AUC 2022 workshop Membrane Proteins

Sedimentation velocity for membrane proteins

Christine EBEL – Caroline Mas

IBS Grenoble FR

christine.ebel@ibs.fr caroline.mas@ibs.fr

AUC 2022 – July 10-15 2022 Lethbridge Canada

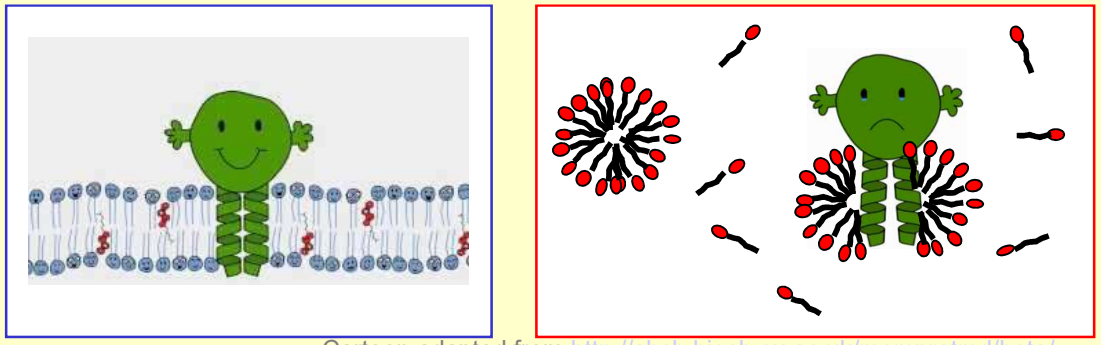
Membrane Proteins

- One third of the expressed proteins
- Essential for the life of the cell
- Target of more than 50% of the drugs.
- Still difficult to obtain high resolution structures

Solubilisation & purification of membrane proteins require detergent

• Associated inactivation & Instability

=> New surfactants / detergents

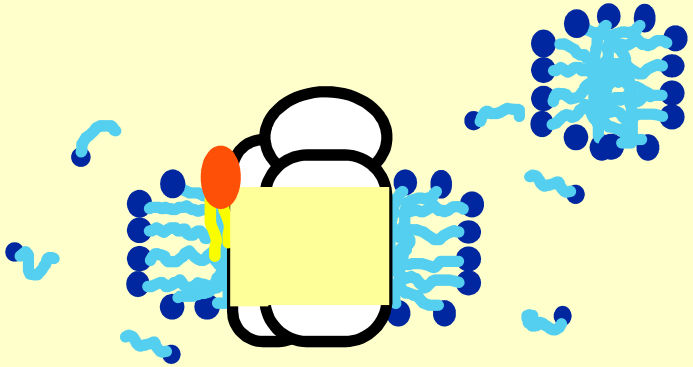


Cartoon adapted from http://sbcb.bioch.ox.ac.uk/memprotmd/beta/

• Heterogeneous and multicomponent systems

=> Specific methods for studying composition, interactions

	Separation	≠ signals	≠ contrasts	Mass Compo	Shape	Structure
SEC-MALS	Y	Y		Y	Y	
AUC	Y	Y	Y	Y	Y	
SANS		Y	Y	Y	Y	Y



Membrane proteins

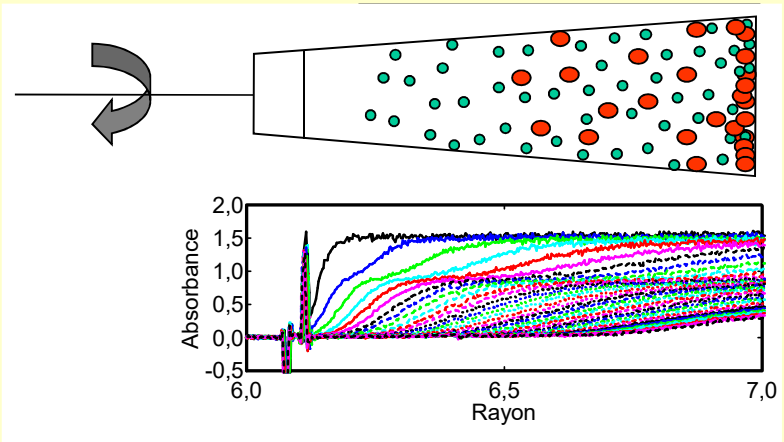
1) AUC, Summary

2) The specificity of membrane proteins

3) Strategy & protocols

4) Examples

Analytical Ultracentrifugation Sedimentation Velocity



homogeneity
shape

density:
composition

Different optics:
composition

$$s = \frac{M(1-\rho\overline{v})}{N_A 6\pi\eta R_H} = \frac{DM_b}{RT}$$

Molar mass &
Association states

Association constants

c(s)

s

0 2 4 6 8 10 12

SV: velocity of the particles

SE particle distribution at equilibrium

buoyant mass= mass + relative density

$$s = \frac{M_b}{N_A 6\pi\eta R_H}$$

$$M_b = M(1-\rho\bar{v}) = \sum M_i(1-\rho\bar{v}_i)$$

shape

R_H, or D, or f, or f/f_{min}

- $c(s)$ analysis => s. Then, if R_H is known => M_b
- Non interacting species analysis: =>s and R_H=M_b
- Sedimentation equilibrium analysis => M_b

Then, information on composition is required for M_b => M_{prot}

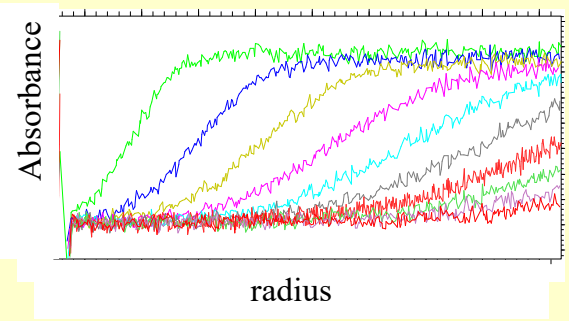
Principle of sedimentation velocity data analysis

Lamm equation, for each type of particles

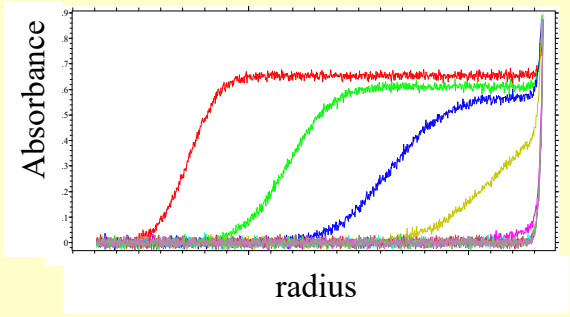
$$(\partial c/\partial t) = -\,1/r\;\partial/\partial r\,[r(c\,s\omega^2 r - D\,\partial c/\partial r)]$$

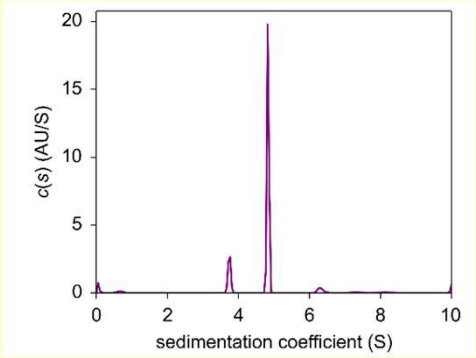
- Data analysis are based on numerical solutions of the transport equation. i.e. simulations
- They compare simulated profiles (with given *s*- and *D*- values) to experimental ones.

Experiment



Simulation





- In the *c*(*s*) analysis, we consider a distribution of particles, for which a plausible relationship between *s* and *D* is established (input : v, f/f_{min}, ρ and η): only concentrations and noises are determined.

  ➔ high resolution distribution of sedimentation coefficients, *s*.

- In the non-interacting species analysis, *s*, *D*, concentrations and noises are adjusted.

  ➔ *s* and *D* (thus R_H), thus *s* and M_b

Interacting system: several or one boundaries

- **In a slow equilibrium** the different species sediment as non-interacting species in proportion related to the dissociation constant for the loading concentration.

- **In a fast or intermediate equilibrium** between different species, the slow boundary reflects the sedimentation of one of the species. The fast boundary is a “reaction boundary” and its value does not reflect the sedimentation of the complex.

s_{mean} can be used for the calculation of the K_D

$s_{mean} = \sum c_i s_i / \sum c_i$

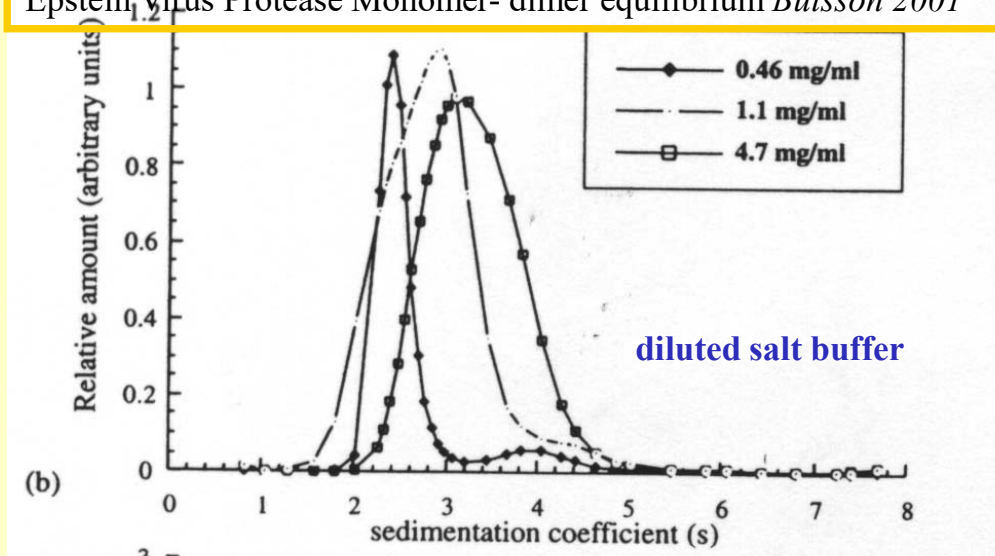
c_i: concentration of component i in g/l,
s_i the corresponding sedimentation coefficient.

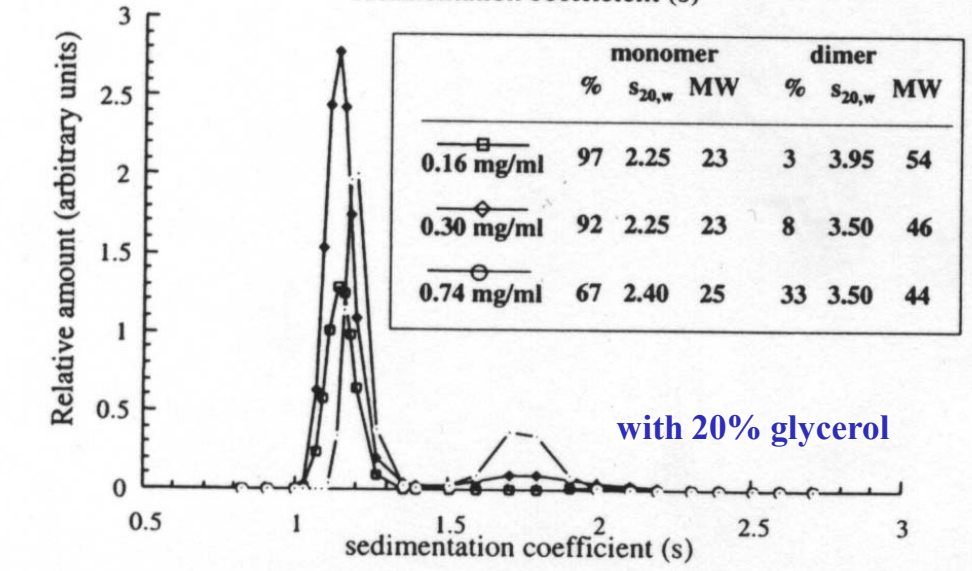
The regime depends on k_{off}= off-rate constant (s^{-1})
$\log_{10}(k_{off})$ =

- -1 : can be considered infinitely fast in the context of SV.
- -3 : still fast, but on the limit where kinetics should significantly influence the boundary pattern.
- -3.4 : intermediate.
- -4 : in the slow regime, but still influenced by kinetics.
- -6 : essentially infinitely slow on the SV time-scale.

Zhao et al Methods 54, 16-30 2011

Epstein Virus Protease Monomer- dimer equilibrium *Buisson 2001*





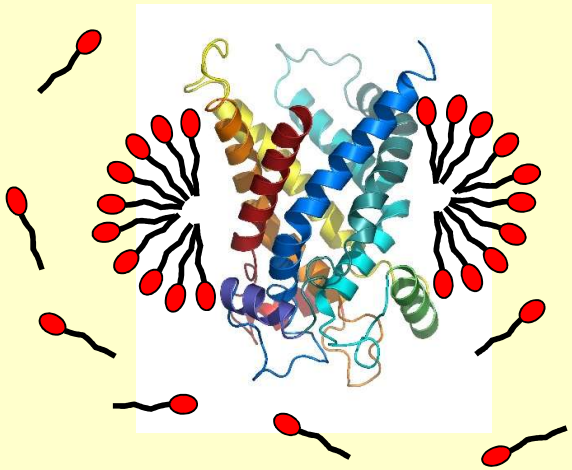
	monomer			dimer		
	%	$s_{20,w}$	MW	%	$s_{20,w}$	MW
0.16 mg/ml	97	2.25	23	3	3.95	54
0.30 mg/ml	92	2.25	23	8	3.50	46
0.74 mg/ml	67	2.40	25	33	3.50	44

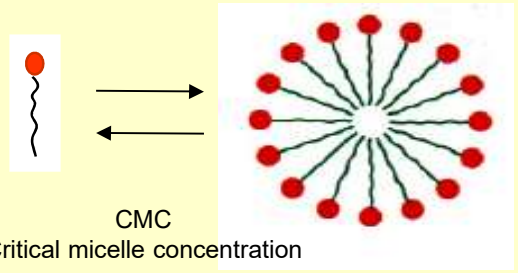
1) AUC, Summary

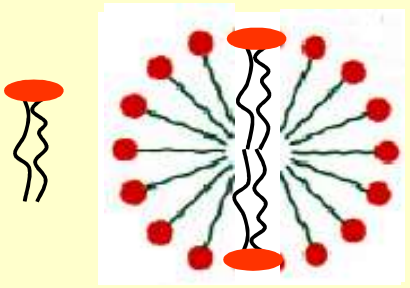
2) The specificity of membrane proteins

3) Strategy & protocols

4) Examples







1: Complex = protein-detergent-lipid.: composition generally unknown
2: Detergent-lipid micelles: concentration often unknown; often they do not absorb at 280nm; lipids content unknown.

Detergent	Bacteriorhodopsin		Ca^{2+}-ATPase	
	Gram/g	Mol/mol	Gram/g	Mol/mol
Triton X-100	2.9	124	0.44	77
$C_{12}E_8$	2.4	119	0.50	103
DM	4.1	207	0.73	152
DDAO			0.5	240

MØller, J. V. & le Maire, M. (1993)

Integration of the $c(s)$ => Signals : A_{280} and ΔJ for each species

$$\Delta J = ((\partial n/\partial c)/\lambda)\, l\, c \qquad A_{280} = \varepsilon_{280}\, l\, c$$

ΔJ: fringe shift, λ: lazer wavelength — A_{280}: Absorbance, ε_{280}: extinction coefficient at 280 nm

l: pathlength ; c: concentration (weigh unit)

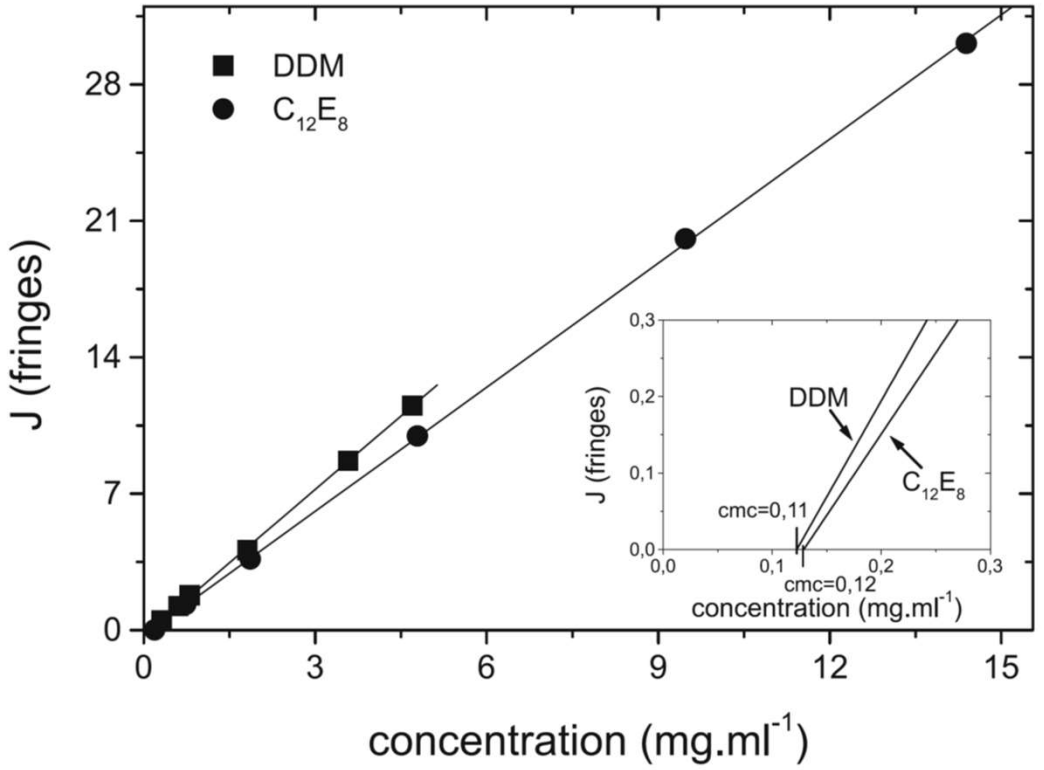
Membrane proteins bound (det.+ lip.) : $\delta_{DL}=(\delta_D + \delta_L)$

	$\partial n/\partial c$ mL g^{-1}	reference	ε_{280} cm^{-1}mg^{-1}mL
Membrane protein	0.187	Hayashi 1989	typically 1
C12E8	0.134		≈ 0
C12E8	0.121	Salvay 2006	≈ 0
DDM	0.143		≈ 0
DDM	0.133	Strop 2005	≈ 0
FOS14	0.133		≈ 0
LDAO	0.148		≈ 0
Triton X100	0.154	Csu´cs 1998	2.31
Lipid	0.083		≈0
DMPG	0.127	Karin 1997	≈ 0
F6-MonoGlu	0.068	Breyton 2009	≈ 0

ε_{280} very often ill-defined for membrane proteins

=> Erroneous δ_{DL} from A_{280} and ΔJ

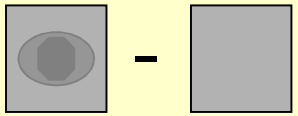
Detergent ($\partial n/\partial c$) and CMC

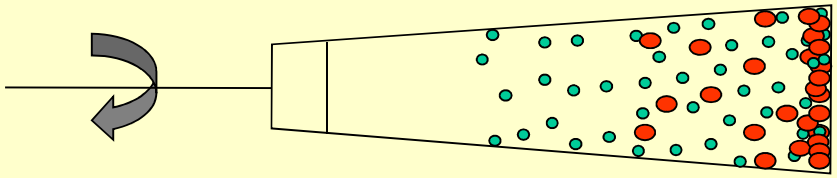


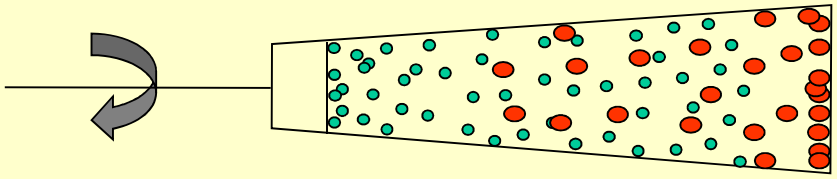
Salvay 2006

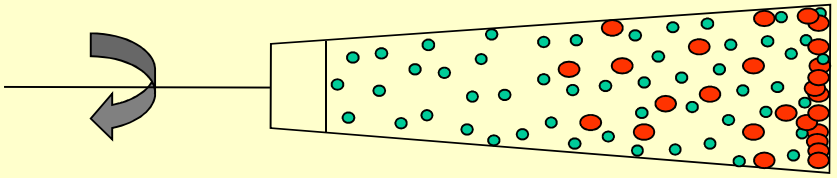
Buoyant properties of the detergent

	Partial specific volume $\bar{v}$* ($ml.g^{-1}$)	Buoyant factor $(1-\rho\bar{v})$ in water
Protein	0.74	0.26
SDS	0.86	0.13
DDM	0.83	0.17
F6-Monoglu*	0.57	0.43
DDAO	1.13	-0.13
LAPAO*	1.002	0
C_8E_5:	0.997	0.003
Octyl POE:	0.99	0.01
$C_{12}E_8$:	0.97	0.03
Lipid	≈ 1; 0.981*	≈ 0 as a mean ; 0.02*



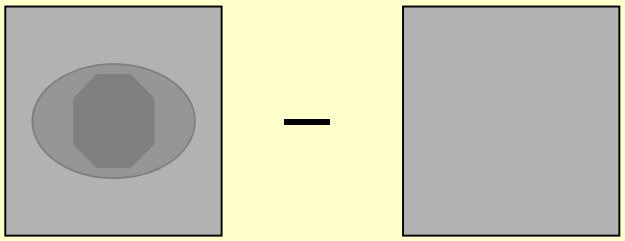






*le Maire et al.(2000), except F6-Monoglu: Breyton 2006; LAPAO: Nury 2008; lipid: Huan 1971.

$$M_b = M_{PD}.(1-\bar{v}_{PD}\rho^\circ)$$



==> compare with calculated M_{PD}, $\bar{v}_{PD}$
==> measurement at two solvent densities => M_{PD}, $\bar{v}_{PD}$

$$M_b = M_P.(1-\rho^\circ\bar{v}_P) + \delta_D M_P.(1-\rho^\circ\bar{v}_D) + \delta_L M_P.(1-\rho^\circ\bar{v}_L)$$

Protein

Detergent

Can be masked if $(1-\rho^\circ\bar{v}_D)=0$

Lipid

Negligeable in water

==>> Compare with calculated *M*b for the components
==>> Contrast density variation in H_2O/D_2O
==>> Estimates of δ_D from SV or other techniques (SEC with radiolabelled detergents...)

$$M_b = M_p.(\partial\rho/\partial c_P)_\mu$$

Can be measured by precise density
(After chromotography /dialysis)

1) AUC, Summary

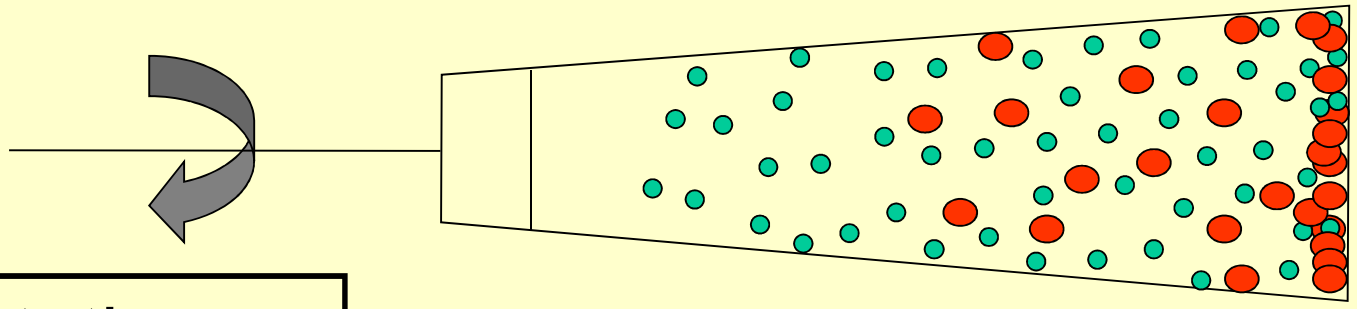
2) The specificity of membrane proteins

3) Strategy & protocols

4) Examples

STRATEGY

First, always: Sedimentation velocity: *c*(*s*)



From sedimentation velocity experiments at 3 concentrations

Distribution of sedimentation coefficients

↓

c(*s*)

↓

- homogeneity?
- interactions?
- possibility of SE experiments?
- bound (detergent+ lipid)
- free detergent concentration
- association state from $s + R_H$ or/and from $s + f/f_{min}=1.25$
- Experiments in $H_2O + D_2O$=> $\overline{v}_{PD}$

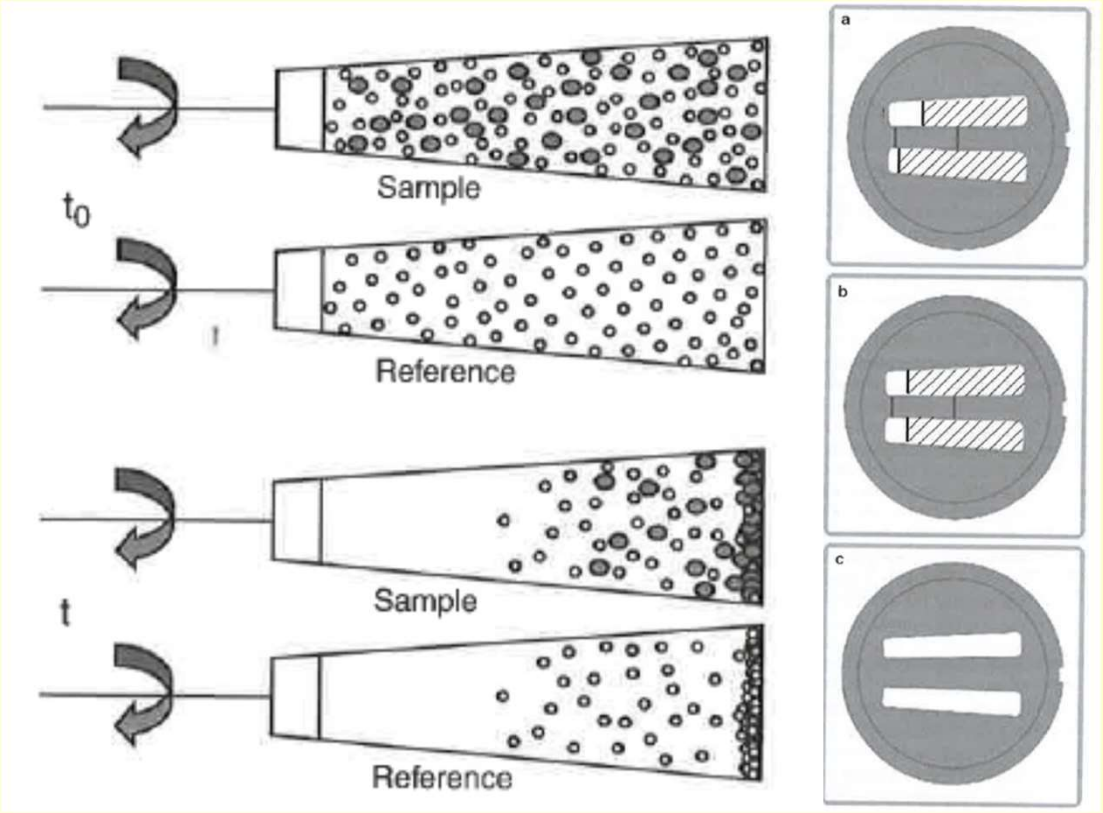
Proposed experimental protocol

- SV at different concentrations: dilution in solvent with and without detergent.

- measurement absorbance + interference.

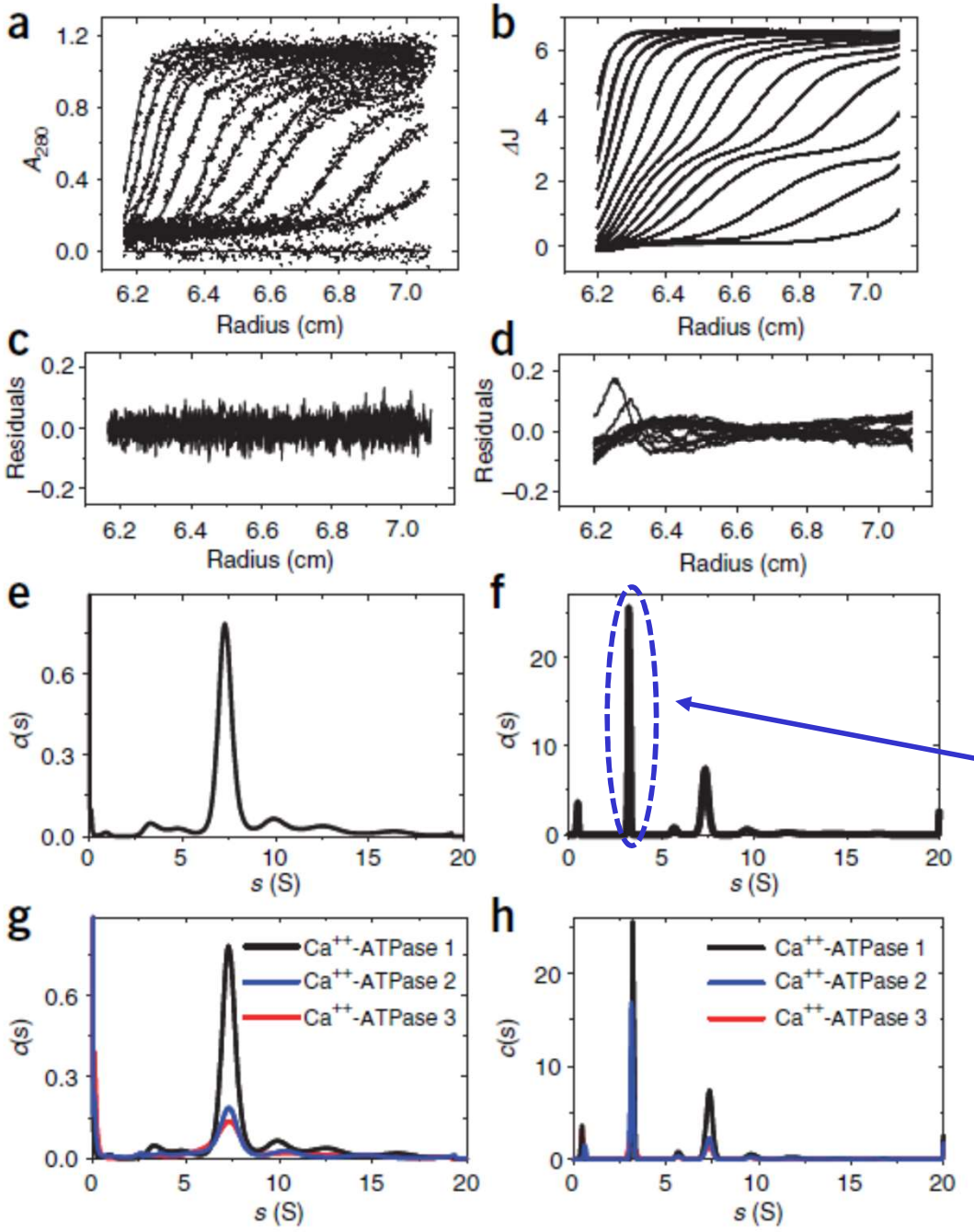
- Recommended : **NO detergent** in the reference compartment:
detergent micelles sediment as particles

- If required (if e.g. glycerol…): exp after dialysis or solvent exchange columns and using double sector capilary type centerpieces to fix the same free detergent concentration in the two channels



For the analysis

Use all knowledge
(bound detergents, lipids, R_H)



Having no detergent in the reference channel allows to measure the free detergent concentration

1) AUC, Summary

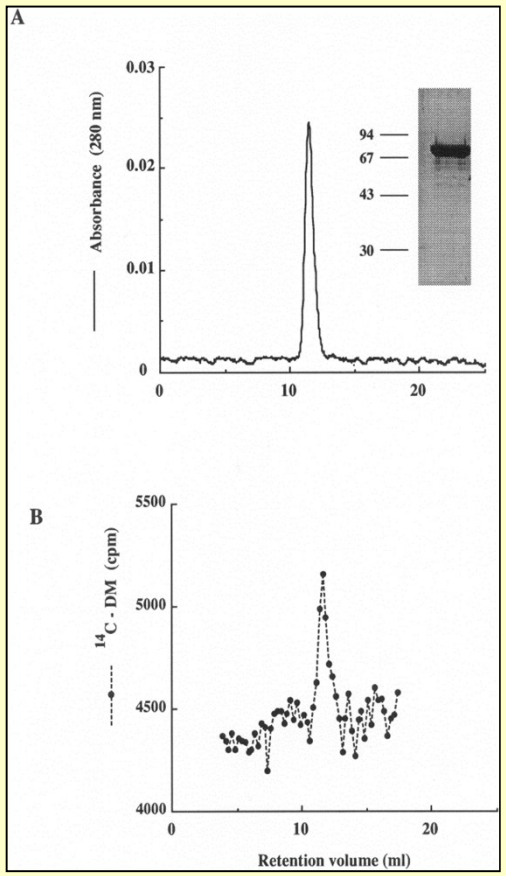
2) The specificity of membrane proteins

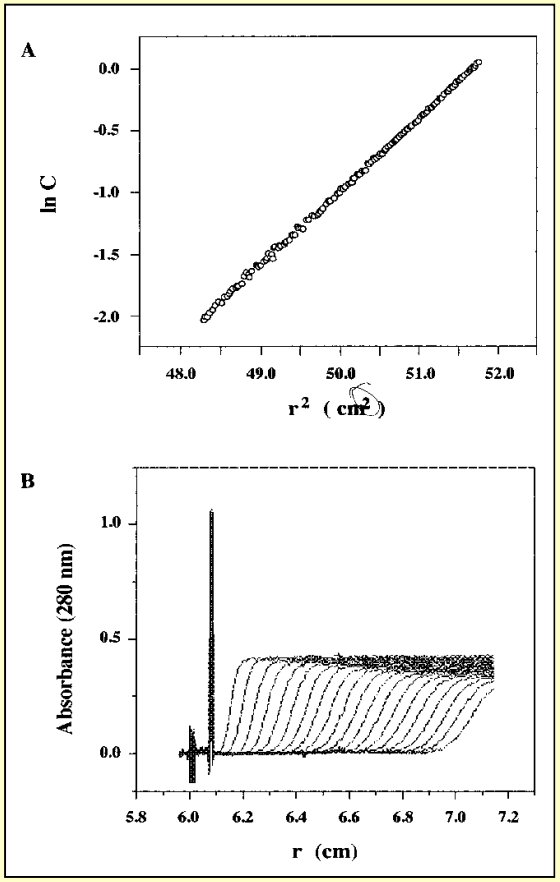
3) Strategy & protocols

4) Examples

- FhuA : SV at 280nm + SEC with radiolabeled DDM
- Change of *c*(*s*) with protein & detergent concentration
- Using different optics
- SV in H_2O and D_2O

FhuA / DDM

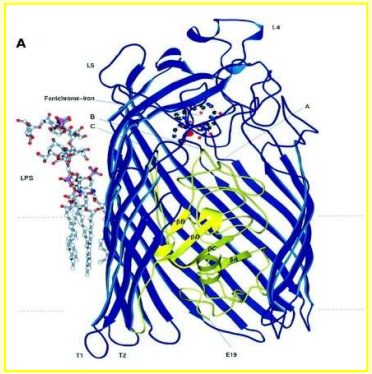




Size exclusion chromatography + *AUC SE and SV at 280nm*
^{14}C labelled detergent

- δ_D=1.2 g/g
- R_H=4.2 nm
- M_b =185 kDa
- $s_{20,w}$=7.8S

Boulanger et al. (1996)
Biochemistry 35, 14216-14224



Ferguson et al.,
Science, 1998, 282, 2215

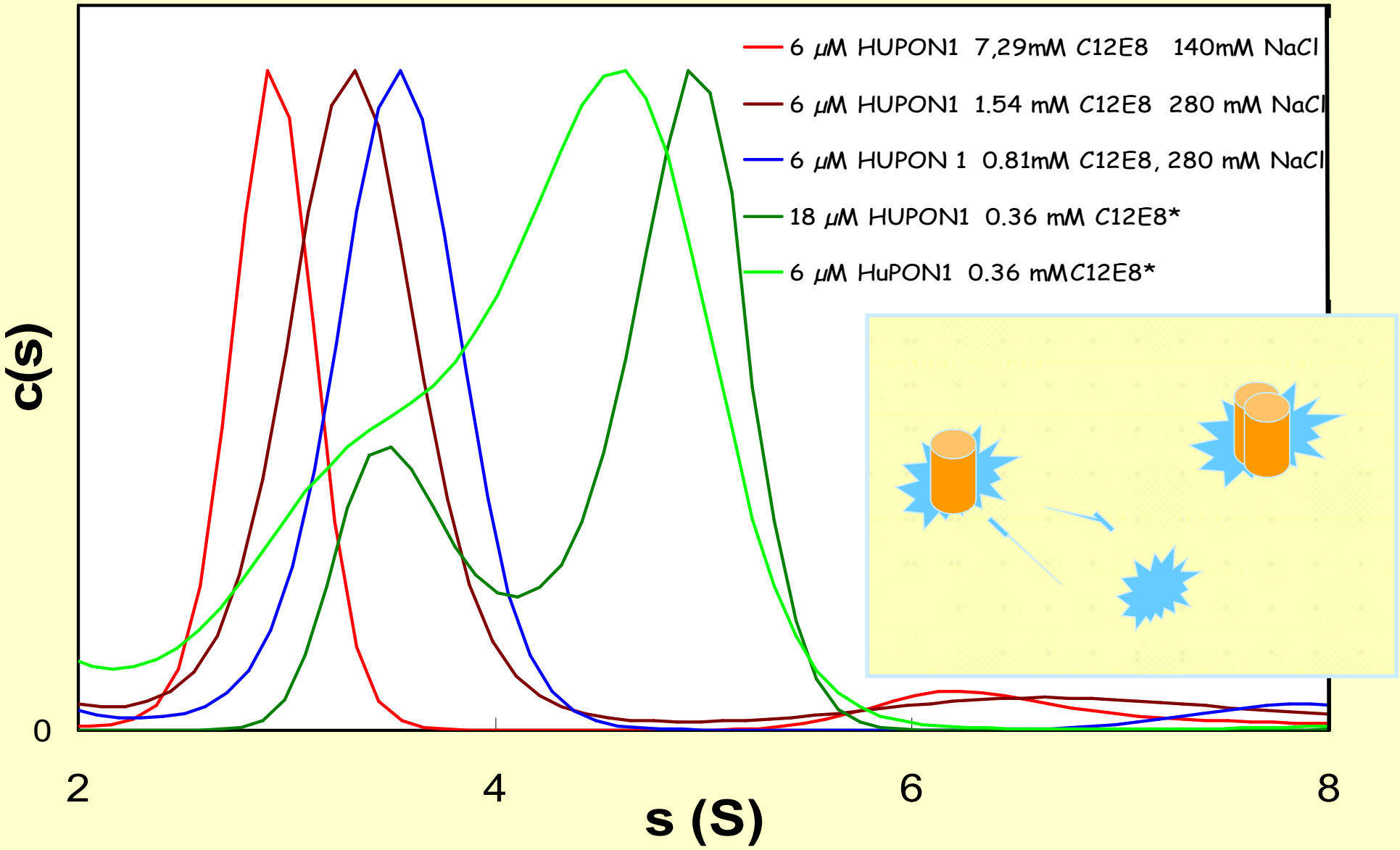
M_b ;δ_D => M_{PD}=185 kDa
M_P=84 kDa

$s_{20,w}$; δ_D; R_H => M_{PD}=172 kDa
M_P=78 kDa

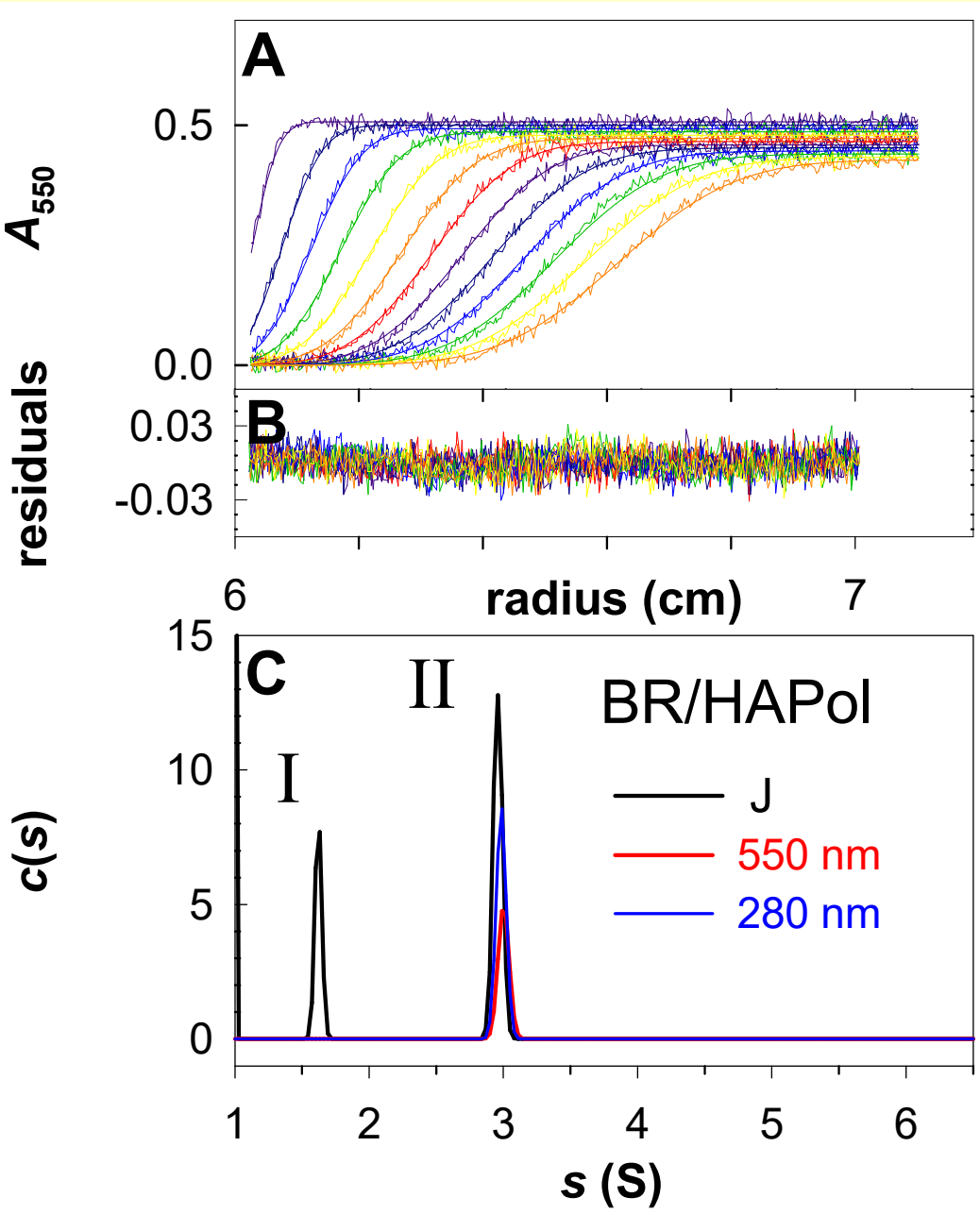
$s_{20,w}$; M_b => R_H=4.5 nm
R_S; compo.=> f/f_{min}=1.18

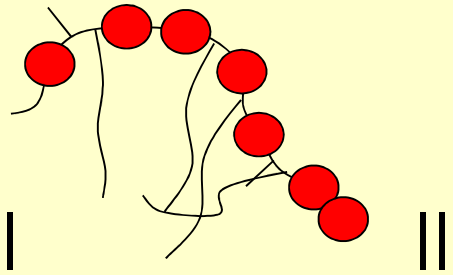
M_{seq}= 79 kDa

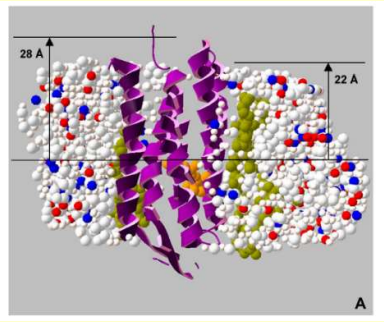
SV AUC Hupon in $C_{12}E_8$
HuPON 1 auto-association is modulated by detergent



Using different optics for characterizing Apol/BR complexes







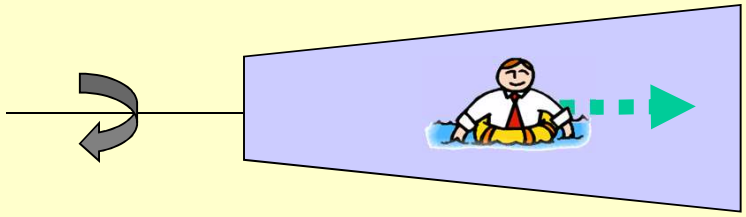
Free APol/BR:
- 0.4 g/g from J

BR Complexes:
- homogeneous or close to homogeneity.
- Native BR from A_{280}/A_{555}
- (APol+lipid)/BR ~ 2.2 g/g from J/A_{280}
- Bound lipids 0.4 g/g from lipid analysis

TOOL: integrating the $c(s)$ from A_{280} and J for determining the bound surfactant + lipid amount now availble in Gussi *Brautigam et al., 2015*

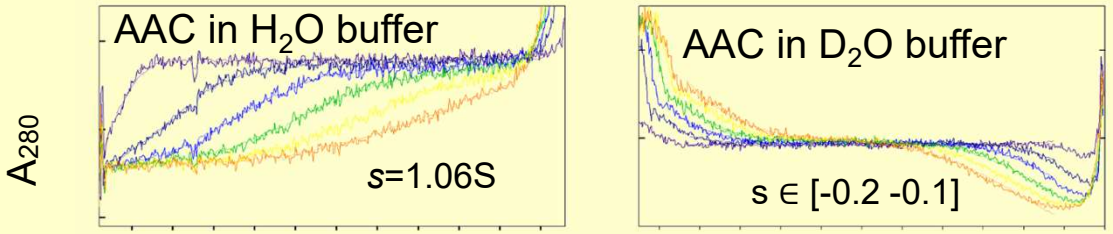
Gohon et al., BJ 2008

SV in H_2O and D_2O solvents



Hypothesis:
same shape; same composition

- *M* changes to M_D (exchangeable H)
- $\bar{v}$ is defined for hydrogenated material
- R_S does not change
- solvent density and viscosity change

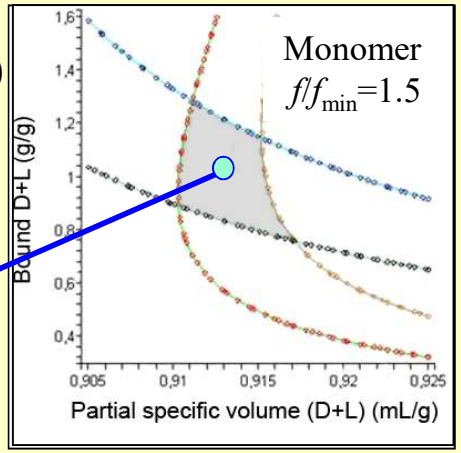


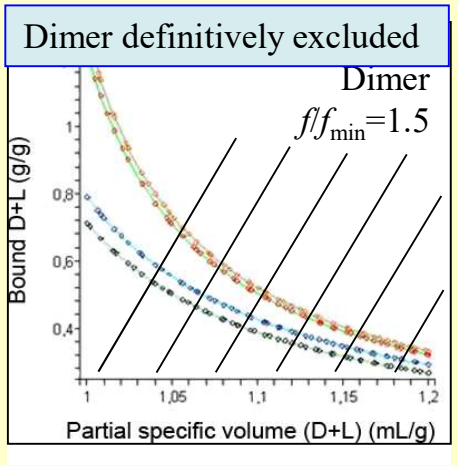
$$s_H = M_p\left[\left(1-\rho_H\bar{v}_p\right)+\delta_d\left(1-\rho_H\bar{v}_d\right)\right]/N_A 6\pi\eta_H R_s;$$

$$s_D = M_p\left[\left((M_D/M)_p-\rho_D\bar{v}_p\right)+\delta_d\left((M_D/M)_d-\rho_D\bar{v}_d\right)\right]/N_A 6\pi\eta_D R_s.$$

The s_H/s_D method

A graphical representation to investigate:
Is a given association state of the protein (monomer, dimer) compatible with the sedimentation coefficients measured in H_2O and D_2O buffer?
Useful when lipid is bound in ill-defined amount





$\bar{v}_{d+l}$: 0.92 ml/g
δ_{d+l} : 1.1 g/g

f/f_{min} : realistic

$\bar{v}_{d+l}$:realistic

TOOL now availble in Sedfit
Le Roy et al., 2015

Nury et al 2008, Dach et al 2012, Le Roy et al 2013, Le Roy et al. 2015

Practical: Ca^{++} ATPase in DDM

Ca^{++} ATPase: $M_{monomer}$=109.49 kDa R_H= 5.5 nm $\overline{v}_p$=0.7425 ml/g M_D/M_H=1.015
ε_{280}=0.966 $cm^{-1}mLmg^{-1}$ $\partial n/\partial c$=0.187 ml/g

DDM: $M_{micelle}\approx$67 kDa $\overline{v}_{det}$=0.82 ml/g M_D/M_H= 1.014
ε_{280}=0 $\partial n/\partial c$=0.143 ml/g

solvents: η_H= 1.00 cp ρ_H= 1.004g/mL η_D=1.23cp ρ_D=1.109g/mL

SV in H_2O (reference buffer without detergent):
absorbance + interference

s_H/s_D

SV of Ca^{++} ATPase in DDM

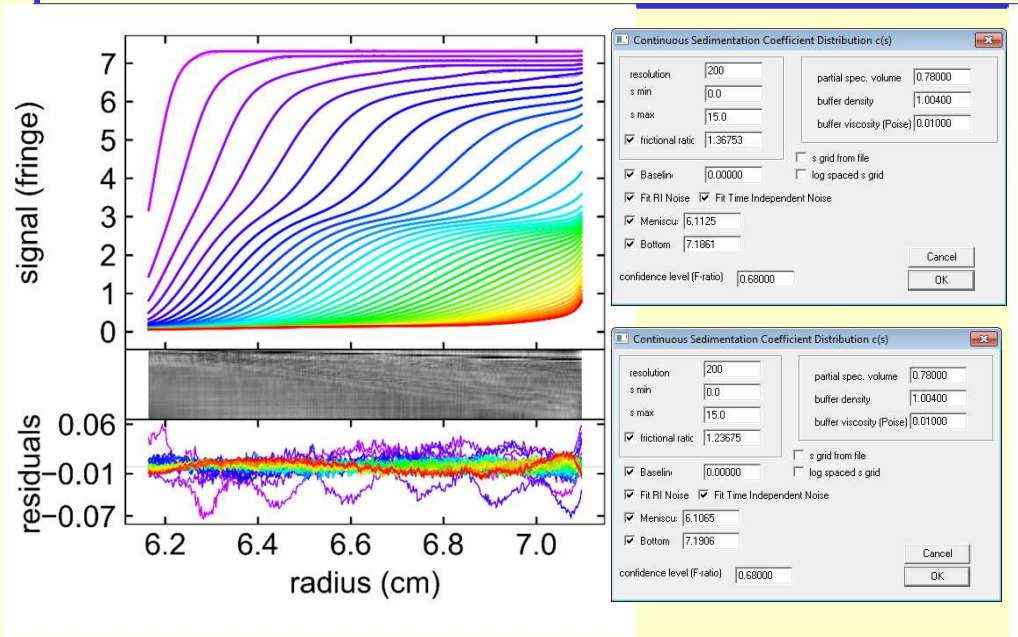
- reference buffer without detergent, $\rho°=1.004$, $\eta°=1.00$

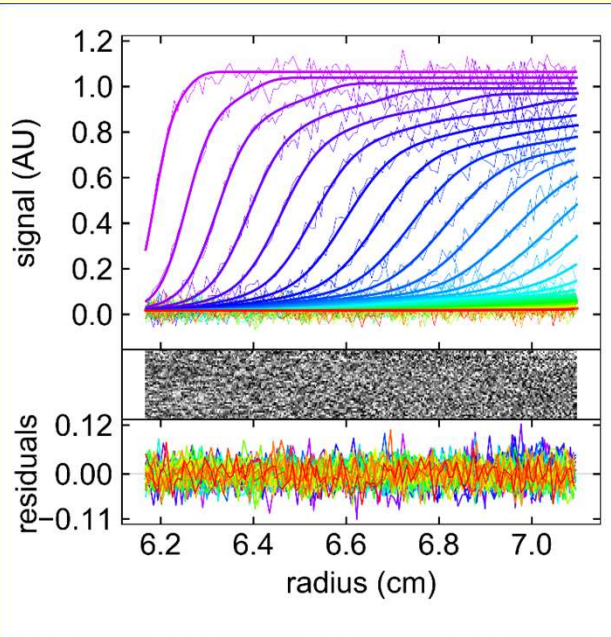
***c*(*s*) analysis of interference than absorbance data** (because detergent micelles moves slower)

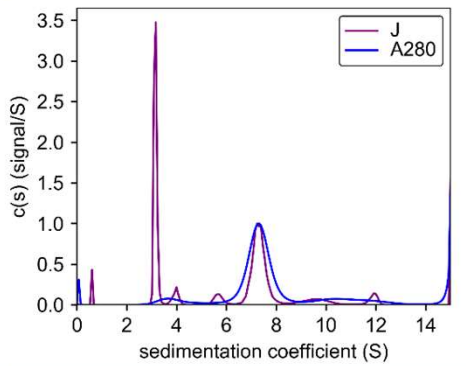
- Selection of the files: => 80 first scans by 2
- Analysis with $\overline{v}$ = 0.78 ml/g, intermediate between detergent and protein, s from 0 to 15S
- Fit f/f_{min}, meniscus, and bottom, with resolution=50, F-ratio=0.5
  Run with resolution=200, F-ratio=0.68
- => report f/f_{min} values: => interference data: f/f_{min} =1.37; absorbance data: f/f_{min} =1.24

Note: f/f_{min} here has no meaning because $\overline{v}$ is not known, and there are different kind of particles

- =>Gussi data fit residual plot (save data only: ra1scans, ip1scans)
- =>Gussi *c*(*s*) plot (save data only: ra1, ip1)
- => use gussi to superpose *c*(*s*) from J and from A280, save as a gussi state (CaATPase)

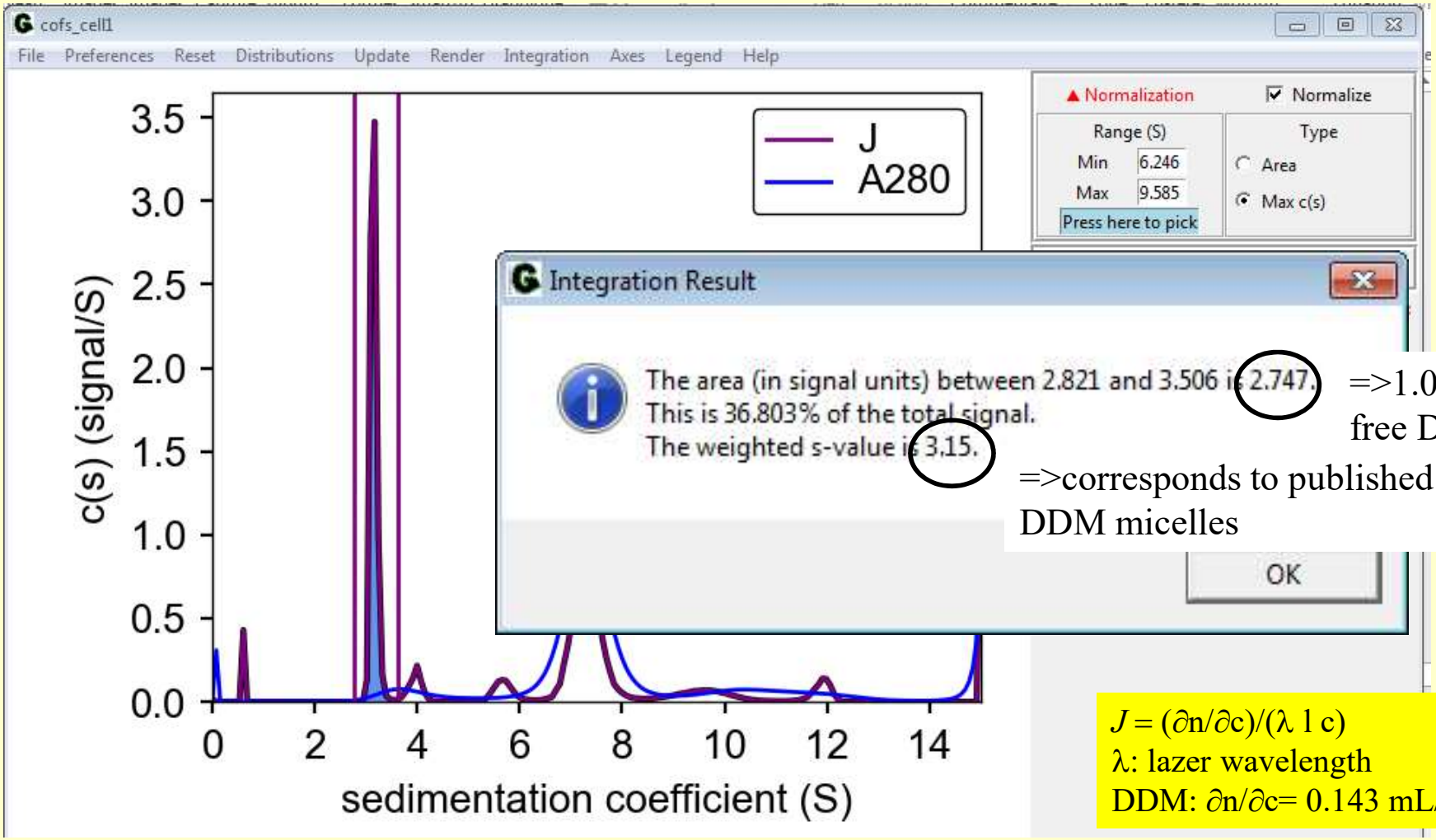






Estimation of free detergent micelle concentration

- Integration in e.g. GUSSI of the detergent signal in the c(s) from interference optics
- Using Excell sheet to calculate c from signal, optical path and ∂n/∂c



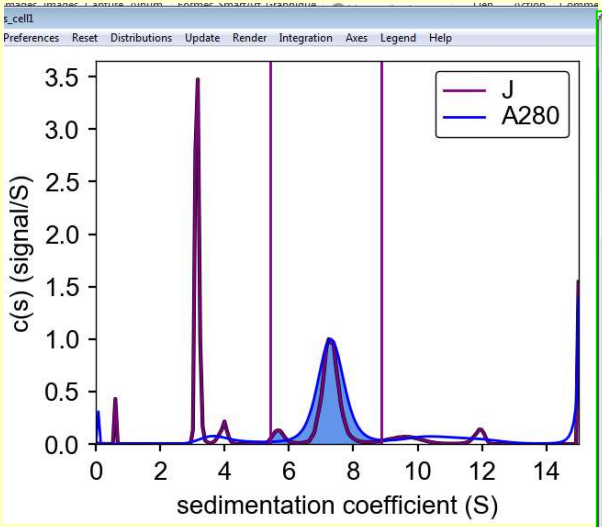
=>1.05 mg/mL of free DDM micelles

=>corresponds to published value for DDM micelles

$J = (\partial n/\partial c)/(\lambda\, l\, c)$
λ: lazer wavelength
DDM: $\partial n/\partial c = 0.143$ mL/g

Estimation of bound detergent and f/f_{min} for a given protein association state

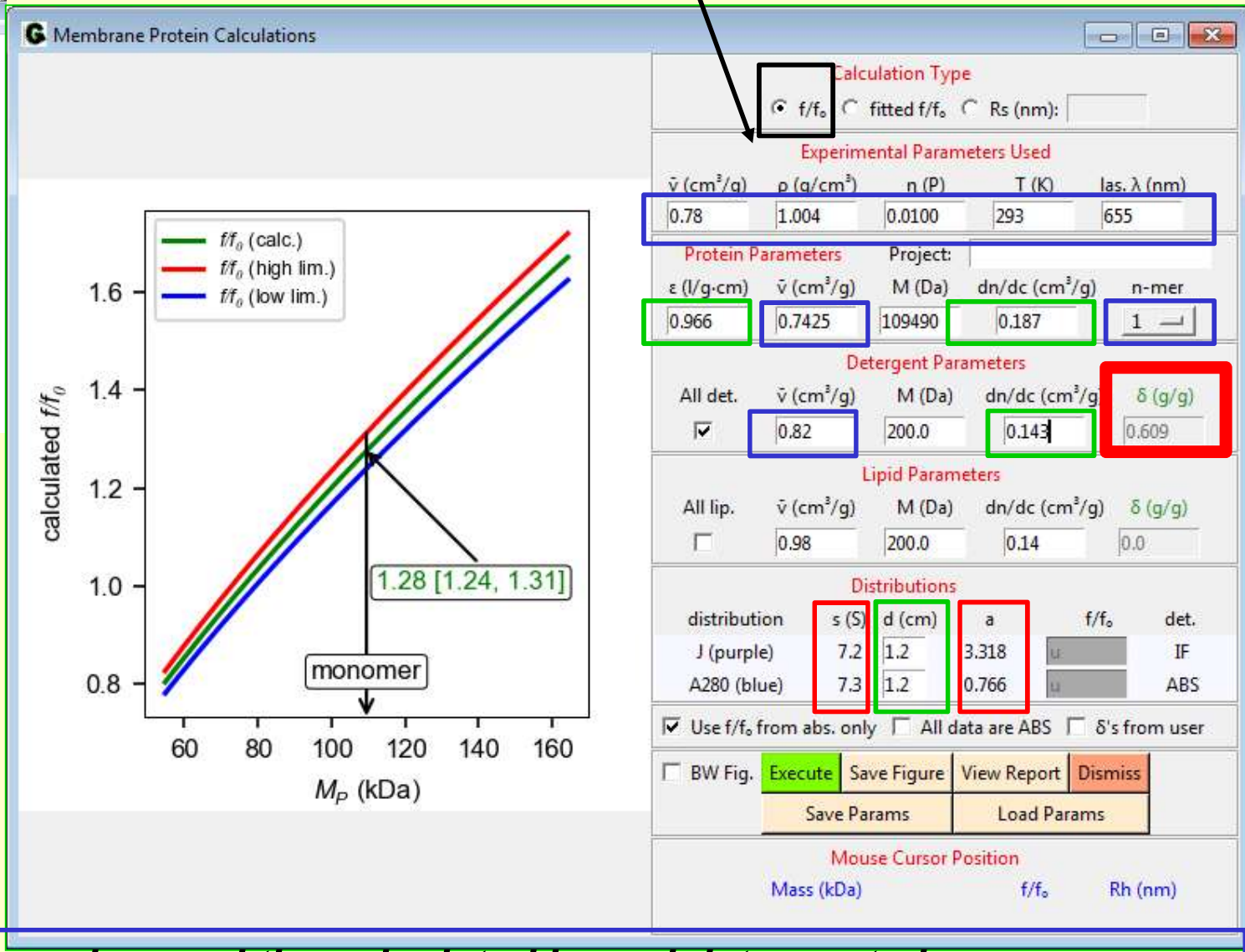
- using gussi with $c(s)$ from Interf first, then from abs.: integrate/membrane protein calculation



Calculated from $c(s)$ integration

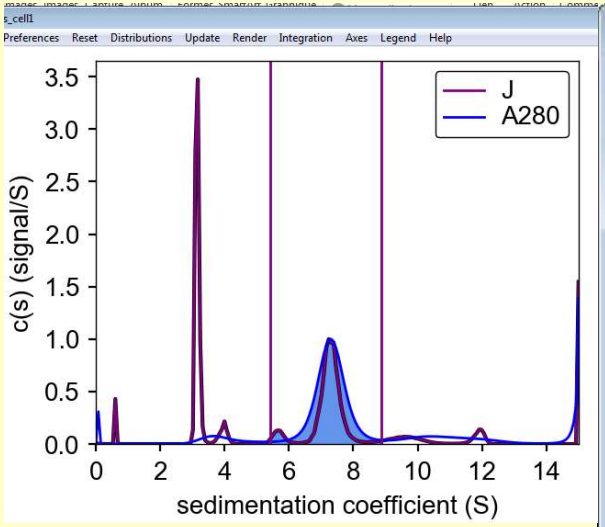
Input used to calcutate bound detergent (ε_{det}=0 by default)

Input used to calcutate f/f_{min}, from s, M, and bound detergent



Results: if monomer: the s-value, and the calculated bound detergent give f/f_{min}=1.28=globular compact RH=4.4 nm. if dimer, f/f_{min}=2 = very elongated, RH=8.9 nm

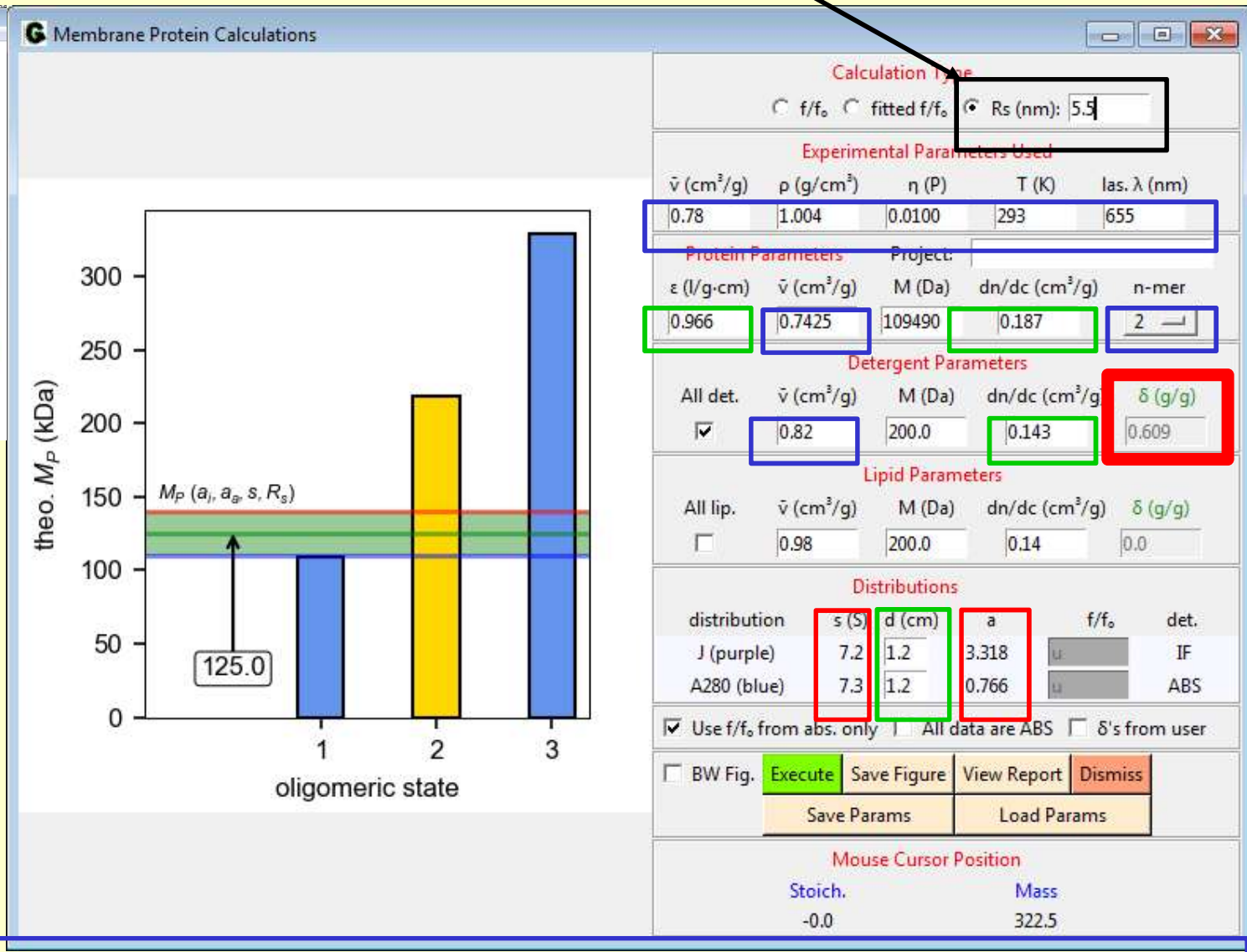
Estimation of bound detergent, then *M* when R_H is known



Calculated from *c*(*s*) integration

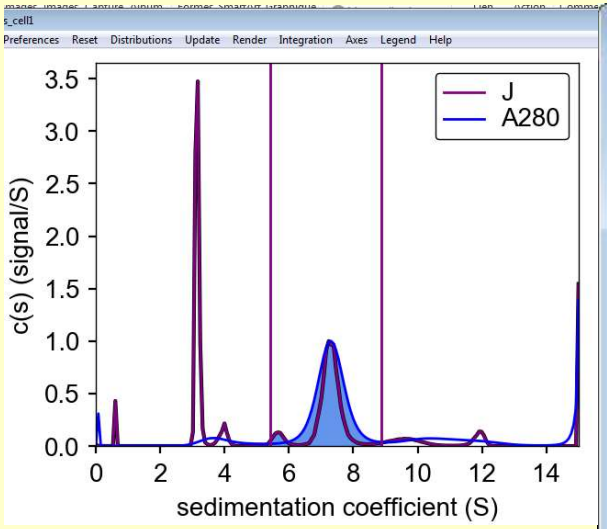
Input used to calcutate bound detergent (ε_{det}=0 by default)

Input used to calcutate *M* from *s*, R_H, and bound detergent



Results: the s-value, and the calculated bound detergent, combined with Rh=5.5nm, gives M=125 kDa, close to the monomer value (109 kDa)

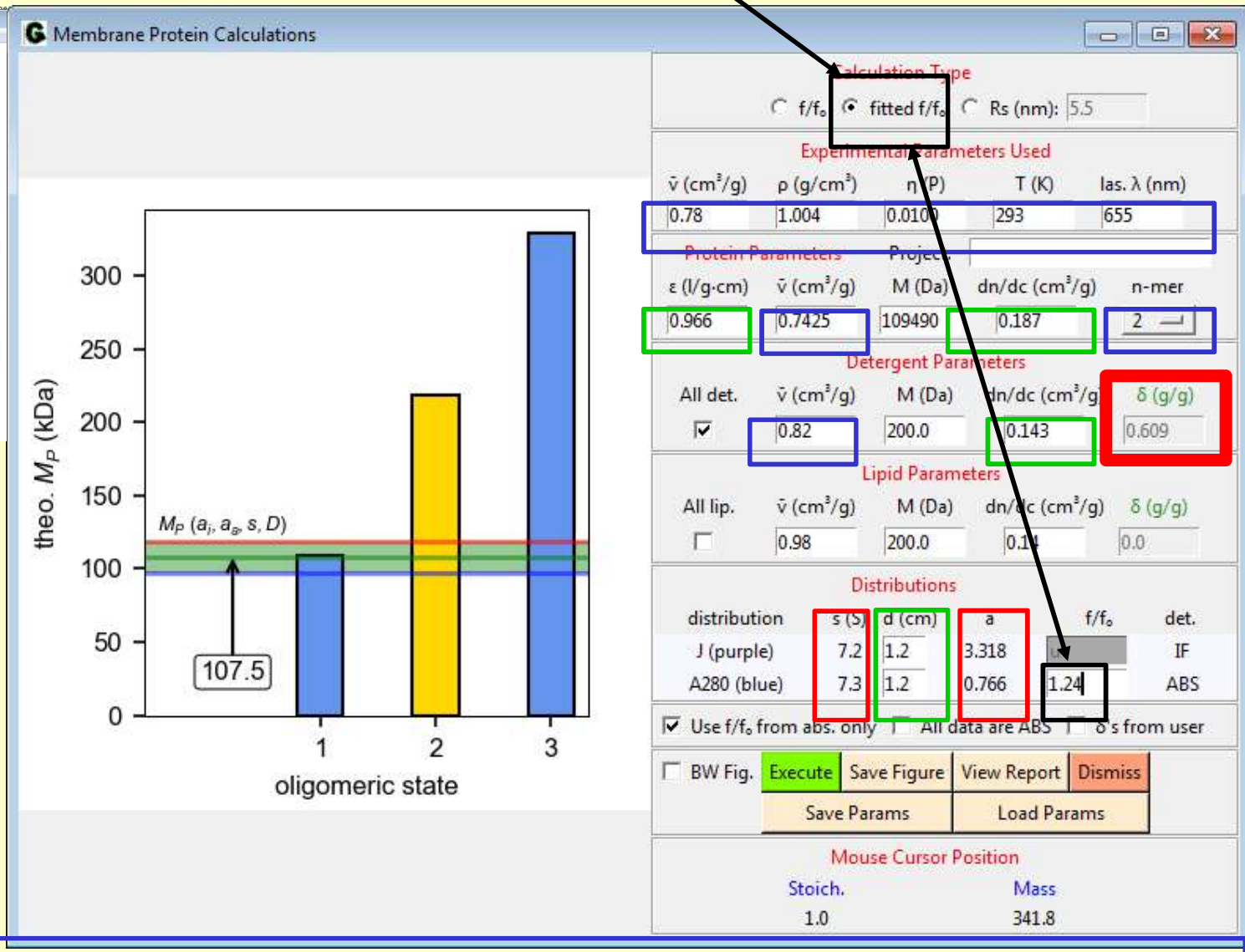
Estimation of bound detergent and *M* given the *f/f*min value calculated in sedfit



Calculated from c(s) integration

Input used to calcutate bound detergent

Input used to calcutate Mfrom s, f/fmin, and bound detergent



Results: using the D-calculated in sedfit (calculated from the apparent f/fmin), M=107 close to the monomer

From sH (usual buffer) and sD (buffer with heavy water, $\eta°$=1.23 cp $\rho°$=1.109 g/mL)

•In sedphat: options/interaction calculator/Ebel B-v Plot of detergent binding from Density contrast SV"

A graphical representation to investigate:
Is a given association state of the protein (monomer, dimer) compatible with the *s*- measured in H_2O and D_2O buffers?
Useful when lipid is bound in ill-defined amount

$$s_H = M_p\left[\left(1-\rho_H\bar{\nu}_p\right)+\delta_d\left(1-\rho_H\bar{\nu}_d\right)\right]/N_A 6\pi\eta_H R_s$$

R_H=R_s is expressed as a function of the other above parameters, and of f/f_{min}.
The program plots the mathematical solutions (δ_d ;ν_d) for $s_{H\,min}$, $s_{H\,max}$, $s_{D\,min}$, $s_{D\,max}$.
The area between these four curves gives the possible solutions (δ_d ;ν_d)
Plots have to be done for different oligomeric states, and f/f_{min}.

In the deuterated buffer, the Svedberg equation is slightly modified to take into account the changes due to protein and detergent H/D exchange, modifying slightly the effective partial specific volume

$$s_D = M_p\left[\left(\left(M_D/M\right)_p-\rho_D\bar{\nu}_p\right)+\delta_d\left(\left(M_D/M\right)_d-\rho_D\bar{\nu}_d\right)\right]/N_A 6\pi\eta_D R_s$$

From sH (usual buffer) and sD (buffer with heavy water, η°=1.23 cp ρ°=1.109 g/mL)

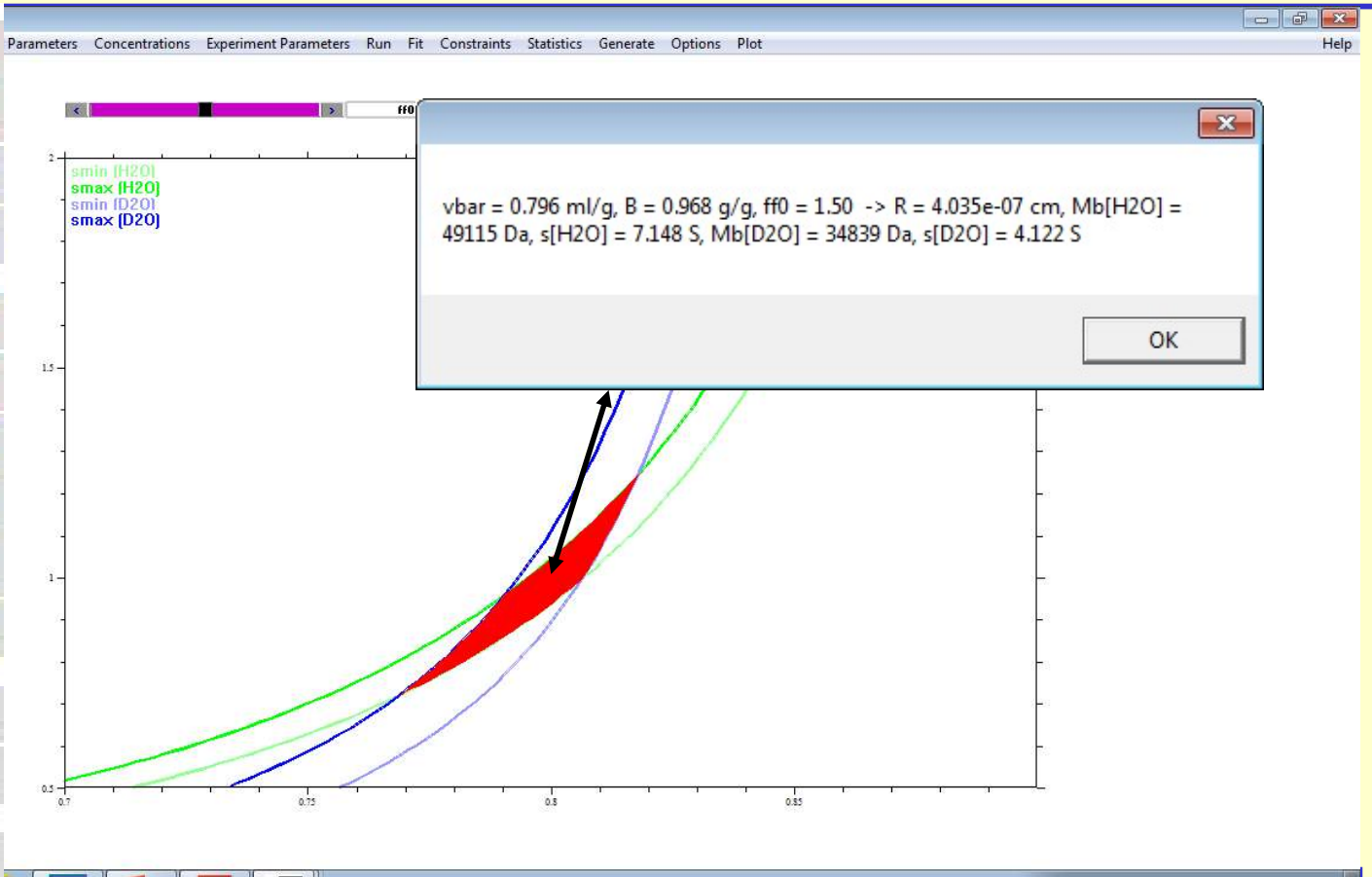
•In sedphat: options/interaction calculator/Ebel B-v Plot of detergent binding from Density contrast SV"

smin (H2O) = 7.0
smax (H2O) = 7.2
smin (D2O) = 4.0
smax (D2O) = 4.2

eta(H2O) = 1.000
rho(H2O) = 1.004
eta(D2O) = 1.230
rho(D2O) = 1.109
MD/M (prot) = 1.0150
MD/M (bound)=1.0140

M (prot) = 109490
vbar (prot) = 0.743

plot vbar(min) = 0.700
plot vbar(max) = 0.900
plot B(min) = 0.500
plot B(max) = 2.000
plot ff0(min) = 1.0
plot ff0(max) = 2.0



***Results:* for a monomer**

With f/f_{min}= 1.25 v_{det}=0.82 +/- 0.02 mL/g, B_{det}=0.45 +/- 0.15 g/g

With f/f_{min} = 1.5: v_{det}=0.80 +/- 0.02 mL/g, B_{det}=0.95 +/- 0.25 g/g

With f/f_{min} = 1.75: v_{det}=0.79 +/- 0.02 mL/g, B_{det}=1.5 +/- 0.3 g/g

Dimer: f/f_{min} > 1.8, with Bdet < 0.5 g/g, v_{det} > 0.82 mL/g, Rs<4.6 nm

Questions?