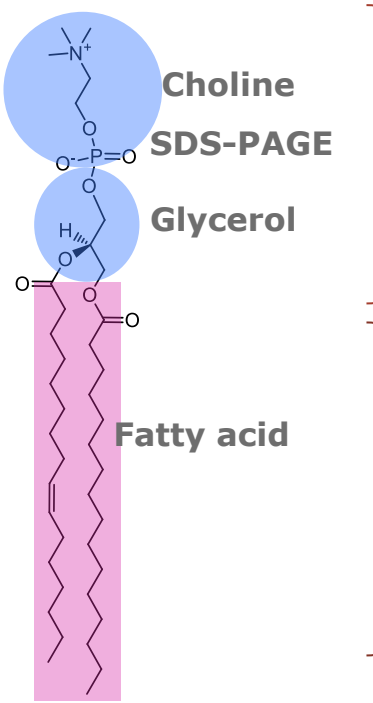
25 kDa

Expression of matched-out MSP1D1

- 85% D-media → 75% D-protein

=> MSP "Invisible" at 100% D_2O

Expression of matched out Phosphatidylcholine POPC



Chemical synthesis

- Expensive and challenging

Bacterial expression

- Normal *E.coli* does not produce PC
- Control of selective deuteration?

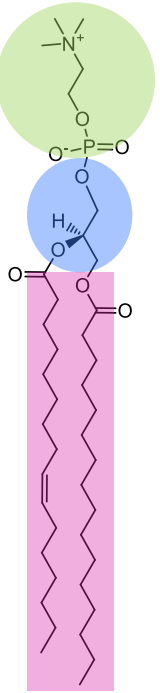
Collaboration with D-Lab, ILL, Grenoble

Selective deuteration of PC in genetically modified *E.coli* by systematic control of the level of D_2O, D-choline and D-glycerol in the growth medium

Choline head group

Glycerol backbone

Fatty acid tails



	Level in growth media (%)		
D_2O	100	100	100
D-glycerol	100	100	100
D-choline	0	50	100

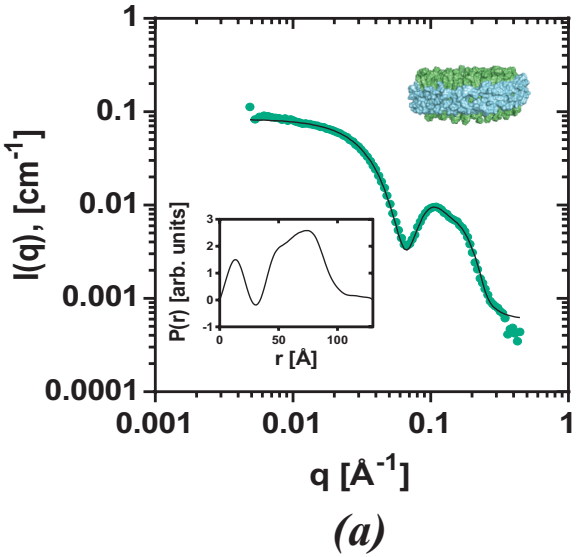
	Level in growth media (%)		
D_2O	100	100	100
D-glycerol	0	50	100
D-choline	50	50	50

	Level in growth media (%)		
D_2O	50	75	100
D-glycerol	0/100	0/100	0/100
D-choline	50	50	50

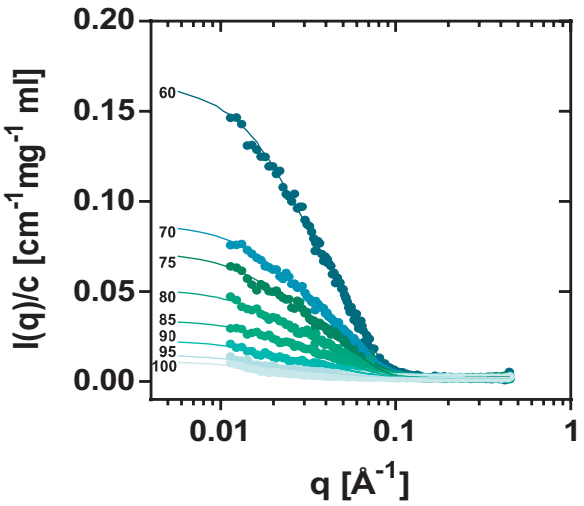
Collaboration with D-Lab, ILL, Grenoble

=> Identification of conditions where the lipids should be matched out.

Stealth Nanodiscs - SANS Contrast Variation

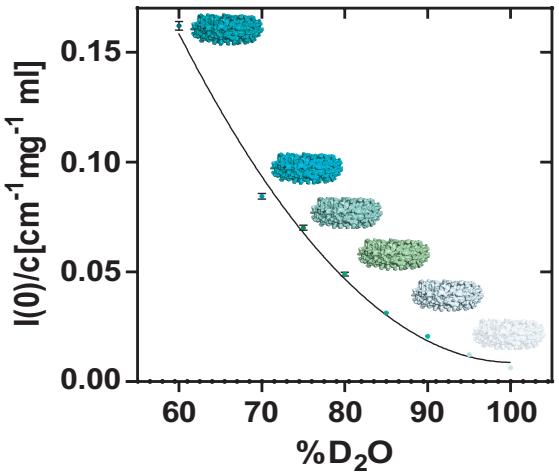


(a)

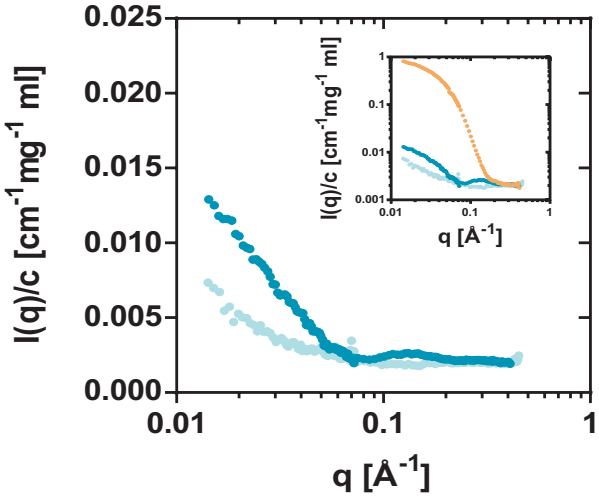


(b)

Maric et al, Acta Cryst D, 2014, in press



(c)



(d)

SANS data from KWS2, FRMII, Munich Sep 2012.
SAXS from BM29, ESRF, Grenoble Sep 2012

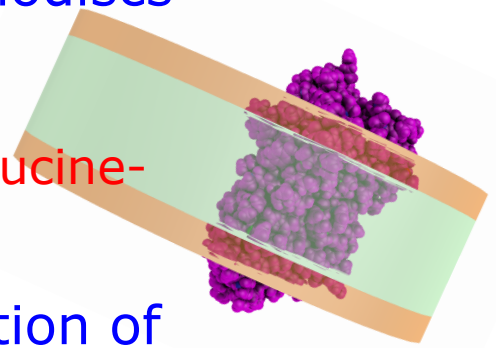
Conclusion: Nanodiscs invisible to neutrons can be prepared.

Next step: To incorporate membrane proteins into the discs

Conclusions

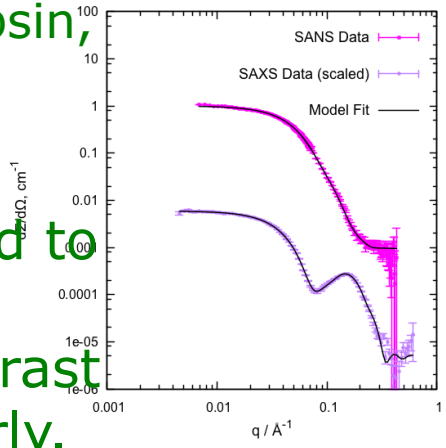
Membrane proteins can be reconstituted into nanodiscs and give structurally well-defined constructs

Systems investigated so far: bR, P450, Proteo-rhodopsin, Sensor-rhodopsin, Aquaporin, CorA, Leucine-Transporter, AHA2-proton-pump systems



The structures can be modelled using a combination of free-form or pdb-based bead modelling and provide information about both protein and lipid environment

Systems analysed so far: bR, P450, Proteo-rhodopsin, Sensor-rhodopsin, Aquaporin, CorA



To do:

-(Much) better understanding of reconstitution need to be established

-Apply stealth-nanodiscs to improve the SANS contrast situation and see the membrane protein more clearly. According to our simulations, this should allow for free form modelling of transmembrane proteins

Limitations of the approach

Assuming that our approach will work:

SAS Structural resolution: Difficult to get below 10 Å. But we would really like to have "atomic" resolution.

Competing techniques:

- Crystallography – Atomic resolution, but hard to crystallize
- NMR – Atomic resolution, but mainly local information, quite time consuming.
- Cryo-TEM – Real space, possibility for filtering out "bad particles". Difficult to get below 10 Å, quite time consuming.

Conclusion: There is at present no perfect solution. It is probably necessary to think of systematic approaches for combining techniques to a much larger extent than today

Acknowledgements

The SAS&Nanodisc group at U. Cph:

- **Nicholas Skar-Gislinge,** PhD student
- **Søren Kynde,** PhD student
- **Martin Cramer Pedersen,** PhD student
- **Selma Maric,** PhD student
- **Søren Roi-Midtgaard,** PhD student
- **Pie Huda,** PhD student
- **Søren Skou Nielsen, Post Doc**
- **Grethe V Jensen, Post Doc**
- ***Lise Arleth,*** *Professor*
- ***Kell Mortensen****, Professor*

UNIK Synthetic Biology

Thank you!

Collaborators:

-Steve Sligar and Ilya Densiov, U. Illinois
-Several UNIK Synthetic Biology project partners at U. Copenhagen
-Javier Perez and Gabriel David Synchrotron Soleil, Paris
-Ralph Schweins, Peter Lindner, D11, ILL Grenoble
-Adam Round, Petra Pernot, ID14-3, ESRF Grenoble
-Martine Moulin, Trevor Forsyth, Michael Haertlein, D-lab, ILL Grenoble
-Henrich Frielinghaus, KWS-2, FRMII, Munich

Funding:

UNIK synthetic Biology project, Lundbeck Foundation, Danish Research Council, Danscatt, ESS